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EDITORIAL

Hygiene hypothesis in inflammatory bowel disease: A critical review of the literature

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Abstract

The hygiene hypothesis is thought to be a significant contributor to the growing incidence of inflammatory bowel disease (IBD) around the world, although the evidence for specific factors that underlie the hygiene hypothesis in IBD is unclear. We aimed to systematically review the literature to determine which hygiene-related factors are associated with the development of IBD. Publications identified from a broad based MEDLINE and Current Contents search between 1966 and 2007 on key terms relevant to the 'hygiene hypothesis' and IBD including *H pylori* exposure, helminths, cold chain hypothesis, measles infection and vaccination, antibiotic use, breastfeeding, family size, sibship, urban upbringing, day care attendance and domestic hygiene were reviewed. The literature suggests that the hygiene hypothesis and its association with decreased microbial exposure in childhood probably plays an important role in the development of IBD, although the strength of the supporting data for each of the factors varies considerably. The most promising factors that may potentially be associated with development of IBD include *H pylori* exposure, helminths, breastfeeding and sibship. However, the vast majority of studies in this area are plagued by serious methodological shortcomings, particularly the reliance on retrospective recall of information making it difficult to truly ascertain the importance of a 'hygiene hypothesis' in IBD. The 'hygiene hypothesis' in IBD is an important area of research that may give clues to the aetiology of this disease. Directions for future research are recommended.

Key words: Inflammatory bowel disease; Hygiene hypothesis; Microbial exposure; Cold chain hypothesis; *H pylori*; Helminths; Measles; Antibiotic; Breastfeeding; Child care

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INTRODUCTION

Inflammatory bowel disease (IBD) is a chronic inflammatory disorder of the gastrointestinal tract[1]. Crohn's disease and ulcerative colitis represent the two most common forms of the condition, with both associated with significant morbidity[2,3]. While the aetiology of IBD remains obscure, it is thought to be the result of a combination of genetic and environmental factors[4]. Although genetic factors including NOD2, IL-23R and ATG16 genes have been implicated in Crohn's disease (CD) and to a lesser degree in and ulcerative colitis (UC)[5,6], they do not account for the striking rise in the incidence of IBD seen over the course of the 20th century[7], raising a strong possibility for an environmental hypothesis in IBD. Only two environmental influences (smoking and appendectomy)[8,9], however, are well established risk factors for IBD, although many others (including oral contraceptives and diet)[10,11] have been proposed to be important in the condition but with inconsistent results[12]. One promising group of environmental factors that may be potentially associated with IBD are those related to the "hygiene hypothesis"[13], which is closely linked to decreased microbial exposure in childhood.

In this review we aim to critically review the evidence for a hygiene hypothesis in IBD by examining factors that are directly linked to microbial exposure, factors that modify the response to infection and proxy measures for microbial exposure. Understanding the role of environmental factors such as these is critical for the possible prevention of IBD in genetically predisposed individuals as well as potentially offering disease improvement to those already suffering with the disease.

THE HYGIENE HYPOTHESIS

The hygiene hypothesis as a potential explanation for IBD comes from observations that the rise in the incidence of IBD, both in developed and developing countries[14,15], has coincided with improvements in hygiene over the 20th century. These improvements in hygiene include access to clean water, a hot water tap, a smaller family size and

thus less crowding, non-contaminated food and hygiene products such as toothpaste[16,17]. There are suggestions however that the rise in allergic and autoimmune disorders[18] over the last century, particularly in developing countries may not be accurate. Based on data from the World Health Organisation it is evident that there is extremely poor coverage of even basic demographic information such as vital registration of deaths[19]. Moreover, so-called Third World economies cannot provide the necessary public infrastructure necessary for diagnosis and reporting of these types of conditions, thus raising doubts as to the importance of a hygiene hypothesis in these disorders.

Nonetheless, Strachan (1989) was the first to link the hygiene hypothesis to the rise in allergic diseases[13]. He reported an inverse relationship between family size and the development of atopic disorders. The hygiene hypothesis is based on the possibility that a child could be overprotected from exposure to common infectious agents in the environment owing to improved hygiene[20]. If the child then comes into contact with a pathogenic infectious agent later in life (delayed exposure), an inappropriate immunologic response is triggered that could lead to the development of an abnormal or ineffective inflammatory process and possibly even IBD.

MICROBIAL EXPOSURE

Improved hygiene is believed to result in a limited exposure to micro-organisms[21]. Such exposure is thought to be necessary in programming the immune system of the gut and mitigating its future inflammatory responses, perhaps even resulting in CD when the immune system is challenged[21]. The underlying premise is that early childhood infection helps to establish the immunological balance between pro-inflammatory Th1 and tolerance-inducing regulatory T cells, preventing the subsequent untoward responses to allergens, microbial or other antigenic stimuli[22]. Thus various childhood circumstances such as day care attendance, presence of siblings, and domestic hygiene-related factors can influence the probability of contracting a "viral infection" at a vulnerable time in immunological development.

We performed a broad based MEDLINE and Current Contents search between 1966 and 2007 on key terms relevant to the "hygiene hypothesis" and IBD including *H pylori* exposure, helminths, cold chain hypothesis, measles infection and vaccination, antibiotic use, breastfeeding, family size, sibship, urban upbringing, day care attendance and domestic hygiene were reviewed. The reference lists from all relevant studies located in this process were then used to trace other studies to provide systematic coverage of relevant studies in this area.

FACTORS DIRECTLY LINKED TO MICROBIAL EXPOSURE

H pylori exposure

Multiple studies since 1994 have found a significantly lower seroprevalence of *H pylori* in patients with IBD compared to both matched controls, and "disease" controls[23-25]. The prevalence of this bacterial infection is lower in CD compared to UC, in the majority of these studies. Potential confounders include the effect of salazopyrine, other 5-ASA compounds, and antibiotics on the carriage and eradication of *H pylori*[26,27]. However, neither salazopyrine nor the other 5-ASA drugs used in IBD reach the concentrations required in the corpus and antrum of the stomach to effect *H pylori* eradication. In addition, studies of IBD patients and disease control groups including patients with COPD[28], indicate that the *H pylori* rates in those individuals exposed to multiple antibiotics are in fact higher compared to those exposed to either no antibiotics or fewer courses.

This reciprocal relationship, similar to that seen between UC and appendectomy[9], has also been linked to subtle changes in disease natural history. Vare *et al*, investigating the relationship between *H pylori* and CD, found that seropositive patients presented at a significantly later age (40 years) compared to seronegative CD patients (30 years, $P < 0.001$)[29]. Modification of phenotype was also identified in a separate study in which seropositive non-smoking CD patients had significantly fewer relapses and a lower risk of bowel resection compared to seronegative non-smoking patients ($P < 0.01$ and $P < 0.05$, respectively)[30].

These observations support a protective role for *H pylori* in both the development and natural history of CD, and need further investigation to determine whether this organism is acting as a surrogate marker of childhood exposure, or may have a more direct and wide-ranging effect on gastrointestinal immune development.

Helminths and IBD

With greater urbanisation and other hygiene practices introduced over the last century has come the decreased acquisition of soil-borne helminths in infants. Helminths are thought to play an important immunoregulatory role in the intestinal flora and as such have been linked to the development of IBD[31]. Firstly helminthic infection is associated with a strong Th2 response, which opposes the Th1 response associated with autoimmune disease and CD[32-34]. Secondly, chronic infection with these organisms may generate a network of regulatory T (Treg) cells that secrete transforming growth factor (TGF)-B and interleukin (IL)-10[33,35]. These cytokines may not only regulate aggressive Th1 responses but also control heightened Th2 responses that contribute to chronic allergic diseases. While there is a wealth of data from animal models to support an immunological role for helminths[32-34] there are limited data to confirm these pathways in the human gut. Moreover helminths infection may bring other anti-colitis mechanisms into play, including increases in mucus and water secretion into the gut lumen[36,37], which may influence the interaction between gut bacteria, their products, and a diseased epithelium, as well as impacting on intestinal motility. Helminths may affect microbial ecology[38] and the neuroendocrine response[39], but none of these factors however have been assessed in human studies. While preliminary results

from human trials in IBD patients using *Trichiuris suis*, or pig whip worm, demonstrate clinical efficacy[40] and no significant allergic disease post infection[41]. Long-term data, particularly after repeated exposure however are urgently needed to confirm these results. Thus, helminths may offer a novel therapeutic avenue in IBD patients, but much more research is needed to fully understand the mechanism behind any potential human association.

Cold chain hypothesis

The cold chain hypothesis shows temporal and geographical coincidences between the development of the refrigerator and the outbreak of Crohn's disease[42]. It is also possible that external factors related to the cold chain hypothesis including machine maintenance procedures, food conservation habits and the availability of electricity may also explain these trends[42]. The potential link between the refrigeration of food and Crohn's disease is *via* exposure to psychrotrophic bacteria with pathogenic properties such as *Listeria monocytogenes*, *Yersinia enterocolitica*, *Clostridium botulinum* and *Bacillus cereus* which are bacteria that are capable of surviving or developing at low temperatures[43]. Some of these bacteria have been identified in CD lesions[44,45]. A recent study by Forbes and Kalantiz (2006) found that the average age of having a fridge was more than 4 years earlier in older IBD patients than in age-matched controls, a difference that was significant[46]. While a greater exposure of the gastrointestinal tract to psychrotrophic bacteria in the early years of life may be a contributory factor to the development of Crohn's disease it is not yet considered to be an independent risk factor for the disease.

Childhood infections

An infectious aetiology has been proposed for IBD based on studies finding a significantly higher frequency of infectious events e.g. gastroenteritis during the first 6 mo after birth especially among future cases of CD[47,48]. A questionnaire-based study examining both adults and children with IBD have reported an association between the incidence of gastroenteritis and diarrhoea in infancy and the later development of CD[49]. However, an international multicenter study found no difference in the frequency of gastroenteritis in patients with IBD versus control subjects[50]. While numerous infectious agents such as paramyxoviruses[51] and Mycobacterium paratuberculosis[52] have been implicated in CD without consistency[53], it is the measles virus that has received the most attention.

The evidence on whether the measles virus increases the risk of IBD has come from several sources. The first is based on the biological plausibility of a measles virus in IBD. It is hypothesized that the measles virus is able to persist within the mesenteric endothelium creating an inflammatory reaction characteristic of CD[54]. While several studies have identified the presence of the measles virus in tissue samples[55,56], this finding has not been confirmed in studies using more sensitive methods such as the polymerase chain reaction (PCR)[57,58].

Another group of studies have examined the association of *in utero* and perinatal measles exposure and the development of IBD. Ekbom *et al* (1996) reviewed the hospital charts from 25 000 deliveries at a Swedish hospital between 1940 and 1949[59]. They found 4 cases of measles infection during pregnancy and of these, three of the offspring had developed CD. This study however suffered from some important biases including the selection of a period of time when it was known that in two cases of measles in the mother were followed by CD in the offspring. Moreover the diagnoses of maternal measles was based on clinical criteria only and the retrospective design makes it impossible to determine whether it is the measles exposure or some other factor during pregnancy that led to the subsequent development of CD in these children. Moreover studies from Denmark[60] and the UK[61] have failed to detect any cases of IBD after exposure to measles virus *in utero*. A history of perinatal infection has been reported to increase the risk of developing CD by five times[62], although the risk for IBD from measles infection specifically is much lower at 1.5 and is not significant[63]. Moreover Haslam *et al* (2000) did not find an increased risk for Crohn's among children born in years with high measles incidence rates versus children born in other years[64].

In summary the evidence supporting an association between measles infection in terms of in situ detection of measles virus, in utero and perinatal exposure and ecological evidence is discordant, but does not appear to be a causative factor in this condition.

FACTORS MODIFYING THE RESPONSE TO INFECTION

Childhood vaccinations

Childhood vaccinations have the ability to alter the maturation of the intestinal and systemic immune system and as such have been implicated in IBD[65]. Thompson *et al*, 1995) were the first to raise the possibility of a link between measles vaccination and IBD[65]. In a cohort analysis of 3545 individuals in the U.K. they showed that individuals with a history of exposure to measles vaccination were 3 fold and 2.5 fold more likely to develop CD and UC, respectively, compared with unvaccinated controls. Methodological shortcomings such as selection of participants from different populations, differential loss to follow-up between the two cohorts, and different ascertainment of outcome by exposure category, however, all dampened the strength of their findings. Subsequent cohort studies were also unable to confirm these initial findings[66,67].

Further controversy was fuelled when Wakefield *et al* reported 8 out of 12 children with non-specific colitis had symptoms attributable by the parents to the measles, mumps and rubella (MMR) vaccination[68]. Several issues related to selection and recall bias, no control group and lack of a clear case definition raised concerns as to the validity of these claims. In response to public concern, a case control study from the Vaccine Safety Datalink Project was conducted to address the link between MMR and risk of IBD[69]. Cases were 142 patients with chart confirmed IBD born 1958 to 1989 who were compared

to 432 controls matched by birth year, gender and health maintenance organisation. There was no evidence that either monovalent measles vaccine or the combination of MMR vaccination was associated with risk of IBD. One study found a significantly increased risk for CD with increasing age, but this was based on three children vaccinated at 2 years or older[67].

Evidence from ecological studies has generally failed to find a link between the incidence of measles vaccination and IBD[70-72]. The incidence of CD has been reported to be increasing since the 1940's, twenty years before the introduction of the measles vaccine.

Although this is a controversial area of research, the findings from well-designed studies do not support a link between vaccines containing measles and the subsequent development of IBD.

Antibiotics and development of IBD

The critical relationship between the human gut and its microflora has been highlighted in the field of IBD, particularly with the development of molecular models of the disorder such as the IL-10 knockout mouse[73]. These models clearly indicate that IBD does not develop in a germ-free environment. The role of gut bacteria has been further underlined by recent genetic discoveries that point to disturbances in the recognition and handling of bacteria in CD[74].

Antibiotics can potentially influence and disturb this relationship, and therefore have been the subject of several case-control studies in IBD[50,75,76]. In addition, some authors have pointed to the temporal relationship between the introduction of antibiotics on a wide scale after the Second World War, and the significant increase in the incidence of IBD seen in the second half of the last century[77].

There are a limited number of studies that address the role of antibiotics in the aetiology of IBD. Two small early studies were both heavily influenced by recall bias and the potential for reverse causation[50,75]. These found a positive association between antibiotic use and CD. In view of the potential lead-in time between onset of symptoms and actual diagnosis of CD, it is perhaps not surprising then that there may be increased prescriptions for antibiotics given to CD patients in the 1-2 years prior to diagnosis. With this confounder in mind, Card *et al* analysed the General Practice Research database (GPRD) in the UK to pull out prospectively collected data on CD cases and controls[76]. This study assessed antibiotic usage, other medication use, and smoking status as well as addressing the above issue of lead-in time, by analysing data 2-5 years pre-diagnosis. The investigators found that 71% of 587 CD patients were prescribed antibiotics 2-5 years prior to diagnosis compared to 58% of 1460 controls (Adjusted OR 1.32 [1.05-1.65], $P < 0.001$). Although there was no obvious confounding by gender, age, or smoking status, there was a lack of specificity with respect to the type of antibiotic prescribed, and the finding of associations with other drug groups including those for neurological conditions and oral contraceptive pills. This indicated that the association with antibiotics may not be causal. However, the strong interaction between cases and use of tetracyclines, particularly in those subjects with no prior gastrointestinal symptoms nor gastrointestinal medications, was intriguing and may provide some guidance as to future studies in this area.

Breastfeeding

The role of infant feeding practices may play a role in the development of IBD by affecting the early exposure to dietary antigens. Some of the important differences between human breast milk and infant formula include immunoglobulins (IG's), lactoferrin, lysozyme, growth factors, allergic factors, carnitine and DHA & ARA. For example Lactoferrin is an iron-binding protein found in human milk but not in formulas. It limits the availability of iron to bacteria in the gut and alters which "healthy" bacteria will thrive in the gut. It has a direct antibiotic effect on bacteria such as *Staphylococcus spp.* and *E. coli*[78].

The majority of the evidence supports a protective effect of breastfeeding in UC and CD[79-81]. A recent study based on an Italian population of 819 IBD patients suggested lack of breastfeeding to be associated with an increased risk of both CD and UC[82]. These results were supported by paediatric studies in Canada and the United States that found paediatric CD patients were less likely to have been breastfed[83,84]. Gilat *et al* performed a 14-center, 9-country study looking at more than 400 IBD patients whose disease started prior to 20 years of age and who were younger than 25 years at the time of the study[50]. These investigators found no significant difference between patients and control subjects in the frequency of breastfeeding as well as consumption of cereal or refined sugar during infancy.

A major problem with studies in this area are their retrospective design and dependence on patient or maternal recall to complete questionnaires. Moreover no studies have examined the potential confounding effects on the development of IBD of environmental factors such as maternal exposure to endocrine disrupting chemicals and the subsequent levels in human breast milk[85]. Further prospective studies using larger sample sizes is necessary to strengthen the validity of these observations.

PROXY MEASURES OF MICROBIAL EXPOSURE

A range of factors including family size, sibship, birth order, urban upbringing, day care attendance and domestic hygiene such as presence of a hot water tap and flush toilets have been examined in IBD studies as a proxy marker of environmental exposures in early life. Table 1 summarises the methodologies of key studies in this area. It can be seen that there is a heavy reliance on adult case-control retrospective designed studies and while many variables have been studied; only a few are significantly consistent.

Family size/ sibship/ birth order

Family size can be used to indicate the level of over-crowding in a home, which has been associated with poor

Table 1 Key studies examining the association between IBD and proxy measures of the 'hygiene hypothesis'

Author	Sample	Study design	Variables examined
Gilat, 1987[50]	14 centres from 9 countries UC = 197; CD = 302 Diagnosis before 20 yr, all patients < 25 yr old Age-sex match controls	Case control Questionnaires	Siblings, birth order, breast feeding, infection, eczema, family history, vaccination, pregnancy factors
Rigas, 1993[84]	New York, USA Diagnosed between 1986-1990 CD = 68; UC = 39 Pediatric gastroenterology controls = 202	Case control Questionnaires	Sibship size, maternal age at birth, month of birth, breastfeeding, maternal smoking one more
Gent *et al*, 1994[92]	UK UC = 231; CD = 133 16-87 yr Age-sex matched controls from same general practice as cases	Case control Home interview	Housing in infancy, presence of a lavatory, hot water tap, separate bathroom
Duggan *et al*, 1998[93]	UK Consecutive weekly attendees at IBD clinics UC = 213, CD = 110 (aged over 15 yr) Aged-Sex match controls from hospital patients undergoing elective surgery	Case control Questionnaire	Previous surgery, childhood domestic circumstances before age 11 yr (heating, day care, toilets, fixed bathroom, bedroom sharing)
Sicilia *et al*, 2001[87]	Spain Gastroenterologists recruited incident cases of CD patients aged 10-79 yr = 103 Outpatient clinic patient controls matched for age, sex and urban/rural.	Case control Population based study Structured interview 95% response rate	Number of persons in home, number of bathrooms, availability of hot water
Montgomery *et al*, 2002[88]	Swedish inpatient register UC = 15 823; CD = 12 668 Controls from Swedish Census, Births & Deaths Register: age and location matched 79 546; 63 035, respectively	Case control Swedish Multi-generation register linking cases and controls with family history information	Siblings
Feeney *et al*, 2002[16]	UK (Grew up in UK) 16-45 yr CD = 139; UC = 137 attendees at Gastroenterology clinics	Case control General hospital Questionnaire	Hp seroprevalence, family, hot water tap, nursery attendance, indoor toilets, swimming pool use, number or cars, number of house moves, urban/rural location, pets
Hampe *et al*, 2003[89]	Germany Consecutive IBD patients identified from German Crohn's & Colitis association = 2351 Controls unaffected first degree relatives = 3364	Case controls Questionnaire	Availability of tap water, toilet, central heating, siblings and community size
Amre *et al*, 2006[22]	Canada Newly diagnosed CD < 20 yr old = 194 Orthopaedic patients controls matched for timing of diagnosis & area of residence	Hospital based Case control Structured questionnaires - mothers mostly answered for patients	Siblings, breastfeeding, day care, place of residency, hot water tap, toilets, number of inhabitants, number of rooms, availability of private bed, personal hygiene, pet ownership, infection, smoking history

hygiene and potential exposure to infection[86]. A small family size and thus a less propensity for exposure to infection has been associated with a lower risk for IBD[86]. Amre *et al* (2006) created a 'crowding index' which refers to the number of rooms in the home divided by the number of inhabitants living in the home and found the mean 'crowding index' to be lower in cases than controls and thus protective against CD[22]. No differences however were found between the numbers of people residing in the home during infancy (< 18 years) in IBD patients versus controls[87], although this study had limited power.

Siblings may influence the development of IBD by altering exposure patterns to microorganisms in early life by affecting acute manifestation of infection by influencing age of transmission and severity[88]. In a large Swedish case control study, Montgomery *et al* (2002) found having older siblings increased the risk for UC, even after controlling for multiple births, sex, maternal age, year of birth, region and socioeconomic class. This may be because older siblings risk exposing younger siblings to infection at a higher dose or at an earlier age. In contrast, having younger siblings was significantly associated with a decreased risk of CD. This effect was most pronounced when younger siblings were born within two years of the patient's birth. It is hypothesised that having younger siblings may prolong or re-expose the older sibling to the presence of micro-organisms, conferring some protective effect against CD through appropriate priming for mucosal immune function[88]. These findings however have not been replicated in smaller studies[22].

Birth order as a potential marker for the hygiene hypothesis in IBD has been examined in several studies with inconsistent results[50,89]. For example, Hampe *et al* (2003) found birth later in the sibling order was significantly associated with a lower risk for IBD (included patients with CD, UC and indeterminate colitis)[89]. This is because first-born children are usually exposed to infection later in life than younger siblings who come into contact with common viral and bacterial infection through older siblings. Rigas *et al* (1993) however did not find birth order to be significantly associated with either disease based on medical records[84].

Urban upbringing

It has been suggested that an urban upbringing represents a more "hygienic" childhood compared with a childhood spent on a farm or a rural location. Several studies report that a higher incidence of IBD in urban as opposed to rural areas[90,91]. Others however have failed to replicate this finding[16,87].

Day care attendance

Day care attendance in young children has the potential for exposing children to greater infections. Amre *et al* (2006) in a hospital- based case control study found day care attendance in the first 6 mo of life to be associated with an increased risk for CD[22]. Feeney *et al* (2002) however found no significant difference between IBD cases and controls in regards to pre-school attendance[16].

Domestic hygiene

Several domestic hygiene-related exposures have been investigated in relation to IBD. This is because good domestic hygiene may protect an individual from exposure to a full range of agents that programme the immune system in the gut during infancy, causing an inappropriate response, perhaps even CD, when exposure occurs at a later age[92]. Gent *et al* (1994) found CD to be 5 times more common in IBD patients whose first house had a hot water tap and a separate bathroom, but no association was reported for mains drainage or access to a flush toilet[92]. Moreover domestic hygiene variables were not associated with UC[93]. A similar finding was reported by Duggan *et al* (1998) who found availability of hot water before age 11 to be significantly associated with CD but not UC[93]. Others however have failed to find a significant association between availability of hot water, toilet facilities, bed sharing, source of drinking water and number of bathrooms and risk for CD[22,50,89] but some of these studies did not evaluate controls that were representative of the case population or had power problems[50]. Pet ownership[22] and animal exposure during childhood[94] have been associated with an increased risk for CD but are not consistent findings[16]. The frequent use of a swimming pool was significantly associated with CD, although the mechanisms behind the association remain unclear and could be more to do with socioeconomic status rather than hygiene related[16]. Most of the studies except one[50], however, rely on the retrospective collection of information *via* questionnaires making it difficult to rule out recall bias (Table 1).

SOCIOECONOMIC STATUS

Earlier studies cited socioeconomic status as an important factor in the development of IBD, in that a higher socioeconomic status was more prevalent in IBD patients versus controls[95,96]. A higher socioeconomic status may also confer less household crowding and better domestic hygiene (hot tap) and thus increase the risk for CD by limiting exposure to micro-organisms[86]. As hygiene practices have improved over the course of the 20th century, socioeconomic status has become less important in IBD. In a recent study by Bernstein *et al* (2001) no differences in socioeconomic status were found between IBD patients and controls[97].

FUTURE DIRECTIONS

Based on a systematic review of the literature it is evident that there is a strong need for future studies in this area to adopt a prospective design in a paediatric sample to avoid the pitfalls associated with retrospective studies such as recall bias. This is crucial as many factors proposed to be important in the hygiene hypothesis such as breastfeeding date back to birth and infancy. Population based studies are also important to overcome problems inherent with selection biases which may be present in the many patient based studies that dominate this area. The evidence suggests that it is also important to look at Crohn's disease and ulcerative colitis separately since exposure risks do not appear to be the same for both disorders. The need to establish and validate better markers for hygiene related factors is crucial as it is impossible to rule out the possibility that the relationship between IBD and a hygiene related factor such as sanitation for example is not an indirect effect that may serve as a mechanism for other yet unknown lifestyle factors. Future research should also be directed towards trying to understand the potential mechanisms underlying associations between hygiene related factors and IBD in the hope of providing clues to the aetiology of this disease.

CONCLUSION

IBD is a growing disease that is costly to the individual and society. The underlying premise of the hygiene hypothesis is that decreased microbial exposure in childhood may lead to the subsequent development of IBD appears to be plausible, although the strength of the evidence supporting this varies between many hygiene-related factors. Helminthic infection, *H pylori* exposure, antibiotic use, breastfeeding and sibship represent the most promising factors supporting the hygiene hypothesis in IBD, but carefully designed prospective evaluation is urgently needed.

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OBSERVER

Hugh Freeman, MD, Professor, *Series Editor*

The spirit of Henry Norman Bethune and gastroenterology

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From the Series Editor: A unique column, The Observer, will be initiated soon in *World Journal of Gastroenterology* (*WJG*). Professor Hugh James Freeman, a gastroenterologist from the Department of Medicine, University of British Columbia, Vancouver, has been appointed as a Series Editor for The Observer. Serving as a forum for both gastroenterologists and/or hepatologists worldwide, Professor Freeman will periodically invite experts in particular research fields to examine important progress made in both Gastroenterology and/or Hepatology. The Series will examine challenging issues faced at the fundamental level from the research laboratory to the bedside. The Observer is an invited editorial and will not accept independent uninvited submissions. For more information, please do not hesitate to contact Professor Freeman at hugfree@shaw.ca.

Abstract

Henry Norman Bethune was a physician and surgeon from Canada. He had a highly impressive medical career in Montreal but did his most important work in China where he cared for soldiers on the battlefield. He died in 1939 and was recognized as a hero, but only much later received recognition in Canada. He was a skilled doctor, both as a physician and as a surgeon. However, he was much more and will serve as an inspiration for this series. He was an innovator, an idealist and a perfectionist. It is hoped that this series will gather expert commentaries on a range of issues critical to the subspecialty from fundamental science to clinical care so that future directions can be defined.

Key words: Bethune; Gastroenterology; China; Canada; Battlefield surgery

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INTRODUCTION

Developing this new editorial feature presents a challenge, but also an important opportunity. There is a broad range of critical issues that gastroenterologists must deal with on a daily basis. Some relate specifically to fundamental scientific aspects of our specialty as well as clinical elements of "cutting edge" patient investigation, treatment and ongoing care, particularly for chronic illnesses. Several subspecialty journals in gastroenterology, hepatology and nutrition already provide new and updated information, from time to time, on many of these issues. Although being informed about the past and present state of knowledge is very important, there is also an increased need to visualize, even speculate about future directions in all of these related fields. It is hoped that this column will help to share such insights through editorials from individual experts focused on specific areas of our discipline.

Other critical areas include the impact of industry and the role that different global or multinational corporations already have in our subspecialty, especially those involved in the production of new pharmaceuticals as well as biological agents. In addition, there are ongoing changes and developments in endoscopic technology and imaging devices that will continue to impact our highly procedure-driven specialty. Moreover, there are critical concerns related to manpower needs to screen for different forms of cancer. Finally, there are demanding needs in developing nations to provide optimal care in our subspecialty. This journal is unusual in its absence of advertising and will certainly provide a different setting for evaluation of many of these issues. Leading experts in the new frontiers of our rapidly expanding discipline will contribute updates in their field along with their insights. Candid editorial comments on the present state of knowledge, controversies, and, perhaps, their predictions related to the future will be encouraged.

BETHUNE

At McGill University in Montreal, it seemed customary during my student years for each new medical class to

become informed about the important efforts of many outstanding physicians that preceded our own experience. Like many other schools, their photographs and sculptures adorned the hallways of the medical school buildings. This feature of the curriculum probably had many purposes, including the development of a form of local pride for the contributions that these early faculty made to clinical and investigative medicine. Some, intimately linked to McGill University, seemed very impressive, including William Osler and Wilder Penfield. Many in my class, however, were most intrigued with the exploits of a rather curious Canadian physician, Henry Norman Bethune, who had a most amazing career ending with his final months in China. His life was first revealed to us in a low budget black-and-white film. Later, it appeared on an almost annual basis, witnessed by our own class, the result being that it seemed to become indelibly imprinted on our brains. Perhaps, in retrospect, this was done purposefully, before and during our initial clinical exposures and encounters on the wards where these ancestral giants had actually worked and toiled in previous times. We were being inspired.

Bethune was born in Gravenhurst, Ontario in 1890. He entered medical school at the University of Toronto, and then spent time as a stretcher bearer in a field ambulance unit for the Canadian Army in France in 1915. In the interim, his medical studies were temporarily interrupted, but eventually, he graduated in 1916. He was reported to have suffered a severe bout of pulmonary tuberculosis leading to surgical treatment using the technique of surgically-induced pneumothorax. This experience inspired his interest in surgery, particularly thoracic surgery.

He eventually came to Montreal and became a member of the McGill Teaching Faculty where he served on the surgical team at the Royal Victoria Hospital. There, he personally developed over a dozen new surgical instruments, including the Bethune Rib Shearer. Remembered by some for his intellect and surgical inventions, Bethune was also a realist, almost painfully so. In spite of a highly impressive career in Montreal, he became very disillusioned as many of his own patients, especially the poor, often re-developed tuberculosis after treatment. In large part, it seemed to Bethune to be due to their almost immediate return to their former poor living conditions after discharge from hospital. As a result, he developed a persisting passion for socialized medicine and, at this early stage in his career raised the issue of universal health care. As time has shown, however, he was a true revolutionary and, initially, was clearly not popular with his medical colleagues in Montreal. Indeed, in Canada, socialized medicine in its current form did not actually appear for almost another half century.

After visiting the Soviet Union, Bethune joined the Communist Party of Canada in 1935. During the Spanish Civil War in 1936, he worked on the Republican side against the Fascists, organizing the first mobile blood transfusion unit and treating battlefield injuries. In 1938, he joined the Chinese army of Mao Tse-Tung (Mao Ze-Dong) and the Communist Party during the Japanese invasion of China. There, he went to Yenan in 1938 and formed the first mobile army surgical hospital (MASH) unit that was carried on mules. Bethune performed numerous surgeries in the Taihang Mountains. He worked with tradesmen to develop new surgical instruments and trained physicians and nursing staff. Bethune died in November 1939 in Tanghsien (Tang xian County), Hopei, during the Sino-Japanese War, apparently of sepsis reported to have resulted from an accidental wound suffered during a surgical procedure without surgical gloves. In December 1939, Mao Tse-Tung (Mao Ze-Dong) wrote an essay entitled "*In Memory of Norman Bethune*" and concluded:

"...We must all learn the spirit of absolute selflessness from him. With this spirit everyone can be useful to the people. A man's ability may be great or small, but if he has this spirit, he is already noble-minded and pure, a man of moral integrity and above vulgar interests, a man who is of value to the people..."

Although Bethune became well known in China, recognition in Canada came only decades later, even after a medical school had been named in his honor, located in Jilin. In 1976, the Bethune Memorial House, a National Historic Site in Canada, was dedicated in Gravenhurst, Ontario. In 1990, a biographical film entitled "Bethune: The Making of a Hero", starring Donald Sutherland, was produced as a joint venture that included Telefilm Canada, the Canadian Broadcasting Corporation and China Film Co-production. During the same year, Canada and China each issued two national postage stamps, identical in design, to honor Bethune. In 1998, Bethune was inducted into the Canadian Medical Hall of Fame. In 2006, China Central Television developed a 20-part drama series directed by Yang Yang, entitled "Dr. Norman Bethune", reported to be the most expensive Chinese television series ever produced.

LOOKING FORWARD

This new editorial section, The Observer, will be dedicated to Henry Norman Bethune and his important contributions as a Chinese doctor. Bethune became a hero in China, not only because he was a very good physician and a skilled surgeon, but also because he was an innovator, an idealist and a perfectionist. These qualities seem especially important today in gastroenterology. It is sincerely hoped that this new journal column will be able to gather expert commentaries on a range of issues critical to the subspecialty from fundamental science to clinical care. Here, it is hoped that current specialty data can be deciphered and its future directions defined. The spirit of this man must be carried forward.

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REVIEW

Overwhelming postsplenectomy infection syndrome in adults - A clinically preventable disease

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Abstract

Overwhelming postsplenectomy infection (OPSI) syndrome is a rare condition, but is associated with high mortality. However, recognition and clinical management of OPSI is not well established. The prevalence of splenectomy increased recently because it was a clinically effective treatment for hepatitis C virus-associated thrombocytopenia before the introduction of the interferon/ribavirin combination therapy. We reviewed the literature characterizing the clinicopathological features of OPSI and assessed the most effective and feasible administration of the condition. A Medline search was performed using the keywords 'overwhelming', 'postsplenectomy infection', 'postsplenectomy sepsis', 'chronic liver disease', and/or 'splenectomy'. Additional articles were obtained from references within the papers identified by the Medline search. Durations between splenectomy and onset of OPSI ranged from less than 1 wk to more than 20 years. Autopsy showed that many patients with OPSI also had Waterhouse-Friderichsen syndrome. Although the mortality rate from OPSI has been reduced by appropriate vaccination and education, the precise pathogenesis and a suitable therapeutic strategy remain to be elucidated. Protein energy malnutrition (PEM) is commonly observed in cirrhotic patients. Since the immune response in patients with PEM is compromised, a more careful management for OPSI should therefore be applied for cirrhotic patients after splenectomy. In addition, strict long-term follow up of OPSI patients including informed consent will lead to a better prognosis.

Key words: Overwhelming postsplenectomy infection; Splenectomy; Overwhelming; Postsplenectomy sepsis; Chronic liver disease; Postsplenectomy infection

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INTRODUCTION

Splenectomized patients are a significant infection risk, because the spleen is the largest accumulation of lymphoid tissue in the body[1]. Overwhelming postsplenectomy infection (OPSI) is a serious fulminant process that carries a high mortality rate[2-4]. OPSI cases have been well documented, and more recently the syndrome was reviewed in the literature[1,5,6]. The pathogenesis and risk of developing fatal OPSI remain ill-defined, however, especially in the normal adult host.

Anti-hepatitis C virus (HCV) therapy with pegylated interferon has proven effective for virus clearance in recent years[7,8]. Prior to that, treatment of HCV-associated thrombocytopenia was an important and unresolved problem[9,10]. Splenectomy in patients with hepatitis C cirrhosis is now a safer prelude to antiviral treatment[11-14]. However, protein energy malnutrition (PEM) is a common manifestation in patients with liver cirrhosis, with incidences as high as 65%-90%[15-17], putting them at a higher risk of OPSI, because patients with PEM generally have a reduced immune response. This review describes the clinical features of OPSI and discusses management strategies for patients with OPSI, particularly those with chronic liver disease.

Symptoms of OPSI

King and Shumacker first described bacterial sepsis following splenectomy in infants and children in 1952[18]. It emerged subsequently that a comparable syndrome occurs in asplenic adults REF. Fulminant bacterial sepsis in asplenic patients will be termed OPSI in this review in keeping with common usage in the literature.

Aspecific and mild physical symptoms of postsplenectomy appear in the early stages of OPSI. These include fatigue, colored skin, body weight loss, abdominal pain, diarrhea, constipation, nausea, and headache (Table 1)[6,19]. Pneumonia and meningitis are frequent more severe concomitants. The clinical course may rapidly progress to coma and death within 24 to 48 h, due to the high incidence of shock, hypoglycemia, marked acidosis, electrolyte abnormalities, respiratory distress, and disseminated intravascular coagulation[20,21]. The mortality

rate is 50%-70% despite aggressive therapy that includes intravenous fluids, antibiotics, vasopressors, steroids, heparin, packed red blood cells, platelets, cryoprecipitates, and fresh frozen plasma[2,19,22-24]. The later clinical course frequently mirrors that of Waterhouse-Friderichsen syndrome (WFS), and bilateral adrenal hemorrhage may be found at autopsy[25,26]. The mechanism that connects splenectomy to WFS is unknown but the possible causes of OPSI include loss of splenic phagocytic function, decreasing serum immunoglobulin levels, suppression of lymphocyte sensitivity, or a change in the opsonin system[27,28].

Table 1 Clinical features of overwhelming postsplenectomy infection

Cryptic infection (no obvious focus)
Short, nonspecific prodrome
Massive bacteremia with encapsulated organism
Septic shock with disseminated intravascular coagulation
Marked virulence: 50% to 70% mortality
Death ensues in 24 to 48 h

Overwhelming postsplenectomy infection had been defined as septicemia and/or meningitis, usually fulminant but not necessarily fatal, and occurring at any time after removal of the spleen.

Risk factors

Splenectomized patients are at risk of life-threatening sepsis. Major factors for stratifying risk include the age at which splenectomy occurs, the subsequent time interval from splenectomy, the reason for splenectomy, and the overall immune status of the patient REF.

Infants do not acquire specific antibodies against encapsulated organisms until relatively late in the development of antibody responses. Although OPSI can occur at any age, children are therefore at greater risk of developing the condition, especially those under the age of 2 years[29,30]. However, estimates of the incidence and timing of OPSI vary widely in the literature, and some authors report the greatest risk of developing OPSI in the first two years after splenectomy[31]. Recent reports recommended that all asplenic patients should receive optimal advice and protection against OPSI regardless of the underlying etiology, based on evidence that the increased risk of severe sepsis after splenectomy is lifelong[32,33].

Splenectomy performed for a hematological disorder, including thalassemia, hereditary spherocytosis, auto-immune hemolysis, immune thrombocytopenic purpura, or lymphoma, appears to carry a higher risk than splenectomy performed as a result of trauma[34,35]. In addition, patients with hematological and autoimmune disorders, or lymphoma show impaired immunity, and patients undergoing treatment such as chemotherapy often show decreased serum immunoglobulin levels; both groups may therefore have an increased susceptibility for OPSI.

Hyposplenism occurs when splenic functions are reduced by disease or are absent congenitally or after splenectomy. Howell-Jolly bodies are small, round remnants of the original erythrocyte nucleus. Increased numbers of Howell-Jolly bodies in a peripheral blood smear, although not overly sensitive, can identify the degree of hyposplenism that presents a risk for developing OPSI[2,19,36].

Infecting organisms in OPSI

Outside the splenic circulation, polysaccharide antigens are poorly immunogenic in comparison with protein antigens. This contributes to polysaccharide-coated bacteria evading the immune response and subsequent phagocytosis[28]. Host defenses against bacteria are therefore critically dependent on humoral immunity and production of type-specific antibodies. While liver Kupffer cells clear most well-opsonized bacteria, encapsulated organisms resist antibody binding and are primarily removed by the spleen. Sepsis in asplenic patients can occur with any organism, be it bacteria, virus, fungus, or protozoan, however encapsulated organisms are frequently associated with sepsis in splenectomized patients. Encapsulated organisms such as *Streptococcus pneumoniae* are particularly resistant to phagocytosis, but is quickly overcome in the presence of even a small amount of type-specific antibody[24,37]. Without the spleen, prompt antibody production against a newly encountered antigen is impaired and bacteria proliferate rapidly. Therefore, the risk of invasive pneumococcal disease in patients without a spleen is 12-25 times greater than that in the population at large[38,39]. Invasive disease in the asplenic patient due to such encapsulated organisms as *Streptcoccus pneumoniae* (50%-90%), *Neisseria meningitides*, *Hemophilus influenzae*, and *Streptococcus pyogens* (25%) leads to uninhibited bacterial overgrowth[24,29,33,37].

Prevention of OPSI

Treatment of OPSI is generally aggressive due to the serious nature of the condition and associated mortality. Comprising intravenous fluids, antibiotics, vasopressors, steroids, heparin, packed red blood cells, platelets, cryoprecipitates, and fresh frozen plasma, it may fail to alter the course of this fulminant septic syndrome[25]. Therefore, prevention of OPSI is extremely important for immunocompromised patients who have undergone splenectomy.

Preventive strategies including vaccination and education are also important for splenectomized patients. Functionally or anatomically asplenic patients are at increased risk of infection from encapsulated organisms compared to the general population. Vaccines available for the most common organisms include the 23-valent pneumococcal polysaccharide vaccine, a 7-valent protein-conjugated pneumococcal vaccine, the *Hemophilus influenzae* type B vaccine, and the meningococcal vaccine[28]. The polysaccharide-based pneumococcal vaccine is recommended for all adults at increased risk of pneumococcal infection, and particularly the asplenic patients[40]. The Centers for Disease Control and Prevention in the US (revaccination every 6 years) and the British Committee for Standards in Haematology (revaccination every 5-10 years) recommended revaccination for the prevention of OPSI, at the same time emphasizing the rather frequent need for shorter intervals between revaccinations to keep antibody concentrations at a level sufficient by probability to confer protection[41,42]. Unfortunately, fatal pneumococcal sepsis has been

reported in asplenic, vaccinated patients. It remains advisable, nevertheless, to offer splenectomized patients protection due to their increased risk of developing severe disease and because the vaccine itself poses minimal risk. Jockovich[43] reported no OPSI among patients vaccinated before splenectomy; however, 10.4% of patients who did not receive vaccination developed OPSI. In addition, 5% of patients who were given vaccination after splenectomy developed OPSI. For elective splenectomy, the vaccine should be given at least 2 wk before surgery[43,44].

Finally, patient education represents a mandatory strategy for preventing OPSI. Studies have shown that from 11% to 50% of splenectomized patients remain unaware of their increased risk for serious infection or the appropriate health precautions that should be undertaken[45,46]. Patients should understand the potential severity of OPSI and the possibility of rapid progression. Physicians should inform any new healthcare professionals, including dentists, of asplenic status. In particular, the presence of increased Howell-Jolly bodies on a peripheral blood smear should be highlighted on the laboratory report to inform the patients' physicians of possible hyposplenism[2,19,36], This information and its significance should be in turn relayed to the patient. Moreover, advice for asplenic individuals to be issued with a form of medical alert, such as a card or a bracelet, has two purposes. First, it should provide a constant reminder to the individual of their condition and, second, knowledge of their state might be vital for medical attendants in the event of a medical emergency. This aspect of patient management needs increased attention as only one patient was found to hold such a card. The possibility that OPSI episodes in asplenic patients may have been avoided with improved knowledge cannot be excluded[5].

Future directions

HCV is a major public health problem and a leading cause of chronic liver disease. Various studies have suggested that 3%-20% of chronically infected patients will develop cirrhosis over a 20-year period, and are at significant risk of developing hepatocellular carcinoma[47]. Recent therapeutic trials in well-defined, selected populations revealed that combinations of interferons and ribavirin are more effective than monotherapy for HCV treatment. Moreover, trials of pegylated interferons yielded improved sustained virus response rates with similar toxicity profiles. As with all clinical decisions, selection of patients for HCV treatment requires an accurate assessment of therapeutic risk *vs* benefit, a determination that is complicated by exclusion from registration trials of persons with conditions that might increase risk and diminish benefit. Application of these principles to individual patients can be challenging, and the relative strength of recommendation of treatment varies accordingly. However, HCV is also a possible cause of chronic thrombocytopenia in these patients, by a mechanism that remains unclear. Moreover, treatment for HCV often leads to anemia, neutropenia, and thrombocytopenia. These conditions prevent safe application of treatment in patients with baseline pancytopenia, due to the cirrhosis-related hypersplenism[14]. The various treatments used, including steroids and intravenous immunoglobulin, have generally produced only transient responses[48,49]. Nevertheless, partial splenic embolization and laparoscopic splenectomy may enable anti-HCV therapy to be given. Recently, Hayashi *et al*[14] reported that splenectomy for severe thrombocytopenia with the intent to apply interferon therapy can be beneficial. It is therefore feasible that splenectomy prior to anti-HCV treatment for HCV-related chronic thrombocytopenia will occur more frequently in the near future. In addition, physicians should follow the occurrence of OPSI in these patients not only during the treatment for HCV clearance but also for the entire follow-up period.

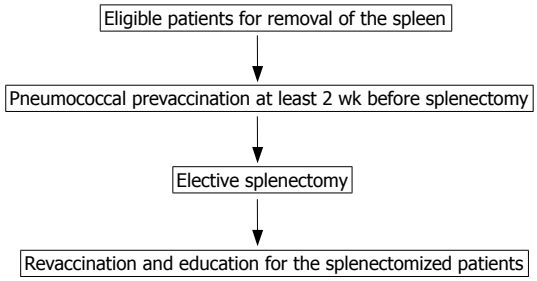

Figure 1 A proposed prevention strategy for splenectomized patients in management algorithm.

CONCLUSION

The spleen is crucial to the host response to infection by clearing polysaccharide-encapsulated bacteria. This response involves the clearing of pathogens from the bloodstream as well as the rapid production of specific antigens. Splenectomy results in an increased risk of septic complications associated with a high mortality rate, the most serious being the development of OPSI. Nonspecific symptoms, including nausea, vomiting, fever, and unconsciousness, followed by a rapid progression to coma and shock characterizes OPSI, and it is treated aggressively.

Eradication of HCV with the recently introduced therapy of pegylated interferon and ribavirin would be particularly beneficial in those with advanced liver fibrosis or cirrhosis. Splenectomy might be useful also to raise platelet counts so that pegylated interferon-based therapy can be performed safely in patients with cirrhosis due to HCV. Prevention strategies such as vaccination and education are also potentially important parts of the strategy for splenectomized HCV patients (Figure 1). Finally, reduction of the immune response in patients with chronic liver disease and/or PEM necessitates careful follow up of splenectomized patients with chronic liver disease to monitor the appearance of OPSI.

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REVIEW

Adenoviral gene therapy in gastric cancer: A review

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Abstract

Gastric cancer is one of the most common malignancies worldwide. With current therapeutic approaches the prognosis of gastric cancer is very poor, as gastric cancer accounts for the second most common cause of death in cancer related deaths. Gastric cancer like almost all other cancers has a molecular genetic basis which relies on disruption in normal cellular regulatory mechanisms regarding cell growth, apoptosis and cell division. Thus novel therapeutic approaches such as gene therapy promise to become the alternative choice of treatment in gastric cancer. In gene therapy, suicide genes, tumor suppressor genes and anti-angiogenesis genes among many others are introduced to cancer cells *via* vectors. Some of the vectors widely used in gene therapy are Adenoviral vectors. This review provides an update of the new developments in adenoviral cancer gene therapy including strategies for inducing apoptosis, inhibiting metastasis and targeting the cancer cells.

Key words: Gastric cancer; Adenovirus; Gene therapy; Vector; Apoptosis; Metastasis

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INTRODUCTION

Gastric cancer is one of the most common malignancies in the world with an estimated 934 000 cases reported globally in 2002[1], and the second most common cause of death from cancer. The prognosis of gastric cancer is poor with an estimated relative 5 years survival rate of less than 20%[2].

In Iran, 26.1% of cancers reported in 2002 in men and 11.1% of cancers in women were due to gastric cancer. Gastric cancer is the first leading cause of Cancer related death in Iranian men and the second in Iranian females. The incidence rates of gastric cancer in Iran are well above the world average; it is the fourth common cancer in the world however it is the most common malignancy in Iran[3]. Comparing gastric cancer rates with the data of 30 years ago, shows that the overall incidence of gastric cancer in Iran has gradually increased over the years[4]. This is in contrary to the global trend which has seen a steady decrease in the incidence and mortality rates for gastric cancer.

The current best approach for treating gastric cancer is complete surgical removal of the tumor with the adjacent lymph nodes however the efficacy of this therapeutic approach as well as hormone, radio and chemotherapy is very limited. Thus new therapeutic approaches are needed[5,6].

Gastric cancer is a genetic disease developing from a multi-step process[7]. Single or multiple mutations in genes related to growth control, apoptosis, invasion and metastasis form the molecular genetic basis of malignant transformation and tumor progression[8], so a better understanding of the molecular basis of tumor host interactions leads to significant progress in the development of new therapeutic agents. Based on this theory, cancers can be curable if the regulating mechanisms are reintroduced to the cancer cells; this can be achieved using gene therapy, which is an emerging alternative for treatment of cancers[9,10].

In gene therapy vectors are used to insert new genes into cells, or to switch off existing genes[11]. Some of the most efficient vectors used for gene therapy are viral vectors, as they naturally insert their genes into cells, they are well adopted and equipped for such a task. In recent years adenoviruses have been extensively used as vectors to deliver foreign genome into mammalian cells. Adenoviruses have certain features, which make them attractive vectors for gene transfer to target cells. Some of these characteristics include their ability to infect a broad range of cell types, including dividing as well as non-dividing cells, the ease with which adenovirus genome can be manipulated, and the ability to obtain high titers. To achieve an adenoviral vector, foreign cDNAs are inserted into the adenovirus genome, resulting in recombinant adenoviruses containing the gene of choice.

Cancer gene therapy has 3 essential goals: the first is

to suppress cancer growth and induce apoptosis in cancer cells, the second goal is to inhibit metastasis of malignancy to other sites and finally the effects of gene therapy must be limited to cancer cells. These goals can be achieved using some strategies, which we will further explain below[12-15].

TUMOR SUPPRESSOR GENES AND APOPTOSIS INDUCING GENES

As mentioned earlier, one of the ways for stopping cancer growth is to reintroduce the regulating mechanisms into cells, these mechanisms are supervised in the first place by tumor suppressor genes, so by inserting these genes into cells, tumor growth can be suppressed. Reintroduction of tumor suppressor genes also has the additional advantage of making targeted cells susceptible to chemotherapy[16].

The most famous tumor suppressor gene is P53, which is mutated in 60% of gastric cancers and in almost all other cancers[17]. Introduction of the p53 gene *via* a recombinant adenovirus has been shown to inhibit the growth of gastric cancer cells with mutated p53 *in vitro* and *in vivo*[18,19]. P53 not only regulates cell cycle and cell growth but it also has a crucial role in activating proapoptotic genes such as Bax, Apaf-1, Fas and PTEN thus not only limiting cancer growth but also inducing apoptosis in cancer cells (if the proapoptotic genes are not mutated themselves). Other tumor suppressor genes useful in gastric cancer include: P16 gene and Fhit[20-23].

There are two approaches for inducing apoptosis in cancer cell lines, the first is to introduce native proapoptotic genes into cancer cells, this has the additional advantage of minimum toxicity of novel gene for non cancer cells (which already have a copy of native proapoptotic genes). The second approach on the other hand is to introduce genes of non-mammalian enzymes into cancer cells, so that they can convert non toxic prodrugs into highly toxic substances that will kill the cell[24]. This approach has the advantage of bystander effect, which means not only the infected cell will be killed but it will release the highly toxic drug which will kill the adjacent cells as well[25].

Among genes introduced into cells to induce apoptosis, Bax is a good example, as it independently of cell's P53 activity can make the cell commit suicide; it also has the further advantage of sensitizing the malignant cells to conventional anti-tumor treatments[26]. Some other genes that can be used in inducing apoptosis in gastric cancer cells include genes, which are effective in Caspase 3 common pathway including Caspase 8[27,28].

Another method of killing cancer cells is to transfect them with genes of non-mammalian enzymes which can convert non toxic prodrugs into highly toxic agents, destroying the infected cell and nearby cells. The most widely used suicide gene/prodrug system is the herpes simplex virus (HSV) thymidine kinase (HSV-tk)/ ganciclovir (GCV) system that can convert the prodrug GCV into phosphorylated GCV. The phosphorylated GCV inhibits cellular DNA synthesis and leads to the killing of cancer cells *via* apoptotic and non-apoptotic mechanisms[29,30]. A similar approach involves the expression of recombinant *E. coli* cytosine deaminase (CD) in gastric cancer cells together with the administration of 5-fluorocytosine (5-FC). 5-FC is given orally and converted to 5-fluorouracil in the tumor cells expressing CD[31] (Table 1).

Table 1 A review of recent methods used in gastric cancer adenoviral gene therapy

Apoptosis inducers & tumor suppressors	
Gene	**Conclusion**
FasL	Infection of human gastric carcinoma cells (SGC-7901) with Ad-FasL showed increased expression of FASL, resulting in apoptosis[32].
HDAC inhibitor	Induces apoptosis in cancer cells expressing wild and pseudo-wild type p53 *via* activating the p53 through acetylation[35].
E2F-1	E2F -1 is a transcription factor that regulates cell cycle progression into S-phase. Combining E2F-1 overexpression with cyclin-dependent kinase inhibitors results in an enhanced apoptotic response, causing nearly complete gastric tumor cell death[33].
Bax	In a study, Ad/Bax made marked Bax protein expression and effective apoptosis in MKN-1, MKN-7, MKN-28 gastric cell lines[34].
p51 and p53	p51 (p73L/p63/p40/KET), a p53 homologue, binds to p53-responsive elements to upregulate some p53 target genes and has been suggested to share partially overlapping functions with p53. p53 is an apoptosis promoting gene which could be used to eliminate tumor growth in gastric cancer. Adenovirally transduced p51A cDNA into human gastric cancer cells promotes apoptosis just like p53[36].
Caspase 8	Adv-Caspase 8 can selectively induce apoptosis in detached carcinoma cells[37].

ANTI ANGIOGENESIS GENES

Angiogenesis is essential for tumors in order to grow and form metastases. It is a multi step process, which is initiated by release of growth factors from cells, these growth factors include growth factors such as the vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF) and hepatocyte growth factor (HGF)[38-40]. Inhibiting angiogenesis in tumors can in theory stop a tumor from functioning and growing, it is especially useful in limiting the dissemination of cancer[41-43]. Anti angiogenesis treatment do not need to specifically target cancer cells to inhibit metastasis as creating an anti angiogenesis environment around the cancer is equally effective. An example of anti angiogenesis genes is NK4, an HGF antagonist. It has been proved that transfecting gastric cancer cells with NK4 can stop the formation of Intra tumor vessels as well as peritoneal metastasis[44]. Another anti angiogenesis gene which can be introduced into cells using adenoviruses is soluble VEGF receptor (sFlt1) (Table 2).

TARGETED GENE THERAPY

One of the challenges of current gene therapy vector development, concerns targeting a therapeutic gene

Table 2 A review of recent methods used in gastric cancer adenoviral gene therapy

Metastasis inhibitors	
Gene	**Conclusion**
NK4	Peritoneal tumors in mice treated early with adenoviral mediated NK4 were significantly reduced. In another study Ad.CMV.NK4 resulted in efficient production and secretion of NK4 and significantly inhibited proliferation, migration and invasion of gastric cancer cells[45].
Flt-1	When administered intra peritoneally it markedly reduced the number of metastatic nodules larger than 1 mm in diameter on the peritoneal surface[46].
Caspase 8	In a study it was determined that Caspase 8 can augment anoikis in MKN-45 cells and suppress its peritoneal dissemination in nude mice with xenograft gastric cancer transplants and consequently increase survival, Caspase 8 has also proved to be useful in limiting metastasis in other carcinomas originating from epithelial tissues[37].

Table 3 A review of recent methods used in gastric cancer adenoviral gene therapy

Targeted gene therapy	
Method	**Conclusion**
HDAC inhibitor	Increase expression of the Coxackie adenovirus receptor and subsequent transfection efficiency of the adenovirus in cancer cells[35].
COX-2	COX-2 promoter shows the strongest cytocidal effect in gastric cancer cells when it is used in a conditionally replicating adenovirus (CRAD) context & with adenoviral vectors displaying 5/3 chimeric fibers[56].
EPCAM	It has been demonstrated that there is a marked difference in expression of the human epithelial cell adhesion molecule (EpCAM) between normal and (pre)malignant lesions of the stomach and esophagus. Based on this, using EpCAM to achieve gastric and esophageal adenocarcinoma selective gene transfer.may be a feasible choice for cancer-specific gene therapy[57]. In a study using EPCAM antibody adhered to adenovirus, transduction of normal gastric epithelium and liver tissue was reduced at least 10-fold in comparison with native adenovirus however tumor transduction levels remained the same[58].
Carcinoembryonic antigen (CEA)	A remarkable degree of targeted gene delivery to gastric cancer cells was obtained with Adv-FZ33 with the fully human anti-carcinoembryonic antigen (CEA) monoclonal antibody, C2-45[59-62].

to diseased cells with the aim of achieving sufficient gene expression in the affected tissue, while minimizing toxicity and expression in other tissues[47,48]. The use of recombinant adenoviruses as vectors for gene therapy is restricted by the widespread distribution of the coxsackie and adenovirus receptor (CAR), which allows infection of a range of tissues and precludes specific *in vivo* targeting[49-51].

Targeting can be achieved at the level of capsid binding or at later transduction events by the use of tissue-specific promoters. Targeting at the level of binding is preferred because even the interaction of cells with empty capsids leads to toxic effects. However a combination of both strategies has its obvious advantages.

Manipulating capsid binding can be achieved by direct genetic modifications of the capsid proteins, or it is possible to add ligands to the capsid *via* polymers like polyethylene glycol (PEG) and poly-[N-(2-hydroxypropyl) methacrylamide] (HPMA). Using these approaches, the native tropism of the virus is ablated either by the addition of polymer to fiber knob or the use of an anti fiber neutralizing antibody in the context of a bi-functional conjugate and creating targeted tropism by adding ligands. For example, for targeting ovarian cancer cells, it is possible to attach FGF to PEG bonded to viral capsid thus increasing specificity and also efficiency of transfection.

Targeting can also be achieved using tissue-specific promoters (TSP)[52-54], for example in alpha-fetoprotein (AFP)-producing gastric tumors, the adenovirus-mediated expression of HSV-tk by an AFP enhancer/promoter element selectively eliminates AFP positive, but not AFP-negative cell lines when treated with ganciclovir[55] (Table 3).

CONCLUSION

During the past 10 years, much has been learnt about molecular alterations in gastric cancer. Based on the understanding of the molecular mechanisms underlying gastric carcinogenesis, new therapeutic strategies targeted at the molecular defects in the tumor cells have been designed and many promising therapy results have been obtained from *in vitro* or *in vivo* studies. Among these approaches, Adenoviral gene therapy has proven to be the most promising however the efficiency of gene therapy for treatment of cancer in humans has remained low, this may be due to low rates of transduction and specificity. There may be many reasons for this, but it is widely agreed that this is mainly due to the relative resistance of cancer cells to introduce foreign material combined with low transgene expression *in vivo*. One of the most important issues affecting the possible clinical application of gene therapy is the need to ensure the highest possible safety levels developing protocols and targeting adenoviral vectors for gastric cell lines may yield the answer to this problem. Another prospect worth mentioning is the benefit of combining several approaches in order to achieve the highest possible therapeutic effect, examples of combined therapy approaches include combining Ad-p53 with HDAC inhibitor and sodium butyrate (SB), this has shown a significantly higher growth suppressive effect than single treatments of each. Another example shows combining NK4 with Cysplatin has enhanced inhibitory effect on peritoneal metastasis, suggesting that the combination of intra peritoneally chemotherapy with NK4 might be effective[63,64].

Recent studies have shed light on pathogenesis of gastric cancer, it has been long argued that *H Pylori* acts as a carcinogenic factor in inducing gastric adenocarcinoma, recent studies have supported this hypothesis, thus an area of future investigation in treatment of gastric cancer could involve *H Pylori* modification and also the intracellular

pathways altered by *H Pylori* in gastric mucosa. Although it has been reported that simple eradication of *H Pylori* without reversing the effects *H Pylori* has caused on gastric mucosa has limited efficacy in preventing gastric cancer, however there is a need for further investigation in this field[65-67].

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TOPIC HIGHLIGHT

Jamal A Ibdah, MD, PhD, *Series Editor*

Non-alcoholic fatty liver disease and the metabolic syndrome: An update

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Abstract

Sedentary lifestyle and poor dietary choices are leading to a weight gain epidemic in westernized countries, subsequently increasing the risk for developing the metabolic syndrome and nonalcoholic fatty liver disease (NAFLD). NAFLD is estimated to affect approximate 30% of the general US population and is considered the hepatic manifestation of the metabolic syndrome. Recent findings linking the components of the metabolic syndrome with NAFLD and the progression to nonalcoholic steatohepatitis (NASH) will be reviewed; in particular, the role of visceral adipose tissue, insulin resistance, and adipocytokines in the exacerbation of these conditions. While no therapy has been proven effective for treating NAFLD/NASH, common recommendations will be discussed.

Key words: Nonalcoholic fatty liver disease; Metabolic syndrome; Insulin resistance; Cytokines; Inflammation

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INTRODUCTION

Sedentary lifestyles and poor dietary choices are contributing to a weight gain epidemic in westernized societies. Recent epidemiological studies suggest an increased risk of cardiovascular disease (CVD) and type Ⅱ diabetes in overweight and obese individuals. Unfortunately, incidence of the metabolic syndrome and nonalcoholic fatty liver disease (NAFLD), which can precede the development of CVD and type Ⅱ diabetes, are also increasing.

The metabolic syndrome, a cluster of metabolic abnormalities with abdominal adiposity and insulin resistance as its central components, affects approximately 25% of the American adult population[1] and is associated with an increased risk of CVD and type 2 diabetes[2]. NAFLD, hepatic steatosis not due to alcohol consumption, is the most common cause of chronic liver disease[3]. It is estimated that about 30% of the general US population has excessive fat accumulation in the liver[3], reaching levels as high as 75%-100% in obese and morbidly obese individuals[4].

The relationship between NAFLD and the metabolic syndrome is becoming increasingly recognized. Approximately 90% of patients with NAFLD have ⩾ 1 characteristic feature of metabolic syndrome and about 33% have the complete diagnosis[5], placing NAFLD as the hepatic representation of the metabolic syndrome[6,7]. In addition, presence of the metabolic syndrome predicts higher risk for the development of NAFLD in both men and women[8]. NAFLD encompasses a histological spectrum ranging from simple hepatic steatosis to nonalcoholic steatohepatitis (NASH), advanced fibrosis, and cirrhosis. The majority of individuals with NAFLD have no symptoms with a normal physical examination; however, about 2%-6% of adult Americans and 20% of those who are obese may develop steatosis with inflammation (NASH), fibrosis, and cirrhosis[9]. Furthermore, there appears to be a close link between the metabolic syndrome, low grade inflammation, and oxidative stress[10].

In this editorial, we will review the clinical links between the components of the metabolic syndrome with

the manifestations of NAFLD and the progression to nonalcoholic steatohepatitis (NASH). In addition, we will discuss the potential role of adipocytokines and oxidative stress in the exacerbation of these conditions. Further, we will briefly discuss commonly prescribed therapeutic treatments.

DIAGNOSIS AND PREVALENCE OF THE METABOLIC SYNDROME

Although an association between different metabolic abnormalities had been noted for several years, the metabolic syndrome was first publicly described in 1988 by Reaven[11]. Then called Syndrome X, the metabolic syndrome consisted of a cluster of metabolic abnormalities, including obesity (especially abdominal obesity), insulin resistance, impaired glucose metabolism, dyslipidemia, and elevated blood pressure[11]. The current definition of the metabolic syndrome varies depending on the position of different regulating bodies[12], and it is not within the scope of this review to delineate the best available definition.

The metabolic syndrome is estimated to affect about 25% of the American adult population[1], and the prevalence will continue to increase dramatically. Regardless of the precise definition, the hallmark features of the metabolic syndrome are impaired glucose tolerance/insulin resistance, hypertension, central adiposity, and dyslipidemia consisting of low high-density lipoprotein-cholesterol (HDL-C) and elevated plasma triglycerides (TG). It is largely believed that insulin resistance is the central feature in the development of the metabolic syndrome.

DIAGNOSIS AND PREVALENCE OF NAFLD

It is estimated that about 30% of the US adult population has NAFLD, approximately 16%-20% of non-obese individuals and as high as 76% and 100% prevalence in the obese and morbidly obese individuals, respectively[4]. Overall, NAFLD is estimated to affect over 90 million Americans. NAFLD is characterized by increased accumulation of triglycerides (TG) in hepatocytes (hepatic steatosis). The diagnosis of NAFLD requires that hepatic steatosis be $\geqslant$ 5% by weight in the absence of excess alcohol consumption (> 20 g/d)[13]. Most people with NAFLD, especially those with fatty liver but no inflammation, experience little to no problems from the condition. The diagnosis usually is first suspected in an overweight or obese person who is found to have mild elevations in specific liver enzymes measured in circulation including elevated alanine aminotransferase (ALT) and aspartate aminotransferase (AST), with ALT levels being greater than AST levels.

Currently, the liver biopsy is considered the "gold standard" for the direct measurement of hepatic fat and is the only reliable method for diagnosing fatty liver and/or NASH. However, the liver biopsy has limited applicability in epidemiological and clinical studies and is not routinely performed because of the invasiveness of the procedure. Several non-invasive methods are currently available, which include ultrasound, computed tomography (CT), magnetic resonance imaging (MRI), and ^{1}H-magnetic resonance spectroscopy (^{1}H-MRS). Each has its limitations and is only semi-quantitative, but is more easily employed for routine screening.

LINKING THE METABOLIC SYNDROME AND NAFLD

Hyperinsulinemia and insulin resistance play a major role in the pathogenesis of NAFLD[14]. Because insulin resistance is the key component of the metabolic syndrome (often referred to as the insulin resistance syndrome or pre-diabetes) and is also commonly found with obesity, and because these same factors are commonly associated with NAFLD, there is a plethora of accumulating evidence supporting an association between obesity, the metabolic syndrome, and NAFLD[6,7]. In fact, NAFLD is now considered to be the hepatic representation of the metabolic syndrome[14,15].

The metabolic syndrome, in part through glucose intolerance and insulin resistance, is strongly associated with steatosis, fibrosis, and cirrhosis of the liver in severely obese adults[16]. In addition, central fat distribution, fatty liver, and glucose intolerance are noted in mildly obese and in normal weight subjects[17,18]. Further, numerous studies have demonstrated that obesity, type 2 diabetes, dyslipidemia, hypertension, and insulin resistance are strongly associated with NAFLD[12,19-22]. NAFLD also is strongly associated with hepatic, adipose tissue, and whole body reductions in insulin sensitivity, increased rate of gluconeogenesis, impaired insulin response to suppress gluconeogenesis, and impaired fatty acid oxidation[23-25]. However, the question about whether hepatic insulin resistance is a cause or a consequence of hepatic steatosis is unresolved[26-28].

The metabolic syndrome predicts higher risk of NAFLD in men and women, independent of weight gain[8], and those individuals with the metabolic syndrome are less likely to experience regression of NAFLD[8]. In addition to insulin resistance, body mass index and waist circumference, indices of obesity and central adiposity, also are associated with the metabolic syndrome, insulin resistance, fibrosis, and steatohepatitis[29], and both liver fat and visceral adipose tissue stores are associated with metabolic risk[30]. Moreover, HDL-C concentrations are significantly reduced[24] and serum TG and cholesterol levels are elevated with NAFLD[24,30]. Collectively, it appears that insulin resistance is not the only contributing factor in the development of NAFLD, but rather, a combination of multiple stimuli.

IS THE METABOLIC SYNDROME MEDIATING THE PROGRESSION OF NAFLD?

Physical inactivity and poor nutritional choices are leading to a surge in obesity, insulin resistance, and metabolic syndrome in industrialized nations. Day and James[31] proposed the "two-hit hypothesis" to explain the presence of bland steatosis and the progression to inflammation

(NASH), fibrosis, and cirrhosis. Recent findings suggest that components of the metabolic syndrome are integrally involved in the pathogenesis of this two hit model.

The first "hit" is hepatic steatosis, NAFLD in its simplest form, develops from an imbalance in triglyceride formation *vs* turnover. We and others have shown that mitochondrial dysfunction (i.e. impaired mitochondrial β-oxidation) is a primary cause of hepatic steatosis (discussed in detail by Wei *et al* in this issue)[23,32,33]. Insulin resistance is a near essential requirement and is believed to influence the first "hit" of NAFLD in the following ways: (1) activate the secretion of harmful adipocytokines from adipocytes, (2) alter rates of hepatocyte TG synthesis and transport, (3) and finally increases lipolysis [hydrolysis of TG to free fatty acids (FFA)] rates in central adipose which are dumped into the portal vein where they are processed in the liver, exposing the liver to excessive FFA levels[12]. In addition to impaired mitochondrial function and conditions exacerbated by insulin resistance, elevated hepatic *de novo* lipogenesis and TG synthesis strongly contributes to hepatic steatosis. In fact, a recent study found that about 26% of hepatic TG accumulation in NAFLD patients could be accounted for by *de novo* lipogenesis[34].

Once hepatic steatosis is present, other factors, such as inflammation and oxidative stress, are thought to promote progression to NASH, fibrosis, and necrosis. The metabolic syndrome is linked to inflammation and oxidative stress[10], and it has been demonstrated that individuals with the metabolic syndrome have increased lipid peroxidation[35]. The second "hit" likely is comprised of a secondary insult, particularly by adipocytokines and reactive oxygen species, which activate stellate cells and increase fibrogenesis and lipid peroxidation. Hepatic inflammation and fibrosis are associated with the presence and severity of the metabolic syndrome[36]. Furthermore, the presence of steatohepatitis with fibrosis, confirmed with liver biopsy, is associated with increased waist circumference and body mass index[29]. Hyperglycemia and hypertriglyceridemia also are associated with NASH[37], and insulin resistance is more severe in individuals with NASH *vs* simple fatty liver[37]. If the insults are great enough and the patient develops cirrhosis, about 33% of these patients will die or develop morbid conditions[38].

THE METABOLIC SYNDROME AND COMMON LIVER MARKERS

Circulating concentrations of the liver transaminases, ALT, AST, and to less extent γ-glutamyltransferase (GGT) are commonly used as markers of NAFLD for many years. Elevated levels are considered a consequence of liver damage due to fatty acid infiltration and inflammatory stimuli, and recent findings indicate that serum levels of these enzymes are associated with multiple components of the metabolic syndrome.

Increases in ALT are positively associated with each component of the metabolic syndrome, increased TG, glucose, waist circumference, diastolic blood pressure, and reduced HDL-C levels[19]. In addition, insulin resistance, the prevalence of severe liver steatosis, and ALT values have been found to be significantly higher in subjects with the metabolic syndrome compared to those with less than three of the five clinical features considered for its diagnosis[39]. Recent data published from NHANES Ⅲ found significant association with increased ALT and insulin resistance, type Ⅱ diabetes, and the metabolic syndrome[40]. GGT, a less specific marker of liver function, is linked to obesity, hypertension, sedentary lifestyle, hyperinsulinemia, dyslipidemia, inflammation, and oxidative stress[41-45]. Furthermore, GGT concentrations have been found to be associated with hypertension in individuals with central adiposity[46], suggesting the potential for a pathogenic link among fatty liver disease, endothelial dysfunction, and cardiovascular risk.

ROLE OF ADIPOSE TISSUE, FREE FATTY ACIDS, AND ADIPOCYTOKINES

Adipose tissue

Adipose tissue is now recognized as not simply a storage depot for excess energy, but rather, an active endocrine organ that secretes a number of molecules termed, adipocytokines. A number of these adipocytokines have been linked to alterations in insulin sensitivity, including adiponectin, leptin, resistin, and tumor necrosis factor-α (TNFα). In addition, many of the same signaling molecules have been shown to be associated with suppressed hepatic insulin sensitivity[47,48], and it is thought that adipocytokines may contribute to the development of liver fibrosis.

Visceral adipose tissue is more biologically active than subcutaneous adipose tissue and is known to release greater quantities of adipocytokines. Visceral adipose tissue is a better predictor of altered liver function and insulin resistance than obesity defined by body mass index[49,50]. FFAs lipolyzed from visceral adipocytes are dumped directly into the portal vein where they circulate through the liver and with suppressed oxidative capacity seen in insulin resistance will overload hepatocytes with lipid and promote hepatic TG storage.

Free fatty acids

The liver represents a major site of glucose uptake and storage and the major site for insulin clearance. Under normal insulin sensitive conditions, insulin inhibits glycogenolysis and gluconeogenesis by the liver, suppressing glucose production. However, both the metabolic syndrome and NAFLD commonly lead to hepatic insulin resistance in which the most common feature is the inability of insulin to cease hepatic glucose production. Boden *et al*[51] demonstrated that hepatic insulin resistance could be induced in rats by infusing lipids during a euglycemic-hyperinsulinemic clamp, in part by elevating hepatic diacylglycerol concentrations and increasing the activation of protein kinase C, IKκB, and NF-κB. Moreover, free fatty acids (FFA) and lipid intermediate flux from adipocyte lipolysis or from excess dietary intake activate protein kinase C and phosphorylate serine residues on the insulin receptor and insulin receptor

substrates, impairing tyrosine phosphorylation and increasing hepatic insulin resistance which likely lead to elevated glucose production and persistent hyperglycemia.

The liver also responds to elevated FFA by increasing cholesteryl ester synthesis, production of very low density lipoproteins[52], and *de novo* TG synthesis, exacerbating dyslipidemia. In addition, elevated FFA also have toxic effects on the liver due to lipid peroxidation, resulting in fibrogenesis and progression to cirrhosis[53]. Furthermore, lipid-laden hepatocytes also can swell and this architectural distortion can influence hepatocyte health[54].

Leptin

The role of leptin and leptin resistance in NAFLD has been under recent investigation. Under normal, healthy conditions, leptin is thought to play a role in regulation of body weight. However, in human obesity, leptin concentrations are elevated but there is reduced responsiveness to available leptin presumably from reduced leptin receptor expression, resulting in leptin resistance either centrally or locally at the level of the liver. Leptin is thought to play a major role in hepatic TG accumulation[55], and hepatic steatosis is observed in ob/ob and db/db mice that have leptin and leptin receptor mutations, respectively. Elevated leptin levels are associated with increased serum ALT levels and could be involved in promoting hepatocellular injury[56]. However, leptin therapy has been shown to reverse insulin resistance and hepatic steatosis in individuals with lipodystrophy[57]. In addition, existing data from animal studies also support the role of leptin replacement in reducing TG synthesis and inducing β-oxidation[58]. Future studies should focus on leptin receptor expression and the bioavailability of leptin and associations with NAFLD and the metabolic syndrome.

Resistin

Resistin has recently been identified as a signaling molecule that is induced during adipogenesis and secreted by adipocytes, particularly visceral adipose tissue stores[48,59]. Resistin was identified on the basis of an adipocyte specific protein that played a role in insulin resistance. Little attention has been given to the role of resistin in the metabolic syndrome and NAFLD, but available findings targeting resistin appear promising. Resistin levels are increased in NAFLD patients and levels are related to the histological severity of the disease[60]. Resistin gene expression and protein secretion are markedly reduced by anti-diabetic thiazolidinediones (TZDs), and administration of anti-resistin antibody has been shown to improve blood glucose and insulin action in mice with diet-induced obesity[59]. In addition, weight loss interventions in humans have demonstrated significant reductions in circulating concentrations of resistin[48].

TNFα

Adipose tissue represents a site for significant macrophage accumulation, the major source of local TNFα expression[61]. TNFα is an adipocytokine with a well-known role in antagonizing the effects of adiponectin and contributing to insulin resistance[62]; however, recent observations suggest that it also is involved in the metabolic syndrome and progression of NAFLD. In particular, TNFα has been shown to play an important role in mouse models of obesity and insulin resistance, and there also appears to be a link between TNFα levels and liver mitochondrial dysfunction that contributes to NAFLD and NASH. TNFα is elevated in individuals with central adiposity and other components of the metabolic syndrome[63]. It is thought that overproduction of TNFα in liver plays a pathogenic role in NAFLD, as TNF mRNA and TNF receptor 1 mRNA levels are increased in patients with NASH[64]. Furthermore, TNFα alters apolipoprotein metabolism by suppressing apoE secretion and apoA1 expression in HepG2 cells[65] and rapidly down-regulates the anti-oxidative protein associated with high-density lipoprotein, paraxonase-1 (PON1)[66].

Adiponectin

Adiponectin is an adipocyte-specific secretory protein that is found in relatively high circulating levels (5-10 mg/L), but decreased concentrations predict the incidence of CVD, the metabolic syndrome, and NAFLD[67-71]. Adiponectin concentrations are decreased in patients with obesity, insulin resistance, type 2 diabetes, and NAFLD[69,72] and correlate negatively with hepatic fat content[28]. In addition, hyperinsulinemia down regulates adiponectin receptor expression[73], reducing overall biological activity. Adiponectin has antilipogenic effects that may protect non-adipocyte tissues like liver and muscle. Adiponectin stimulates mitochondrial β-oxidation by activating AMP-dependent protein kinase (AMPK)[74] and down regulates a key transcription factor in *de novo* fatty acid synthesis, sterol regulatory element binding protein 1-c (SREBP-1c)[75]. This, in turn, reduces malonyl-CoA levels and the inhibition on carnitine palmityl transferase-1, leading to increased fatty acid oxidation and reduced hepatic TG accumulation[76].

Adiponectin also displays anti-oxidative/inflammatory properties as it may antagonize the effects of inflammatory mediators like TNFα and attenuate the progression of NAFLD by reducing hepatic stellate cell proliferation and increases apoptosis[77]. Furthermore, administrations of recombinant adiponectin in ob/ob mice, a model of NASH, attenuates serum ALT levels and drastically reduces hepatic steatosis[78].

Regardless whether adipocytokines are merely markers of the presence of NAFLD or are playing pivotal roles in the pathogenesis of the disease, existing evidence supports further study of adipocytokines as possible therapeutics for human NAFLD. It appears that alterations in adipocytokines secretion are exacerbating insulin resistance in skeletal muscle and liver and promoting hepatic steatosis and the development of NASH.

THERAPIES OF NAFLD AND THE METABOLIC SYNDROME

With no proven treatment for NAFLD/NASH, the focus of previous investigations has been on the treatment of components of the metabolic syndrome (obesity, hypertension, dyslipidemia, and diabetes). This strategy has

been met with some success and available therapies appear to slow NAFLD development and progression.

Pharmacological interventions

The strong relationship between insulin resistance and NAFLD suggests that insulin sensitizing therapies (TZDs and metformin) might be beneficial in the prevention or improvement in NAFLD. TZDs and metformin are oral glucose-lowering medications used to treat type 2 diabetes that enhance insulin sensitivity and signaling. TZDs bind to the peroxisome proliferator-activated receptors (PPARs) and improve insulin sensitivity, in part, by facilitating enhanced TG storage by adipocytes, suppressing the ectopic storage of lipids into liver and skeletal muscle. In addition, TZDs appear to have anti-inflammatory properties, inhibiting adipocyte gene expression and reduce circulating levels of TNFα and resistin[79], and increase adiponectin concentrations[80]. However, it should be noted that a common side effect of TZDs in clinical trials is weight gain.

Metformin is the drug of choice for treating obesity and type Ⅱ diabetes, and additional findings support its use for treating NAFLD, although the precise mechanism(s) of action are not known. It is known, however, that metformin targets and activates AMPK[81], which has multiple beneficial effects. Administration of metformin reduces hepatic gluconeogenesis, decreases absorption of glucose from the gastrointestinal tract, and increases insulin sensitivity. It also has been shown to reduce hepatic lipogenesis, increase fatty acid oxidation, and reduce serum ALT levels, steatosis, and inflammation in the ob/ob mouse model of NAFLD[82]. Furthermore, metformin use in non-diabetic NAFLD patients has been shown to reduce liver fat by 50% and decrease liver inflammation and necrosis[83].

Lipid lowering agents also can lower risks of the metabolic syndrome and NAFLD. It is well known that statins combat dyslipidemia, a hallmark of the metabolic syndrome, by reducing serum TG and increasing HDL-C levels; however, statins also have been shown recently to improve liver enzymes and hepatic inflammation (reviewed in[84]). Fibrate administration also has been met with marginal success. Furthermore, the angiotensin system is known to play a role in hypertension, and it is thought to be involved in the pathogenesis of hepatic fibrosis and insulin resistance. Although only preliminary evidence exists, treatment with the angiotensin Ⅱ receptor antagonist losartan beneficially improved blood markers of hepatic fibrosis and serum ALT levels[85]. Large, randomized control trials are lacking, but future studies are warranted.

Lifestyle modifications

Lifestyle modifications targeted at increasing physical activity and reducing energy intake are recommended by health care providers for optimal health and are the most common prescribed therapy for individuals diagnosed with NAFLD. In addition, recent cross-sectional studies in humans provide evidence that increased habitual physical activity[86] and cardiorespiratory fitness[87] are inversely associated with NAFLD. However, there are relatively few prospective studies that have examined the effects of aerobic training on the development and prevention of hepatic steatosis in humans or animal models.

Managing body weight is likely the best overall method in the treatment of conditions in both the metabolic syndrome and NAFLD. Since insulin resistance and obesity play a central role in NAFLD, weight loss is the mainstay of treatment. The weight loss program should be centered on the caloric restriction rather than alteration of dietary contents, with a prescription of both aerobic and resistance training exercise. Initial goals from the patient should be a 10% reduction in body weight by the combination of exercise training and energy restriction. This should be targeted over a 6 mo period, and it is important that individuals continue exercising to maintain the lost weight and for overall cardiometabolic health.

Weight loss by energy restriction significantly reduces hepatic TG content, rates of endogenous glucose production, and increases insulin suppression of endogenous glucose production in type 2 diabetics[88]. Surgical weight loss has been reported to reduce prevalence of the metabolic syndrome, liver steatosis, inflammation, and fibrosis[89]. However, these findings are not universal and it has been suggested that rapid weight loss may in fact, significantly increase hepatic steatosis, presumably from increased FFA flux from elevated rates of lipolysis. Exercise training in combination with energy restriction significantly reduces fatty liver and serum ALT concentrations in obese individuals with NAFLD[90]. Furthermore, weight loss (10%) by diet and exercise significantly elevates adiponectin concentrations[72]. Moreover, we recently have shown that weight loss by diet and exercise significantly reduces circulating leptin concentrations[91] and improves insulin sensitivity and lipid peroxidation in overweight and obese adults with components of the metabolic syndrome[92].

CONCLUSION

Most experts consider NAFLD to be the hepatic manifestation of the metabolic syndrome. The progression from hepatic steatosis to steatohepatitis may worsen insulin resistance and dyslipidemia, or the worsening of peripheral factors may mediate alterations in the liver. Regardless of the initiating pathophysiological event(s), recent findings emphasize the important links between NAFLD and the metabolic syndrome. Future investigations targeting the components of the metabolic syndrome as they exacerbate conditions of NAFLD are warranted. Furthermore, weight loss and insulin-modulating pharmacologic agents are the most effective treatment thus far, but combination therapies of lifestyle modifications and pharmacological agents could prove to be more effective than the individual prescription of specific drugs.

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TOPIC HIGHLIGHT

Jamal A Ibdah, MD, PhD, *Series Editor*

Nonalcoholic fatty liver disease and mitochondrial dysfunction

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Abstract

Nonalcoholic fatty liver disease (NAFLD) includes hepatic steatosis, nonalcoholic steatohepatitis (NASH), fibrosis, and cirrhosis. NAFLD is the most common liver disorder in the United States and worldwide. Due to the rapid rise of the metabolic syndrome, the prevalence of NAFLD has recently dramatically increased and will continue to increase. NAFLD has also the potential to progress to hepatocellular carcinoma (HCC) or liver failure. NAFLD is strongly linked to caloric overconsumption, physical inactivity, insulin resistance and genetic factors. Although significant progress in understanding the pathogenesis of NAFLD has been achieved in years, the primary metabolic abnormalities leading to lipid accumulation within hepatocytes has remained poorly understood. Mitochondria are critical metabolic organelles serving as "cellular power plants". Accumulating evidence indicate that hepatic mitochondrial dysfunction is crucial to the pathogenesis of NAFLD. This review is focused on the significant role of mitochondria in the development of NAFLD.

Key words: Nonalcoholic fatty liver disease; Mitochondria; Fatty acid oxidation; Liver

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INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) encompasses a disease spectrum ranging from simple hepatic steatosis to steatohepatitis (NASH), fibrosis, and cirrhosis. NAFLD has a very high prevalence in the US and worldwide[1]. It is becoming the leading cause for referral to liver clinics in most areas. In the US, it occurs in about 20%-35% of the population in over 60 million of the general adult population. Further, NAFLD occurs in about 2.6% of children and up to 53% of obese children are diagnosed with NAFLD[2,3]. The prevalence of NAFLD will likely continue to rise. Obesity, hyperglycemia, type 2 diabetes and hypertriglyceridemia are most important risk factors. Genetic factors undoubtedly predispose to NAFLD, as supported by higher prevalence of steatosis in Hispanics than Caucasians and African-Americans[4]. NAFLD has the potential to progress to hepatocellular carcinoma (HCC) or liver failure, both events that ultimately lead to early death.

DEFINITION AND CHARACTERISTICS OF NAFLD

NAFLD is defined as an excess of fat in the liver in which at least 5% of hepatocytes display lipid droplets[5] that exceed 5%-10% of liver weight[6,7] in patients who do not consume significant amounts of alcohol (140 g ethanol/week for men and 70 g ethanol/week) for women[8]. However, this definition is still controversial, because there is only 1.7% ± 0.2% of liver fat content in some healthy men[9] and liver fatty accumulation is absent in hepatic cirrhosis[10]. Morphologically, hepatic steatosis manifests as accumulation of macrovesicular or microvesicular intracytoplasmic fat droplets in hepatocytes. In macrovesicular steatosis, a large single fat vacuole fills the cytoplasm of hepatocytes and displaces the nucleus to the periphery, causing a characteristic signet ring appearance. In microvesicular steatosis, numerous small lipid droplets occupy the cytoplasm of hepatocytes and do not displace the nucleus to the periphery. Hepatic steatosis can be reversible or progress to NASH depending on the cessation or persistence of the underlying provocative cause, respectively. NASH represents steatosis, inflammation, fibrosis, ballooning hepatocytes, apoptotic cells and Mallory's hyaline. The inflammatory extent varies considerably and does not always correlate with the degree of steatosis. Infiltrating inflammatory

cells consist of lymphocytes and polymorphonuclear leukocytes. Apoptotic ballooned and Mallory hyaline hepatocytes may indicate the onset of NASH. Liver cell death and inflammation activate stellate cells leading to the development of hepatic fibrosis that often commences in zone 3 and manifests as perisinusoidal, perivenular (around terminal hepatic veins), and pericellular fibrosis. Hepatic steatosis, inflammation and aggressive fibrogenesis as well as sustained hepatocellular proliferation contribute to the development of liver cirrhosis.

DIAGNOSIS OF NAFLD

The majority of patients with NAFLD are asymptomatic or may complain of fatigue, exercise intolerance, or unspecific, vague abdominal pain in the right upper quadrant[5]. Physical examination can be unremarkable, although a palpable enlarged liver is frequent. Liver tests usually show minor nonspecific abnormalities. Alanine aminotransferases and gamma-glutamyl transpeptidase levels are elevated in most cases, but may be normal in some patients with advanced NASH and hepatic cirrhosis[11]. Imaging techniques including ultrasonography and magnetic resonance imaging are useful to detect degrees of steatosis, but can not distinguish inflammatory activity and fibrosis. Liver biopsy is still the gold standard for the diagnosis of NASH and fibrotic severity[12-14] which can be used to evaluate the degree of steatosis, inflammation, and fibrosis and also can help exclude other causes of liver disease[15].

PATHOGENESIS OF NAFLD

A currently favored hypothesis is that "two hits" are required for a subject to develop NASH[16]. The first hit leads to hepatic steatosis, and the second to hepatocyte injury and inflammation. However, the primary metabolic abnormalities leading to lipid accumulation within hepatocytes has remained poorly understood. Accumulating evidence indicates that mitochondrial dysfunction plays a central role in the pathogenesis of NAFLD, and that NAFLD is a mitochondrial disease[17].

Liver and metabolism

The liver, as a super metabolic achiever in the body, plays a critical role in metabolism of carbohydrate, protein and fat to maintain blood glucose and energy homeostasis.

Hepatic mitochondria: Mitochondria serve as the cellular powerhouse that generates ATP or heat by using substrates derived from fat and glucose. Hepatocytes are normally rich in mitochondria and each hepatocyte contains about 800 mitochondria occupying about 18% of the entire liver cell volume. Mitochondria play an important role in hepatocyte metabolism, being the primary site for the oxidation of fatty acids and oxidative phosphorylation. A mitochondrion contains inner and outer membranes composed of phospholipid bilayers and proteins. The outer mitochondrial membrane contains numerous integral proteins called porins, which contain a relatively large internal channel that is permeable to all molecules of 5000 daltons or less. Larger molecules can only traverse the outer membrane by active transport through mitochondrial membrane transport proteins. Unlike the outer membrane, the inner membrane does not contain porins, and is highly impermeable; almost all ions and molecules require special membrane transporters to enter or exit the matrix. The inner mitochondrial membrane contains proteins with four types of functions: the oxidation reactions of the respiratory chain; ATP synthase; specific transport proteins that regulate the passage of metabolites into and out of the matrix; protein import machinery. The matrix contains a highly concentrated mixture of hundreds of enzymes. Of the enzymes, the major functions include oxidation of pyruvate and fatty acids, and the citric acid cycle. The mitochondrial respiratory chain (MRC) is extremely important for energy generation and it consists of multiple polypeptides. Most of the respiratory chain polypeptides are encoded by nuclear DNA, but some are encoded by mitochondrial DNA (mtDNA). mtDNA is a circular double-stranded molecule located in the mitochondrial matrix. mtDNA is extremely sensitive to oxidative damage due to its proximity to the inner membrane, the absence of protective histones, and the incomplete DNA repair mechanisms in mitochondria. Therefore, any factors that affect mitochondrial integrate will cause reduced mitochondrial function.

Hepatic free fatty acids sources: (a) dietary triglycerides (TG) as chylomicron particles from the intestine; (b) *de novo* synthesis in the liver; (c) Free fatty acids (FFA). influx into the liver from lipolysis of adipose tissue; (d) diminished export of lipids from the liver; and (e) reduced oxidation of fatty acids. Imbalance of these metabolic steps will increased TG accumulation within the cytoplasm of hepatocytes.

Hepatic mitochondrial fatty acid oxidation: Fatty acid oxidation (FAO) is the major source of energy for skeletal muscle and the heart, while the liver oxidizes fatty acids primarily under the conditions of prolonged fasting, during illness, and during periods of increased physical activity. FAO also plays an essential role in the intermediary metabolism of the liver. The oxidation of fatty acids in the liver fuels the synthesis of ketone bodies, 3-hydroxy butyrate and acetoacetate, which are utilized as alternative sources of energy by extrahepatic organs, like the brain when blood glucose levels are low. FAO occurs in three subcellular organelles, β-oxidation in mitochondria and peroxisomes, and ω-oxidation in the endoplasmic reticulum[18,19]. Mitochondrial β-oxidation is the dominant oxidative pathway for the disposal of fatty acids under normal physiologic conditions and is primarily involved in the oxidation of short-chain ($< C_8$), medium-chain (C_8-C_{12}), and long-chain (C_{12}-C_{20}) fatty acids. Short-chain and medium-chain FFAs freely enter the mitochondria, while long-chain FFAs entry into the mitochondria is regulated by the activity of the enzyme carnitine palmitoyl transferase I (CPT- I). Glycolysis generates pyruvate, which is transformed by mitochondria into acetyl-CoA, an intermediate that goes through the citric acid cycle for the production of reducing agents and ATP. However, when glucose and energy levels are

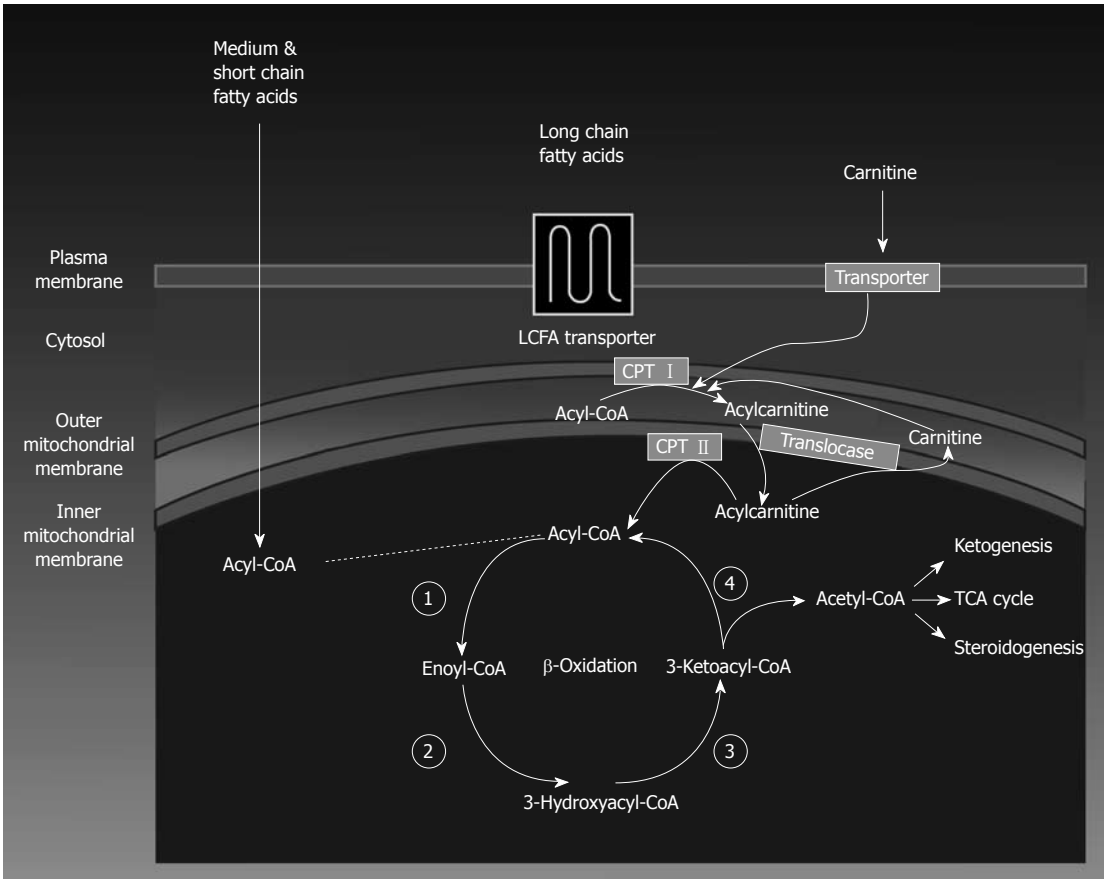

Figure 1 An illustration of mitochondrial fatty acid β-oxidation. LCFA: long-chain fatty acid; TCA: tricarboxylic acid.

elevated, acetyl-CoA is converted to citrate which can leak out of the mitochondrial matrix into the cytosol through the tricarboxylate carrier. In the cytosol, citrate regenerates acetyl-CoA which is converted to malonyl-CoA by acetyl-CoA carboxylase. Malonyl-CoA plays important roles in both hepatic fatty acid oxidation and lipid synthesis. Malonyl-CoA is the initial component for fatty acid synthesis. High malonyl-CoA levels also actively inhibit CPT- I enzyme activity thus robustly decreasing fatty acid oxidation by reducing the rate of fatty acid entry into the mitochondria. Thus, periods of caloric overconsumption, and excessive energy supply, increase malonyl-CoA levels which promotes hepatic fatty acid synthesis (storage) and suppresses fatty acid oxidation (catabolism). Conversely, in the fasting state, hepatic malonyl-CoA levels are low, allowing extensive mitochondrial import of long-chain FFAs and high rates of β-oxidation. Figure 1 depicts the pathway for mitochondrial fatty acid oxidation. During β-oxidation in mitochondria, FFAs undergo a dehydrogenation, then hydration, followed by a second dehydrogenation, and finally thiolysis, releasing one 2-carbon acetyl-CoA molecule and a shortened fatty acid (Figure 1). The cycle is repeated to split the fatty acid into acetyl-CoA subunits. The acetyl-CoA units enter the citric acid cycle to produce reducing agents which can be converted to ATP in the electron transport chain. Under fasting conditions, acetyl-CoA moieties can be converted into ketone bodies (acetoacetate and β-hydroxybutyrate), which are released by the liver to be oxidized in peripheral tissues by the tricarboxylic acid cycle.

MITOCHONDRIAL DYSFUNCTION IN NAFLD

Although the mechanisms responsible for fatty liver are still not fully elucidated, decreased capacity to oxidize fatty acids, increased delivery and transport of FFAs into the liver, and augmented hepatic fatty acid synthesis are likely to play a significant role in the pathogenesis of NAFLD. We and others have shown that mitochondrial abnormalities are closely related to the pathogenesis of NAFLD which raised the possibility that NAFLD is a mitochondrial disease[17,20-22]. The mitochondrial abnormalities associated with NAFLD include ultrastructural lesions, depletion of mitochondrial DNA (mtDNA), decreased activity of respiratory chain complexes, and impaired mitochondrial β-oxidation. Abnormal morphologic changes in liver mitochondria have been observed in patients and animal models with NASH[18,20-24]. Electronic microscopy revealed that mitochondria in NAFLD are big and swelled, scarce in number, and that the mitochondrial matrix has paracrystalline inclusions and hypodensity. Figure 2 shows an example of the ultrastructural changes in the mitochondrial in a mouse model with a fatty acid oxidation defect and hepatic steatosis that we have generated and reported earlier[22]. We have also found similar mitochondrial lesions in other rodent models that develop hepatic steatosis/NASH (data not shown). These same mitochondrial lesions are found in liver biopsy specimens from patients treated with 4,4'-diethylaminoethoxyhe xestrol, a drug that inhibits mitochondrial respiratory

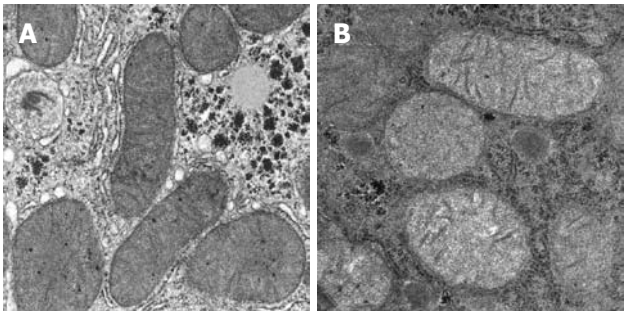

Figure 2 Representative electron micrograph of hepatocytes from control wild-type mice (MTPa$^{+/+}$) (**A**) and mice heterozygous for a mitochondrial trifunctional protein defect (MTPa$^{+/-}$) (**B**) at 11 700 × magnification. The MTPa$^{+/-}$ mice develop hepatic steatosis (see Figure 3). The mitochondria from the MTPa$^{+/-}$ mice were swollen with hypodense matrix and disrupted cristae. Reproduced with permission from *Gastroenterology*. 2005; 128: 1381-1390.

chain (MRC) activity and mitochondrial β-oxidation[25]. Prolonged treatment with this agent is associated with hepatic steatosis and steatohepatitis that is histologically indistinguishable from NAFLD in humans[25]. The ultrastructural mitochondrial defects in patients with NAFLD may be indicative of defective mitochondrial functions, e.g. reduced MRC activity[26] and impaired ATP synthesis[27]. NAFLD is often found in patients with insulin resistance, obesity and type 2 diabetes, the same metabolic conditions in which there is decreased oxygen consumption and ATP production, reduced total mtDNA and mtDNA transcription factor A, and reduced content of respiratory proteins in the fat, muscle and liver[28]. Many genes encoding mitochondrial proteins in skeletal muscle and fat are negatively correlated with body mass[29-33]. mtDNA depletion in hepatocytes impairs mitochondrial function and causes hepatic steatosis and other liver injury[34,35]. Patients with NASH have decreased expression of mtDNA-encoded polypeptides[36] and low activity of complexes Ⅰ, Ⅲ, Ⅳ and Ⅴ[26]. Mice with genetic deletion of NEIL1 DNA glycosylate have increased mtDNA damage and deletions and develop fatty liver disease[37].

Multiple enzymes are involved in mitochondrial β-oxidation and deficiency of these enzymes may lead to the development of hepatic steatosis, e.g. mice with disrupted medium-chain and very-long-chain acyl-CoA dehydrogenase genes manifest defects in fatty acid oxidation that likely lead to the witnessed micro- and macrovascular hepatic steatosis found in these mice. Mitochondrial trifunctional protein (MTP) is a heterotrimeric protein that consists of four α-subunits and four β-subunits and catalyzes long-chain fatty acid oxidation. MTP defects in humans are recessively inherited, and children with defects of any of the three enzymatic activities exhibit mostly microvesicular hepatic steatosis. We have generated a mouse model for a null mutation causing complete MTP deficiency and demonstrated that homozygous mice develop hepatic steatosis immediately after birth[38]. In subsequent report, we have documented that aging mice heterozygous for the MTP defect also become insulin resistant and develop hepatic steatosis as shown in Figure 3[22]. Further, the activity of mitochondrial respiratory chain complex is decreased in liver tissue of patients and animal models with NAFLD[26,39].

A number of mechanisms can be considered to explain the mitochondrial dysfunction found in NAFLD patients and animal models. Possible mechanisms include (a) excessive reactive oxygen species (ROS) production, (b) increased TNF-α expression, and (c) altered PGC-1 expression.

Reactive oxygen species

MRC dysfunction can directly lead to the production of ROS. If electron flow is interrupted at any point in the respiratory chain, the preceding respiratory intermediates can transfer electrons to molecular oxygen to produce superoxide anions and hydrogen peroxide[40,41]. Malondialdehyde and 4-hydroxynonenal, two byproducts of lipid peroxidation, can inhibit mitochondrial cytochrome *c* oxidase by forming adducts with this enzyme. ROS-induced depletion in mtDNA can severely lower mitochondrial number and function leading to steatosis and liver lesions[34]. Such depletion can impair the synthesis of complexes Ⅰ, Ⅲ, Ⅳ and Ⅴ of the MRC, because mtDNA encodes for 13 of the MRC polypeptides. Evidence of oxidative stress has been found in patients with NASH[21]. Decreased activity of the MRC in ob/ob mice is in part attributable to the oxidative stress, because treatment of ob/ob mice with antioxidant MnTBAP, a mimic of manganese superoxide dismutase, improved the activity of several complexes of the MRC. Surprisingly, activity of complex Ⅱ, which polypeptides are only encoded by nuclear DNA, decreased even more in ob/ob mice treated with MnTBAP. Furthermore, liver histology improved markedly in mice treated with MnTBAP. MRC complexes' activity was reduced by 30% to 50% versus control activity. These complexes' activity was inversely correlated to blood TNF-α levels, body mass index, and HOMA index[26].

TNF-α

Another important factor to consider in the pathogenesis of mitochondrial dysfunction is TNF-α. High blood TNF-α levels have been found in patients with NASH[26,42,43]. In ob/ob mice TNF-α concentrations in liver tissue were some 20-fold higher than in normal mice[39]. The likely sources of the hepatic TNF-α are hepatocytes and Kupffer cells[44]. TNF-α induces mitochondrial swelling with a lighter matrix and a loss of septa. In addition, TNF-α induced swelling of the mitochondria causes a bursting of the mitochondrial membrane leading to an interference between MRC complexes Ⅰ and Ⅲ[45,46]. Anti-TNF-α treatment in ob/ob mice improve complex Ⅰ, Ⅱ, Ⅲ and Ⅴ activity, β-oxidation activity, and liver histology[39].

PGC-1

Nuclear receptors are pleiotropic regulators of glycolytic and oxidative metabolism[47]. Mitochondrial activity is transcriptionally controlled, in part, by the nuclear receptors and the peroxisome proliferator-activated

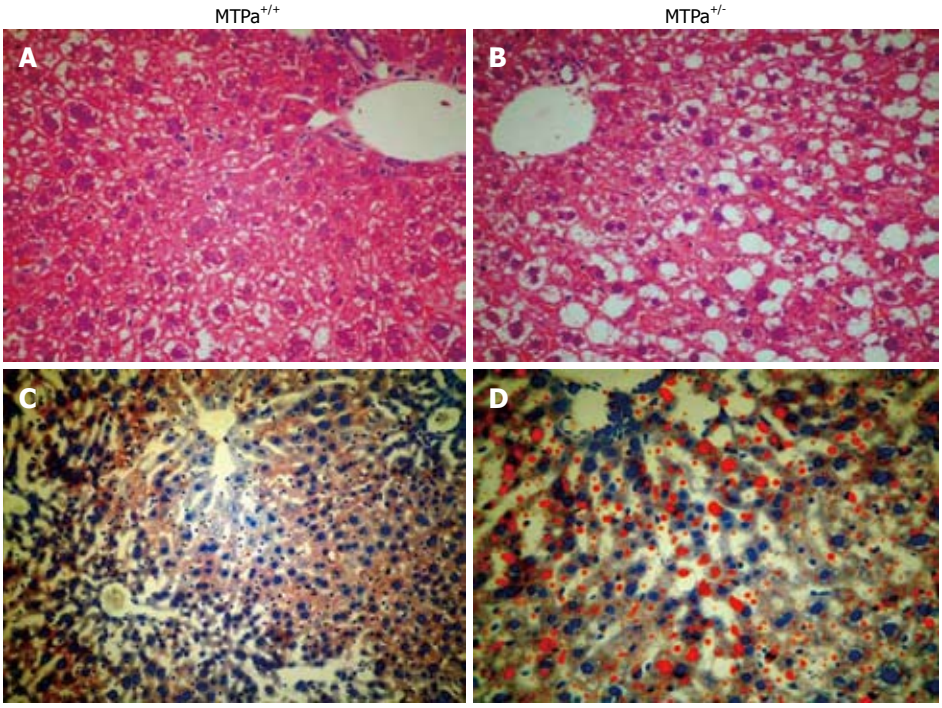

Figure 3 Representative liver sections obtained from control wild-type mice ($MTPa^{+/+}$) (**A** and **C**) and mice with a defect in mitochondrial trifunctional protein ($MTPa^{+/-}$) (**B** and **D**) littermates stained with hematoxylin-eosin (**A** and **B**) and oil red O (**C** and **D**) (20 ×). Reproduced with permission from *Gastroenterology*. 2005; 128: 1381-1390.

receptor-γ coactivator 1 (PGC-1)-related protein family such as PGC-1α and PGC-1β[48,49]. PGC-1α and PGC-1β are preferentially expressed in tissues with high oxidative capacity such as heart, skeletal muscle and brown adipose tissue, where they serve critical roles in the regulation of mitochondrial functional capacity and cellular energy metabolism[50-53] PGC-1α potently induces the expression of genes implicated in energy homeostasis in almost every cell type through known mitochondrial regulators such as the estrogen-related receptors (ERRs), peroxisome proliferator-activated receptor δ, or nuclear respiratory factor (NRF-1, 2)[48,49,54,55]. Overexpression of PGC-1α in skeletal muscle cells results in an increased energy expenditure, mitochondrial biogenesis[52,53], whereas loss of PGC-1α results in reduced muscle performance, cardiac defects, and other metabolic and behavioral defects[56,57]. Liver expresses low levels of PGC-1α and PGC-1β at normal condition, however, their expression is upregulated at fasting[58-60]. PGC-1α and PGC-1β activate hepatic fatty oxidation by inducing expression of PPARα[59,60]. Hepatocytes from PGC-1α deficient mice have diminished FAO activity and mitochondrial respiration rates[49].

CONCLUSION

Genetic, environmental and nutritional factors are involved in the pathogenesis of NAFLD, which is nowadays a major public health problem. In the absence of proven pharmacological therapy of NAFLD, it is critical to explore its pathogenesis and novel therapeutic pathways. In this Editorial, we have reviewed evidence that implicates mitochondrial dysfunction as a primary mechanism for development of NAFLD. Mitochondrial dysfunction may not only cause fat accumulation, but also may lead to the generation of ROS and cytokine production contributing to progression of NAFLD by inducing hepatic inflammation and fibrosis.

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TOPIC HIGHLIGHT

Jamal A Ibdah, MD, PhD, *Series Editor*

Role of the JNK signal transduction pathway in inflammatory bowel disease

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Abstract

The c-Jun NH2-terminal Kinase (JNK) pathway represents one sub-group of the mitogen-activated protein (MAP) kinases which plays an important role in various inflammatory diseases states, including inflammatory bowel disease (IBD). Significant progress towards understanding the function of the JNK signaling pathway has been achieved during the past few years. Blockade of the JNK pathway with JNK inhibitors in animal models of IBD lead to resolution of intestinal inflammation. Current data suggest specific JNK inhibitors hold promise as novel therapies in IBD.

Key words: JNK pathway; Inflammatory bowel disease; Inflammation

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INTRODUCTION

Although our understanding of the pathogenesis of inflammatory bowel disorders (IBD), especially Crohn's disease (CD) and ulcerative colitis (UC) has greatly improved, the specific causes are still not known. Cytokines such as TNF-α, IL-1 and IFN-γ play an important role in the pathogenesis of IBD[1,2]. Elucidating the mechanisms of cytokine induced inflammation in IBD could lead to novel therapies. Recent studies have focused on the identification of intracellular signaling pathways and transcription factors through which cytokines mediate their effects. Mitogen-activated protein kinases (MAPKs) are components of the signaling cascades where diverse extracellular stimuli converge to initiate inflammatory cellular responses. MAPKs are made of three subgroups-p42/44 extracellular signaling kinase (ERK), Jun-N-terminal Kinase (JNK), and p38 MAP Kinase[3]. Recent studies have highlighted the importance of the JNK pathway in IBD. This review will focus on the role of the JNK signaling pathway in IBD.

THE JNK-MAPK PATHWAY

The mammalian JNK were initially called stress activated protein kinase (SAPK) because they were activated by a variety of environmental stresses[3,4]. Later, the JNK pathway was also discovered to respond to cytokines, such as TNF-α and IL-1, and growth factors. JNK is a multi-factorial kinase involved in several physiological and pathological processes. Specific stimuli trigger the activation of MAP3Ks, which then phosphorylate and activate the MAP2K isoforms MKK4 and MKK7, which in turn phosphorylate and activate JNK[5]. JNK was discovered to phosphorylate c-Jun at the NH2-terminal Ser63 and 73 residues, and thus termed JNK. However, several recent studies have shown that JNK can phosphorylate a variety of substrates, including additional transcription factors and some non-nuclear proteins[3,6]. In addition to c-Jun, JNK can phosphorylate transcription factors such as JunB, JunD, c-fos, ATF2 and ATF3. These transcription factors along with c-Jun, make up the Activator Protein-1 transcription factor (AP-1), which regulates the expression of several stress-responsive genes. The JNK class of enzymes comprises of three main types: JNK1, JNK2, and JNK3[4,7]. The first two are ubiquitous, whereas the third is restricted to the brain, heart and testis. Differential splicing and exon usage results in multiple isoforms of JNK1, 2 and 3 genes. Each JNK is expressed as a short form (46 kDa) and long form (54 kDa)[3]. The alternative forms of each JNK1, 2, and 3 appear to differ in their ability of bind and phosphorylate different substrate proteins. Targeted gene disruption of each JNK has also defined differential functions for JNK1, JNK2 and JNK3 in many different cell types[7]. Deletion of JNK1 or JNK2 resulted in defective T cell differentiation and activation[8].

ROLE OF JNK IN IBD

The JNK pathway is considered to be a potentially relevant target for therapy inflammatory disease states. JNK regulates the maturation and activity of T cells and synthesis of pro-inflammatory cytokines such as interleukin-2 (IL-2), IL-6 and TNF-α. Several recent studies have demonstrated the importance of JNK pathway in chronic inflammatory disorders involving the expression of specific proteases and cytokines. For example, JNK pathway appears to be involved in the expression of TNF-α in rheumatoid arthritis[9]. JNK inhibitors such as SP600125 protected mice from joint damage in rheumatoid arthritis animal models[9]. Additionally, the JNK pathway also plays a role in artherosclerosis[10,11]. These findings led to the investigation of the role of JNK pathway in intestinal inflammation.

JNK activation in human intestine in patients with IBD was shown in 4 studies[12,13]. Increased activation of JNK along with ERK and p38 MAPK in human colonic tissue from 27 patients with moderate to severe CD or UC was first shown by Waetzig et al. Hommes *et al* also noted increased activation of p38 and JNK in their study involving 12 patients. Mistsuyama *et al* subsequently confirmed these findings in their recent study. They examined whether JNK phosphorylation was greater in sites of active inflammation compared to normal intestine in patients with IBD. Both ELISA and immunostaining demonstrated that JNK was highly activated in colonic tissue with active disease. Phospho-JNK was present in the intestinal cells, macrophages and lymphocytes, localized pre-dominantly in the nucleus. These findings validated the results from *in-vitro* cell culture studies[14]. Interestingly, increased JNK activation has also been shown in steroid-resistant patients[15]. The significance of this finding is currently unclear. The detection of enhanced activation of JNK in intestinal tissue in patients with IBD may serve as a diagnostic tool for early recognition of steroid unresponsiveness. Role of the different isoforms of JNK in IBD was investigated in a recent study[16]. Deletion of either JNK1 or JNK2 did not prevent the development of colitis in animals. However, deletion of JNK2 was associated with deterioration of disease activity. Further studies examining the role of different isoforms of JNK in IBD are needed.

The role of JNK inhibitors as potential therapies for IBD has been studied in both animal models of IBD and in humans. There are at least 40 different small-molecule JNK inhibitors that have either published or patented[3]. These inhibitors either affect JNK signaling pathway indirectly (e.g. CEP 1347) or block the catalytic domain of JNK (e.g. SP 600125). Unfortunately, most of these compounds only have a moderate specificity for JNK and may also interfere with other signaling pathways. Peptide inhibitors of JNK pathway, which have a higher specificity for their targets, are currently being developed. However, one of the major obstacles with peptide drugs is their rapid degradation and difficulty with delivery across cell membranes. These obstacles have been reportedly overcome by a recently described cell-permeable peptide that contains the JNK-binding domain of human c-Jun. Two studies assessed the effect of JNK inhibitor, SP 600125, on dextran sodium sulphate (DSS) colitis animal model[12,17]. SP 600125 is a reversible ATP-competitive inhibitor of protein kinases. It targets all the three different isoforms of JNK. At higher concentrations, it inhibits other protein kinases upstream of JNK (namely MKK3, and MKK6). One study evaluated SP 600125 in a rat model (Sprague-Dawley rats) of DSS colitis while the other used a mice model (C57BL/6) of DSS colitis. Both studies demonstrated the activation of JNK pathway in inflamed intestinal tissue in DSS induced colitis. JNK inhibition showed a marked protective effect against experimental colonic injury in animals. Specifically, treatment with SP600125 led to attenuation of weight loss and macroscopic damage. A beneficial effect was also noted on the histological severity of colitis. Destruction of the epithelial layer and glandular architecture, inflammatory infiltrates in the lamina propria, and edema of the submucosa in the colon was less severe in the SP600125 treated animals. Treatment with SP 600125 also resulted in a significant reduction in the levels of TNF-α, IL-6 and IFN-γ. Additionally, SP 600125 inhibited cytokine production by activated CD3/CD28 mesenteric lymphocytes[17]. One major limitation of these studies is that a more specific inhibitor of JNK was not investigated. Animal studies utilizing a peptide inhibitor or SiRNA against the JNK pathway are needed. Human studies have also suggested similar benefits of JNK blockade to those seen in animals. CNI-1493, a guanylhydrazone that inhibits the phosphorylation of both JNK and p38 MAP kinase, was studied in an open- label pilot study in 12 patients with moderate to severe Crohn's disease. Two different doses of CNI-1493 (8 or 25 mg/m^2) were given intravenously once daily for 12 d. A significant change in CDAI from baseline was noted at wk 2 and persisted up to wk 16. CRP levels decreased significantly during the first weeks of treatment. Endoscopic improvement was observed in all but one patient. Five patients had active fistulizing CD, and closure of the fistula was observed in 4 patients. A steroid sparing effect was seen in 89% of patients maintained on steroids. Additionally, CD-related arthralgia/arthritis resolved in all patients. Although the small sample size in this study precludes any significant conclusions, this study suggests CNI-1493 has significant therapeutic potential in CD. Further studies using JNK specific inhibitors in IBD are currently needed.

CONCLUSION

The JNK pathway plays an important role in various inflammatory disorders. Recent data suggest that JNK activation plays an important role in the intestinal inflammation in patients with IBD. However, the role of the different JNK isoforms in IBD has not been elucidated. Additionally, the mechanism by which JNK activation leads to intestinal inflammation is unclear and deserves further study. Cross talk of JNK pathway with other signaling pathways also needs to be investigated. Recent studies suggest a role for JNK blockade in IBD therapy. However, JNK inhibitors which could also inhibit other kinases were used. Studies using JNK specific inhibitors (e.g. peptide inhibitors) are needed. To increase

the likelihood of success, it may be important to develop isoform-specific JNK inhibitors, as they are likely to have increased efficacy and specificity resulting in fewer potential side effects.

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Methylation of TIMP3 in esophageal squamous cell carcinoma

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Abstract

AIM: To measure the frequency of DNA methylation of the tissue inhibitor of metalloproteinase 3 (TIMP3) promoter and relate this to any change of gene expression in esophageal squamous cell carcinoma in patients from a region of high incidence in China.

METHODS: Cancer cell lines were treated with or without the demethylating reagent 5-aza-2′-deoxycytidine. Methylation of the TIMP3 promoter was assessed in three regions by melt curve analysis and its expression was assessed by real-time RT-PCR. Tumors and proximal resection margins were obtained from 64 patients with esophageal squamous cell carcinoma from a region of high incidence in China. Methylation was assessed by melt curve analysis and expression by immunohistochemistry.

RESULTS: Methylation in one of the three promoter regions assessed correlated with gene silencing in esophageal cell lines. A degree of methylation of TIMP3 was found in only four esophageal squamous cell carcinomas, and partial loss of TIMP3 protein expression in just one.

CONCLUSION: Methylation and loss of expression of TIMP3 occurs infrequently in esophageal squamous cell carcinoma in a region of high incidence in China.

Key words: Esophageal squamous cell carcinoma; Immunohistochemistry; Methylation; TIMP3

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INTRODUCTION

Tumor invasion, metastasis and angiogenesis require proteolysis and remodeling of basement membranes and extracellular matrix (ECM) by enzymes such as matrix metalloproteinases (MMPs). The MMPs can degrade all components of the ECM, which makes regulation of these enzymes important in the development and dissemination of cancer. Many factors, including inflammatory cytokines, growth factors, hormones, and cell-cell and cell-matrix interactions can alter the transcription of the MMP genes[1], while enzymes can be specifically inhibited by TIMPs, which bind covalently to the active site of the enzyme. Altered expression of MMPs is associated with a poor prognosis in a range of solid tumors[2].

TIMP3, which binds to the ECM, is a multifunctional secreted protein with properties not just limited to the inhibition of MMPs. These roles of TIMP3 in cancer progression are highlighted in reports that its vector-mediated expression in cancer cells reduces metastasis[3], induces apoptosis, augments drug sensitivity in prostate cancer cell lines[4], and inhibits tumor growth in lung cancer cells in an *in vivo* model[5].

A reduction in TIMP3 expression has been reported to correlate with poor outcome in a number of cancers. DNA methylation of gene promoters is one method of silencing transcription, and TIMP3 methylation has been noted in a wide range of tumors. Reports indicate that the reduced expression of TIMP3 is a common occurrence in esophageal adenocarcinoma (EAC), is associated with methylation of the promoter, and correlates with poor outcome[6,7]. TIMP3 is also frequently methylated in Barrett's esophagus (BE), and has been investigated as a prognostic indicator for progression to EAC[8,9]. In contrast to the many studies of EAC, there is a study of esophageal squamous cell carcinoma (ESCC) in a cohort of patients

from Japan that has shown a decrease in TIMP3 protein expression, as measured by immunohistochemistry (IHC), which correlates with invasive activity and metastasis[10]. However, the mechanism responsible for the reduction of this expression has not been investigated. This study aimed to measure the frequency of methylation of TIMP3 in ESCC in patients from a region of high incidence in China, and to determine if this correlated with a reduction of TIMP3 expression.

MATERIALS AND METHODS

Patient samples

Primary tumors and, when available, non-cancerous proximal resection margins from 64 consecutive patients undergoing resection for ESSC at the Department of Thoracic Surgery, Fourth Hospital, Hebei Medical University, China were preserved in RNAlater (Ambion, Austin, TX, USA). Patient gender, age at the time of operation, and tumor stage, differentiation and volume were recorded (Table 1). Survival data were available for 45 patients. The study complied with all appropriate institutional guidelines.

Table 1 Characteristics of patients with ESCC

Variable	*n*
Male	45
Female	19
Age, median (range)	57 (42-76)
Males	57 (42-70)
Females	62 (49-76)
Tumor volume (cm^3), median (range)	57 (4-300)
Histological differentiation	
Well/moderate	57
Poor	4
Not recorded	3
Tumor stage	
T1N0M0	3
T2N0M0	9
T2N1M0	4
T3N0M0	29
T3N1M0	10
T3N1M1	1
T4N0M0	2
T4N0M1	1
T4N1M0	2
Not recorded	3

Demethylation of cell lines with 5-aza-2'-deoxycytidine (aza-dC)

Demethylation studies of TIMP3 were performed on nine cancer cell lines, OE19, OE21, OE33, TE7, DU145, LNCAP, T47D, ZR75.1, and KCL22. The cell lines were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum at 37℃ in 5% CO_2. Cells were seeded into flasks and cultured for 24 h before they were treated with 0 or 1 μmol/L aza-dC (Sigma-Aldrich, Sydney, NSW, Australia). To determine the length of time required for the cells to undergo at least two divisions in the presence of aza-dC, selected cell lines were labeled with PKH-26 (Sigma-Aldrich), as described previously[11]. Cell lines were treated for either 72 or 96 (OE19) h with 0 or 1 μmol/L aza-dC. The medium was then replaced with fresh medium not containing aza-dC, and the cells incubated for a further 24 h before harvesting.

Preparation of bisulfite-modified DNA

Genomic DNA was isolated from normal donor lymphocytes, cultured cells, and RNAlater-stabilized tissues as previously described[11]. DNA (2 μg) was bisulfite-modified as previously described[11], except that bisulfite-modified DNA was purified using an UltraClean PCR Clean-up DNA Purification Kit (MO BIO Laboratories, Carlsbad, CA, USA), and resuspended in 100 μL ultra pure water (Fisher Biotec Australia, Wembley, WA, Australia).

Methylation analysis of the TIMP3 promoter

Bisulfite-modified DNA was amplified using primers that amplified three overlapping regions designated R1, R2 and R3 (Figure 1). The primer sets did not discriminate between methylated and unmethylated sequences. The primers and PCR conditions were specific for bisulfite-modified DNA, and did not amplify unmodified DNA. All methylation-analysis PCRs were performed using the QuantiTect SYBR Green PCR Kit (Qiagen, Hilden, Germany) in a final volume of 15 μL, containing 1 μL bisulfite-modified DNA and a final concentration of 0.5 μmol/L forward and reverse primers (GeneWorks, Therbarton, SA, Australia) (Table 2). Bisulfite-modified lymphocyte and CpG methylase-treated lymphocyte DNA were included in each PCR run, and served as unmethylated and methylated controls, respectively. Reactions were incubated in a Rotor-Gene 3000 (RG-3000) (Corbett Life Science, Sydney, NSW, Australia) at 95℃ for 15 min, then 45 cycles of 95℃ for 30 s and 55℃ for 60 s, and a final extension of 72℃ for 4 min. Methylation was determined by analyzing the melt curve of the PCR product at the end of the amplification cycle. The temperature was ramped from 60 to 95℃, rising 0.5 or 1℃ at each step, waiting 30 s on the first step, then 5 s for each step thereafter. The dF/dT was determined for each PCR product using the RG-3000 application software v6 (Corbett Life Science). The dF/dT curves of the samples were compared to those of the unmethylated and methylated controls. A sample was considered methylated when there was a shift in its dF/dT curve away from that of the unmethylated control. The degree of methylation was graded as -, +, ++, +++ according to the degree of the shift to the right, as assessed by two independent observers.

Expression of TIMP3 mRNA in cell lines by quantitative real-time RT-PCR

Cell line RNA was isolated using an RNeasy kit with on-column DNase I digestion (Qiagen). The cDNA was synthesized from 2 μg RNA using SuperScript Ⅱ (Invitrogen, Mount Waverly, Vic, Australia). Quantitative real-time RT-PCR (qRT-PCR) was performed using the QuantiTect SYBR Green PCR Kit in a final volume of 10 μL, containing 1 μL cDNA and a final concentration of 0.5 μmol/L forward and reverse primers (Table 2). Triplicate reactions were incubated in an RG-3000 at 95℃ for 15 min, then 45 cycles of 95℃ for 30 s and 60℃ for

Table 2 Primer sequences for methylation analysis and quantitative real-time RT-PCR

	Forward primer	Reverse primer	Length (bp)
TIMP3 R1	TTTGGGTTAGAGATATTTAGTGGTT	RAATCRTTCAAATCCTTATAAAAAATAATA	175
TIMP3 R2	GGYGGTATTATTTTTTATAAGGATTTG	AAACCCCRCCTCRAACTATTAAA	158
TIMP3 R3	TTTGAGGGGGYGGGTTTTAATAGTT	AACRACCTCCCRACGAAAAAACAAA	174
TIMP3 qRT-PCR	CCAGGACGCCTTCTGCAAC	CCTCCTTTACCAGCTTCTTCCC	71

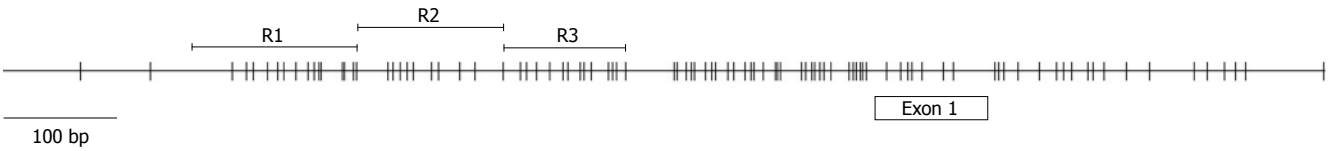

Figure 1 Schematic representation of the TIMP3 promoter. The schematic was generated by downloading the TIMP3 CpG island genetic element from http://genome.ucsc.edu/, using the Human March 2006 Freeze. The relative location of TIMP3 exon 1 is indicated. Each vertical line represents the location of a CpG. The three overlapping regions (R1, R2 and R3) amplified for methylation analysis are shown.

60 s, and a final extension of 72℃ for 4 min. The PCR products were electrophoresed on 1.5% (w/v) agarose gels and stained with ethidium bromide to confirm expected product sizes. The expression of TIMP3 was normalized to that of porphobilinogen deaminase (PBGD)[11].

Analysis of TIMP3 expression in ESCC by immunohistochemistry

Primary tumors and non-cancerous proximal resection margins preserved in RNAlater were fixed in formalin and embedded in paraffin using routine histopathology protocols. Sections (4 μm) of the formalin-fixed paraffin-embedded tissue were mounted on to polylysine-coated slides, de-waxed and rehydrated. Antigen retrieval was performed by heating the sections for 5 min in 10 mmol/L citrate buffer (pH 6) in a microwave pressure cooker. After cooling to below 30℃, sections were immunostained at room temperature using a Dako Autostainer Plus (Dako, Glostrup, Denmark). Sections were incubated for 60 min with a 1:750 dilution of mouse anti-human TIMP3 monoclonal antibody (Chemicon International, Temecula, CA, USA), and then with the MACH 4 Universal HRP Polymer (Biocare Medical, Concord, CA, USA). Visualization was performed with liquid 3, 3-diaminobenzidine (Dako) as the chromogen. Sections were counterstained with Meyer's hematoxylin.

Statistical analysis

Statistical comparison of the expression of TIMP3 mRNA following treatment with and without aza-dC for each cell line was performed using unpaired *t* tests. All statistical analysis was performed using InStat version 3.0a (GraphPad Software, San Diego, CA, USA).

RESULTS

Expression and methylation of TIMP3 in cell lines

The expression of TIMP3 mRNA was measured by qRT-PCR in cell lines without (0 μmol/L) and with (1 μmol/L) aza-dC demethylation treatment (Figure 2). Without aza-dC treatment, no TIMP3 expression was detected in

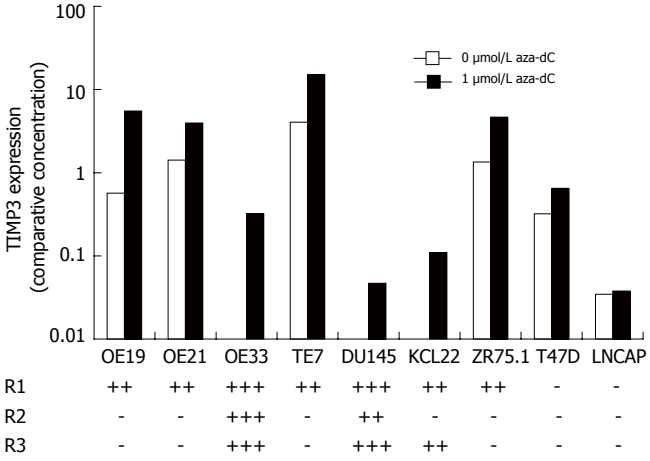

Figure 2 Expression and methylation of TIMP3 in cancer cell lines. The level of TIMP3 mRNA in cell lines treated with either 0 or 1 μmol/L aza-dC was determined by qRT-PCR and normalized using PBGD. Methylation was determined by melt curve analysis in three overlapping regions R1, R2 and R3, and is summarized.

OE33, DU145 and KCL22 cells, but was detected in all other cell lines tested. A significant increase in expression was induced by aza-dC in all cell lines except LNCAP.

The methylation in three overlapping regions of the TIMP3 CpG island (Figure 1) was evaluated by melt curve analysis and is summarized in Figure 2. Methylation in R1 was observed in OE19, OE21, OE33, TE7, DU145, KCL22, and ZR75.1 cells. Methylation in R2 was observed in OE33 and DU145 cells. Methylation in R3 was observed in OE33, DU145 and KCL22, the cell lines that did not express TIMP3 prior to treatment with aza-dC. Treatment with aza-dC caused a reduction in methylation in all regions in which methylation was observed (Figure 3). These data suggested that in the cell lines evaluated in this study, methylation in R3 was the one associated with transcriptional silencing of TIMP3 expression.

Expression and methylation of TIMP3 in ESCC

Methylation of the TIMP3 CpG island was evaluated in

Table 3 TIMP3 methylation and expression in methylated ESCC samples

Patient	M/F	Age	Tumor			Methylation			Expression
			Volume (cm^3)	Differentiation	Stage	R1	R2	R3	
28	F	65	60	Moderate	T3N1M1	++	-	-	Strong heterogeneous
41	M	65	30	Moderate	T3N0M0	++	-	++	Strong heterogeneous
44	M	66	18	Moderate	T3N0M0	++	-	++	Strong heterogeneous
75	F	72	27	Moderate	T3N1M0	++	+	++	Focal loss

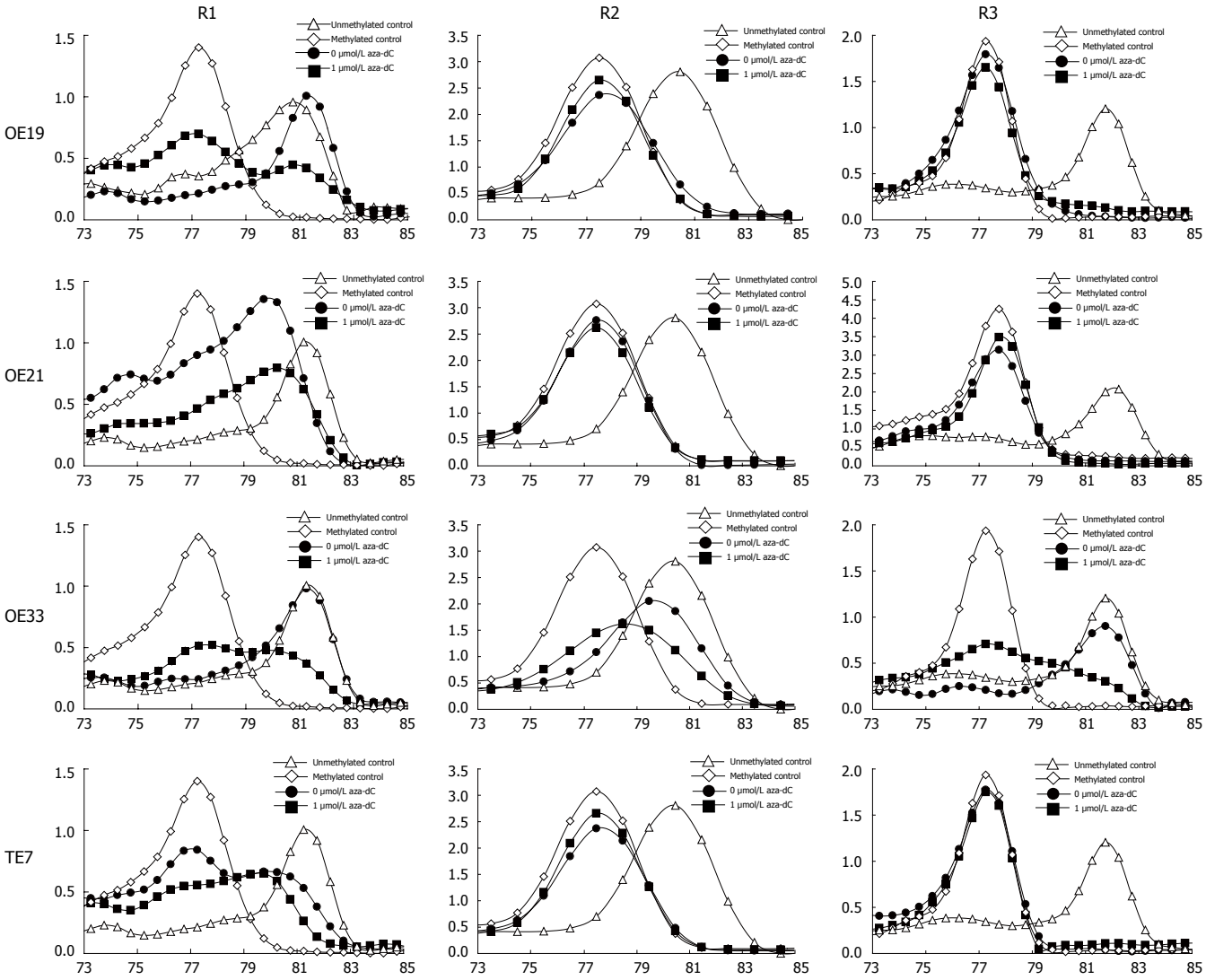

Figure 3 Methylation of TIMP3 in esophageal cancer cell lines. The X axis is temperature, the Y axis is fluorescence dF/dT.

tissues resected from patients with ESCC. Methylation was not detected in R1, R2 or R3 in the cancer-free proximal resection margins from these patients. However, methylation was detected in tumors from 4/64 (6%) patients. In the four tumors in which methylation was detected, low levels of methylation were present in R1 but not R2 or R3 in one patient, in R1 and R3 but not R2 in two patients, and in all three regions in one patient (Figure 4). TIMP3 protein expression was assessed in patient tissues by IHC. Strong heterogeneous cytoplasmic expression of TIMP3 was limited to the basal layers of the squamous mucosa (Figure 5A). All ESCCs that were unmethylated in all three regions analyzed displayed strong heterogeneous cytoplasmic staining of TIMP3 in cancer cells (Figure 5B and C). A significant loss of TIMP3 staining was not observed in the ESCCs that were methylated in either R1 or R1 and R3. By contrast, focal loss of TIMP3 staining was observed in the tumor that was methylated in all three regions (Figure 5D). TIMP3 methylation or loss of expression was not associated with patient age, gender, tumor volume, differentiation or stage (Table 3), or survival.

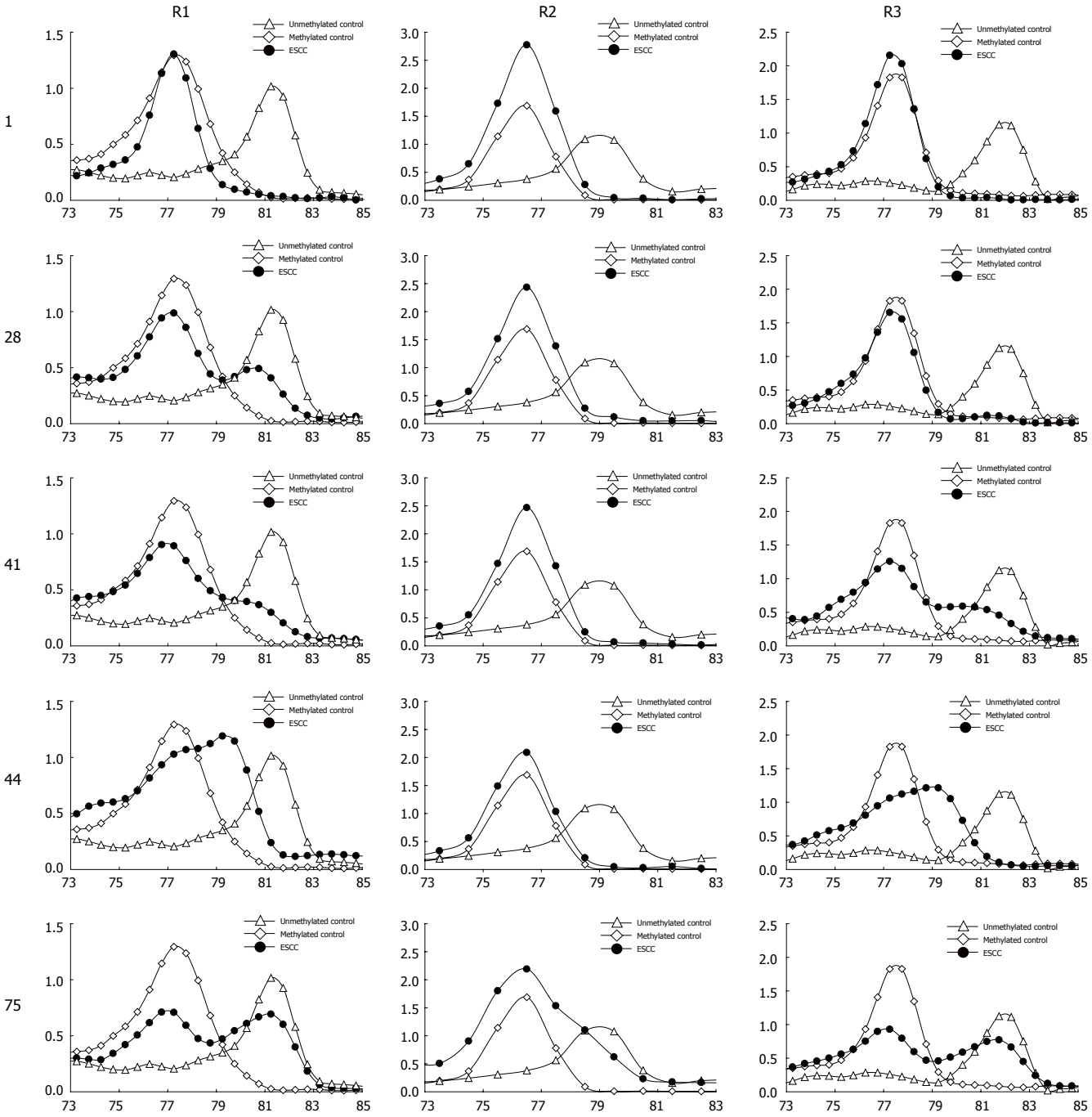

Figure 4 Methylation of TIMP3 in ESCC samples. The X axis is temperature, the Y axis is fluorescence dF/dT.

DISCUSSION

TIMP3, as an inhibitor of the proteolytic activity of MMPs, is believed to reduce tumor invasiveness and metastasis. Down-regulation of TIMP3 expression occurs in a number of cancers and has been linked to a poor outcome. One possible mechanism for the reduction in TIMP3 expression is DNA methylation of its promoter region. We first determined which region of the promoter CpG was associated with gene silencing by measuring gene expression and methylation in three overlapping regions of the TIMP3 CpG island in nine cancer cell lines. We found that methylation in only one of these regions appeared to be important in gene silencing. We then measured methylation and expression of TIMP3 in ESCC specimens resected from 64 patients from an area of China that has a very high incidence of this disease. Methylation was present in any of these three regions in just four of the 64 ESCC samples examined. Significant focal loss of TIMP3 protein expression was only observed in the tumor of one patient with ESCC, which was methylated in all three regions.

TIMP3 not only inhibits MMPs but also inhibits tumor growth and angiogenesis[12,13], and induces apoptosis in cancer cells[4,14]. Clinical studies have reported a reduction in TIMP3 expression that correlates with clinical

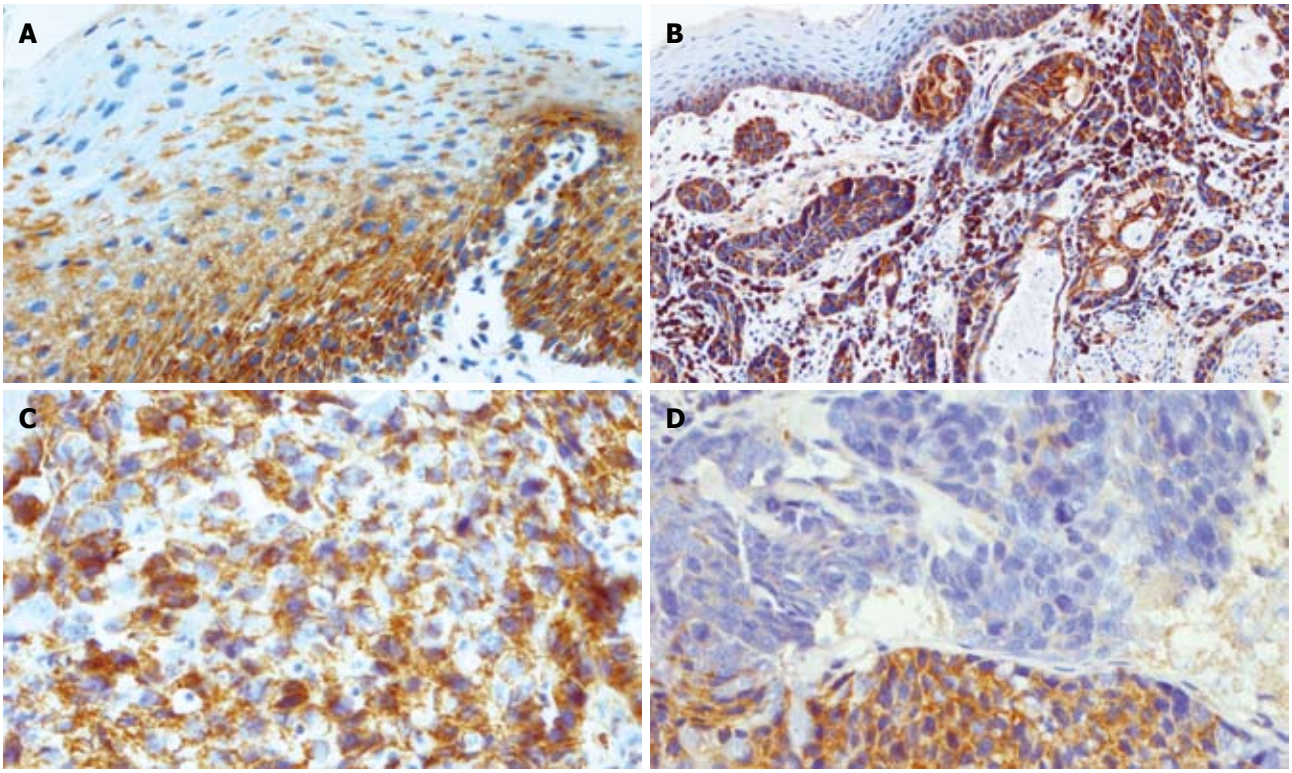

Figure 5 Expression of TIMP3 in esophageal tissues. **A**: Strong heterogeneous cytoplasmic expression of TIMP3 was limited to the basal layers in normal squamous mucosa; **B**: TIMP3 expression in unmethylated ESCC underlying normal squamous mucosa; **C**: Unmethylated ESCC showing strong heterogeneous staining; **D**: Methylated ESCC showing the region of reduced expression.

parameters in a number of cancers, including colon[15], lung[16,17] and breast[18,19] cancer and choriocarcinoma[20]. Methylation of the TIMP3 promoter is associated with silencing of transcription and has been noted in tumors of the thyroid[21,22], lung[16,17], bladder[23,24], stomach[25,26], uterus[27,28], bone[29], breast[30], liver[31] and colon[32,33].

Several studies have suggested that there is regional variation in TIMP3 expression within a tumor. Powe *et al* reported that TIMP3 mRNA expression decreases at the invasive edge of poorly differentiated colon carcinoma, and suggested that a regional loss of TIMP3 may contribute to increased invasiveness[34]. In normal esophagus, TIMP3 expression is restricted to the cytoplasm of basal, parabasal and stromal cells. Darnton *et al*[6] reported that TIMP3 protein expression was observed in all of 79 EACs examined, but staining intensity varied throughout the tumor. Whilst superficial areas of the tumors were always intensely stained, reduced expression was observed in central regions and variable expression, from pale to intense, was observed at the invading edges. Reduced TIMP3 expression was more often observed in an advanced tumor stage and was associated with reduced patient survival, but not lymph node positivity or tumor differentiation. TIMP3 mRNA and methylation were detected in 16/16 and 19/21 primary EACs, respectively, but correlation with reduced staining was not reported[6].

Most interest in methylation in cancer has focused on regions with relatively high densities of CpG islands, which are associated with gene promoter regions. It is common for methylation to vary between different regions of a particular CpG island when comparing DNA isolated from different cell lines or from different human tissues. For some genes, methylation of particular regions of the CpG island appears to be more important as a cause of silencing than methylation of other regions. Using established cancer cell lines, we analyzed three overlapping regions of the CpG island and correlated the methylation in each with expression, to determine if some regions of the island were more important than others in regulating gene expression.

The first detailed analysis of DNA methylation of the CpG island at the TIMP3 promoter was performed by Bachman *et al*[35]. They have demonstrated methylation in R1 in colon cancer cell lines, irrespective of whether or not these cells expressed TIMP3 mRNA. Their data are consistent with our findings in cell lines, and together, these findings suggest that methylation in this region by itself is not sufficient for transcriptional silencing. Bachman *et al* have observed dense methylation in R2 and R3 in cell lines that do not express TIMP3, and less methylation in these two regions in cell lines that do express the TIMP3 transcript. Significantly, our results demonstrated that in cell lines, complete silencing of TIMP3 transcription was only observed when there was significant methylation in R3, and that this complete silencing occurred even though there was no detectable methylation in R2.

A consistent characteristic of methylation of the TIMP3 CpG island that we observed was that methylation in R2 only occurred when there was methylation in R3, which in turn only occurred when there was methylation in R1. These findings indicate that aberrant methylation in the TIMP3 CpG island is progressive, beginning in

R1, before progressing to R3 and then R2. Progressive methylation has been observed in the p16 CpG island of primary human mammary epithelial cells escaping from growth arrest[36]. Regional methylation within a CpG island that is sufficient to silence transcription has also been reported for other genes, including MGMT[37] and MT3[11].

More than 90% of esophageal cancers are either adenocarcinomas or squamous cell carcinomas. In EAC, a number of reports have indicated that reduced expression of TIMP3 is common, and is associated with methylation and poor outcome. Darnton *et al* reported methylation of TIMP3 in 90% of EAC and 72% of BE patients, and that reduced expression of TIMP3 protein in EAC is associated with increased tumor invasiveness and reduced patient survival[6]. By contrast, Brock *et al*[38] reported that only 19% of EAC tissues showed TIMP3 methylation, and that this by itself was not a prognostic indicator. Eads *et al* reported TIMP3 methylation in 86% of EAC, and in 78% of dysplastic BE patients, and low methylation in 33% of metaplastic BE patients[7]. Clement *et al* found that TIMP3 was methylated in 65% of EAC patients, and that a significantly higher percentage of patients with BE who progressed to EAC had methylated TIMP3 than those who did not progress, which suggests its value as a prognostic indicator in BE[8]. Schulman *et al*, however, reported TIMP3 methylation in 56% of EAC and 59% of BE tissues, and concluded that hypermethylation of TIMP3 is not an independent risk factor for progression from BE to EAC[9].

In contrast to the many studies of TIMP3 in EAC, there is only one in ESCC. Miyazaki *et al*[10] reported that in 90 patients who underwent surgery for ESCC at Gunma University Hospital (Japan), TIMP3 protein expression was always observed in cells in the shallow areas of the tumor, but was not expressed by cells at the invasive front of the same tumor. The percentage of cells with reduced TIMP3 expression at the invasive front varied between patients; in 33% of patients, 80% of the cells expressed TIMP3; in 30% of patients, 30%-80% of the cells expressed TIMP3; and in 37% of patients, < 30% of the cells expressed TIMP3. The patients that had the greatest percentage of cells with reduced expression had significantly reduced postoperative survival, increased depth of invasion, more metastatic lymph nodes, and higher disease stage. However, they did not analyze methylation of TIMP3. The low frequency of methylation that we observed in ESCC was not due to our methods, as we have found methylation in 88% (22/25) of EAC patients (unpublished observations); this is consistent with other reports of this cancer[7-9]. There is no obvious explanation for the discrepancy between our results in this Chinese cohort and the Japanese study. The disease may be biologically different, perhaps due to lifestyle, environmental or genetic differences. Further studies are required to investigate this issue.

We found that methylation of the TIMP3 CpG island was detectable in only 4% (4/64) of ESCC patients from a region in China that has a high incidence of this disease. The number of patients with methylation in R3, which we showed in cell lines correlated with transcriptional silencing, was 3/64. We found a loss of TIMP3 expression by immunohistochemistry in only one patient, and then this was only a focal loss. There is no obvious explanation for the presence of TIMP3 protein in patients with R3 methylation. Our results clearly showed that the loss of expression or methylation of TIMP3 is uncommon in ESCC from this region of China.

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COMMENTS

Background

Increased knowledge of the underlying biology of the cancer may lead to improvements in diagnosis and treatment. TIMP3 inhibits matrix metalloproteinases (MMPs), which can inhibit tumor growth and metastasis. There is one report suggesting that TIMP3 is reduced in esophageal squamous cell carcinoma (ESCC). We measured the frequency of methylation of TIMP3 in ESCC in patients from a region of high incidence in China, and determined if this correlated with a reduction of TIMP3 expression.

Research frontiers

Methylation which silences genes is generally found in regions with relatively high densities of CpG, known as CpG islands, which are associated with gene promoter regions. Measuring methylation in these critical regions of DNA may assist in diagnosis or prognosis in cancer.

Innovations and breakthroughs

We defined the region of the TIMP3 CpG island which is associated with the silencing of the gene. We found that reduction in expression of TIMP3 is uncommon in ESCC from a region of high incidence in China, and correspondingly methylation was infrequent as well.

Terminology

MMPs: enzymes which break down and reshape the molecules which surround a cell and hold it in its place. Methylation of DNA: one way to silence a gene. Gene promoter: the part of the DNA where the cell begins to make gene product.

Peer review

This is an excellent and well written contribution.

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Clinical significance of type VI pit pattern subclassification in determining the depth of invasion of colorectal neoplasms

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Abstract

AIM: To clarify whether subclassification of the type VI pit pattern on the basis of magnifying colonoscopy findings is useful in determining the type and depth of invasion of colorectal neoplasms.

METHODS: We retrospectively analyzed 272 colorectal neoplasms (117 dysplasias and 155 submucosal invasive carcinomas; 228 patients) with a type V pit pattern [type VI, *n* = 202; type VN, *n* = 70 (Kudo and Tsuruta classification system)]. We divided lesions with a type VI pit pattern into two subclasses, mildly irregular lesions and severely irregular lesions, according to the prominent and detailed magnifying colonoscopy findings. We examined the relation between these two subclasses and histology/invasion depth.

RESULTS: One hundred and four lesions (51.5%) were judged to be mildly irregular, and 98 lesions (48.5%) were judged to be severely irregular. Ninety-seven (93.3%) mildly irregular lesions showed dysplasias or submucosal invasion of less than 1000 μm (SM < 1000 μm). Fifty-five (56.1%) severely irregular lesions showed submucosal invasion equal to or deeper than 1000 μm (SM ⩾ 1000 μm). Mild irregularity was found significantly more often in dysplasias or lesions with SM < 1000 μm than in lesions with SM ⩾ 1000 μm ($P < 0.01$).

CONCLUSION: Subclassification of the type VI pit pattern is useful for identifying dysplasias or lesions with SM < 1000 μm.

INTRODUCTION

Pit pattern classification (Figure 1) of colorectal lesions, initially proposed by Kudo[1] and modified by Kudo and Tsuruta[2], is reported to be related to the histologic characteristics of the lesions[3-9]. Magnifying colonoscopy is used for differential diagnosis between non-neoplastic and neoplastic lesions[5,9-16] and for assessing the depth of invasion of early colorectal carcinoma[9,17-21].

Several studies have suggested that there is little risk of lymph node metastasis from early colorectal carcinoma that involves the superficial layer of the submucosa, less than 1000 μm from the muscularis mucosae[9,17,22-24]. Recently, the Japanese Society for Cancer of the Colon and Rectum proposed the following new criteria for curative histopathologic conditions after complete endoscopic mucosal resection (EMR) of submucosal carcinoma: (1) a submucosal invasion depth of less than 1000 μm (SM < 1000 μm), (2) well to moderately differentiated adenocarcinoma including the invasive portion, and (3) no vessel involvement[22,24]. In accordance with these proposed criteria, it has become important to distinguish submucosal invasion equal to or deeper than 1000 μm (SM ⩾ 1000 μm) from SM < 1000 μm prior to treatment of submucosal carcinoma, to reduce the number of needless surgical resections.

Many studies have shown that the type VN pit pattern is an indicator of massive submucosal invasion of colorectal neoplasm[9]. However, colorectal neoplasms with a type VI pit pattern include various lesions, such as dysplasia and submucosal carcinoma, with either SM < 1000 μm

or SM ⩾ 1000 μm[9]. Thus, it is difficult to decide upon a therapeutic strategy for colorectal neoplasm on the basis of the current type VI pit pattern classification. In this study, we assessed the clinical usefulness of type VI pit pattern subclassification in determining the histology/invasion depth of colorectal neoplasms.

MATERIALS AND METHODS

We analyzed 272 colorectal neoplasms with a type V pit pattern (type VI, n = 202; type VN, n = 70). The colorectal neoplasms comprised 117 dysplasias and 155 submucosal invasive carcinomas, 129 of which were deeper than 1000 μm, resected endoscopically or surgically from 228 patients at Hiroshima University Hospital during the period January 1999 through March 2005. All lesions in this study were analyzed by five colonoscopists who were well trained in magnifying colonoscopy and blinded to the pathology findings, retrospectively.

To evaluate pit patterns, we used a magnifying colonoscope (EC-410CM, EC-450ZM, EC-450ZH or EC-450ZW, Fujinon Toshiba, Omiya, Japan; or CF-240Z or CF-H260AZI, Olympus, Tokyo, Japan) with zoom functions ranging from × 17 to × 126. When a lesion was detected by standard colonoscopic observation, the surface mucus was washed away with lukewarm water, and indigo carmine dye was spread over the lesion. This dye enhances the colonoscopic appearance because it is retained within the pits and grooves of the mucosal surface. For more precise assessment, crystal violet stain was applied to the margins of the pits, rendering each pit pattern clearly visible in all cases. The type V pit pattern was classified as one of two subtypes according to the Kudo and Tsuruta classification system[1,2] (Figure 1): type VI, irregularly arranged and similar to type IIIL, IIIS, or IV patterns in size; and type VN, an area of obvious non-structure (as per the Hakone consensus meeting in April 2004)[9,25].

Each resected neoplasm was fixed routinely with 10% buffered formalin and embedded in paraffin, after which the entire tumor was cut into serial 2- to 3-mm thick slices. Microscopic examination of hematoxylin and eosin-stained sections was performed by one pathologist unaware of other features of the case. Dysplasia was defined according to the Vienna criteria[26]. According to previously proposed measuring methods[22,24], the depth of submucosal invasion was determined using a micrometer under a microscope, and taken as the distance from the muscularis mucosae to the point of the deepest invasion (tumor apex).

We first analyzed the relation between the type V pit pattern subtype (VI or VN) and histology/invasion depth. We then examined the relation between SM ⩾ 1000 μm and histology/invasion depth and the following five detailed magnifying colonoscopy findings: (1) irregularity of the pit margins, (2) staining characteristics of the areas between pits, (3) area diameter of the type VI pit pattern (< 5 mm or ⩾ 5 mm), (4) density of the pits, and (5) width of the intervening membrane between the pits (Figure 2).

We divided the lesions with a type VI pit pattern into two subclasses (mildly irregular lesions and severely irregular lesions) according to the prominent and detailed magnifying colonoscopy findings of the first analysis. Mildly irregular lesions were defined as lesions with one or no significant magnifying colonoscopy findings, and severely irregular lesions were defined as lesions with two or more significant magnifying colonoscopy findings. This was done to diagnose SM ⩾ 1000 μm on the basis of cluster analysis[27,28]. Using these data, we examined the relation between the type VI pit pattern subclassifications and histology/invasion depth, the status of the muscularis mucosae, and the presence of desmoplastic reactions at the surface of the lesion (Figure 3). The muscularis mucosae were classified as detected, partially disappeared, or disappeared, as reported previously[29]. Desmoplastic reaction of the submucosal layer was classified as absent (-), mild to moderate (+), or severe (++), as reported previously[18,30].

The associations of dysplasia, SM < 1000 μm, and SM ⩾ 1000 μm with the type V pit pattern subtypes, detailed magnifying colonoscopy findings, and the type VI lesion subclasses, were analyzed by chi-square test. $P < 0.05$ was accepted as statistically significant. In addition, to identify predictors of SM ⩾ 1000 μm, we performed multivariate logistic regression analysis. All statistical analyses were performed using JMP statistical software, version 5.0.1 J (SAS Institute Inc, Cary, NC).

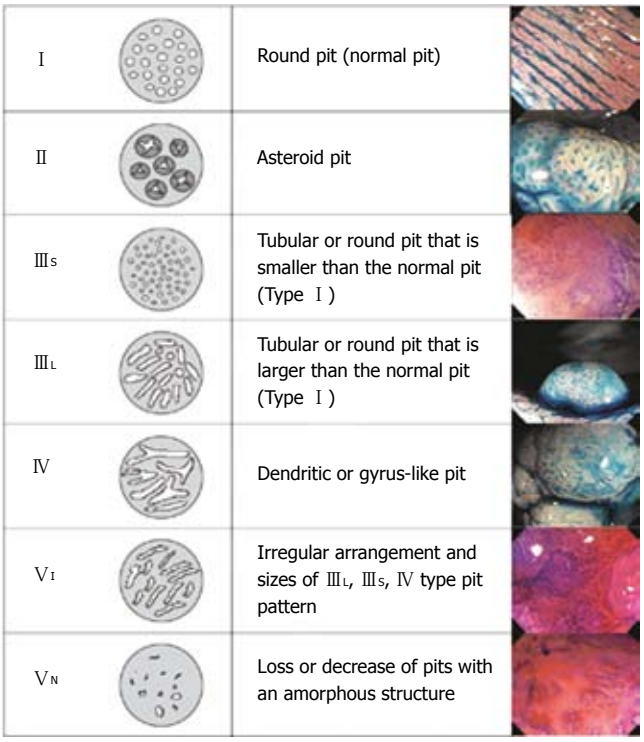

Figure 1 Classification of pit patterns of colorectal lesions.

RESULTS

Histology/invasion depth of colorectal neoplasm in relation to type V pit pattern subtypes

Dysplasia, SM < 1000 μm and SM ⩾ 1000 μm were found

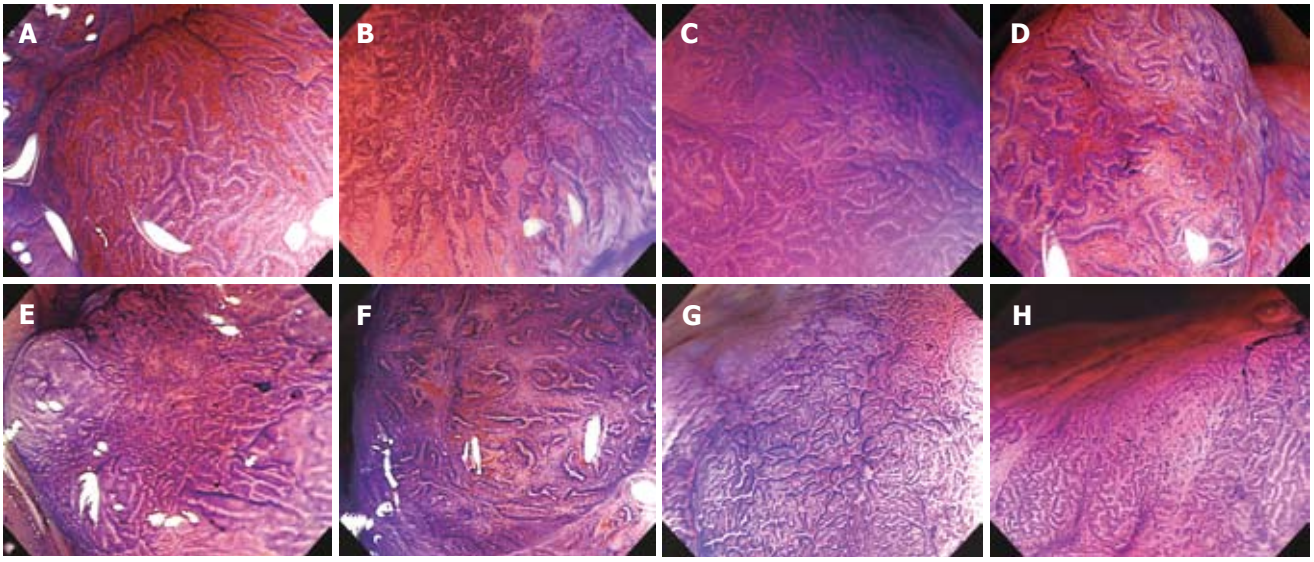

Figure 2 Magnifying features of colorectal neoplasm (crystal violet): **A**: Regular pit margins; **B**: Irregular pit margins; **C**: Clear staining characteristics of the areas between pits; **D**: Unclear staining characteristics of the areas between pits; **E**: High residual pit density; **F**: Low residual pit density; **G**: Narrow intervening membrane between pits; **H**: Wide intervening membrane between pits.

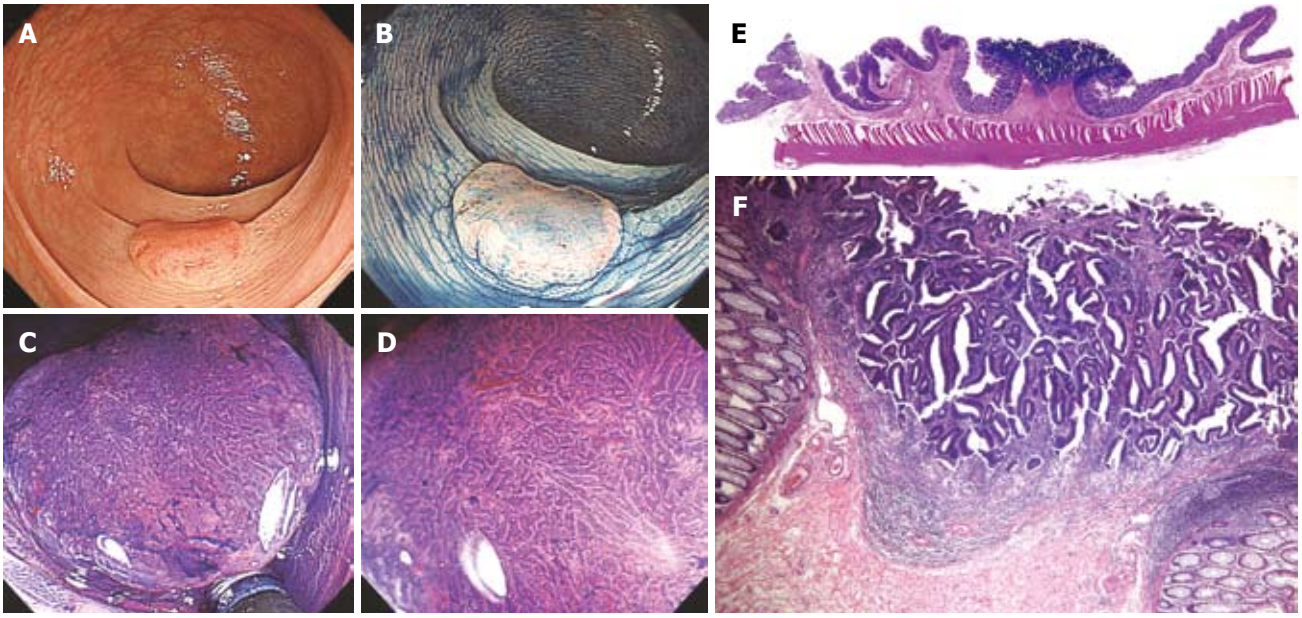

Figure 3 Type Ⅱa + 10Ⅱc lesion, 12 mm in diameter. **A**: Standard colonoscopic view; **B**: Standard colonoscopic view with indigo carmine spraying; **C**, **D**: Magnifying colonoscopic picture with crystal violet staining reveals type V_I pit pattern. Irregular pit margins, unclear staining characteristics of the areas between pits, > 5 mm area of type V_I pit pattern, high residual pit density, and narrow intervening membrane between pits is revealed; **E**: Cross-section (hematoxylin-eosin, × 8) of a surgically resected specimen showing submucosal invasion (1800 μm); **F**: Low-power view (hematoxylin-eosin, × 40) of type V_I pit pattern. Muscularis mucosae have disappeared. Desmoplastic reactions are mild to moderate. In this case, lymph node metastasis was not detected.

in association with 57.9% (117/202), 11.4% (23/202), and 30.7% (62/202) of the neoplasms with type V_I pit patterns, respectively (Table 1). Dysplasia, SM < 1000 μm, and SM ≥ 1000 μm were found in association with 0% (0/70), 4.3% (3/70), and 95.7% (67/70) of the neoplasms with type V_N pit patterns, respectively. The type V_N pit pattern was found significantly more often in lesions with SM ≥ 1000 than in dysplasias or lesions with SM < 1000 μm ($P < 0.01$). Sensitivity and specificity of the type V_N pit pattern for a diagnosis of SM ≥ 1000 were 51.9% (67/129) and 97.9% (140/143), respectively.

Histology/invasion depth of colorectal neoplasm with a type V_I pit pattern in relation to detailed magnifying colonoscopy findings

SM ≥ 1000 μm was found in association with 58.2% (46/79) of the lesions with irregular pit margins, 57.5% (50/87) of the lesions with unclear staining characteristics of the areas between pits, 41.3% (57/138) of the lesions with a type V_I pit pattern area ≥ 5 mm in diameter, 26.6% (29/109) of the lesions with high residual pit density, and 31.9% (44/138) of the lesions with a wide intervening membrane between pits (Table 2). SM ≥ 1000 μm was

Table 1 Histology/invasion depth of colorectal neoplasm in relation to type V pit pattern subtypes, *n* (%)

Type V pit pattern subtypes		Histology/invasion depth		
		Dysplasia	SM < 1000 μm	1000 μm ≤ SM
V_I	202 (100)	117 (57.9)	23 (11.4)	62 (30.7)
V_N[1]	70 (100)	0 (0)	3 (4.3)	67 (95.7)

[1]Dysplasia. SM < 1000 μm *vs* SM ≥ 1000 μm, $P < 0.01$. SM: Submucosa.

Table 2 Histology/invasion depth of colorectal neoplasm with type V_I pit pattern in relation to detailed magnifying colonoscopy findings, *n* (%)

Magnifying colonoscopy finding		Histology/invasion depth		
		Dysplasia	SM < 1000 μm	1000 μm ≤ SM
Irregular pit margins[1]	79 (100)	23 (29.1)	10 (12.7)	46 (58.2)
Unclear staining characteristics of the area between pits[1]	87 (100)	21 (24.1)	16 (18.4)	50 (57.5)
Area diameter of type V_I pit pattern ≥ 5 mm[1]	138 (100)	65 (47.1)	16 (11.6)	57 (41.3)
High residual pit density	109 (100)	64 (58.7)	16 (14.7)	29 (26.6)
Wide intervening membrane between pits	138 (100)	81 (58.7)	13 (9.4)	44 (31.9)

[1]Dysplasia. SM < 1000 μm *vs* SM ≥ 1000 μm, $P < 0.01$. SM: Submucosa.

found significantly more often in association with irregular pit margins, unclear staining characteristics of the areas between pits, and a type V_I pit pattern area ≥ 5 mm in diameter than in association with regular pit margins, clear staining characteristics of the areas between pits, and a type V_I pit pattern area < 5 mm in diameter.

Results of multivariate logistic regression analysis for predictors of SM ≥ 1000 μm

In multivariate logistic regression analysis, unclear staining characteristics of the areas between pits, irregular pit margins, and a V_I pit pattern area diameter of ≥ 5 mm were shown to be significant predictors of SM ≥ 1000 μm (Table 3). High residual pit density and a wide intervening membrane between pits were not significant.

Histology/invasion depth of colorectal neoplasm in relation to type V_I pit pattern subclassifications

One hundred and four lesions (51.5%) were judged to be mildly irregular, and 98 lesions (48.5%) were judged to be severely irregular (Table 4). Ninety-seven (93.3%) mildly irregular lesions showed dysplasias or SM < 1000 μm. Fifty-five (56.1%) severely irregular lesions showed SM ≥ 1000 μm. Mild irregularity was found significantly more often in dysplasias or in lesions with SM < 1000 μm than in lesions with SM ≥ 1000 ($P < 0.01$). Sensitivity and specificity of mild irregularity for dysplasias or SM < 1000 μm were 69.3% (97/140) and 88.7% (55/62), respectively.

Table 3 Results of multivariate logistic regression analysis for predictors of submucosal invasion deeper than 1000 μm (*n* = 202)

Magnifying colonoscopy finding	Odds ratio (*P* value)	Relevant finding
Unclear staining characteristics of the areas between pits	6.24 (< 0.0001)	Clear staining characteristics of the areas between pits
Irregular pit margins	4.89 (< 0.0001)	Regular pit margins
Area diameter of type V_I pit pattern ≥ 5 mm	4.14 (0.0132)	Area diameter of type V_I pit pattern < 5 mm
High residual pit density	1.51 (0.3335)	Low residual pit density
Wide intervening membrane between pits	1.02 (0.9740)	Narrow intervening membrane between pits

Table 4 Histology/invasion depth of colorectal neoplasm in relation to type V_I pit pattern subclassifications, *n* (%)

Type V_I pit pattern subclassification		Histology/invasion depth		
		Dysplasia	SM < 1000 μm	1000 μm ≤ SM
Mildly irregular[1]	104 (100)	89 (85.6)	8 (7.7)	7 (6.7)
Severely irregular	98 (100)	28 (28.6)	15 (15.3)	55 (56.1)

[1]Dysplasia. SM < 1000 μm *vs* SM ≥ 1000 μm, $P < 0.01$. SM: Submucosa.

Table 5 Status of muscularis mucosae in relation to type V pit patterns, *n* (%)

Type V pit pattern		Status of muscularis mucosae		
		Detected	Partially disappeared	Disappeared
V_I				
Mildly irregular[1]	104 (100)	97 (93.2)	6 (5.8)	1 (1.0)
Severely irregular[a]	98 (100)	38 (38.8)	31 (31.6)	29 (29.6)
V_N[b]	67 (100)		8 (11.9)	59 (88.1)

[1]Detected *vs* partially disappeared, disappeared, [b]$P < 0.01$, [a]$P < 0.05$.

Status of the muscularis mucosae in relation to type V pit patterns

The muscularis mucosae was detected in 97 (93.2%) mildly irregular lesions (Table 5). Partial disappearance or disappearance of the muscularis mucosae was seen in 60 (61.2%) severely irregular lesions and 67 (100%) lesions with a type V_N pit pattern. Severe irregularity was found significantly more often in association with partial disappearance or disappearance of the muscularis mucosae than in association with detection of the muscularis mucosae ($P < 0.05$). The type V_N pit pattern was found significantly more often in association with partial disappearance or disappearance of the muscularis mucosae than in association with detection of the muscularis mucosae ($P < 0.01$).

Desmoplastic reactions at the surface of the lesion in relation to type V pit patterns

No desmoplastic reaction of the superficial layer was observed in 100 (96.2%) mildly irregular lesions (Table 6). Desmoplastic reactions (+)/(++) were observed in 50

(51.0%) severely irregular lesions and 67 (100%) lesions with a type VN pit pattern. The type VN pit pattern was found significantly more often in lesions with a desmoplastic reaction (+)/(++) than in lesions with desmoplastic reaction (-) ($P < 0.01$).

DISCUSSION

Endoscopic treatment, such as EMR, is both a therapeutic technique and an important diagnostic technique. Therefore, it is important to be able to identify lesions for which endoscopic resection would be curative to avoid meaningless endoscopic resection for lesions that should be treated surgically. Pit pattern classification is used clinically to help determine the best treatment for colorectal tumors[9]. Type Ⅰ and Ⅱ pit patterns predict nonneoplastic lesions, whereas type Ⅲ, Ⅳ, and Ⅴ pit patterns predict neoplastic lesions. Lesions with a type Ⅲ or Ⅳ pit pattern are almost always dysplasias and are thus indications for endoscopic resection. Almost all lesions with a type VN pit pattern show SM ⩾ 1000 μm. The reported accuracy of detection of massive submucosal invasion on the basis of the type VN pit pattern is 97%[23]. In our study, SM ⩾ 1000 μm was found in 95.7% of lesions with a type VN pit pattern. Therefore, surgical resection is indicated for such lesions. By contrast, lesions with a type VI pit pattern include dysplasia and various submucosal carcinomas; thus, it is difficult to decide upon a therapeutic strategy on the basis of the current pit pattern classification system. It is necessary to analyze the type VI pit pattern in detail to determine the appropriate therapeutic strategy. The present study revealed that irregular pit margins, unclear staining characteristics of the areas between pits, and a type VI pit pattern area diameter ⩾ 5 mm are significant predictors for submucosal invasion of colorectal neoplasms of 1000 μm or more.

Lesions that were subclassified as mildly irregular lesions were mainly dysplasias or lesions that showed SM < 1000 μm (93.3%). Therefore, endoscopic resection is indicated for mildly irregular lesions. On the contrary, lesions that were classified as severely irregular lesions included not only dysplasias or lesions with SM < 1000 μm (43.9%), but also lesions with SM ⩾ 1000 μm (56.1%). For severely irregular lesions, the therapeutic strategy should be determined on the basis of standard endoscopic findings in conjunction with those of other modalities, such as contrast enema radiography or endoscopic ultrasonography[29,31,32]. New diagnostic modalities, such as narrow band imaging, are expected to provide more information about the invasion depth of colorectal carcinomas[33-36].

Our results revealed that there is a significant histologic difference between mildly irregular lesions and severely irregular lesions. The degree of disappearance of the muscularis mucosae increased as the pit patterns changed from VI with mildly irregularity to VI with severely irregularity to VN. If we could determine the status of the muscularis mucosae by magnifying colonoscopy, the pit pattern would be helpful in determining the depth of submucosal invasion depth by endoscopic ultrasonography. The muscularis mucosae had disappeared in all lesions with a type VN pit pattern; thus, we can measure the invasion depth from the surface of a lesion of this type to the deepest portion[29]. It has been reported that desmoplastic reactions are related to massive submucosal invasion[18]. In the present study, the incidence of desmoplastic reactions increased as the pit patterns changed from VI with mildly irregularity to VI with severely irregularity to VN. There were no desmoplastic reactions in mildly irregular lesions. These results indicate that changes in the appearance of the pits are caused by the process of submucosal infiltration of the colorectal neoplasm. Although the mechanism underlying this process is not clear, it is possible that irregular pit margins and unclear staining characteristics of the areas between pits may involve several molecular markers. We reported previously that the proliferation, infiltration and lymph node metastasis of submucosal colorectal carcinoma are significantly related to the expression of markers such as Ki-67, E-cadherins, MUC1, cathepsin D and MMP-7 at the deepest portion[37-43]. We also reported previously that MUC1 expression at the superficial layer may be related to colorectal tumors with a type V pit pattern[42]. However, there are few reports pertaining to the relation between the expression of specific molecular markers and morphogenesis of the type VI pit pattern. There may be a relation between the expression of molecular markers and detailed magnifying colonoscopy features of the type VI pit pattern. Further investigation will clarify the relation between molecular morphogenesis at the lesion surface and type VI pit pattern subclassifications.

We conclude that type VI pit pattern subclassification is useful for identifying dysplasias or lesions with SM < 1000 μm. Subclassifications can be applied to decisions about whether endoscopic treatment is indicated for colorectal neoplasms. However, we cannot identify lesions with SM ⩾ 1000 μm on the basis of type VI pit pattern subclassifications.

Table 6 Desmoplastic reaction at the lesion surface in relation to type V pit pattern, *n* (%)

Type V pit pattern		Desmoplastic reaction		
		Absent (-)	Mild to moderate (+)	Severe (++)
VI				
Mildly irregular	104 (100)	100 (96.2)	4 (3.8)	
Severely irregular	98 (100)	48 (49.0)	29 (29.6)	21 (21.4)
VN[b]	67 (100)		16 (23.9)	51 (76.1)

[b]$P < 0.01$ (-) *vs* (+), (++).

COMMENTS

Background

Colorectal neoplasms with a type VI pit pattern include various lesions, such as dysplasias and submucosal carcinomas, with either SM < 1000 μm or SM ⩾ 1000 μm. Thus, it is difficult to decide upon a therapeutic strategy for colorectal neoplasm on the basis of the current type VI pit pattern classification.

Research frontiers

In this study, we assessed the clinical usefulness of type VI pit pattern subclassification in determining the histology/invasion depth of colorectal neoplasms. There has been little study on type VI pit pattern subclassification.

Innovations and breakthroughs

Type V_I pit pattern subclassification is useful for identifying dysplasias or lesions with SM < 1000 μm.

Applications

Subclassifications can be applied to deciding whether endoscopic treatment is indicated for colorectal neoplasms.

Terminology

Type V_I pit pattern subclassification: We divided the lesions with a type V_I pit pattern into two subclasses (mildly irregular lesions and severely irregular lesions) according to the prominent detailed magnifying colonoscopy findings of the first analysis. Mildly irregular lesions were defined as lesions with one or no significant magnifying colonoscopy findings, and severely irregular lesions were defined as lesions with two or more significant magnifying colonoscopy findings.

Peer review

The authors retrospectively investigated whether subclassification of the type V_I pit pattern on the basis of magnifying colonoscopy findings was useful in determining the type and depth of invasion of colorectal neoplasm. They concluded that subclassification of the type V_I pit pattern is useful for identifying dysplasias or lesions with SM < 1000 μm.

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RAPID COMMUNICATION

Detection of hMSH2 and hMLH1 mutations in Chinese hereditary non-polyposis colorectal cancer kindreds

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Zhang CH, He YL, Wang FJ, Song W, Yuan XY, Yang DJ, Chen CQ, Cai SR, Zhan WH. Detection of hMSH2 and hMLH1 mutations in Chinese hereditary non-polyposis colorectal cancer kindreds. *World J Gastroenterol* 2008; 14(2): 298-302

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Abstract

AIM: To establish and validate the mutation testing for identification and characterization of hereditary non-polyposis colorectal cancer (HNPCC) in suspected Chinese patients.

METHODS: Five independent Chinese kindreds with HNPCC fulfilling the classical Amsterdam criteria were collected. Genomic DNA was extracted after informed consent was obtained. The coding region of hMSH2 and hMLH1 genes was detected by polymerase chain reaction (PCR) and denaturing high-performance liquid chromatography (DHPLC). Mutations identified in the proband by DHPLC were directly sequenced using a 377 DNA sequencer, analyzed with a basic local alignment tool (BLAST), and tested in the corresponding family members by direct DNA sequencing.

RESULTS: Mutations were identified in two Chinese HNPCC kindreds. One was the missense mutation of hMSH2 c.1808A→G resulting in Asp 603 Gly identified in the proband of the fifth HNPCC (HNPCC5) kindred. In the HNP5 kindred, three family members were found to have this mutation and two of them had colorectal cancer. The other mutation of hMLH1 c.1882A→G was identified in the HNP2 kindred's proband, which might be the nonsense mutation analyzed by BLAST.

CONCLUSION: Pedigree investigation and mutation testing of hMSH2 and hMLH1 are the practical methods to identify high-risk HNPCC patients in China.

INTRODUCTION

Hereditary non-polyposis colorectal cancer (HNPCC) syndrome is characterized by autosome-dominantly inherited predisposition to early colorectal carcinoma and extracolonic epithelia-derived tumors most often located in the gastrointestinal and urogenital tracts[1]. The mean age of HNPCC and sporadic colorectal cancer (CRC) patients at diagnosis is 42 years and 65 years, respectively. HNPCC, accounting for approximately 5%-15% of all CRCs, is categorized as Lynch I or Lynch Ⅱ syndrome (according to revised Amsterdam criteria)[2]. Germline mutations of the mismatch repair (MMR) genes identified in HNPCC kindreds, including *MSH2*, *MLH1*, *MSH6*, *PMS1* (promotion of mutS homology 1), *PMS2*, and *MLH3*, have been proved as the major cause for HNPCC by linkage analysis. Mutations in two of these MMR genes, *MSH2* and *MLH1*, account for the majority of the kindreds with HNPCC[3]. Thus, pedigree investigation and MMR gene testing, as the basis for efficient prevention and treatment of HNPCC, are most often used in early diagnosis of at-risk family members and in confirmation of the diagnosis of HNPCC.

CRC is the third life-threatening cancer in China. However, HNPCC pedigree and its predisposition gene have not been extensively studied. We have established a CRC database since 1994 and the follow-up rate is above 90%. We have recently paid attention to hereditary colorectal cancer in South China and found that about 3% of CRC patients have multiple CRCs to which the young are vulnerable[4,5]. Thus, we collected those CRC families and finally obtained eleven independent Chinese kindreds with HNPCC by deep pedigree investigation until January,

2004. Five of them fulfilled the classical Amsterdam criteria. To identify high-risk populations with HNPCC, we tested hMSH2 and hMLH1 mutations in these classical kindreds.

MATERIALS AND METHODS

Patients

The clinical diagnosis of classical HNPCC was established and verified at the Department of Gastrointestinopan creatic surgery, the First Affiliated Hospital of the Sun Yat-Sen University[2]. Five independent Chinese kindreds with HNPCC fulfilling the classical Amsterdam criteria were collected. The study was approved by the Ethical Committee of Sun Yat-Sen University.

DNA isolation

Peripheral blood samples were collected from both patients and their family members in each Chinese kindred after informed consent was obtained for genetic analysis. DNA was extracted directly from leukocytes following the standard procedures.

All exons of *MLH1* and *MSH2* were amplified for mutation testing. Samples used were amplified in 20 mL reaction volume containing approximately 100 ng DNA in 50 mmol/L KCl, 10 mmol/L Tris-HCl, 1.5 mmol/L $MgCl_2$, 200 mmol/L each dNTP, 0.01% gelatin, 1 U Taq-polymerase, and 20-40 pmol each primer. The PCR amplification conditions were as follows: denaturation at 95℃ for 5 min, followed by 30 cycles of denaturation at 95℃ for 30 s, annealing at fragment-specific annealing temperature for 1 min, extension at 72℃ for 1 min, and a final extension at 72℃ for 7 min. The primers, annealing temperature, and size of the PCR products for each of the investigated hMLH1 and hMSH2 exons are described elsewhere[6-8]. The primers were synthesized by Shanghai Bio-Chemical Corporation.

DHPLC analysis

The amplified PCR fragments were screened for sequence variants by denaturing high pressure liquid chromatography (DHPLC) on a WAVE DNA fragment analysis system (Transgenomic, San Jose, CA, USA). The running conditions for each amplicon (available upon request) were determined by the Wavemaker 3.4.4 software (Transgenomic, San Jose, CA, USA) based on the DNA sequence. Five mL of each PCR product (containing 50-100 ng of DNA) was denaturated at 95℃ for 3 min and then gradually reannealed by decreasing the sample temperature from 95℃ to 65℃ over 30 min. PCR products were then separated at a flow rate of 0.9 mL/min with a linear acetonitrile gradient. Generally, analysis took approximately 10 min including column regeneration and re-equilibration to starting conditions. The column mobile phase consisted of a mixture of 0.1 mol/L triethylamine acetate (pH 7.0) with (buffer B) or without (buffer A) 25% acetonitrile. Samples displaying variant elution peaks in each run were chosen for sequence analysis. For some amplicons displaying variations in elution profiles of control samples, all samples were sequenced.

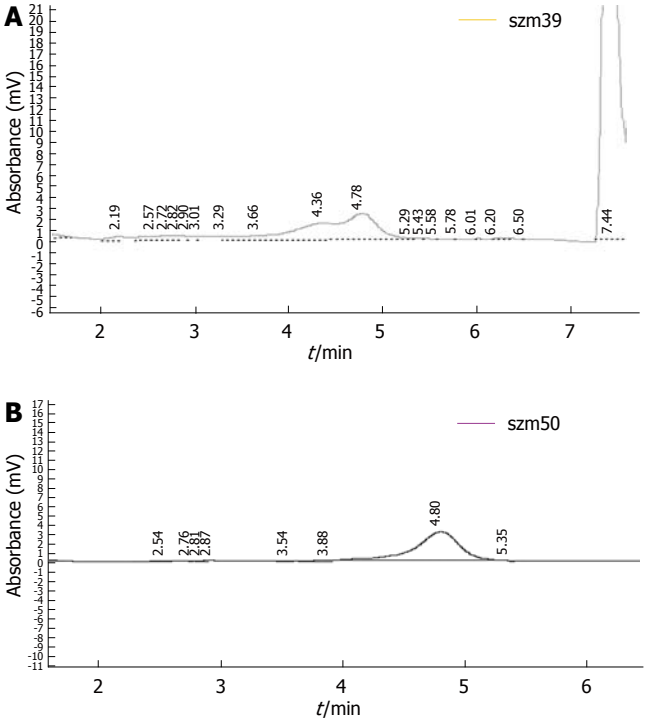

Figure 1 DHPLC showing variant elution peaks (**A**) and normal elution peaks (**B**) in exon 12 of hMSH2 gene.

DNA sequencing

DHPLC variants were confirmed by direct sequencing of independently amplified PCR products of the amplicons, in both sense and antisense direction, using the same primers. Sequencing was performed with Big Dye™ terminator cycle sequencing ready reaction kit (Applied Biosystems, Inc., Foster City, CA) on an ABI Prism™ 377 DNA sequencer following the standard conditions recommended by the manufacturer. Sequences obtained were aligned and compared to published wild-type sequences by Sequencher 3.1.1 analysis software.

Mutations identified in the kindred's proband were tested among their family members by direct DNA sequencing.

RESULTS

Among the 5 probands of the five Chinese HNPCC kindreds, 180 PCR products were screened by DHPLC. Two PCR products found with variant elution peaks by DHPLC were identified to have mutations. One was hMSH2 c.1808A→G resulting in Asp 603 Gly identified in the HNP5 kindred's proband and tested among the family members (Figures 1-3). The other was hMLH1 c.1882A →G identified in the HNP2 kindred's proband (Figures 4 and 5), which was not tested among the family members, because it might be the nonsense mutation analyzed by BLAST.

As shown in Figure 3, in the HNPCC5 kindred, there were four colorectal carcinoma patients in two successive generations, and three of these were diagnosed before the age of 45 years. The proband developed endometrial carcinoma at the age of 61 years, bladder carcinoma at the age of 66 years and colorectal carcinoma at the age of 72

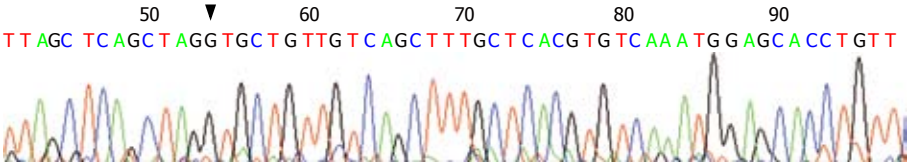

Figure 2 HPLC variants confirmed by direct sequencing displaying a single nucleotide substitution of c.1808A→G in exon 12 of hMSH2 gene.

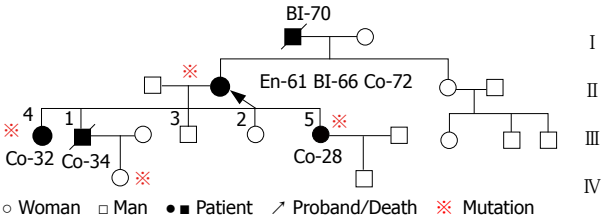

Figure 3 Pedigree tree of HNPCC 5 kindred. Co: Colon; Bl: Bladder; En: Endometrium.

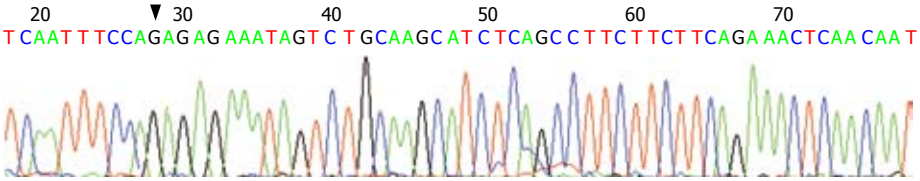

Figure 4 DHPLC variants confirmed by direct sequencing revealing a single nucleotide substitution of c.1882A→G in exon 16 of hMLH1 gene.

years, while his father developed bladder carcinoma at the age of 70 years. In addition, patient Ⅲ-1 had colorectal carcinoma at the age of 34 years and died of synchronous hepatic metastasis.

In the HNP5 kindred, each PCR product from nine proband's family members was tested by direct DNA sequencing. Only three of them were found to have the missense mutation in hMSH2 at position A1808G. The missense mutation sequence variant was found in exon 12 of hMSH2 gene. It was a single nucleotide substitution of c.1808A→G (Figure 2), which resulted in Asp 603 Gly of hMSH2 (NCBI Ref. Seq. NM 000251 and NP 000242 for mRNA and protein, respectively). Proband and patient Ⅲ-4 and -5 had this mutation and developed colorectal carcinoma. Patient Ⅲ-1's daughter had no colorectal disease even though she had this mutation, and was still under follow-up. No mutation was found in the others.

DISCUSSION

CRC is one of the most common cancers and its clinical selection criteria for cancer families were first established in Amsterdam in 1990 by the International Collaborative Group on HNPCC and modified in 1999[2]. Detection of constitutional mutations in genes associated with predisposition to cancer is practical in molecular diagnosis. Data suggest that molecular testing is much more efficient, if analyses are focused on a limited number of alterations[9-13]. Detection of germline mutation carriers is an efficient method to define high-risk CRC patients. Identification of germline mutations of either hMSH2 or hMLH1 could be performed in 50%-70% of families meeting the Amsterdam criteria for HNPCC, whereas the families not complying with these criteria show a much lower frequency of the MMR gene mutations[14-17]. Thus, hMSH2 or hMLH1 gene testing is most often used in early diagnosis of at-risk family members with HNPCC.

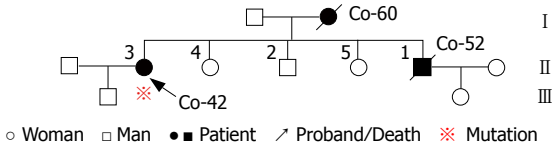

Figure 5 Pedigree tree of HNPCC 2 kindred. Co: Colon.

To identify a population-specific panel of mutations, it is crucial to describe them in all ethnic groups.

Even though CRC is common in China, few studies on HNPCC pedigree and its predisposition gene are available[4,17]. In our study, HNPCC kindreds were collected and hMSH2 and hMLH1 gene mutations were tested in order to find high-risk CRC populations. Two mutations were found among the five Chinese HNPCC kindreds. In the 5HNPCC kindreds, missense mutation was found in four members, three of them developed colorectal carcinoma. Although it has not been confirmed as a germline mutation yet, it may be an important factor for CRC development. Thus, the patient Ⅲ-1's daughter with this mutation should be closely followed up.

Approximately 20% of patients with colorectal cancer have a genetic component and HNPCC is the most common autosome dominant hereditary syndrome predisposing to colorectal cancer[18,19]. Various methods have been described to screen for HNPCC and directly test for mismatch repair gene mutations[20-27]. For patients with available tumor specimens, MSI and IHC are widely performed. Ruszkiewicz *et al*[28] reported that immunohistochemistry is an alternative method for assessment of MSI status, which is fast and relatively inexpensive compared with MSI testing. Some reports[26,29,30] suggest that detection of MSI and IHC for hMSH2/hMLH1 proteins is a reliable pre-screening test for hMLH1/hMSH2 germline mutations in families suspected of having HNPCC. Because China is a developing country with a large population and the incidence rate of CRC

increases, a screening protocol specific for the Chinese population is necessary to detect the high-risk populations with HNPCC. hMSH2 and hMLH1 gene mutation testing is a practical method for detecting HNPCC in Chinese population. Meanwhile, further research should be performed in Chinese HNPCC kindreds with germline mutations.

COMMENTS

Background

Approximately 20% of colorectal cancer patients have a genetic component and hereditary non-polyposis colorectal cancer (HNPCC) is the most common autosome dominant hereditary syndrome predisposing to colorectal cancer. Various methods have been described to screen for HNPCC and directly test for mismatch repair gene mutations. Even though CRC is common in China, few studies on HNPCC pedigree and its predisposition gene are available.

Research frontiers

Data suggest that molecular testing is much more efficient when analyses are focused on a limited number of alterations. Detection of germline mutation carriers is an efficient method to define high-risk CRC patients. Germline mutations of either hMSH2 or hMLH1 can be identified in 50%-70% of families meeting the Amsterdam criteria for HNPCC. Thus, hMSH2 or hMLH1 gene testing is most often used in early diagnosis of at-risk family members with HNPCC.

Innovations and breakthroughs

Five independent Chinese kindreds with HNPCC fulfilling the classical Amsterdam criteria were collected. Mutations were identified in two Chinese HNPCC kindreds. One was the missense mutation of hMSH2 c.1808A→G resulting in Asp 603 Gly, the other was the mutation of hMLH1 c.1882A→G .

Applications

Molecular pathological tests should be performed in order to identify individuals and at-risk family members with HNPCC. Close follow-up and intensive surveillance should be performed.

Terminology

Denaturing high-performance liquid chromatography (DHPLC) is a method for separating DNA duplexes that differ in the identity of one or more base pairs. The method is believed to be most efficient at the site of mutation. Basic local alignment search tool (BLAST) is powerful to compare novel sequences with previously characterized genes. Both functional and evolutionary information can be obtained from well designed queries and alignments. BLAST 2.0 can rapidly search for nucleotide and protein in their databases.

Peer review

The results of this clinical study indicate that mutation testing for hMSH2 and hMLH1 by PCR and direct sequencing is a preferable method to identify high-risk HNPCC patients.

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BASIC RESEARCH

Expression of thymidine kinase mediated by a novel non-viral delivery system under the control of vascular endothelial growth factor receptor 2 promoter selectively kills human umbilical vein endothelial cells

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Abstract

AIM: To investigate the killing efficiency of a recombinant plasmid containing a thymidine kinase (TK) domain insert driven by the vascular endothelial growth factor receptor 2 (VEGFR2) promoter (KDR) on vascular endothelial cells.

METHODS: The KDR-TK fragment was extracted from pBluescript Ⅱ KDR-TK plasmid by enzymatic digestion with *Xho*I and *Sal*I. The enhanced green fluorescence protein (EGFP) carrier was extracted from pEGFP by the same procedure. The KDR-TK was inserted into the pEGFP carrier to construct pEGFP-KDR-TK. Using ultrasound irradiation and microbubble, pEGFP-KDR-TK was transferred into human umbilical vein endothelial cells (HUVECs). The transient infection rate was estimated by green fluorescent protein (GFP) expression. Transfected HUVECs, non-transfected HUVECs, and HepG2 cells were cultured in the presence of different concentrations of ganciclovir (GCV), and the killing efficacy of HSV-TK/GCV was analyzed by 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyl tetrazolium bromide (MTT) assay.

RESULTS: The recombinant pEGFP-KDR-TK was successfully constructed by inserting the KDR-TK fragment into the pEGFP carrier. Transfected HUVECs showed cytoplasmic green fluorescence, and the transient transfection rate was about 20.3%. Pools of G418-resistant cells exhibited a higher sensitivity to the prodrug/GCV compared to non-transfected HUVECs or non-transfected HepG2 cells, respectively.

CONCLUSION: KDR promoter and the suicide gene/prodrug system mediated by diagnostic ultrasound combined with microbubble can significantly kill HUVECs. Such therapy may present a novel and attractive approach to target gene therapy on tumor vessels.

Key words: Microbubble; Ultrasound; Gene therapy; Vascular endothelial growth factor receptor 2; Human umbilical vein endothelial cells

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Wang Y, Xu HX, Lu MD, Tang Q. Expression of thymidine kinase mediated by a novel non-viral delivery system under the control of vascular endothelial growth factor receptor 2 promoter selectively kills human umbilical vein endothelial cells. *World J Gastroenterol* 2008; 14(2): 224-230

http://www.wjgnet.com/1007-9327/14/224.asp

INTRODUCTION

Angiogenesis coincides with increased tumor cell entry into the blood circulation and thus facilitates metastasis. Previous reports revealed that targeting angiogenesis represents a new strategy for antitumor therapy[1].

Vascular endothelial growth factor-A (VEGF-A) is the major player in tumor angiogenesis, which activates two tyrosine kinase receptors VEGFR1 (Flt-1) and VEGFR2 (KDR in humans/Flk-1 in mice). KDR was found to be overexpressed on activated endothelial cells of newly formed vessels and strongly associated with invasion and metastasis in human malignant diseases[2].

Gene therapy has made significant progress in tumor therapy[3]. The effectiveness of suicide gene strategy directed against cancer cells has been shown for various tumor cell types[4]. Among several suicide gene prodrug combinations, the herpes simplex virus-thymidine kinase (HSV-TK) gene is a prototype. However, non-selective, ubiquitous expression of suicide genes may

cause unexpected adverse effects such as bone marrow suppression[5,6]. Thus, prerequisite for achieving effective and safe gene therapy against cancer is the induction of a strong, tumor vascular-selective expression of suicide genes. The success of gene therapy largely depends on the development of a gene delivery vector that is safe, easy-performed, and efficient. Ultrasound exposure in combination with microbubble is a new non-viral delivery system that might improve transfection efficiency. Nie *et al*[7] have shown that a combination of microbubble contrast agents and ultrasound irradiation might be a beneficial and much easier way for non-viral gene therapy.

In the present study, we established a KDR promoter to drive TK gene expression in human umbilical vein endothelial cells (HUVECs). Transfection was achieved by diagnostic ultrasound combined with a microbubble contrast agent. Our purpose was to analyze the cytotoxic effect of TK/ganciclovir (GCV) under the control of the KDR promoter on HUVECs.

MATERIALS AND METHODS

Cell isolation and culture

Primary HUVECs were isolated using the modified "irrigative digestion" technique as described by Jaffe *et al*[8]. Briefly, a transfusion silica gel tube was inserted into one end of new born aseptic umbilical cords after abdominal delivery from healthy pregnant women who had normal routine urinalysis and blood tests (women with any complications of pregnancy or viral infections were excluded) to flush the bloodstain remaining in the cavity repeatedly with PBS, and then 15-20 mL collagenase Ⅱ (Sigma, St. Louis, MO, USA) was injected into the vein cavity and retained 10-12 min for digestion. The collection of umbilical vein was flushed with PBS and the endothelial cells were collected and centrifuged at 1000 rpm for 8 min. Cells were resuspended and seeded in human endothelial-serum free medium (SFM) (Gibco, California, USA) supplemented with 100 mL/L fetal bovine serum (FBS, Gibco), 2 ng/mL vascular endothelial cell growth factor (Sigma), 90 μg/mL heparin (Sigma), 100 U/mL penicillin, and 50 μg/mL streptomycin. Culture medium was changed after 24 h and then every three days (3 mL) till the cells became a confluent monolayer. The primary cells were allowed to grow for 3 passages (1:2 split). Viability was assessed by trypan blue dye exclusion staining (Sangong Biological Engineering technology, Shanghai, China). HUVECs were identified by staining with rabbit anti-human von Willebrand factor (Boshide Company, Wuhan, China), as well as by performing the acetylated low-density lipoprotein color reagent test (DiI-Ac-LDL) (Molecular Probes, Novato, Canada, Europe BV). Experiments were performed on cultures with a viability > 95%. The human hepatoma cell line HepG2 was obtained from Laboratory of Surgery of our institution and cultured in Dulbecco's modified Eagle medium (Sigma) supplemented with 10% FBS. The level of KDR expression in HUVEC and HepG2 cells was determined immunohistochemically.

Preparation of plasmid DNA

The pBluescript Ⅱ KDR-TK plasmid, which contains Herpes simplex virus thymidine kinase (HSV-TK) and the restriction sites for *Xho*I and *Sal*I and which is driven by the KDR promoter, was kindly provided by Dr Li Baojin. The 2.2-kb KDR-TK fragment was extracted from pBluescript Ⅱ KDR-TK by a double digestion with *Xho*I and *Sal*I (Takara Biotechnology Co.,Ltd, Dalian, China) and identified by electrophoresis and DNA sequencing. The pEGFP 4.7 kb carrier was released from EGFP plasmid DNA (donated by Doctor Zhang Ge, Laboratory of Molecular Medicine, Sun Yat-Sen University, China) containing the restriction sites for *Xho*I and *Sal*I using the same technique. The KDR-TK was inserted into the same clone location of the pEGFP vector with T4 ligase (Takara) to construct a plasmid termed pEGFP-KDR-TK. Restriction enzyme digestion and DNA sequencing (Bioasia Biotech Ltd, Shanghai, China) were used to confirm correct construction. The plasmid EGFP-KDR-TK was propagated in *E. coli* strain DH5α and purified using a large-scale plasmid DNA purification kit (Plasmid Maxi Kit; Qiagen, Hilden, Germany). The purity of EGFP-KDR-TK plasmid DNA was assessed by ultraviolet spectrophotometry.

Preparation of microbubble contrast agent

The ultrasound contrast agent SonoVueTM (Bracco SpA, Milan, Italy) that was used in this study consists of 59 mg of sulfur hexafluoride gas (SF6) and 25 mg of a freeze-dried white powder. Reconstitution of the material with 5 mL saline, yielded phospholipidstabilized microbubbles filled with sulfur hexafluoride with a diameter of less than 8 μm (mean, 2.5 μm)[9].

Gene transfer into cells with microbubble-enhanced ultrasound irradiation

HUVECs were seeded in 24-well plates at a density of 1.5×10^5 cells/well together with 15.0 μg of pEGFP-KDR-TK (50 μg/mL) in a total volume of approximately 300 μL and incubated for 20 min at 37℃. The 24-well plates were randomly divided into three groups with 8 samples in each group: group A, sham-exposure to ultrasound without addition of SonoVue; group B, exposure to ultrasound without addition of SonoVue, and group C, exposure to ultrasound with the addition of 2% SonoVue (600 μg/well). The ultrasound machine used was HP-Viewpoint 2500 scanner (Phillips Medical System, Bothell, MA, USA). An E94K213 transcranial vector transducer probe (12 mm in diameter) was placed on a polyester film (thickness, 6 μm) that covered the 24-well plate with 10 mm thickened contact gel between the probe and the polyester film, and the cells were exposed to 1.9 MHz continuous ultrasound for 5 min at an 80.0 mW/cm^2 output intensity (Figure 1). Five minutes after the first exposure, another 3 min exposure was performed under the same conditions. Cell viability was tested immediately after exposure by trypan blue dye exclusion staining. Cells were then incubated in SFM, 10% FBS, and 2 ng/mL vascular endothelial cell growth factor in 5% CO_2 at 37℃ for 48 h. Transfection efficiency was determined by fluorescence microscopy (Leica, Danaher Corporation, Wetzlar, Germany).

Subsequently, the cells were incubated for another 4 h

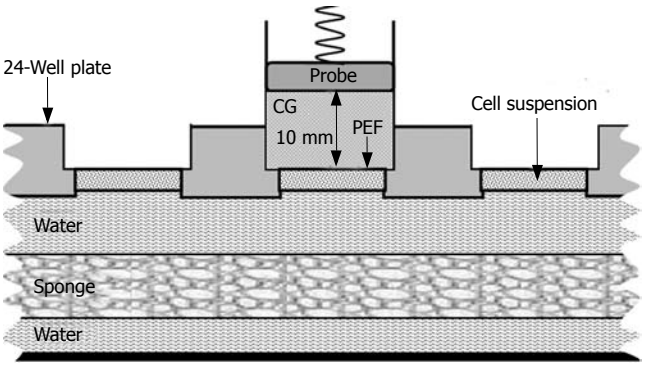

Figure 1 Schematic diagram of gene transfection. Transducer with contact gel (CG) was placed on the cell suspension covered with a 6-μm poly-ester film (PEF) under 1.9 MHz continuous ultrasound with a 80.0 mW/cm^2 output intensity. The 24-well plate was kept in a water bath at 37℃, and a sponge mat was placed in the water bath.

before they were subjected to selection with 600 μg/mL G418 (Gibco). At that time point when most of the cells in group A had died, G418 concentration was decreased to 200 μg/mL to maintain filtrate efficacy. After around three weeks of selection, a stable G418-resistant cell pool has formed as visualized by fluorescence microscopy. Cell colonies named KDR-TK/VEC were transferred with filter paper dipped trypsin to anther 24-well plate and cultured in an humidified atmosphere with 5% CO_2 at 37℃ for 3 d for subsequent experiments.

Sensitivity of cells to prodrug

KDR-TK /VEC, HUVEC, and HepG2 cells in logarithm growth term were detached by amylopsin (Sigma), the number of viable cells was determined by trypan blue dye exclusion, and viable cells were seeded in 96-well plates at a density of 2×10^3 cells/well. One day later, the cells were treated with 500 μL of fresh medium in the absence or presence of varying concentrations of GCV (0, 1, 10, 50, or 100 μg/mL). After incubation for 3 d, the sensitivity of cells to GCV was assessed using conventional 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyl tetrazolium bromide (MTT) assay[10]. The percentage of suppressive cells was calculated with the following formula:

Relative suppressive rate = (1 - average OD570 of experiment/OD570 of control) × 100%.

Statistical analysis

Data were expressed as mean ± SD. The statistical significance of the data was compared by using two paired Student *t* test. ANOVA was used to analyze the differences among the different groups. Results were considered significant at $P < 0.05$.

RESULTS

Identification of HUVEC and KDR expression in cell lines

The average amounts of the endothelial cells isolated from umbilical cords using modified "irrigative digestion" technique were 1×10^6 cells. Light microscopic studies found that after 5 d in culture, HUVECs grew in confluent monolayer without a definable whirling pattern. The cells

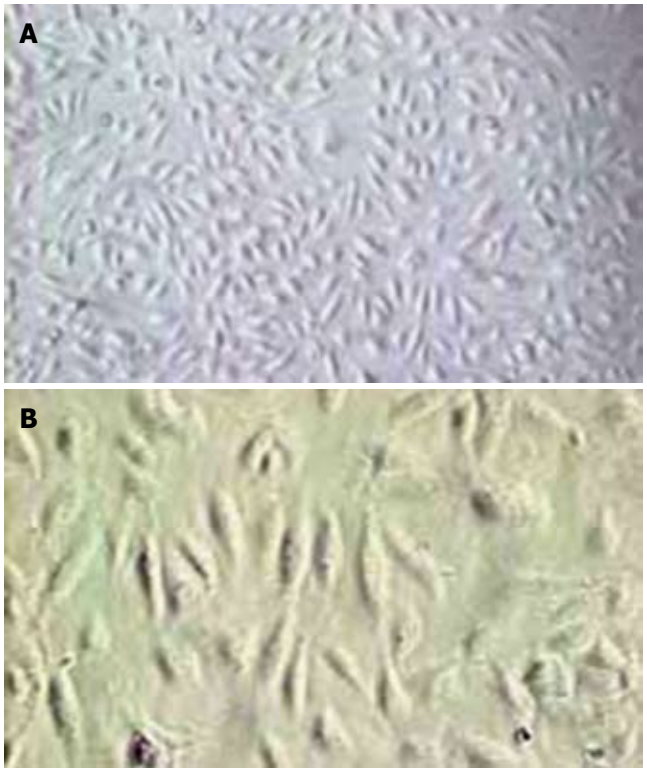

Figure 2 Photomicrographs show cultured human endothelial cells on d 7. The cells appeared to be a monolayer of very flat, polygonal-shaped cells. **A**: × 100; **B**: × 200.

were homogenous, closely opposed, large, and polygonal with an oval centrally coated nucleus and indistinct cell border (Figure 2). Factor Ⅷ is an important plasma marker of endothelium damage/dysfunction, which can also be detected in death or dysfunctional endothelial cells. The positive dye was brown in cytoplasm of HUVEC stained with the factor Ⅷ related antigen (Figure 3A). Compared with the factor Ⅷ, DiI-Ac-LDL can be taken up only by the living endothelial cells. The living HUVECs incubated with DiI-Ac-LDL are found to be red fluorescence under the fluorescent photomicroscope (Figure 3B), while the dead cells are uncolored. In the present study, the DiI-Ac-LDL stain revealed that more than 95% HUVEC were alive. To estimate the selectivity of the strategy using the KDR promoter, it is necessary to identify KDR-positive and KDR-negative cell lines. As shown in Figure 4, immunohistochemical analysis revealed KDR expression in HUVECs but not in HepG2.

Identification of recombinant plasmids

The positive clone of recombinant pEGFP-KDR-TK, which contains the neomycin resistance gene and the independent KDR promoters controlling the expression of TK, was identified by restriction endonuclease cleavage and sequencing. DNA sequencing and electrophoresis showed correct KDR-TK insertion into the pEGFP carrier (Figure 5).

Transfection of HUVECs with pEGFP-KDR-TK

The transient transfection rate was assessed by fluorescence microscopy 48 h after transfection. Fluorescent microscopy

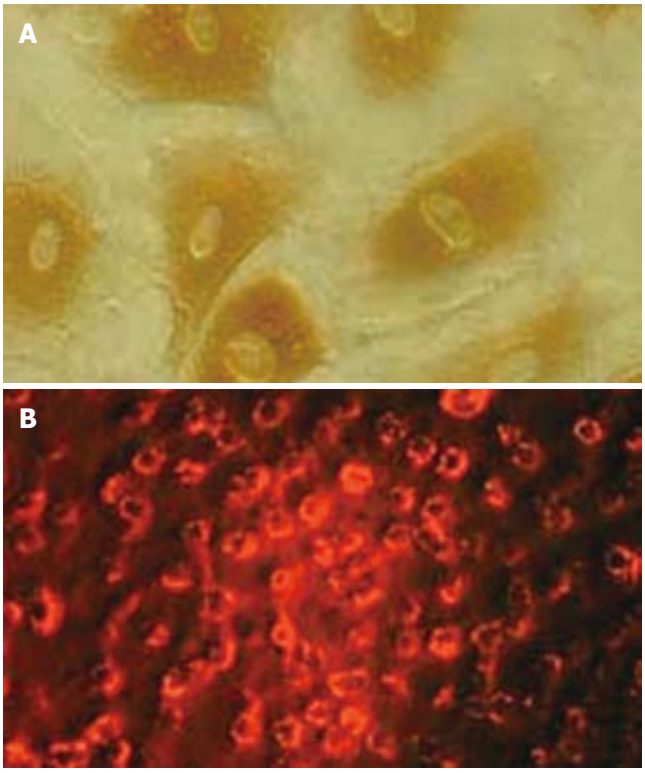

Figure 3 Immunofluorescence study of cultured human endothelial cells. **A**: Shows immunofluorescence positive-stained cells treated with Factor Ⅷ related antigen. The cells were stained brown (× 400); **B**: Shows positive-stained cells *in vitro* treated with DiI-Ac-LDL. More than 95% HUVECs are found to be red fluorescence (× 400).

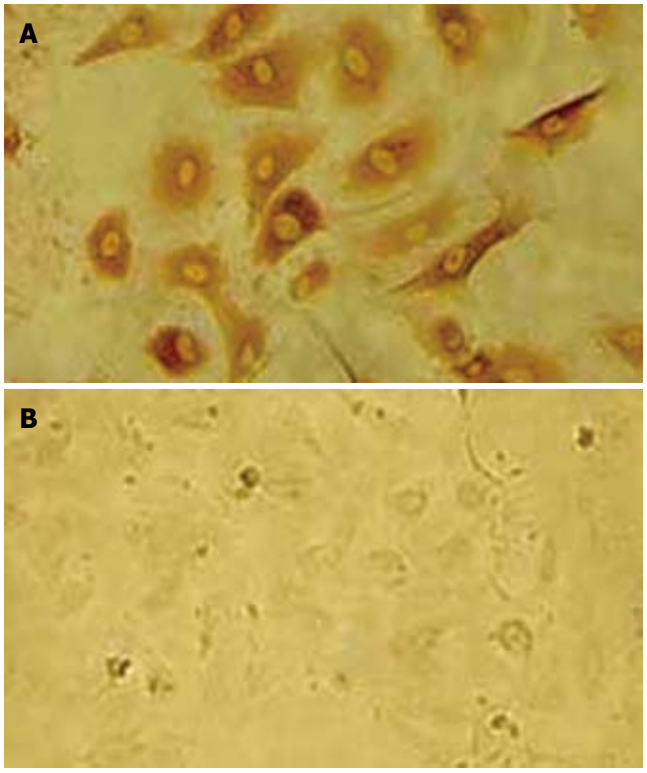

Figure 4 Immunofluorescence study (× 400) of cultured human endothelial cells. **A**: Shows positive-stained human endothelial cells with KDR receptor; **B**: Shows negative-stained human hepatoma cells without KDR receptor.

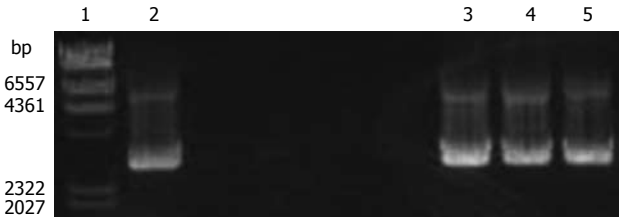

Figure 5 Identification of recombinant pEGFP-KDR-TK by restriction enzyme digestion (*Sal*Ⅰ/*Xho*Ⅰ) and electrophoresis. Lane 1: DNA markers (λDNA/*Hind*Ⅲ); Lane 2-5: pEGFP-KDR-TK was digested with *Sal*Ⅰ/ *Xho*Ⅰ.

revealed that the cells in group A had no obvious coloration (Figure 6A). Fluorescent staining was observed in few cells (1.7%) in group B (stimulated by ultrasound only, Figure 6B), whereas the HUVECs transfected by the pEGFP-KDR-TK in group C (stimulated by both SonoVue and ultrasound exposure) showed clear green fluorescence within 20.3% HUVECs indicating EGFP expression (Figure 6C and D). EGFP and TK were co-expressed in HUVECs by microbubble-enhanced ultrasound irradiation, followed by selection of permanent transfectants in the presence of G418. The G418-resistant cells were named KDR-TK/VEC.

Cell survival rate

The percentage of surviving cells was examined by trypan blue dye exclusion after ultrasound exposure. The cell survival rates were 94.0% ± 1.1% in the group A, 93.5% ± 0.9% in the group A and 90.2% ± 0.9% in group B (Figure 7).

Sensitivity of genetically modified cells to GCV

We selected GCV as a prodrug and examined the sensitivity of KDR-TK/VEC, HUVEC, and HepG2 to GCV. As shown in Figure 8, HepG2 cells were found to be resistant to GCV, and the value of IC 50, defined as the dose required for 50% cytotoxicity, was > 100 μg/mL (the highest concentration tested). HUVECs exhibited a higher sensitivity to GCV in comparison to HepG2, when the concentration tested was above 50 μg/mL, the IC 50 value for HUVECs was still more than 100 μg/mL. Conversely, KDR-TK/VEC cells were susceptible to GCV in a dose-dependent manner and exhibited approximately 75% suppression at a GCV concentration of 100 μg/mL, with the IC 50 value being approximately 10 μg/mL. KDR-TK/VEC cells also showed significantly higher sensitivity to GCV compared to non-transfected HUVECs, resulting in 2 to 5-fold higher suppression to GCV.

DISCUSSION

Acquired drug resistance is a major problem in cancer therapy. Many deaths from cancer may follow the development of resistance to chemotherapy. The emergence of resistance depends in part on the genetic instability, heterogeneity, and high mutational rate of tumor cells[1]. In contrast, endothelial cells have relative genomic stability. Therefore, antiangiogenic therapy targeting tumoral endothelial cells may avoid the problem

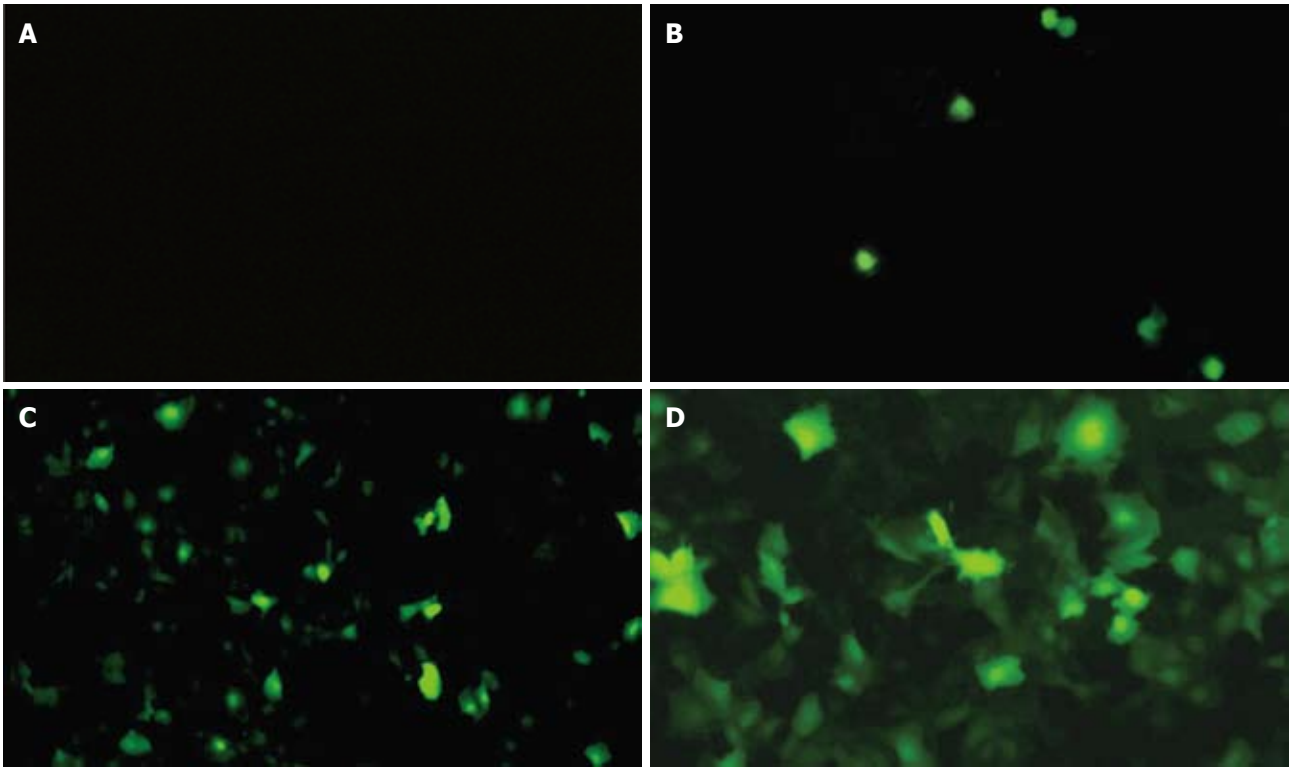

Figure 6 Fluorescent microscopy showed EGFP expression 48 h after pEGFP-KDR-TK transfection. **A**: Shows no obvious EGFP expression in cells without microbubbles and ultrasound (× 200); **B**: 1.7% cells exposed to ultrasound only shows EGFP expression (× 200); **C, D**: Significant increase in EGFP expression in HUVECs exposed to both the ultrasound and SonoVue (**C**: × 200, **D**: × 400). About 20.3% HUVECs had EGFP expression.

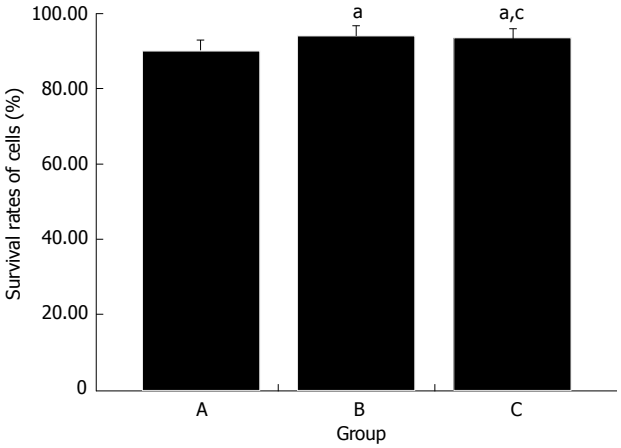

Figure 7 HUVECs survival rate examined by trypan blue dye exclusion after transfection. Values are expressed as the average percentage of viable cells, $n = 5$ from 3 independent experiments. $^{a}P < 0.05$ *vs* group A; $^{c}P > 0.05$ *vs* group B.

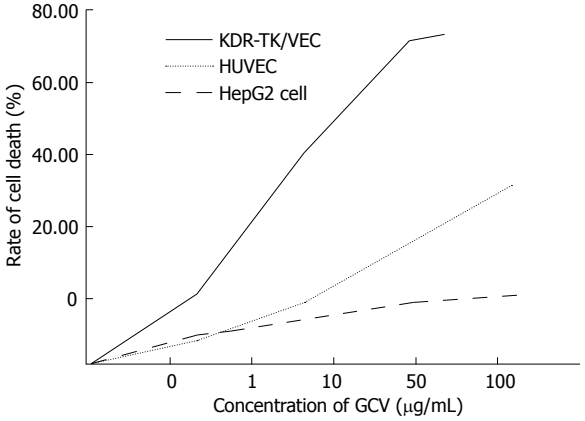

Figure 8 *In vitro* cytotoxic effect of GCV on KDR-TK transfected HUVECs (KDR-TK/VEC), non-transfected HUVECs, and HepG2 cells. KDR-TK/VECs, non-transfected HUVECs, and HepG2 cells were cultured with various concentrations of GCV for 3 d and viability was estimated by MTT assay.

of drug resistance. In addition, the growth and persistence of solid tumors and their metastases are angiogenesis dependent[11], thus angiogenesis provides a target for therapeutic approaches to solid tumor, and the inhibition of angiogenesis is very promising.

Most angiogenic inhibitors are biologically active peptides. It is difficult to express proteins persistently and stably *in vivo*, and the complexity and time requirement for protein purification make their clinical application infeasible[12]. Gene therapy is an attractive means of delivering these antiangiogenic agents to tumor site, and targeting the tumor vessels could avoid potential side effects. Although several previous studies have shown the effectiveness of TK targeting in endothelium as antiangiogenic strategy, few of them have reported with suicide gene therapy by targeting TK in the vasculature and not in the human tumor cells[13,14]. Ozalki *et al* have shown that vWF2 promoter combined with HSV-TK suicide gene can exert an endothelial-preferential killing effect on the transduced HUVECs *in vitro*[15]. Von Willebrand factor (vWF) is a blood glycoprotein which is excreted by endothelium and megakarcytes, and the elevation of vWF

is not only found in cancer, but also in other cardiovascular diseases[16]. Consequently, tumor vessels are not accessible targets for antiangiogenic gene therapy, when the vWF2 promoter and HSV-TK/prodrug are administrated systemically. Compared with vWF, KDR has a major role in tumor angiogenesis, and in adulthood, KDR appears mostly restricted to vascular endothelial/lymph endothelial cells[2]. Because of the overexpression of KDR in tumors vasculature, KDR-promoter did not yield high TK protein expression in normal vasculature, whereas in endothelial cells within tumors. Dancer and colleagues indicated that expression of TK driven by an endothelial-specific promoter can cause significant decrease in microvessel density and inhibit tumor growth[17].

The suicide gene used in this study has been widely used in gene therapy of animal tumors, and it encodes an enzyme that can effectively phosphorylate the antiviral nucleoside analogue ganciclovir (GCV) to its monophosphase form, which is further phosphorylated by cellular kinases to DNA polymerase inhibitors[18]. Apoptosis has been demonstrated as the major mechanism of cell death caused by the HSV-TK/GCV system in different cells[19,20]. The present study demonstrated that KDR promoter and the suicide gene/prodrug system showed a targeted killing effect on transfected HUVECs. The sensitivity of KDR-TK-transfected HUVECs to GCV was found to be about 2 to 5-fold higher when compared to non-transfected HUVEC and HepG2 cells.

Viral delivery systems have been developed as highly efficient carriers for gene delivery to a variety of tissues. However, viral gene carriers have some major disadvantages such as possible immune response, severe inflammation reactions, the risk for insertional mutagenesis, and interference with expression of cellular genes[21,22]. Recently, ultrasound together with microbubble has been recognized as a useful means for controlled delivery of drugs. Although the mechanism of enhanced transfection efficiency by ultrasound is not yet fully understood, cavitation is an important event that increase capillary permeability and produce transient nanopores in cell membranes. SonoVue as well as other microbubble can be rapidly destroyed by the sonication, and the threshold of cavitation decreases after addition of microbubbles. In this study, we demonstrate that widely used diagnostic ultrasound and microbubble-based ultrasound contrast agent can also be applied for the delivery of DNA. The expression of EGFP in HUVECs was just 1.7% in the group only treated with ultrasound, whereas the expression rate significantly increased to 20.3% ($P < 0.001$) when treated with ultrasound and SonoVue. In contrast, therapeutic or high-intensity focused ultrasound has been used for most of the described ultrasound-facilitated gene delivery and gene transfection experiments[23-25]. An advantage of using diagnostic ultrasound instead of therapeutic ultrasound allows visualization of the site of interest and microbubble possible by using conventional ultrasound imaging systems. In this study, more than 90% of the cells treated with ultrasound and SonoVue were viable. Though the longer exposure time used in our experiment, the cell viability in this study is also higher than that using therapeutic ultrasound[26]. Although the transient transfection rate of KDR-TK in this study is relatively low compared with other studies using viral vectors, we believe this disadvantage can be offset by lower acoustic frequency, higher ultrasound output, or longer exposure time.

Our observation also suggested that non-transfected HUVECs have more sensitivity to GCV, compared with non-transfected HepG2 cells. The mechanism was not yet understood; we speculated that the lower sensitivity to GCV of non-transfected HepG2 cells might partly involve the deficiency of TK in tumor cells[27].

In summary, our study revealed that, on the basis of the KDR-mediated invasion, TK/GCV can accomplish a selective killing effect on HUVECs *in vitro*. KDR promoter and the suicide gene/prodrug system mediated by diagnostic ultrasound combined with microbubble may present a novel and attractive approach for gene therapy to target tumor vessels.

COMMENTS

Background

Tumor angiogenesis plays a critical role in the development and progression of the solid tumor. Targeting angiogenesis represents a new strategy for antitumor therapies. Ultrasound exposure in combination with microbubble is a new non-viral delivery system.

Research frontiers

Gene therapy to target tumor vessels can cause a significant decrease in microvessel density and inhibit tumor growth. Just as therapeutic ultrasound, diagnostic ultrasound in conjunction with microbubbles can also be a useful gene delivery system.

Innovations and breakthroughs

Diagnostic ultrasound-facilitated gene delivery on targeting the vascular endothelial cells has not been used. This study established a specific promoter in order to target gene expression in human umbilical vein endothelial cells and demonstrated that diagnostic ultrasound combined with microbubble can be a useful gene delivery system.

Applications

This study showed KDR promoter and the suicide gene/prodrug system mediated by diagnostic ultrasound can significantly kill human umbilical vein endothelial cells. This may present a novel and attractive approach to target gene therapy on tumor vessel.

Terminology

Microbubble: microscopically gas bubbles encapsulated by an elastic shell, can be used in diagnostic and therapeutic applications.

Peer review

This study is well-designed and very interesting, and the manuscript is well-written.

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CLINICAL RESEARCH

Clear cell colitis: A form of microscopic colitis in children

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Abstract

AIM: To describe a new clinical and pathological subtype of microscopic colitis in children.

METHODS: A selected group of children with abdominal pain, constipation and/or diarrhoea showing discrete or no macroscopic abnormalities on endoscopy was described.

RESULTS: Multiple biopsies of colon showed large mononuclear clear cells in lamina propria of mucous membrane provided that good quality histological sections were performed and observed under a higher magnification. Otherwise, they could be misinterpreted as artefacts. Their presence in routine histology might suggest a systemic storage disease (Whipple's disease), and neuronal intestine dysplasia. Using immunohistochemical staining and electron microscopy we confirmed their origin from CD68 positive mononuclear macrophages.

CONCLUSION: The presence of large clear cells is a constant microscopic feature. Failure of transient large bowel stationary macrophages plays a role in the pathogenesis of this benign microscopic clear cell colitis, sometimes coexisting with allergy.

Key words: Microscopic colitis; Clear cell colitis; Diarrhoea; Constipation; Abdominal pain; Children; Allergy; Endoscopy

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INTRODUCTION

Microscopic colitis constitutes a group of diseases with unknown aetiology and causes neither destruction nor rebuilding of large bowel mucosa. Microscopic colitis is rarely recognized in children[1,2] and may be misinterpreted as inflammatory bowel disease[3]. Various forms of microscopic colitis have been identified in adults[4-11], including lymphocytic, collagenous, multinucleated giant cells. No description of microscopic colitis with the presence of giant mononuclear clear cells in lamina propria in children is available. Several staining methods can be used to identify the phenotype of these cells. This is probably not a new disease but it is frequently overlooked due to routine use of low magnifications and poor quality of histological staining for paraffin-embedded materials. The presence of large amounts of these cells in our series of biopsies is characteristic in children with chronic abdominal pain, diarrhoea or constipation. In three cases, the preliminary diagnosis was Whipple's disease, neuronal dysplasia and even Gaucher disease. Definitive diagnosis of clear cell colitis (CCC) was made by immunohistochemistry methods and electron microscopy. A higher diagnostic profile is possible for identification of various forms of microscopic colitis.

Clinical, endoscopic and morphological analyses of CCC were described in series of large bowel biopsies. Characteristic giant mononuclear clear cells were found in sections by routine hematoxylin and eosin staining. Magnifications larger than 200 × must be used and high quality paraffin histology sections must be obtained to detect the cells. Otherwise, CCC may be overlooked. Using immunocytochemical and electron microscopic procedure, these clear cells were identified as activated macrophages which were CD68 positive and contained multiple vacuoles with characteristic amorphic homogenous lipid like material. The cells were found only in various parts of large intestine but not in small intestine or gastric or duodenal mucosa. Ten-year observation of patients in whom repeated biopsies were performed, allowed us to conclude that CCC is a form of microscopic colitis with chronic persistent benign course and persistent constant histological pattern. During the last 3 years, 20 patients underwent a prolonged (6-mo to a 1-year) therapy with probiotics. Clinical symptoms improved and the number of clear cells decreased in microscopic examinations. In 6 cases the cells disappeared

completely from mucous membranes. The remaining patients received a continuous long-term follow-up.

MATERIALS AND METHODS

Patients

Clinical and morphological study on CCC involved 81 children aged between 17 mo and 17 years selected out of 5000 large bowel biopsies taken from children who underwent treatment in paediatric centres between 1994 and 2005. The group included 63% boys and 18% girls. The majority of patients (68%) were inhabitants of urban areas, whilst the rest (32%) lived in the countryside. Colon dysfunction was the reason for diagnostic investigation. The majority of patients reported recurring abdominal pain, diarrhoea, constipation, and poor appetite. Their stools were of various consistency and contained mucus. Each child had at least two endoscopic examinations of the final part of the alimentary tract (23 had rectoscopy and 58 had colonoscopy).

Clinical, laboratory and endoscopic evaluations were performed carefully. Clinical diagnosis was established based on the number of stools per week, the presence of mucus, general comfort of a child, intensity of stomach aches, the presence of fever, ESR, haemoglobin concentration, state of nutrition and the presence of parenteral manifestations. Evaluating the rate for changes in endoscopic image, we took into consideration vascular drawing, granulation of mucous membrane.

Bacteriology and virology

The presence of intestinal pathogens (Salmonella, Shigella, Campylobacter, Escherichia coli, Yersinia, Clostridium difficile) and Giardia infection was verified. Diagnostic research for the presence of enteroviruses was performed in the Center of Virology of the National Institute of Hygiene on the Hep-2 and RD cell lines supplied by the WHO. The presence of retrovirus was checked in order to obtain a full virological evaluation. Tests of the latex reaction with Slidex Rota-kit 2 set and ELISA serological examination (using the DAKO rabbit anti-rotavirus (Human) reactants recommended by the WHO) were performed.

Morphological examination

During colonoscopy 3 specimens were collected from each of the 58 patients. Colonoscopy was performed 2 times and a total number of 248 biopsies were examined. A comparison of long-term changes occurring in the course of CCC was possible. Rectoscopy was performed 3 times in 23 cases. A total number of 317 biopsies were collected. Paraffin-embedded specimens were cut into serial sections which were stained with hematoxyllin and eosin. In 81 cases, 162 immunological staining procedures were performed with 16 monoclonal antibodies (to CD3, CD4, CD22, CD34, CD31, CD68, PCNA, Ki67, chromogranin, synaptophysin, neurofilament, actin, p53, GFAP, IL-beta, TNF alpha) and 4 histological staining procedures were performed for mucicarmin, PAS, PAS diastase. A total of 2916 staining procedures were reviewed (Table 1). The phenotypes of clear cells were identified.

Table 1 Expression of immunocytochemical markers in the CCC of the colon submucosa including some histological staining

Marker	Clear cells
CD3 (T cell)	Negative
CD4 (T cell)	Negative
CD22 (B cell)	Negative
CD34 (precursor cell)	Negative
CD31 (Endothelial)	Negative
CD68 (Macrophages)	+++ Strongly positive
PCNA-proliferation antigen	Negative[2]
Ki67 mitotic activity	Negative[1]
Chromogranin	Negative
Synaptophysin	Negative
Naurofilament	Negative
Actin	Negative
P53 mutations	Negative
GFAP glial marker	Negative
TNF alpha	Negative
IL2	Negative
Mucicarmin	Negative
PAS	Strong positive
PAS diastase	Digested
Osmium tetraoxide	Positive

[1]1-3 per crypt; [2]Positive in the cryptic epithelium.

In 10 cases, examination under an electron microscope (Jeol 100CX) was also performed. Preparations of intestinal mucosal biopsies were fixed in 4% glutaraldehyde in cacodylate buffer and then in OsO_4, followed by a typical Epon procedure. Ultra-thin sections were counterstained with lead citrate and uranyl acetate.

RESULTS

Clinical observation

The incidence of CCC as a form of microscopic colitis (MC) was 2% in children with abdominal pain, diarrhoea and constipation. ESR < 100 mm and MHC/hemoglobin concentration > 8.5 g/dL were found in all children. During the 10 years of observation, no clinical or endoscopic changes in IBD were noticed. Before analysis, children did not take any medicines or drugs that could caused pathological changes in mucous membrane of the large intestine. No treatment was administered. In some cases, a pre-treatment with salazopyrine for a few months did not result in regression of endoscopic or histological changes. Clinical symptoms were improved when probiotics was administered in 20 patients. Clear cells disappeared on mucosal membranes in the second biopsy from 6 patients. The remaining patients remained on treatment with Saccharomyces boulardi.

The dominant symptoms in both girls and boys included recurrent abdominal pain, stiff and (or) loose stools, even diarrhoeal stools with mucus additive. Some patients were admitted to gastroenterology hospital departments mainly because of suspected IBD. Some showed atopic skin inflammation and (or) recurring upper respiratory system inflammation. Five children with bronchial asthma were treated. Laboratory peripheral blood and urine tests and microbiologically parasitological stool tests did not reveal any pathological changes.

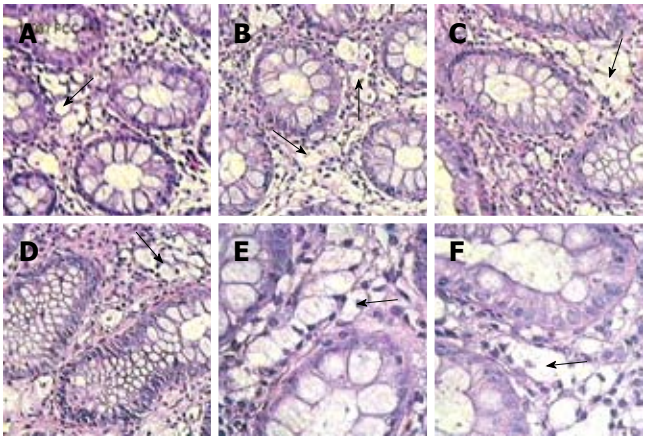

Figure 1 Microscopic features of CCC (**A-F**) (HE, × 320).

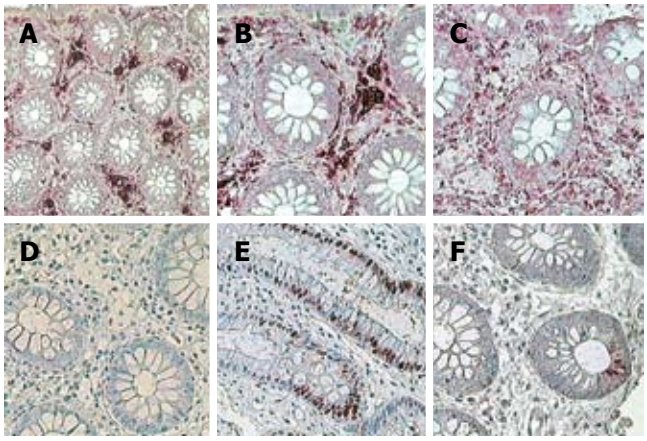

Figure 2 Immunocytochemistry of CCC using monoclonal antibodies and APPAP method showing positive expression of mononuclear phagocytic cell line (**A** and **B**), negative T3 and T4 lymphocytes (**C**), negative neurofilament (**D**), Normal PCNA expression in cryptal epithelium (**E**), and normal mitotic activity of positive CD67 (**F**) (× 320).

Abdominal pain occurred in all cases. The pain was mild or moderate. Less than 18 stools per week were counted in 7 children, 18-35 stools in 59 cases and 36-60 stools per week in the remaining 15 cases, constipation in 19 cases. A subjective evaluation of general feeling of children was good. Parenteral symptoms were noted in 19 out of 81 children, mainly upper respiratory tract infection. Forty-three children had ESR less than 40 mm, 20 had 50-100 mm ESR after 1 h. Haemoglobin concentration was above 10 g/dL in all cases. The state of nutrition (Cole and Stanfield) was over 85% in 27 cases, 80%-85% in 46 cases and under 80% in 8 cases. Bacteriological and virological examination of stools gave negative results.

Endoscopic examination

Endoscopic examinations were done with a stiff rectoscope. Eighty-two percent of the patients had also a supplementary colonoscopy. Macroscopically, all patients appeared to have normal mucosa or granulated mucosa, with sharply outlined vascularization and a thin layer of mucus in the rectum and sigmoid colon. In children with CCC undergoing colonoscopy, characteristic fine granulation and increased sharpness of vascular outline in the descending and transverse colon were found and changes in the ascending colon were also observed in 6 children. Susceptibility to bleeding was increased in 9 children. Three to six biopsies were taken during each endoscopic examination of the large intestine, observation was repeated 3 times during the 5-10 years. One specimen was collected at each rectoscopy during the 5-10 years of observation. In the consecutive rectoscopic and endoscopic examinations, the mucosal pattern of CCC was not changed. Persisting granulation and increased sharpness of the vascular pattern were revealed. Endoscopic examination of the upper part of alimentary tract gave normal results.

Histology

Microscopic images of routinely HE-stained sections were analysed. There were large clear cells in the lamina propria of mucosa, appearing either separately or in groups (Figure 1). The cells occupied the entire lamina propria from the covering epithelium to the basis of cryptal glands and the level of muscularis mucosa. Distribution of the clear cells allowed identification of 3 degrees of clear cell density: grade 1-single cells at various levels of lamina propria, grade 2-the presence of aggregates of 3-10 cells, grade 3-clear cells uniformly distributed in lamina propria. The presence of clear cells in patients was permanently observed during the 5-10 years. Clear cells showed a positive reaction with neutral mucopolisacharides in the PAS method with periodic acid and the Shiff's reagent (PAS- positive reaction), and negative reaction after digestion with diastase. Staining with mucicarmine was negative. No intraepithelial lymphocytosis was noted and the base membrane was normal. About 243 serial sections stained with HE were reviewed microscopically.

Immunocytochemistry

Clear cells presented a strong positive expression in the reaction with the CD68 antibody (Figure 2A-F, Table 1). Sixteen immunocytochemical reactions were performed 3 times in 81 patients during the period of observation in order to identify the phenotype of large clear cells.

Electron microscopy

In contrast to primary metabolic diseases, electron microscopic examination of clear cells revealed the presence of single nuclei with nucleoli and multiple secondary phagosomes with accumulation of homogenous osmophilic materials (Figure 3). Other structures of the large intestine mucosa were normal. The material for electron microscopic analysis was taken from 10 patients with CCC confirmed previously by histological examination.

DISCUSSION

Microscopic colitis is the term suggested by some authors for abdominal pain, alternating chronic diarrhoea and constipation and endoscopic image close to normal[4-8]. A characteristic microscopic picture of adults is in the form of collagenous and lymphocytic colitis[9-11]. The cause for microscopic colitis is unknown. Several concepts are

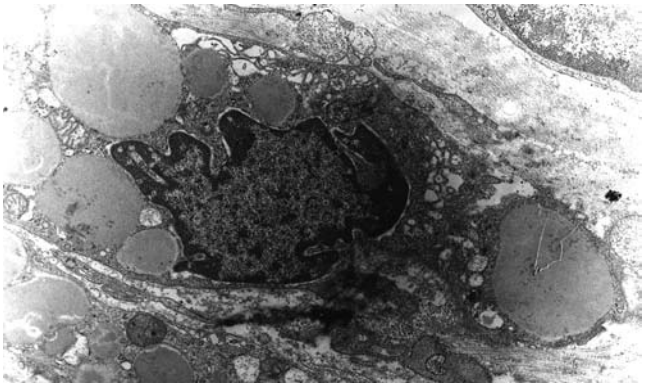

Figure 3 Electron microscopy of clear cells. Giant clear macrophages contain multiple vesicles varying in size filled with homogenous osmiophylic materials (secondary phagosomes) without any structural components in their cytoplasm (OsO_4 staining, × 24 000).

published, including abuse of medications[12], increased concentration of antigens and cytokine expression[13], auto-antibodies in blood[14], prostaglandin level[15]. MC may overlap with indeterminate inflammatory bowel disease[16], but in contrast to CCC, TNF alpha expression may be positive in silent inflammatory bowel disease[17].

CCC in children as a form of microscopic colitis is characterized by the presence of various large mononuclear clear cells in lamina propria. The cells are easily recognized when high magnification microscopy is used and section staining of microscopic slides is good. CCC does not manifest as destruction or reconstruction of glands. In the present study, basic membrane was not damaged, no lymphocytic or calagenous colitis or other forms of microscopic colitis described elsewhere were observed. Clear cells are mononuclear in contrast to other microscopic colitis, where there are giant multinucleated cells[18]. Clear cells described here are different from other muciphages[19]. On the other hand, Hamrock[20] has reported clear cells in patients with AIDS. The cells look like Whipple cells but they can be overloaded with inclusion material (Mycobacterium). Some authors suggested that rectal biopsy should be performed in cases of irritable bowel disease since they may be classified as MC[21].

Differential diagnosis should also consider neuronal dysplasia[22] of intestine, in which large, bright ganglion cells can be very similar to the giant clear cells in CCC. The reaction with neurofilaments, synaptophisine or (and) chromogranine is helpful. Some authors suggest that Salmonella typhimurium type can mobilize phagocytosis in macrophages of the large intestine but we have not found bacterial or viral particles in the phagosomes of clear cells. Different macrophages described in Erdheim-Chester disease exhibit neoplastic characteristics[23]. Possible persistent changes (e.g. after the rotaviral diarrhoea) and the presence of enteroviruses in clear foamy cells can be ruled out on the ground of electron microscopy and negative results of blood and stool test.

Xanthelasma of rectum containing sialomucin, is a different disease rather than CCC. However, patients with similar symptoms suffer from diarrhoea, constipation or haemorrhages. The presence of these symptoms is a result of mechanical injuries of mucosa in the course of chronic constipation. In contrast to muciphages, clear cells were negative for mucicarmine staining in our study.

The mononuclear phagocytic system consists of macrophages widely distributed in the body as circulating or stationary cells. Macrophages of large bowel mucosa belong to stationary cells and have various functions. In some special unknown conditions, they can transform into large clear cells and exhibit transient malfunction with accumulation of lipid material. It should be emphasized that one third of our patients had allergic extra intestinal manifestations. Finally, application of probiotics (Saccharomyces bulardi) has a beneficial effect in children with CCC. Confirmation of this finding requires further long-term observation.

COMMENTS

Background

Clear cell colitis (CCC) is a description of a new form of microscopic colitis in children. This is a benign disease recognized in some children showing symptoms of recurrent alternate diarrhoea and constipation. Patents are directed by physicians to gastroenterology units of district hospitals. Endoscopic examination shows normal or minor changes which do not constitute a qualification for further therapy. Pathologists using usually low magnifications can overlook the presence of large clear cells in lamina propria, and describe mucosa as normal. The lesions are seldom found in academic gastroenterological centers, where selected patients with more serious symptoms are ordered.

Research frontiers

Detection of giant clear cells is very important if no other lesions in large intestine musosa or other organs are found. Otherwise, false diagnosis of primary metabolic disease or neuronal displasia can be made. Before application of immunocytochemical tests, electron microscopy is useful in differential diagnosis, even microscopic examinations of conjunctiva and liver are recommended.

Innovations and breakthroughs

The reason why giant clear cells are aggregated is not clear. The presence of so called muciphages, described previously as a phenomenon in adults can be excluded on the basis of negative mucicarmine staining and different electron microscopic pictures.

Applications

As the children included in the study showed symptoms of allergy, and even asthma, selective activation of lipid and protein storing macrophages is considered to be associated with mucosa auto-immunological response, probably under influence of antigenemia occurring in children suffering from constipation. It should be emphasized that the presence of giant macrophages is chronic and the clinical course is stable. In the present paper, no therapy was needed at the end of a long term clinical and morphological observation period. During the last two years, some improvement was achieved after application of probiotics. Usage of probiotics (among others of Lactobacilus casei) in all children is the aim in future studies.

Terminology

Clear cell colitis is a form of microscopic colitis in children with accumulation of giant clear cells.

Peer review

This manuscript appears to be reasonably well written. The authors described a new form of CCC in children and presented evidence to support their contention.

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RAPID COMMUNICATION

Elevated nitric oxide and 3',5' cyclic guanosine monophosphate levels in patients with alcoholic cirrhosis

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Abstract

AIM: To evaluate whether serum levels of nitric oxide ($NO^{\bullet}$) and plasma levels of cyclic guanosine monophosphate (cGMP) and total glutathione (GSH) are altered in patients with alcoholic cirrhosis and to examine their correlation with the severity of liver disease.

METHODS: Twenty-six patients with alcoholic liver cirrhosis were studied. Serum levels of $NO^{\bullet}$ and plasma levels of cGMP and GSH were measured in 7 patients with compensated alcoholic cirrhosis (Child-Pugh A) and 19 patients with advanced cirrhosis (Child-Pugh B and C). The model for end-stage liver disease (MELD) score was evaluated. Sixteen healthy volunteers served as controls. Liver enzymes and creatinine levels were also tested.

RESULTS: $NO^{\bullet}$ and cGMP levels were higher in patients with Child-Pugh B and C cirrhosis than in Child-Pugh A cirrhosis or controls ($NO^{\bullet}$: 21.70 ± 8.07 *vs* 11.70 ± 2.74; 21.70 ± 8.07 *vs* 7.26 ± 2.47 μmol/L, respectively; $P < 0.001$) and (cGMP: 20.12 ± 6.62 *vs* 10.14 ± 2.78; 20.12 ± 6.62 *vs* 4.95 ± 1.21 pmol/L, respectively; $P < 0.001$). Total glutathione levels were lower in patients with Child-Pugh B and C cirrhosis than in patients with Child-Pugh A cirrhosis or controls (16.04 ± 6.06 *vs* 23.01 ± 4.38 or 16.04 ± 6.06 *vs* 66.57 ± 26.23 μmol/L, respectively; $P < 0.001$). There was a significant correlation between $NO^{\bullet}$ and cGMP levels in all patients with alcoholic cirrhosis. A significant negative correlation between reduced glutathione/glutathione disulfide and the MELD score was found in all cirrhotic patients.

CONCLUSION: Our results suggest a role for oxidative stress in alcoholic liver cirrhosis, which is more significant in decompensated patients with higher levels of $NO^{\bullet}$ and cGMP and lower GSH levels than in compensated and control patients. Altered mediator levels in decompensated patients may influence the hemodynamic changes in and progression of liver disease.

Key words: Oxidative stress; Nitric oxide; Alcoholic cirrhosis; Fibrosis; Model for end-stage liver disease

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INTRODUCTION

Increased production of free radicals has been implicated in several conditions, such as liver damage and fibrosis[1]. Free radicals can damage cellular macromolecules and, therefore, may participate in hepatocellular injury when produced in excess. Endotoxins or cytokines induce macrophages, neutrophils and vascular smooth muscle cells to express a calcium-independent isoform of nitric oxide synthase (NOS). Nitric oxide ($NO^{\bullet}$) is a messenger molecule that functions as a vasodilator and accounts for the biological activity of endothelium-derived relaxing factor (EDRF)[2-4]. The three isoforms of nitric oxide synthase (NOS), the enzyme responsible for the generation of $NO^{\bullet}$, are all found in the liver[4].

Production of $NO^{\bullet}$ activates the soluble guanylate cyclase (sGC) the enzyme with a prosthetic heme group by binding to the ferrous heme iron (Fe^{2+}), resulting in the formation of cyclic guanosine 3'-5' monophosphate (cGMP) from guanosine 5' triphosphate (GTP). This process occurs in both endothelial and vascular smooth muscle cells[5,6]. The increase in concentrations of the second messenger cGMP is stimulated by activation of dependent protein-kinase G (pKG) and confers signalling capacity to the free radical *via* $NO^{\bullet}$-sGC-cGMP-PKG pathway, which in turns leads to the relaxation or dilation of vascular smooth muscle cells (VSMCs), and, subsequently, to inflammation and tissue damage mediated by reactive oxygen species (ROS; for example, $O_2^{\bullet}$ and $OH^{\bullet}$).

$NO^{\bullet}$ promotes tissue toxicity when it reacts with the superoxide anion radical ($O_2^{\bullet}$), which leads to formation of peroxinitrite anion ($ONOO^-$) and hydroxyl radical ($OH^{\bullet}$)[6-9] ($NO^{\bullet} + O_2^{\bullet} \rightarrow ONOO^- + OH^{\bullet}$). Both of these products $OH^{\bullet}$ and $ONOO^-$ are aggressive free radicals and powerful oxidants that can cause membrane lipid peroxidation[5-7,10]. Antioxidants are substances that help to reduce the severity of oxidant stress either by contributing to the formation of a less active radical or by quenching the reaction of $NO^{\bullet}$ which produce peroxinitrite anion and hydroxyl radical. Glutathione (GSH) and its related enzymes are protective effects against oxidative damage[11]. One consequence of GSH depletion, as seen during redox stress, would be enhanced peroxinitrite formation. Glutathione may also be involved in the detoxification of peroxinitrite. Both $O_2^{\bullet}$ and $OH^{\bullet}$ increase production of ROS and GSH depletion, which leads to the abnormal breakdown of fat molecules (that is, lipid peroxidation). This complex process results in the formation of toxic compounds and may contribute to the pathogenesis of alcoholic liver injury.

The aim of this study was to investigate whether serum levels of $NO^{\bullet}$ and 3',5'cyclic guanosine monophosphate (cGMP), and plasma levels of thiol-reduced glutathione (GSHr) and glutathione disulfide (GSSG), are altered in patients with alcoholic liver cirrhosis, and to assess their possible correlation with the severity of liver disease, measured by the Child-Pugh and MELD scores.

MATERIALS AND METHODS

Patients

This study was performed in 26 patients with alcoholic cirrhosis (19 men and 7 women, mean age 56.00 ± 12 years) admitted to the Liver Unit and Division of Gastroenterology, Santa Maria Hospital for evaluation. All patients had a history of alcoholic consumption greater than 80 g/d for at least 5 years. The diagnosis of liver cirrhosis was based on clinical, biochemical and ultrasonographic findings or histological criteria. The patients were classified as having compensated (n = 7) or decompensated liver cirrhosis (n = 19). According to the Child-Pugh scores, all compensated patients belonged to class A. The decompensated group consisted of 8 patients in class C and 11 patients in class B with a Child-Pugh score above 8. At the time of the study no Child A patients showed clinical features of decompensated liver cirrhosis (ascites, edema, encephalopathy or recent portal hypertension associated gastrointestinal bleeding). All patients graded Child-Pugh B-C had moderate to severe ascites without evidence of infection. The main clinical and laboratory findings are shown in Table 1. There was no evidence of hepatocellular carcinoma, cardiac, renal or respiratory failure in the patients studied, and none of them were administered vasoactive drugs or antioxidants during the study.

The control group consisted of 16 healthy subjects (10 men and 6 women, mean age 38 ± 18 years) with normal laboratory findings. Informed consent to participate in the study was obtained from each patient.

Methods

Peripheral venous blood from fasted healthy volunteers and fasted cirrhotic patients was collected in separate tubes, one containing the anticoagulant ethylenediamine tetraacetic disodium (Na_2EDTA) and the other without serum anticoagulant. The blood was allowed to clot for 30 min at 25℃, centrifuged at 2000 × g for 15 min at room temperature, and the serum was then separated and aliquoted into tubes for storage. To obtain plasma samples, the blood was centrifuged immediately at 2000 × g for 10 min at 4℃, and then aliquoted into tubes. The tubes were then stored frozen at -80℃ until they were used to study different biomarkers.

Analytical procedures

Determination of NOx levels: The level of serum $NO^{\bullet}$ was obtained indirectly by measuring the levels of its metabolites nitrate (NO_3^-) and nitrite (NO_2^-). The levels of the final products of $NO^{\bullet}$ oxidation (NO_3^- and NO_2^-) were assessed using the Griess reaction. NO_3^- was enzymatically converted into NO_2^- in the presence of reduced nicotinamide adenine dinucleotide phosphate (NADPH), flavine adenine dinucleotide (FAD) and nitrate reductase. Nitrite reacts with Griess reagent (1 g/L sulphanilamide 0.1 g/L N-(1-naphtyl)-ethylenediamine and 5 g/L phosphoric acid) to give a red-violet diazo dye. The determination of these two elements (NO_3^- and NO_2^-) was measured in triplicate in a microplate reader on the basis of absorbance in the visible range at 540 nm. The concentrations of NO_2^- were determined from a calibration curve obtained using standard NO_2^- solutions. The mean values of the measurements were taken as the final result. The limit of detection of the method is 0.28 μmol/L for nitrate and 0.32 μmol/L for nitrite.

Determination of cGMP: The plasma concentration of cGMP was determined in duplicate by radioimmunoassay (RIA) using a commercial kit according to the manufacturer's protocol (IBL Hamburg, Germany).

Determination of GSHt, GSSG and GSHr: For 1 mL of plasma was used (50 μL of 5% sulphosalicylic acid) to prevent spontaneous oxidation of GSH. After centrifugation at 2000 × g for 10 min, 100 μL of the supernatant was removed for assay of GSH.

The sulfhydryl group of GSH reacts with Ellman's reagent, 5,5'-dithio-bis-(2-nitrobenzoic acid) (DTNB) to produce yellow 5-thio-2-nitrobenzoic acid (TNB). The mixed disulfide, GS-TNB (between GSH and TNB) is subsequently reduced by glutathione reductase and the reduced form of β-nicotinamide adenine dinucleotide phosphate (NADPH), releasing a second TNB molecule and recycling the GSH. The rate of TNB production is directly proportional to this recycling reaction, which, in turn, is directly proportional to the concentration of GSH in the sample. GSH is easily oxidized to GSSG by glutathione reductase (GR), according to the following formula:

$GSSG + NAD(P)H \rightarrow GSH + NADP^+$.

Table 1 Laboratory findings in the patients with alcoholic cirrhosis

	Child-Pugh A (n = 7)	Child-Pugh B (n = 11)	Child-Pugh C (n = 8)	Reference values
LDH U/L	403.71 ± 115.32	453.80 ± 126.10	369.25 ± 151.89	240-480
AST U/L	59.57 ± 51.56	109.20 ± 97	88.88 ± 97.81	0-31
ALT U/L	40.86 ± 27.39	58.50 ± 47.58	43.63 ± 47.43	0-31
γGT U/L	271 ± 190.32	280.12 ± 237.87	196.75 ± 160.35	5-36
AP U/L	134.57 ± 77.62	99 ± 24.61	103.88 ± 44.25	35-104
Leukocytes/μL	5784.29 ± 1677.52	7429 ± 3987.49	5731.25 ± 2187.02	4000-11000
Neutrophils/μL	3289.14 ± 1987.81	4424 ± 3042.67	3555 ± 1781.55	1900-7500
Creatinine mg/dL	1.17 ± 0.47	1.41 ± 0.54	1.91 ± 0.61	0.7-1.1
Bilirubin mg/dL	1.06 ± 0.10	1.56 ± 0.88	2.45 ± 1.37	0.1-1.1
INR	1.02 ± 0.10	1.54 ± 0.71	2.59 ± 0.99	0.8-1.1
MELD	8.29 ± 2.75	16.10 ± 6.77	23.38 ± 7.09	6-8

LDH: Lactate dehydrogenase; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; γGT: Gamma glutamyltransferase; AP: Alkaline phosphatase; INR: International normalised ratio of prothrombin time. The results are expressed as means ± SD and range.

Table 2 Levels of $NO^{\bullet}$, cGMP, GSHt, GSSG, GSHr, MELD and GSHr/GSSG among the controls and patients with alcoholic liver cirrhosis (Child-Pugh A, B and C) studied

Biomarkers	Controls (n = 16)	Child-Pugh A (n = 7)	Child-Pugh B and C (n = 19)	P values
NO_3^- μmol/L	4.16 ± 1.36; (4.21)	6.73 ± 1.32; (6.89)	12.23 ± 4.29; (11.79)	$^b P < 0.001$; $^a P < 0.05$
NO_2^- μmol/L	3.10 ± 1.27; (2.90)	4.97 ± 1.5; (5.76)	9.46 ± 3.93; (7.80)	$^b P < 0.001$; $^a P < 0.05$
$NO^{\bullet}$ (NO_3^- + NO_2^-) μmol/L	7.26 ± 2.47; (7.31)	11.70 ± 2.74; (12.72)	21.70 ± 8.07; (18.22)	$^b P < 0.001$; $^a P < 0.05$
cGMP pmol/L	4.95 ± 1.21; (5.39)	10.14 ± 2.78; (9.0)	20.12 ± 6.62; (21.00)	$^b P < 0.001$; $^a P < 0.05$
GSHt μmol/L	66.57 ± 26.23; (59.88)	23.01 ± 4.38; (22.82)	16.04 ± 6.06; (14.30)	$^b P < 0.001$; $^a P < 0.05$
GSSG μmol/L	18.57 ± 6.39; (18.57)	9.43 ± 2.3; (9.65)	9.47 ± 4.17; (8.13)	$^b P < 0.001$; $^a P < 0.05$
GSHr μmol/L	47.78 ± 23.63; (45.19)	13.58 ± 3.7; (13.88)	6.54 ± 3.31; (6.02)	$^b P < 0.001$; $^a P < 0.05$
GSHr/GSSG	2.68 ± 1.16; (2.95)	1.52 ± 0.63; (1.50)	0.81 ± 0.42; (0.58)	$^b P < 0.001$; $^a P < 0.05$
MELD	6-8	8.29 ± 2.75; (8.00)	20.17 ± 8.45; (20.00).	$^b P = 0.001$; $^a P < 0.05$

Data were analysed by one-way analysis of variance (ANOVA) $^bP < 0.001$ between groups of each biomarker and Student-Newman-Keuls post-hoc test was used for comparison of difference of means between each parameter $^aP < 0.05$. $NO^{\bullet}$: Nitric oxide; cGMP: Cyclic guanosine monophosphate; GSHt: Total glutathione; GSSG: Glutathione disulfide; GSHr: Thiol-reduced glutathione; MELD: Model for end-stage liver disease; GSHr/GSSG: Thiol-reduced glutathione to glutathione disulfide ratio.

The concentrations of GSHr and GSSG in plasma were determined from a calibration curve using standard GSHr and GSSG solutions. Both GSHr and GSSG were measured in duplicate in a microplate reader on the basis of absorbance in the visible range at 405 nm.

Total GSHt is equivalent to the sum of GSSG and GSHr.

MELD score: The model for end-stage liver disease (MELD) score was calculated from the following equation:

$9.57 \times \log_e$ (creatinine mg/dL) + $3.78 \times \log_e$ (bilirubine mg/dL) + $11.2 \times \log_e$ (INR) + 6.43.

The maximal creatinine level considered in the MELD score is 4.0 mg/dL[12].

Statistical analysis

Statistical analyses were performed using the SPSS 12.0 statistical package (SPSS Inc., Chicago). All results are expressed as means ± SD, median values and ranges. Data were analysed by one-way analysis of variance in addition to the Student-Newman-Keuls post-hoc test. Coefficient correlations were evaluated using the linear regression analysis or Pearson correlation. Statistical significance was established at $P < 0.05$.

RESULTS

The baseline laboratory characteristics of the 26 patients with alcoholic liver cirrhosis (classified as Child-Pugh A, B and C) are shown in Table 1. The concentrations of serum $NO^{\bullet}$ and the different plasma biomarkers in the cirrhotic patients and controls are shown in Table 2. There were statistically significant differences between the 4 groups. The minimum $NO^{\bullet}$ level obtained in the control group was 3.19 μmol/L.

Patients with decompensated liver cirrhosis (Child-Pugh B and C) exhibited significantly higher serum concentrations of $NO^{\bullet}$ than both patients with compensated liver cirrhosis (Child-Pugh A) and controls (Figure 1). There was a significant difference in serum $NO^{\bullet}$ concentrations between control subjects and compensated liver cirrhotic patients. The concentrations of plasma cGMP in patients with decompensated cirrhosis were higher than in those in compensated cirrhosis, and this difference was statistically significant ($P < 0.001$). The concentrations of plasma cGMP in patients with compensated liver cirrhosis were higher when compared with those with the controls and this difference was statistically significant ($P < 0.001$) (Figure 2). Significant correlations between $NO^{\bullet}$ and cGMP levels ($r = 0.866$, $P < 0.001$), between $NO^{\bullet}$ levels and MELD scores ($r = 0.627$,

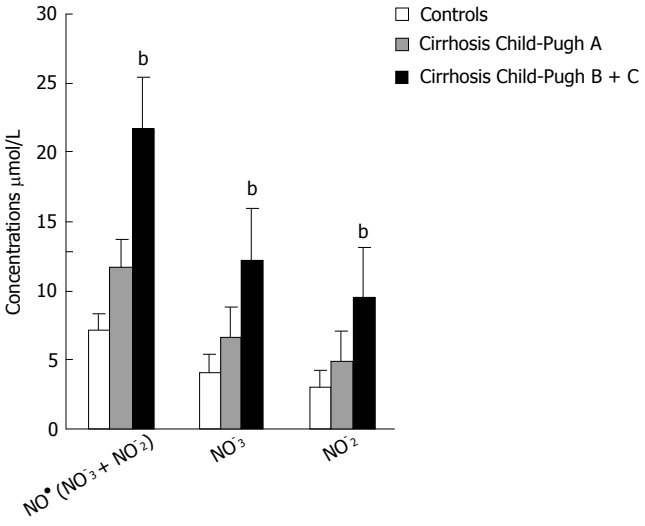

Figure 1 Serum concentrations of nitric oxide NO• (NO_3^- + NO_2^-), nitrates (NO_3^-) and nitrites (NO_2^-), in patients with alcoholic liver cirrhosis classed as Child-Pugh A, B or C, and controls. Data are expressed as means ± SD of triplicate determinations. $^bP < 0.01$ between controls and patients with Child-Pugh A, B or C cirrhosis.

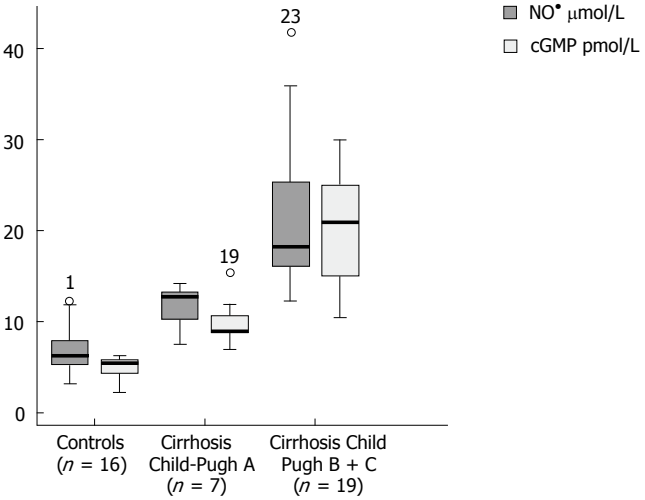

Figure 2 Boxes show the interquartile ranges. Vertical lines show 80% of the values (10^{th}-90^{th} percentiles). The bold horizontal line represents the median. $^bP < 0.01$ between controls and cirrhosis Child-Pugh A, and between controls and cirrhosis Child-Pugh B and C.

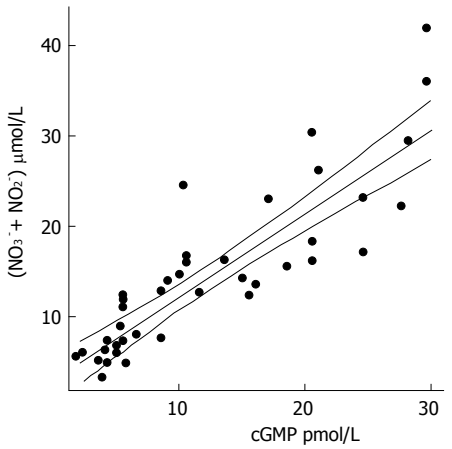

Figure 3 A significant positive correlation between the levels of NO• and cGMP was observed among the 26 patients with alcoholic liver cirrhosis, classed as Child-Pugh A, B and C. Linear regression with 95.00% mean prediction interval, $^bP < 0.01$; NO_3^- + NO_2^- μmol/L = 2.81 + 0.93; *R*-Square = 0.75.

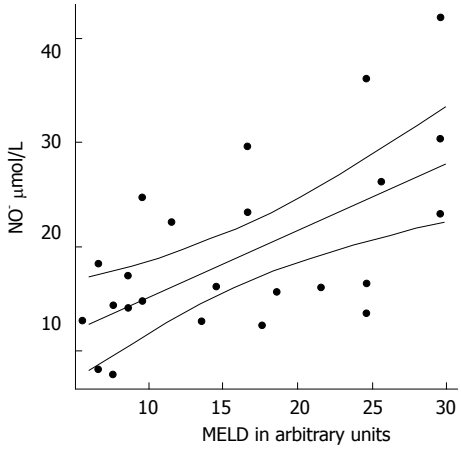

Figure 4 Correlation between NO• levels and MELD scores. Linear regression with 95.00% mean prediction interval, NO• μmol/L = 8.76 + 0.64; $^bP < 0.01$; *R*-Square = 0.39.

$P < 0.01$) and between cGMP levels and MELD scores ($r = 0.452$, $P < 0.05$) were observed among the 26 alcoholic liver cirrhotic patients (Figures 3-5).

Decreases in the plasma levels of GSHt, GSSG and GSHr were observed in all alcoholic liver cirrhotic patients, when compared with controls (Table 2). This decrease in GSHr levels was most prominent in decompensated liver cirrhotic patients. Figure 6 shows the plasma levels of the biomarkers of oxidative stress (GSHr, GSSG and GSHr/GSSG) in controls and in plasma of cirrhotic patients Child-Pugh classes A, B and C. There was a markedly reduced ratio GSHr/GSSG in patients with Child-Pugh B and C cirrhosis compared with patients with Child-Pugh A cirrhosis or controls ($P < 0.001$). The GSHr levels were higher in the controls than in the cirrhotic patients stratified according to Child-Pugh score.

The GSSG levels were higher in patients with Child-Pugh B and C cirrhosis than in those with Child-Pugh A cirrhosis or controls. The ratio between GSHr/GSSG was lower in patients with Child-Pugh B and C cirrhosis than in those with Child-Pugh A.

No correlation between NO• and cGMP levels and creatinine levels was found in patients with decompensated cirrhosis.

The MELD scores were determined in the 26 patients with alcoholic cirrhosis (Table 2). These was higher in the decompensated patients than in the compensated cirrhotic patients ($P < 0.001$). The GSHr/GSSG ratios were lower in cirrhotic patients belonging to Child-Pugh class B and C compared with those in Child-Pugh class A. Significant negative correlations between the MELD score and the GSHr/GSSG ratio ($r = -0.693$, $P < 0.001$) and between NO• level and the GSHr/GSSG ratio ($r = -0.558$, $P < 0.01$) were found in all of the alcoholic cirrhotic patients, as shown in Figures 7 and 8.

DISCUSSION

There is firm evidence of enhanced oxidative stress

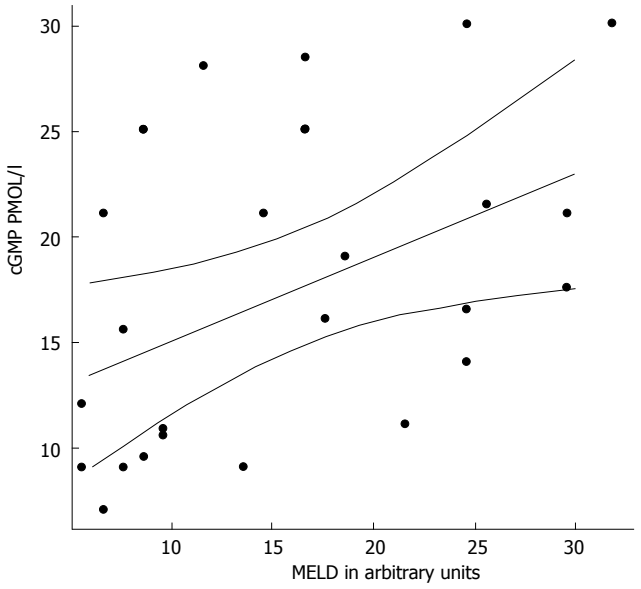

Figure 5 Correlation between cGMP levels and MELD scores. Linear regression with 95.00% mean prediction interval, $^{b}P < 0.01$; cGMP pmol/L = 11.05 + 0.40; *R*-Square = 0.20.

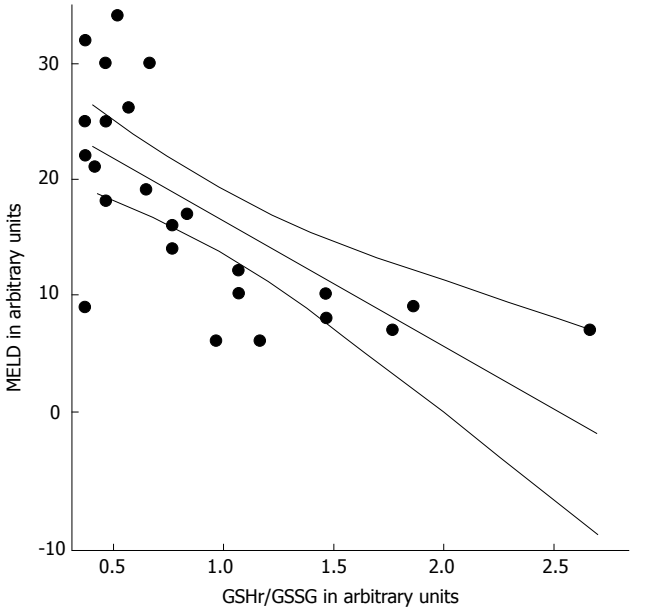

Figure 7 The MELD score increases progressively from Child-Pugh class A to C. A significant negative correlation between MELD scores and GSHr/GSSG ratios was observed. Linear regression with 95.00% mean prediction interval, $^{b}P < 0.01$; MELD in arbitrary units = 27.08 + -10.74; *R*-Square = 0.48.

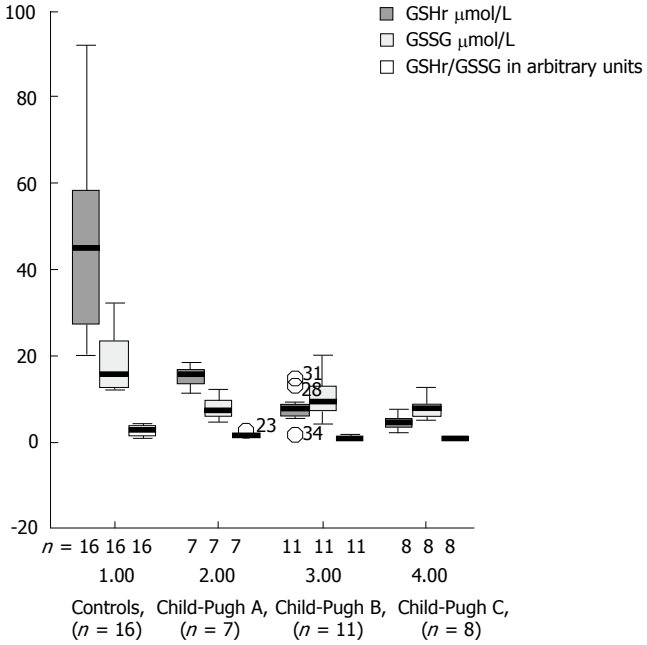

Figure 6 Boxes show the interquartile ranges. Vertical lines show 80% of the values (10^{th}-90^{th} percentiles). The bold horizontal line represents the median. $^{b}P < 0.01$ between controls and patients with Child-Pugh A cirrhosis or between patients with Child-Pugh B cirrhosis or between Child-Pugh C cirrhosis.

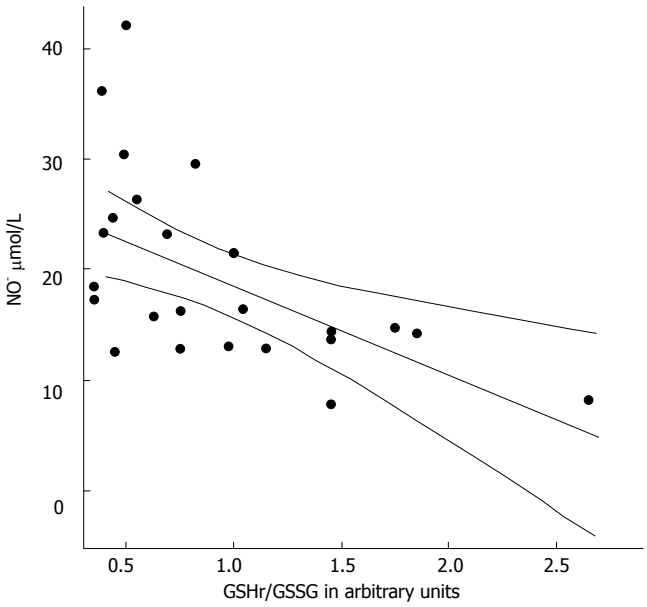

Figure 8 Correlation between NO· levels and GSHr/GSSG ratios. Linear regression with 95.00% mean prediction interval, $^{b}P < 0.01$; NO· μmol/L = 26.52 + -7.99; *R*-Square = 0.31.

in patients with alcoholic liver disease. Studies have shown increased plasma and tissue levels of markers of lipid peroxidation[13-16], and reduced hepatic and plasma antioxidant content[17-20].

In the liver, as in many other organs, NO· has many actions and cellular sources[4,5,16-21]. Determination of the levels of the NO· radical itself is hampered by its radical nature and very short half-life (in the range of seconds). Because NO· activates soluble guanylate cyclase to produce cyclic guanosine 3',5' monophosphate (cGMP) and stable nitric oxide metabolites, we measured the levels of its metabolites, NO_3^- and NO_2^-, in serum, and the levels of cGMP in plasma. The intracellular concentrations of ROS are tightly regulated by multiple defence mechanisms involving ROS-scavenging enzymes and anti-oxidant molecules, which include glutathione and GSH dependent enzymes. We also measured the levels of glutathione in its thiol-reduced (GSHr) and glutathione disulfide (GSSG) forms to better understand the defence mechanisms involving GSH operating in alcoholic cirrhotic patients[22-24].

The data of this study indicate that serum concentrations

of the NO• metabolites NO_3^- and NO_2^-, and those of plasma cGMP, an intracellular second messenger[25] of nitric oxide, in patients with alcoholic cirrhosis, were higher than those in healthy controls. The NO• and cGMP levels were higher in patients with decompensated liver cirrhosis (Child-Pugh B and C) than in those with compensated liver cirrhosis (Child-Pugh A) ($P < 0.001$). Our results also show a decrease in GSHt levels in alcoholic cirrhotic patients compared with healthy controls.

Associations were found between the levels of the biomarkers NO• and cGMP, as well as between the levels of each biomarker and MELD scores ($P < 0.01$). Significant negative correlations were observed between NO• levels and GSHr/GSSG ratios, and between MELD scores and GSHr/GSSG ratios, in the 26 alcoholic cirrhotic patients belonging to all three Child-Pugh classes ($P < 0.01$). The increased concentrations of GSSG in plasma provide a sensitive index of whole-body oxidative stress[26]. A deficiency of hepatic GSH and increased toxic free radical species may contribute to the progression of liver disease[17,18].

One of the more common free radicals produced in response to the ingestion of ethanol is oxygen in a deformed toxic state. These radicals, known as reactive oxygen species, include oxygen radicals and substances closely related to oxygen radical reactions. They can play a role in the development of alcoholic hepatitis by altering the redox state, damaging cell constituents in the liver and causing severe injury[27].

NO• can interact with ROS to form other reactive nitrogen species (RNS). These reactive nitrogen species secondarily promote important reactions such as nitrosation (production of nitrosamines), oxidation (mediating lipid peroxidation, DNA strand breaks and hydroxylation) or nitration (production of nitrotyrosines and nitroguanosines), leading to impaired cellular functions and enhanced inflammatory reactions[28]. On the other hand, ROS stimulates stellate cell activation and fibrogenesis[20,22,27]. Thus, oxidative stress seems to contribute to the pathogenesis of alcoholic liver injury and the progression of alcohol-induced liver disease to alcoholic cirrhosis[22,23].

Patients with advanced liver disease tend to develop a hyperdynamic circulation, which complicates cirrhosis. There is vast experimental evidence for enhanced NO• overproduction and associated cGMP formation in cases of portal hypertension. Moreover, several clinical studies have evaluated NO• synthesis and serum levels in various cirrhotic conditions[29-32]. Our study, including only alcoholic patients of different Child classes, strengthens previous investigations and adds further information with respect to the second messenger cGMP and its relationship with the redox state of GSH.

NO• or nitrite/nitrate measurements can be affected by creatinine levels, as demonstrated recently in acute decompensated cirrhotic patients[32]. In our clinical population, patients with Child C cirrhosis had elevated creatinine values compared to those with Child A cirrhosis, and this could also influence the results. A positive correlation was found between creatinine levels and NO• and cGMP mediators in all three Child-Pugh classes ($P < 0.01$, $P < 0.05$), but no correlation between these mediators and creatinine was found in either decompensated or compensated cirrhotic patients. However, we found an inverse relationship between GSHr/GSSG ratios and creatinine levels, only in decompensated cirrhotic patients ($P < 0.01$). The elevated mediator levels could also be caused by portal hypertension and hyperdynamic circulation where NO• is elevated and, therefore, have no relation to intrahepatic oxidative stress. Recent studies by Lluch *et al*[29] suggest that the serum concentration of asymmetric dimethylarginine (ADMA), an endogenous inhibitor of nitric oxide synthase is elevated in patients with alcoholic cirrhosis. This finding may offer an alternative compensatory mechanism to counteract the excessive visceral vasodilatation caused by excess production of NO• in patient with advanced liver disease.

In summary, our study shows that patients with decompensated alcoholic cirrhosis have higher levels of NO• and cGMP than compensated patients, and that there is a positive correlation between the clinical score of the disease (Child-Pugh classes and MELD scores) and the abnormalities observed. Our findings suggest that NO• and cGMP may contribute to progressive liver disease in these patients and influence the hemodynamic abnormalities. Our findings emphasize the need for further investigations correlating the serum levels of NO•, cGMP or GSH seen in decompensated cirrhotic patients with their clinical outcomes (for example, survival, variceal bleeding, renal failure and septic complications) to develop prognostic parameters and delineate new therapeutic strategies.

COMMENTS

Background

Alcohol accounts for 40%-50% of all deaths due to cirrhosis and remains the most common cause of liver-related mortality. Important pathophysiologic events that mediate alcohol-induced liver injury include increased oxidative stress, hepatocyte apoptosis and necrosis and deposition of collagen, with ensuing fibrosis.

Research frontiers

The correlation of mediators of oxidative stress with the severity of disease in patients with alcoholic liver injury has been studied intensively in recent years, mainly in patients with alcoholic hepatitis. We investigated patients with alcoholic cirrhosis and measured the levels of mediators of oxidative stress (namely, NO• and cGMP), antioxidant defences [thiol-reduced glutathione (GSHr) and glutathione disulfide (GSSG)] and correlated these levels with clinical liver disease severity.

Innovations

In contrast to previous studies, we measured the levels of not only NO•, but also cGMP (an intracellular second messenger of NO•) and correlated these with mediators of antioxidant defence. We found that the levels of nitric oxide metabolites and cGMP were high in alcoholic cirrhotic patients, particularly in those that were decompensated. There was a positive correlation between the clinical score of the disease (Child-Pugh classes and MELD scores) and the oxidative stress abnormalities observed.

Applications

Our findings suggest that NO• and cGMP may contribute to progressive liver disease in these patients. Further investigations correlating the serum levels of NO•, cGMP or GSHr seen in decompensated cirrhotic patients with their clinical outcomes (for example, survival, variceal bleeding, renal failure and septic complications) are needed to develop better prognostic parameters and delineate new therapeutic strategies.

Peer review

The authors measured nitric oxide metabolites in the serum and cGMP, GSH, and GSSG in the plasma with alcoholic cirrhosis patients. They found that nitric oxide metabolites and cGMP levels were high in alcoholic cirrhosis patients, particularly in decompensated alcoholic cirrhosis. Moreover, they found a significant negative correlation between GSH/GSSG ratio and MELD score in all cirrhotic patients.

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RAPID COMMUNICATION

Promoter polymorphism of transforming growth factor-β1 gene and ulcerative colitis

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Abstract

AIM: To elucidate the possible difference in two promoter polymorphisms of the transforming growth factor-β1 (TGF-β1) gene (-800G > A, -509C > T) between ulcerative colitis (UC) patients and normal subjects.

METHODS: A total of 155 patients with established ulcerative colitis and 139 normal subjects were selected as controls. Two single nucleotide polymorphisms within the promoter region of TGF-β1 gene (-509C > T and -800G > A) were genotyped using PCR-RFLP.

RESULTS: There was a statistically significant difference in genotype and allele frequency distributions between UC patients and controls for the -800G > A polymorphism of the TGF-β1 gene ($P < 0.05$). The frequency of the TGF-β1 gene polymorphism at position -800 showed that the AA genotype and the allele A frequencies significantly differed between the patients and healthy controls ($P < 0.05$). At position -509, there was no statically significant difference in genotype and allele frequency between the patients and control subjects.

CONCLUSION: The results of our study indicate that there is a significant difference in both allele and genotype frequency at position -800G > A of TGF-β1 gene promoter between Iranian patients with UC and normal subjects.

Key words: Transforming growth factor-β1; Ulcerative colitis; Promoter; Polymorphism; Iran

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INTRODUCTION

Transforming growth factor-β1 is a cytokine produced by both immune and non immune cells, and it exhibits a broad range of functions. TGF-β1 controls the differentiation, proliferation, and state of activation of all immune cells, wound healing, angiogenesis and is implicated in immune abnormalities linked to cancer, autoimmunity, opportunistic infections, and fibrotic complications[1-5]. It has chemotactic properties and may stimulate cells to produce cytokines such as IL-1, IL6, and TNF-α at the sites of inflammation[1,6]. It is interesting that TGF-β1 plays an important role in promoting and activating extracellular matrix and bone remodeling. Paradoxically it is also involved in several immune suppressive mechanisms particularly as an inhibitor of intestinal epithelial proliferation[7-9]. It is proposed that the TGF-β1 production is under genetic control[7,10]. In the human TGF-β1 gene, which is located on chromosome 19q13 [11-15], eight polymorphisms are presently known. Three of them are localized in the promoter region at positions -988C > A, -800G > A and -509C > T from the first transcribed nucleotide[13].

Polymorphism of TGF-β1 has been investigated in several diseases. For instance, our own investigation indicates that polymorphism of this cytokine is not associated with the repeated spontaneous abortion[16,17]. Polymorphism of TGF-β1 and the risk of hepatocellular carcinoma have been investigated in patients with chronic hepatitis B virus infection[18,19]. In patients with liver cirrhosis, an association has been found between codon 10 and morphology of hepatocellular carcinoma in Korean population[20].

Inflammatory bowel disease is thought to result from inappropriate and ongoing activation of the mucosal immune system due to the presence of normal luminal flora. This aberrant response is most likely facilitated by defects in both the barrier function of the intestinal epithelium and the mucosal immune system[21,22]. As only two previous studies are available on the association of the -509 C > T TGF-β1 polymorphism with inflammatory

bowel disease, especially Crohn's disease[23,24], we investigated whether these two polymorphisms (-509, -800) are associated in south Iranian patients with established UC.

MATERIALS AND METHODS

Subjects

The subjects in this study were comprised of 155 unrelated patients with UC (69 males and 86 females, aged 23-51 years, mean 36.4 years) attending the Department of Gastroenterology, Namazi and Saadi Academic Hospitals of Shiraz Medical University. Disease duration was at least 2 years. Patients were asked about acute phases of the disease after diagnosis. Every patient chart was revisited to complete all available information. A total of 139 age- and sex- matched health volunteers with no history of chronic bloody diarrhea and abdominal pain (50 males, 89 females) served as controls (aged 18-61 years, mean 35.5 years). Diagnosis of UC was established on the basis of conventional endoscopic and histological criteria. Since UC is a dynamic disease and patients can fluctuate into a different phenotype during the course of the disease, we analyzed the clinical records of our patients at two time points: when patients visited the hospital and for the first time he/she was followed up (mean time after diagnosis of 1.5 years). All patients were sub-classified according to gender, age of onset, localization of the disease, need for steroid therapy, and emergent surgical treatment. In UC patients, the localization of gut involvement was defined as proctitis, left-sided (up to the splenic flexure) or pancolitis (beyond the splenic flexure).

DNA extraction and TGF-β1 genotyping

Venous blood was collected into EDTA-coated tubes. DNA was extracted from whole blood using the salting-out method. The following primers (MOLBIOL, Germany) were used for amplification of promoter regions -509 and -800. The sequences of PCR primers for the -509C/T and -800G/A polymorphisms are 5'-CAGTAAATGTATGGGGTCGCAG-3' (forward) and 5'-GGTGTCAGTGGGAGGAGGG-3' (reverse), and 5'-ACAGTTGGCACGGGCTTTCG-3' (forward) and 5'-TCAACACCCTGCGACCCCAT-3' (reverse) respectively. The PCR product sizes from these primers were 153 bp and 388 bp, respectively. PCR was performed in a total volume of 20 μL containing 100 ng genomic DNA, 20 pmol/L of each primer, 0.2 mmol/L dNTPs, 20 mmol/L Tris-HCl (pH 8.8), 10 mmol/L $MgCl_2$, and 1 unit of Taq polymerase (New England BioLabs Ipswich, MA). The PCR cycle conditions consisted of an initial denaturation at 94℃ for 3 min followed by 35 cycles at 94℃ for 60 s, at 61℃ for 60 s, at 72℃ for 60 s for -509 C/T, and at 94℃ for 45 s, at 61℃ for 45 s, at 72℃ for 45 s for -800G/A, and a final elongation at 72℃ for 5 min.

Genotyping was performed by restriction fragment length polymorphism (RFLP) analysis. The following restriction enzymes (Fermantes, Lithuania) were used for the digestion of amplified PCR products. For digestion of PCR products containing positions -800, *Nmu*CI and -509, *Eco811* was applied. The digested PCR products were resolved on 2% agarose gel and stained with ethidium bromide for visualization under UV light.

Table 1 Distribution of TGF-β1 genotype and alleles in UC patients and controls

TGF-β1 genotype and alleles	UC patients *n* = 155 (%)	Controls *n* = 139 (%)	*P* value
Genotype -800			
GG	125 (80.6)	104 (74.8)	< 0.05
GA	29 (18.8)	27 (19.4)	
AA	1 (0.6)	8 (5.8)	
Allele frequency			
G	279 (90)	235 (84.5)	< 0.05
A	31 (10)	43 (15.5)	
Genotype-509			
CC	57 (36.8)	56 (40.3)	
CT	65 (41.9)	63 (45.3)	0.3
TT	33 (21.3)	20 (14.4)	
Allele frequency			
C	179 (57.75)	175 (62.9)	0.19
T	131 (42.25)	103 (37.1)	

Statistical analysis

Allele frequencies at each polymorphic site were calculated by allele counting method. Deviation of the genotype counts from the Hardy-Weinberg equilibrium was tested using χ^2 test with 1 degree of freedom. Differences in the allele frequencies and genotype distributions between the patients with UC and the controls were analyzed by χ^2 or Fisher's exact tests when necessary.

RESULTS

Position -800 (G/A)

In this investigation, the change at position -800G > A of the TGF-β1 gene was studied using PCR-RFLP in 155 cases of UC and 139 normal Iranian subjects. At this position, homozygote GG was found in 125 (80.6%) UC patients and 104 (74.8%) normal subjects, heterozygote GA was observed in 29 (18.8%) UC patients and 27 (19.4%) normal subjects, while homozygote AA was demonstrated in only one of the patients, homozygote AA was shown in eight normal individuals. There was a statistically significant difference in genotype and allele frequency distributions between UC patients and controls for the -800G > A polymorphism of the TGF-β1 gene ($P < 0.05$) (Table 1). A typical genotyping at this position is represented in Figure 1A.

Position -509 (G/A)

In addition, the genotype at position -509C > T of the TGF-β1 gene was studied in the same study groups. At this position, homozygote CC was found in 57 (36.8%) UC patients and 56 (40.3%) normal subjects, heterozygote CT was observed in 65 (41.9%) UC patients and 63 (45.3%) normal subjects, homozygote TT was demonstrated in 33 (21.3%) UC patients and 20 (14.4%) normal subjects. There was no statistically significant difference in genotype distribution and allele frequency at this position between

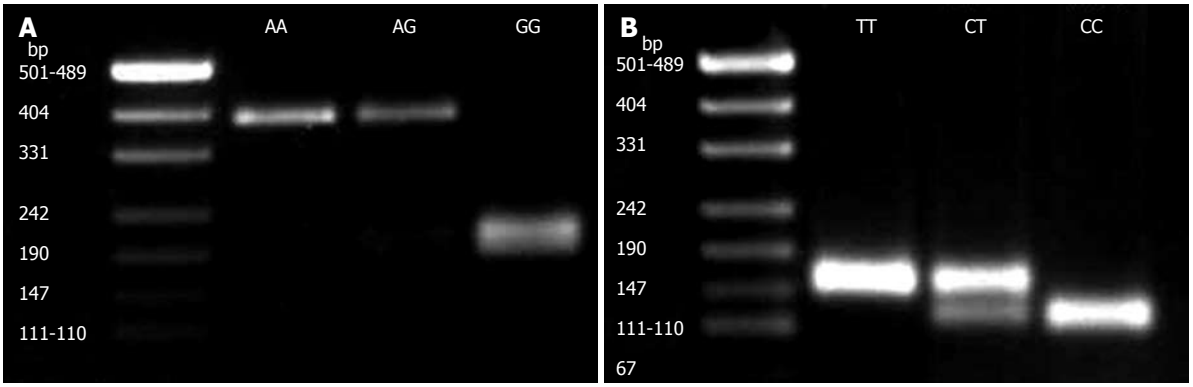

Figure 1 **A**: PCR-RFLP genotyping at position -800 (G/A). PCR product (388 bp) was digested with NmuCI restriction enzyme. From left to right: Lane 1: DNA size marker; lane 2: Homozygote AA; lane 3: Heterozygote AG; lane 4: Homozygote GG; **B**: PCR-RFLP genotyping at position −509 (C/T). PCR product (153 bp) was digested with Eco811 restriction enzyme. Lane 1: DNA size marker; lane 2: Homozygote TT; lane 3: Heterozygote CT; lane 4: Homozygote CC.

UC patients and controls. The distribution of TGF-β1 genotype and allele frequency in UC patients and controls are summarized in Table 1. A typical genotyping at position -509C > T is represented in Figure 1B. A Hardy-Weinberg equilibrium test was performed for the two polymorphisms.

Relation between other factors and polymorphisms

In our study, no significant relation was found between the two polymorphisms and factors such as gender, age of onset, cumulative dose of steroids, need for steroid therapy, localization of the disease, and need for emergent surgical treatment.

DISCUSSION

TGF-β1 is an immunoregulatory and immunosuppressive cytokine. Its role and function have been extensively investigated in inflammatory type of diseases[14]. It has been known that TGF-β1 has various biological and immunological functions, such as decreasing expression of MHC-Ⅱ, reducing synthesis of TH1/TH2 cytokine and suppressing the activation of both T and B lymphocytes. Plasma levels of TGF-β1 have been detected in several inflammatory and malignant diseases and the consensus is that the synthesis of this immunosuppressive cytokine is under the control of genetic factors[3,6,11]. TGF-β1 gene is located on chromosome 19 and contains 7 exons.

Several polymorphisms have been reported in TGF-β1 gene at positions -988, -800 and -509 located in the gene promoter[13-21]. Garcia-Gonzalez and co-workers showed that codon 10 and 25 TGF-β1 polymorphisms in Dutch IBD patients and healthy controls do not participate in defining the susceptibility to and tile nature of the clinical course in IBD[24]. Polonikov AV *et al*[28] reported that TGF-β1 gene polymorphism plays a role in the pathogenesis of gastric ulcer disease. Su Zg and colleagues[16] reported that allele A at position -800 and allele T at position -509 of TGF-β1 gene are increased in Chinese population with chronic obstructive pulmonary disease. Schulte CM[23] showed that IBD susceptibility is not associated with genetic variations in TGF-β1 promoter polymorphism.

In addition to the reports on genetic variations in TGF-β1 polymorphism in IBD, several studies showed that serum level of TGF-β1[17] and mRNA expression[18] are significantly increased in IBD patients.

In the present study, the genotype distribution and allele frequencies of polymorphisms at position -509C > T were not significantly different between UC patients and controls, which is consistent with the previous reports[24,30]. However, at position -800G > A both allele and genotype frequencies were significantly different between UC patients and controls. It has been reported that -800G > A substitution is thought to disrupt a consensus half-site for binding to the nuclear transcription factor (CRE-binding protein), consequently contributing to a lower production of total TGF-β1 in the circulation[7,26].

In our study, AA genotype was reduced in patients with IBD compared with controls. A similar finding was seen in UC patients and controls when the frequency of allele A was analyzed. These findings indicate that individuals bearing allele A and genotype AA are less susceptible to IBD than those lacking allele A and genotype AA.

To the best of our knowledge, polymorphism at position -800 of TGF-β1 gene promoter has not yet been reported in IBD patients. However, both plasma level of TGF-β1 and mRNA expression should be further studied and compared in IBD patients carrying genotype AA at this position.

There are a number of other polymorphisms with known influence on different TGF-β1 expressions[3,4]. To study the role of TGF-β1 haplotype in the pathogenesis of IBD, analysis of other known TGF-β1 gene polymorphisms will strength our current observation.

In conclusion, there is no significant difference in TGF-β1 polymorphisms at position -509 C > T and allele A is significantly associated with genotype AA at position -800 of the promoter region between southern Iranian UC patients and healthy individuals.

COMMENTS

Background

Inflammatory bowel disease (IBD) is a common disorder in our population with an increasing incidence but no clear cut etiology. Cytokines and their receptors,

immunogenetics and host -related factors are all involved in the disease susceptibility. TGF-β1 is produced by regulatory T lymphocytes with a profound suppressive effect on the induction phase of immune response. Plasma levels of TGF-β1 and its local synthesis, in addition to the contribution of genes encoding TGF-β1 have widely been investigated in IBD patients with no convincing and solid ground so far.

Research frontiers

Investigation of cytokine gene polymorphism in health and disease conditions has provided valuable information on the incidence of autoimmune diseases in different ethnics. Current data on TGF-β1 gene polymorphism in IBD are controversial due to the heterogeneity in clinical presentations of the disease and different methods. There is no evidence that TGF-β1 gene polymorphism is associated with IBD in European Caucasian population. Here we report the impact of allele A and genotype AA at -800 of TGF-β1 promoter region and susceptibility to Iranian patients with IBD.

Publications

Schults *et al* and Garcia Gonzalez reported that polymorphism at promoter region has no association with IBD in European individuals. Our data give another view on the significance of polymorphism of TGF-β1 promoter region in IBD patients.

Innovations and breakthroughs

Our findings provide a window for investigation on the mRNA and local secretion of TGF-β1 in IBD patients carrying allele A and genotype AA of TGF-β1 at position -800 of promoter region.

Application

By extending this investigation in a larger sample size within other ethnic groups or in a broader study in a format of meta-analysis, a genetic marker for screening of individual susceptible to IBD can be expected.

Peer review

This is a very interesting and original paper that merits to be fully published without any substantial modifications.It is also in good english written.

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RAPID COMMUNICATION

Prevalence and dietetic management of mild gastrointestinal disorders in milk-fed infants

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Abstract

AIM: To assess the prevalence of mild gastrointestinal disorders in milk-fed infants in paediatric practice, and to evaluate the effectiveness and satisfaction with dietetic treatment.

METHODS: A cross-sectional epidemiological study was first carried out. A total of 285 paediatricians included 3487 children seen during a period of one week. In a second phase an observational, prospective and multicentre study was conducted and 2069 milk-fed infants with mild gastrointestinal disorders (colic, constipation, regurgitation and diarrhoea) were included. There was a baseline visit (start of treatment) and a final visit four weeks later. The effectiveness of the various Novalac formulas, as well as the satisfaction of the parents/tutors and paediatricians with the dietetic treatment were assessed at the final visit.

RESULTS: The prevalence of mild gastrointestinal disorders was 27.8% of all paediatrician consultations (9.2%, 7.8%, 6.1% and 4.6% in relation to colic, constipation, regurgitation and diarrhoea, respectively). The several Novalac adapted milk formulas resolved 88.4% of the mild gastrointestinal disorders. Depending on the type of disorder, differences in response rate were observed. The highest effectiveness was recorded with respect to diarrhoea (92.6%), followed by constipation (91.6%), colic (87.6%) and regurgitation (81%). Overall, 91% of the paediatricians and 88.8% of the parents/tutors were satisfied or very satisfied with the Novalac adapted milk formulas.

CONCLUSION: Mild gastrointestinal disorders show a high prevalence in paediatric practice. The Novalac adapted milk formulas have been shown to be effective in treating mild gastrointestinal disorders in milk-fed infants in the context of routine clinical practice.

Key words: Mild gastrointestinal disorders; Prevalence; Adapted infant formulas; Effectiveness; Satisfaction

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INTRODUCTION

Human milk is a complete and complex species-specific food that provides all the nutrients needed for the growth of infants born to term during the first 4-6 mo of life. There is total agreement that maternal milk is the feeding method of choice for infants. However, at present, 25% of our population does not breastfeed, while 50% of women do so for only a reduced period of time (3-4 mo). In the event of contraindication or refusal to breastfeed, artificial nursing measures must be adopted. In many of these cases the infant may suffer mild gastrointestinal disorders (MGDs) during the period of lactation[1] including colic[2], regurgitation[3], diarrhoea[4] and constipation[5]. These symptoms cause discomfort for the infant and are a source of concern for parents. Acute diarrhoea cannot be considered a dysfunction as such; however, since it can be treated by dietetic measures, it has been included among these MGDs.

Interest among paediatricians to solve these disorders and improve knowledge of their physiopathology has led the infant food industry to develop specific formulas for these conditions. In many cases, dietetic adjustments are the first link in the treatment chain. At present, a broad range of artificial formulas have been developed[6] for feeding "healthy" infants. These formulas can partially or completely replace human milk, covering the normal nutritional requirements of the infant. In the case of infants with MGDs, adapted formulas have been developed[7,8] in which some of the nutrients have been specifically modified with the purpose of minimizing or resolving the digestive disorder while ensuring optimum infant growth. These formulas are manufactured according to the recommendations of the European Society for

Paediatric Gastroenterology, Hepatology and Nutrition (ESPGHAN)[9-11], the European Commission[12,13], and the Royal Decrees of the Spanish Ministry of Health[14,15].

The present study investigates the prevalence of MGDs in Spanish infants under four months of age and seen in paediatric practice. An evaluation is also made of the effectiveness of the specifically elaborated formulas belonging to the Novalac line of products (United Pharmaceuticals, France; Chiesi España S. A, Spain) [Novalac Anti-Colic (low lactose, adapted formula), Novalac Anti-Regurgitation (thickened with starch and enriched amylopectins), Novalac Anti-Diarrhoea (lactose-free and with adapted concentration of electrolytes), or Novalac Anti-Constipation (adapted concentration of magnesium and lactose)], in relation to the number of avoided episodes of colic, regurgitation, diarrhoea and constipation among infants exclusively fed with artificial milk formulas. Lastly, satisfaction with the Novalac line of products among both parents/tutors and paediatricians, was evaluated along with its relation to resolution or reduction of the symptoms associated with MGDs.

MATERIALS AND METHODS

Study design and patients

The present study comprises of two sub-studies. The first sub-study is a cross-sectional data-collecting study involving a 7-d period to estimate the prevalence of infants less than four months of age with MGDs such as colic, constipation, regurgitation and diarrhoea, among the global infant population seen in paediatric practice for any reason. The second sub-study in turn corresponds to a prospective longitudinal survey of the effectiveness of dietetic treatment with the Novalac range of formulas specifically developed for such MGDs.

A total of 285 paediatricians throughout Spain (except Ceuta and Melilla) participated in the study, under real-world conditions of clinical practice. Representation of the nursing infant population by Spanish Autonomous Communities was maintained. To investigate the prevalence of infants with MGDs in paediatric practice, each investigator included all infants less than four months of age seen in the clinic for any reason, during a period of one week. The infants of the cross-sectional study presenting MGDs with artificial milk formulas, and who according to medical criterion could be fed with the Novalac range of formulas, were enrolled in the longitudinal study. During one week, each investigator consecutively included 10 infants that met the selection criteria. This period was prolonged for two weeks until the full quota designated to each investigator was covered.

The selection criteria were: (1) infants up to four months of age fed with artificial milk formulas; (2) the presence of MGDs; (3) the possibility of feeding the infants with some product of the Novalac line of formulas; and (4) continuation of these formulas on an exclusive basis for at least 30 d (with no incorporation of other foods to the diet).

The cross-sectional study included 3487 infants to assess the prevalence of MGDs. The prospective study for evaluating the efficacy of the Novalac line of products involved 2069 patients with MGDs. The patients were visited on two occasions: at the time of inclusion and after four weeks. In the case of infants with diarrhoea, the final visit took place after 4-7 d. A total of 1441 infants completed follow-up.

Study variables

During the initial visit, data were collected relating to sociodemographic (age and sex) and clinical variables (gestational age, weight and height at birth and at the time of the visit, and the type and duration of the disorder). A questionnaire addressing the different symptoms and their intensity was designed for each disorder: (1) Constipation: number of stools/d, consistency, irritability, need for external help to defecate; (2) Regurgitations-vomiting: number/d, duration of the feedings, volume ingested; (3) Colic: excess gases, duration of crying, duration of bottle feeding; (4) Diarrhoea: number of stools and their characteristics.

The effectiveness of the dietetic treatment was evaluated on the final visit. Anthropometric data were collected (weight, height), along with the number of days to disappearance or improvement of the disorder, the evolution of symptoms, adverse events, and satisfaction of the parents/tutors and paediatricians with the treatment. Also documented was paediatrician contact after 15 d with the parents by telephone, to know the condition of the infant (in those cases where the paediatrician considered the call relevant).

The satisfaction of the parents/tutors and paediatrician with the Novalac formulas used for the MGDs was assessed on occasion of the final visit by means of a Likert-type scale with five possible answers (from very satisfied to very dissatisfied).

Statistical analysis

Duplicate data introduction was carried out, with validation of the database. Analysis of the prevalence of MGDs was based on a descriptive analysis of the sociodemographic and clinical characteristics of the study population. Continuous variables were reported as the mean and standard deviation (SD), while categorical variables were expressed as the number and percentage of infants per response category. The description of the characteristics of the infants was made for the total study sample, with stratification according to MGD presence.

A descriptive analysis was made of the number and percentage of infants seen in paediatric practice for MGDs, calculating the confidence interval (CI) of prevalence with a level of significance of 0.05. In order to assess variability in the Spanish setting, comparative studies were made of the prevalence of MGDs among the different regions in the country, based on the chi-square test.

Calculation was made of the number of days needed for disappearance or improvement of the disorder, along with the mean number (95% confidence interval) of days of MGD persistence.

A descriptive analysis was made of satisfaction as expressed by the parents/tutors and paediatricians. To this effect we used descriptive indicators such as the mean,

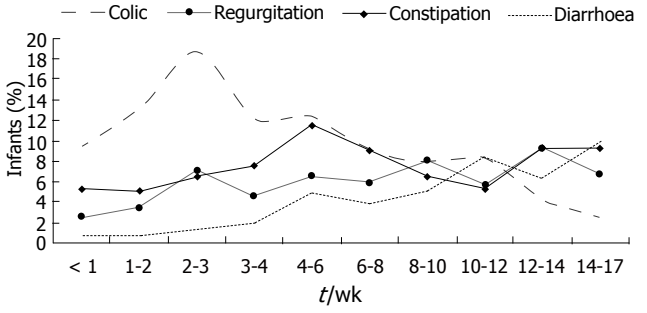

Figure 1 Percentage presentation of mild gastrointestinal disorders (MGDs) according to infant age and mean age at presentation.

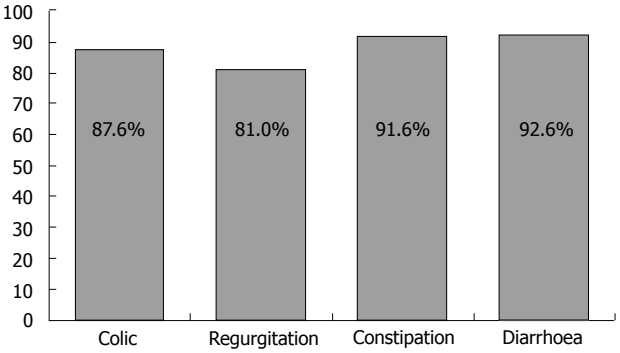

Figure 3 Percentage resolution of disorders with the Novalac formulas.

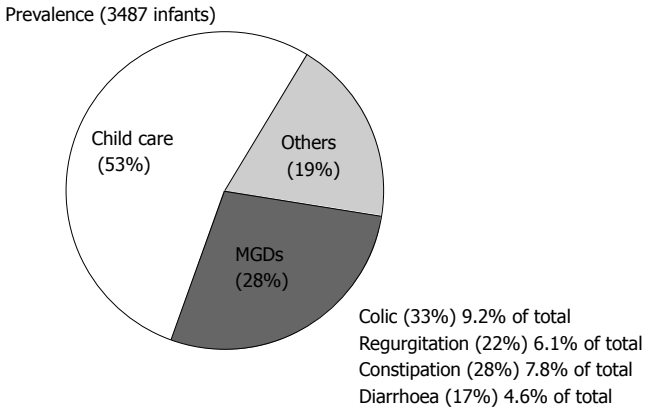

Figure 2 Prevalence of mild gastrointestinal disorders (MGDs) in paediatric practice.

standard deviation, median and percentiles 25 and 75. A study was made of the relationship between parent/tutor satisfaction with the Novalac line of products and their effectiveness, based on the Student t-test for independent data.

The SAS version 8.02 statistical package for Microsoft Windows was used throughout. In all the statistical tests a level of significance of $\alpha = 0.05$ was used.

RESULTS

The results of the cross-sectional study were obtained from the infant population included in the period of one week commented above, and involved 3487 infants. Boys predominated slightly over girls (52.2% *vs* 47.8%). Patient age at consultation was between under one week and 17 wk. Colic manifested at earlier ages (6.2 wk on average), followed by constipation (7.6 wk), regurgitation (8.6 wk) and diarrhoea (10.4 wk) (Figure 1). Gestational age was 39 wk on average (SD: 1.6 wk), with a mean body weight at birth of 3.2 (SD 0.5) kg and a height of 49.5 (SD: 2.3) cm.

The prevalence of MGDs was 27.8% (95% CI: 26.3-29.2); of these disorders, 9.2% (95% CI: 8.3-10.2) corresponded to colic, 7.8% (95% CI: 6.9-8.7) to constipation, 6.1% (95% CI: 5.3-6.9) to regurgitation -vomiting, and 4.6% (95% CI: 3.9-5.3) to diarrhoea. Only 0.6% of the infants presented with other digestive disorders (anorexia/hyporexia and eating disorders), while 19.0% (95% CI: 17.8-20.4) were diagnosed with some other non-digestive problem (upper airways infection, bronchitis and dermatitis). Of the study series, 52.6% consulted for aspects related to child care (Figure 2). The prevalence considering only the 27.8% of infants with MGDs was: colic 31.2% (95% CI: 30.3-36.2), constipation 29.2% (95% CI: 25.3-30.9), regurgitation 21.3% (95% CI: 19.5-24.7) and diarrhoea 18.3% (95% CI: 14.2-18.9).

The longitudinal study included 2069 infants with MGDs: 646 infants with colic, 441 with regurgitation, 604 with constipation and 378 with diarrhoea. The effectiveness of dietetic treatment for these disorders was evaluated among the 1441 infants that completed follow-up. Premature study termination among some infants was due to adverse events in 2.7% of cases, parent decision in 6.9%, loss to follow-up in 1.64%, protocol violations in 2.46%, and non-specified reasons in 16.62%.

Effectiveness in resolving the different disorders, after feeding with the specific Novalac formulas under conditions of routine clinical practice, is reported in Table 1 and Figure 3.

Colic was resolved among 87.6% of the infants after a median of 8 d - the proportion of infants with normal bottle feeding increasing from 33.9% at baseline to 69.2%. Likewise, a considerable reduction was observed in excess gases (from 82.9% at baseline to 25.8%). The duration of crying decreased in all infants.

Regurgitation was resolved in 81% of the infants after a median of 6 d of follow-up. The figure decreased from six regurgitations and/or vomiting episodes a day to only two, and the time required for bottle feeding decreased 53% - with an increase in the volume of daily ingestions in 61.9% of the infants.

A full 91.6% of the cases of constipation were resolved within 7 d. The number of daily stools among the infants with constipation increased from 0.6 (SD 0.7) stools/d to 1.7 (SD 0.8) stools/d. At the end of follow-up, the stools were found to be normal in 95.6% of the infants, while 89.6% presented no pain or discomfort, and 91.2% required no external help in defecating.

The infants with diarrhoea in turn, showed a decrease (mean [SD]) from 5.4 (1.8) to 2.2 (1) stools/d. Fever was absent in 98.5% of the cases, and 79.7% of the infants showed normal defecation at the end of treatment. A full 92.6% of the cases of diarrhoea were resolved within three days (Figure 4).

Development of all infants fed with the Novalac

Table 1 Description of clinical characteristics of patients at baseline and at the end of the study

		Baseline	30 d
Colic	Excess gases	82.90%	25.80%
	Duration of crying		
	< 1 h/d	12.10%	85.20%
	1-3 h/d	53.00%	13.30%
	> 3 h/d	34.80%	1.60%
Regurgitations	Regurgitation and/or vomiting per day [mean (SD)]	6.9 (4.5)	2.4 (1.8)
	Mean number of daily ingestions	6.0	5.6
	Duration of daily ingestions		
	Increases	-	24.50%
	Without change	-	22.40%
	Decreases	-	53.10%
	Volume of daily ingestion		
	Increases	-	61.90%
	Without change	-	18.70%
	Decreases	-	19.40%
Constipation	Type of stools		
	Normal	33.40%	95.60%
	Hard	66.60%	4.40%
	Presence of pain or discomfort		
	Yes	90.00%	10.40%
	No	10.00%	89.60%
	External help needed for defecation		
	Yes	76.10%	8.80%
	No	23.90%	91.20%
		Baseline	7 d
Diarrhoea	Presence of fever		
	Yes	21.40%	1.50%
	No	78.60%	98.50%
	Number of stools per day [mean (SD)]	5.4 (1.8)	2.2 (1)
	Type of stools		
	With mucus and/or blood	5.20%	1.10%
	Liquid	45.00%	4.80%
	Semiliquid	49.80%	14.40%
	Normaly	0.00%	79.70%

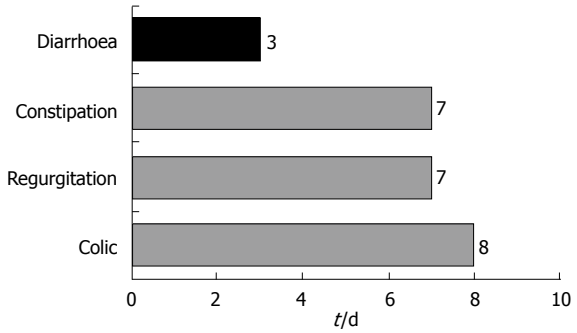

Figure 4 Mean days to resolution of mild gastrointestinal disorders (MGDs).

formulas was within the normal range.

Ninety-one percent (91%) of the paediatricians were satisfied or very satisfied with the dietetic regimen, though statistically significant differences were observed between groups ($P < 0.001$) - increased satisfaction being recorded among the paediatricians that treated diarrhoea and constipation versus those that treated colic and regurgitations. The greatest levels of satisfaction corresponded to the treatment of diarrhoea, with 95.9% of the paediatricians claiming to be satisfied, followed by constipation (92.8%), colic (88.5%) and regurgitation (87.8%).

As to parent satisfaction with the treatment ($P < 0.001$), 88.8% claimed to be satisfied with the course of the disorder - the specific figures being 93.7% in the case of diarrhoea, followed by constipation (90.0%), colic (88.1%) and regurgitation (83.3%).

Regarding the adverse events recorded during the study, only 3.9% of the infants receiving Novalac formulas suffered some adverse event. The most frequent affected the digestive tract (1.4%), including diarrhoea and constipation, and respiratory apparatus (0.7%) (e.g. bronchitis and bronchiolitis). Ten infants (0.5%) required hospital admission for different reasons: septicaemia ($n = 1$), dehydration ($n = 2$), vomiting ($n = 1$), hernia ($n = 1$) and bronchitis or bronchiolitis ($n = 2$).

DISCUSSION

Maternal breast milk is the usual food source for infants, though when breastfeeding is not possible, artificial lactation can be provided in the form of starting formulas, with the introduction of follow-up formulas 4-6 mo later, to ensure optimum nutritional development. During this period mild gastrointestinal disorders (MGDs) are not uncommon, and may be treated with dietetic measures.

This study for the first time provides information on the prevalence of these disorders in Spain, and on

the efficacy of dietetic treatment using the different formulas of the Novalac product range. The results of the prevalence study indicate that colic in infants is the MGD most often seen in paediatric clinics (9.2%), representing 33.3% of all MGDs.

Infant "colic" is an ill-defined term that is often used in reference to different situations found in paediatric practice[16]. Due to the lack of a clear definition, prevalence and treatment studies characteristically include heterogeneous groups of infants. These are usually healthy children that in the first four months of life develop paroxysmal crying without any apparent cause, and that gradually subsides. The underlying aetiology has not been clearly established, though the disorder has been related to alterations in motility[8], prostaglandin dysfunction, abnormal serotonin concentrations, and neuropeptide immaturity. Other authors more inclined to seek behavioural explanations tend to relate infant colic to alterations in the family environment, or even consider it a "normal" form of behaviour in infants with a more irritable temperament.

Human milk contains 7 g of lactose per 100 mL. However, in the first few weeks of life, infants present physiological or functional lactase insufficiency that limits absorption of these amounts. The non-hydrolyzed lactose reaches the colon, where it ferments to yield lactic acid, short-chain fatty acids, methane, carbon dioxide and hydrogen. This exerts a beneficial effect in that the stools become semiliquid as a result. However, is some cases such fermentation gives rise to excess gas[17-19], which favours the development of colic. In effect, different studies have related excess crying with excess intestinal gas[20].

The Novalac Anti-Colic formula offers an important reduction of the symptoms. Colic was resolved in 87% of the cases within one week, with a reduction in crying time, improvement in feeding continuity, and a reduction in the amount of intestinal gas.

In this study, constipation was the cause of consultation in 7.8% of the infants (representing 28% of all MGDs). A recent article published by Quinlan *et al*[21] studied the stool characteristics of 844 infants between 7-15 d old. Hard stools were recorded in 17% of the formula-fed infants, and in none of the breastfed infants. Breastfeeding entails a series of compensating physiological mechanisms that avoid constipation[22]. The Novalac Anti-Constipation formula bases its therapeutic efficacy partially on its high lactose content (8.1 mg/100 mL). The non-hydrolyzed lactose reaches the colon, where it is metabolized by the anaerobic flora, producing an osmotic laxative effect, since it attracts water into the intestinal lumen[17-19]. The magnesium contained in the formula (within the authorized maximum limits: 9.1 mg/100 mL) also enhances the laxative effect due to its osmotic action, and stimulates bowel motility by inducing cholecystokinin (CCK) secretion[23-25]. The efficacy of such treatment is reflected by an increase in the number of stools, and a reduction in the number of hard depositions, associated discomfort and the need for external help in defecation.

The next most prevalent MGD in the present study was regurgitation (22% of the total MGDs). Regurgitation and vomiting are the final manifestation of reflux. The aetiology of reflux in infants is of a multifactorial nature, and has not yet been fully elucidated. Postprandial reflux is accepted to be physiological, and occasionally occurs in almost all infants. However, in some cases the clinical picture is particularly manifest and can produce important discomfort for both the child and family. It is generally agreed that uncomplicated gastro-oesophageal reflux should not receive pharmacological treatment (antacids, prokinetic agents, H2 receptor blockers), though effective measures such as postural and dietetic management are recommended. In any case, the same authors that initially recommended such treatment have recently questioned it[26].

Milk thickening is a therapeutic practice that has been recommended for decades. Increased food viscosity and density reduces the reflux rate but does not modify the rest of parameters indicative of pathological gastro-oesophageal reflux. The products used as thickeners for the most part have been locust bean flour, pectin and cellulose. Cereal starch (rice and corn) has also been proposed, and offers the advantage of avoiding the possible adverse effects associated with the presence of galactomannans[27]. The Novalac Anti-Regurgitation formula contains a specially selected corn starch (having high amylopectin content) in a proportion of 1.9 g per 100 mL. It is pregelatinised and contributes to the increased viscosity in the stomach (on average 10 fold the viscosity in the feeding bottle). The presence of medium-chain fatty acids in turn favours a reduction in gastric emptying[28]. The efficacy of the treatment is reflected by the results obtained in the present study. In effect, the number of regurgitations/vomiting episodes decreased during the 7 d of treatment, and improvements were also seen in the rapidity of bottle ingestion, with a larger ingested volume.

Lastly, acute gastroenteritis represented 16.5% of the global MGDs in our study. Acute gastroenteritis, of a bacterial or viral origin, is characterized by alterations in the normal displacement of water and electrolytes within the intestinal lumen. When diarrhoea is caused by a virus (fundamentally rotavirus) transient lactase deficiency may also result. The duration of this deficiency is usually 7-15 d, though infants that are malnourished or suffer serious intestinal lesions may have persistent diarrhoea for up to 18-24 mo. In those cases in which deficient lactose absorption is suspected, lactose-free formulas are justified[29,30]. In most cases a single week of lactose exclusion suffices, after which the usual formula is reintroduced, while monitoring tolerance.

The most recent developments point to the advisability of introducing soluble fibre[31]. The use of amylase-resistant carbohydrates is an innovation that takes advantage of the functional properties of the colon - specifically, the metabolic activity of the anaerobic flora and the absorption capacity of the colon mucosa. The purported mechanism involves use by the colon mucosa of the short-chain fatty acids produced by bacterial metabolism of non-absorbed carbohydrates, to favour the absorption of water and electrolytes[32]. In the colon, many carbohydrates are fermented by the anaerobic flora, resulting in short-chain fatty acids: propionate, acetate and butyrate. In the mucosa, these fatty acids stimulate the absorption of water and electrolytes.

The Novalac Anti-Diarrhoea formula contains no lactose, and moreover incorporates a series of novelties. The presence of electrolytes within the accepted maximum range (31 mg of Na/100 mL, 83 mg of K/100 mL and 49 mg of Cl/100 mL) ensures an increased mineral supply for those patients who after discontinuing oral rehydration remain at risk of excessive losses. On the other hand, the presence of pectin (1.5 g per 100 mL) offers the advantage versus other fructo-oligosaccharides of being totally fermented in the colon, since it is a larger and more viscous molecule - thereby favouring short-chain fatty acid production.

The present study for the first time offers information on the prevalence of mild gastrointestinal disorders in Spanish infants under four months of age seen in paediatric clinical practice. Dietetic intervention with the Novalac formulas has been shown to be effective in resolving these disorders in the routine clinical setting - with a significant reduction in associated symptoms. A close relationship has been found between satisfaction among the parents/tutors and paediatricians and the effectiveness of the dietetic treatment provided. In turn, the low prevalence of treatment-related adverse events reflects the good tolerability of the formulas belonging to the Novalac range of products.

COMMENTS

Background

Different milk-feed infant formulae have appeared in the market during the last decades for the nutritional treatment of different entities such as colic, constipation, regurgitation and acute diarrhoea, called "mild gastrointestinal disorders" (MGD), There are few data about the prevalence or these MGD and its evolution after the intake of nutritional formulae.

Research frontiers

This study investigated the MGD prevalence, MGD physiopathology, International Committees and Scientific Societies position in front of new formulations composition, as well as clinical response with these new formulae.

Innovations and breakthroughs

In this article we have published, for the first time in our country, relevant data regarding the MGD prevalence and dietetic clinical response in a huge number of milk-fed infants (*n* = 2069). Among international publications one can find lots of information about recommendations of how these formulations have been made and its usages indications and efficacy, but never in a population as big as this.

Applications

This study allows the real acknowledgment about MGD prevalence, and also explains why new formulae composition have to be used and its clinical response. It also increases paediatricians possibility of use of these formulations, giving them confidence regarding clinical efficacy and nutritional safety.

Terminology

Mild gastrointestinal disorders: colic, constipation, regurgitation and acute diarrhoea.

Peer review

The authors are to be congratulated on an important and well presented study.

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RAPID COMMUNICATION

Efficacy and safety of pegylated-interferon α-2a in hemodialysis patients with chronic hepatitis C

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Abstract

AIM: To evaluate the efficacy and safety of pegylated-interferon alpha-2a in hemodialysis patients with chronic hepatitis C.

METHODS: Thirty-six hemodialysis patients with chronic hepatitis C were enrolled in a controlled and prospective study. All patients were treatment naive, positive tested for anti-HCV antibodies, and positive tested for serum HCV-RNA. Twenty-two patients received 135 μg peglyated-interferon α-2a weekly for 48 wk (group A). The remaining patients were left untreated, eleven refused therapy, and three were not candidates for kidney transplantation and were allocated to the control group (group B). At the end of the treatment biochemical and virological response was evaluated, and 24 wk after completion of therapy sustained virological response (SVR) was assessed. Side effects were monitored.

RESULTS: Of 22 hemodialysis patients, 12 were male and 10 female, with a mean age of 35.2 ± 12.1 years. Virological end-of-treatment response was observed in 14 patients (82.4%) in group A and in one patient (7.1%) in group B (P = 0.001). Sustained virological response was observed in 11 patients (64.7%) in group A and in one patient in group B (7.1%). Biochemical response parameters normalized in 10/14 patients (71.4%) at the end of the treatment. ALT levels in group B were initially high in six patients and normalized in one of them (25%) at the end of the 48 wk. In five patients (22.7%) therapy had to be stopped at mo 4 due to complications of weakness, anemia, and bleeding.

CONCLUSION: SVR could be achieved in 64.7% of patients on hemodialysis with chronic hepatitis C by a treatment with peglyated-interferon α-2a. Group A had a significantly better efficacy compared to the control group B, but the side effects need to be concerned.

Key words: Peglyated-interferon; Hemodialysis; Chronic hepatitis C; Treatment; Efficacy; Safety

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INTRODUCTION

Hemodialysis patients are at high risk of infection by hepatitis C virus (HCV) because the hemodialysis unit is a medical environment where exposure to blood is frequent. Therefore, the prevalence of HCV infection, from less than 5% to over 70% in some countries, is greater than the prevalence of HCV infection in the general population[1]. HCV infection is an important cause of morbidity and mortality among patients with end-stage renal disease (ESRD)[2]. HCV infection in patients on maintenance hemodialysis was reported in 10%-59% of patients, in comparison to 0.3%-1.5% observed in the general population[3]. The prevalence of HCV infection is 10%-20% in dialysis patients in developed countries[4,5] and much higher in less developed countries[6]. The prevalence of anti-HCV antibodies among dialysis patients was 40.3% in Turkey[7], 30% in India[8], and 43.9% in Saudi Arabia[9]. In United States of America in 2000, 8.4% of haemodialysis patients were anti-HCV positive[10].

The main mechanisms involved in nosocomial infection with HCV in haemodialysis patients are filter re-use, the use of contaminated haemodialysis machines, and contamination of medical staff's hands. It has been shown that the incidence of HCV infection in haemodialysis patients increases if the medical staff member does not change her/his gloves before injecting each patient and if hepatitis C patients undergo haemodialysis in the same room[11].

The eradication of HCV infection is thought to be valuable for patients with ESRD, especially those who are candidates for kidney transplantation[12]. To prevent the development of these complications and to make these patients suitable for transplantation, standard interferon-α

was used in various doses or regimes for the treatment of these patients[13].

The supplement of a polyethyleneglycol molecule to interferon produces a biologically active molecule with a longer half-life time and more favorable pharmacokinetics; these characteristics enable for a more appropriate once-weekly dosing. When pegylated-interferon α-2a (PEG-IFN) alone is given to chronic hepatitis C patients with normal renal function for 48 wk, the sustained virological response (SVR) rate is approximately twice that with standard interferon[14,15].

This study evaluated the tolerability and efficacy of PEG-IFN in patients with chronic hepatitis C. Therefore, we carried out a controlled prospective longitudinal study to assess the biochemical and the virological response at 48 wk of treatment with PEG-IFN and its tolerability in hemodialysis patients with chronic HCV infection.

MATERIALS AND METHODS

Study design and patients

The present controlled and prospective study was carried out in the Department of Infectious Diseases in Dicle University Hospital, and in one Private Dialysis Center in Diyarbakir, Turkey. In total, 58 among the 161 patients with total hemodialysis in this center were anti-HCV positive (36%). Of the 58 patients, 38 were HCV-RNA positive (65%). Two patients were excluded because they had decompensated liver disease (n = 1), coinfection with hepatitis B virus (n = 1), or because they were lost to follow-up. Thirty-six HCV-RNA positive patients were informed about the benefits and possible risks of PEG-IFN treatment. Fourteen patients were excluded from the study, eleven refused the therapy, and three were not candidates for kidney transplantation and were allocated to the control group (group B). The remaining 22 patients were allocated to the PEG-IFN treatment group (group A). All patients underwent chronic hemodialysis treatment for ESRD during the study period. Hemodialysis was carried out routinely 2-3 times weekly in the patient population. All patients were anti-HCV antibody positive and had detectable HCV-RNA by polymerase chain reaction for at least 6 mo. It has been reported that liver biopsy (histology) is not suggested in the patient with chronic hepatitis C and end-stage renal diseaese because of high bleeding risk.

Inclusion criteria

PEG-IFN therapy was performed in patients meeting the following inclusion criteria: (1) Age < 65 years; (2) absence of pregnancy and agreement to avoid pregnancy during therapy; (3) informed consent; (4) lack of autoimmune, thyroid, psychiatric, or malignant disorders; (5) negative HIV antibody test; and (6) thrombocyte count > 70 000/mm^3 and white blood cell count > 3000/mm^3.

Exclusion criteria

Patients meeting at least one of the following criteria were excluded: (1) Age < 18 or > 65 years; (2) presence of coinfection with HBV or HIV; (3) receiving immunosuppressive therapy or other treatments, namely antihistaminics, non-steroidal anti-inflammatory drugs, aciclovir, or amiodarone; (4) previous treatment for HCV infection; (5) alcohol consumption > 40 g/d; (6) active drug addiction; (7) evidence of hepatocellular carcinoma (α-fetoprotein > 100 ng/mL); (8) hemophilia; or (9) contraindication to interferon therapy.

Study protocol

Patients (group A) enrolled in the study received 135 μg PEG-IFN (40 kDa) (PEGASYS; F. Hoffmann-La Roche, Basel, Switzerland) weekly for 48 wk at the end of dialysis session. All treated patients were evaluated at the end of wk 12 of treatment. The antiviral treatment was continued if the patient had at least a 2-log decline from baseline HCV-RNA level. Patients were followed up and evaluated for 24 wk after completion of treatment. Therapy was monitored weekly by complete blood count and liver function tests (alanine aminotransferase [ALT; U/L], aspartate aminotransferase [AST; U/L]) for 3 mo, then monthly. HCV-RNA testing was carried out before treatment and then every 3 mo. Anti-HCV antibody was measured by a third generation commercial ELISA (Innotest HCV Ab IV; Innogenetics NV, Ghent, Belgium). Liver biopsy was not performed in hemodialysis patients. Serum HCV-RNA was quantified using a reverse transcriptase-polymerase chain reaction assay (Amplicor HCV ver. 2.0; Roche Diagnostic Systems, Branchburg, NJ) with a dynamic range being between 600 and 500 000 IU/mL. All samples were blindly tested in duplicate.

Virological and biochemical response criteria

In group A virological early response (virological EAR), virological end-of-treatment response (virological EOR), and sustained virological response (SVR) were defined as negative HCV-RNA by PCR at 12 and 48 wk of the therapy, and 6 mo after completion of therapy, respectively. In the treatment group, biochemical early response (biochemical EAR), biochemical end-of-treatment response (biochemical EOR), and sustained biochemical response (biochemical SR) were defined as the normalization of serum ALT activity at wk 12 and 48 and 6 mo after completion of therapy, respectively. Although group B patients did not receive PEG-IFN, biochemical and virological recovery at 12, 48, and 72 wk after the beginning of the study were categorized as early response (EAR), end-of-treatment response (EOR), and sustained response (SR), too.

Statistical analysis

Student's t test was used to compare mean values between groups, and the χ^2 test and Fisher's exact test were performed to analyze qualitative data. Parametric data are expressed as mean ± SD. A value of $P < 0.05$ was considered statistically significant. Statistical analysis was performed by using SPSS version 10.0 (SPSS Inc; Chicago, IL).

RESULTS

Enrollment started in November 2004 and the study was finished in July 2006. Seventeen of 22 patients finished therapy. The mean serum viral load before treatment was

Table 1 Demographic and clinical features of study patients

Variables	Group A (n = 22)	Group-B (n = 14)	P
Age (yr)	35.2 ± 12.1	37.1 ± 14.6	0.629
Male (%)	12 (54.5)	10 (71.4)	0.448
BMI (kg/m^2)	25.6 ± 3.3	26.1 ± 3.9	0.78
ALT (IU/L)-Range	59.2 ± 22.4-(33-109)	44.8 ± 20.9-(21-71)	0.489
Viral load (x 10^5 copy/mL)	7.9 ± 4.8	8.1 ± 4.5	0.89
Genotype 1b (%)	86.4	92.9	0.56
HD duration (mo)	52.4 ± 24.7	49.8 ± 21.1	0.95

Table 2 Virological response rates

	Group A (n = 22)	Group-B (n = 14)	P
Early response			
Virological (%)	82.4	0	< 0.001
End-of-treatment response			
Virological (%)	82.4	0	< 0.001
Sustained response			
Virological (%)	64.7	0	< 0.001

2.4 × 10^5 copy/mL. At the beginning of therapy, ALT levels were found to be elevated in fourteen patients (63.6%). In nine of these patients, ALT activity decreased to normal levels within 12 wk of treatment (biochemical EAR 64.3%). At the end of the treatment, four patients still had high ALT levels (biochemical EOR 71.4%). In this group, the mean serum ALT activity at initiation was 59.2 ± 22.4 IU/L (range, 33-109 IU/L). This significantly decreased to 29.9 ± 13.7 IU/L and 21.8 ± 10.9 IU/L at wk 12 (P = 0.017) and at the end of the treatment (P = 0.001), respectively. At the beginning of the study, ALT levels were high in six patients in group B. One of the patients' levels became normal at 12 wk resulting in a biochemical EOR of 16.7%. In the control group, the mean ALT level was 44.8 ± 20.9 IU/L at the beginning. This value declined to 33.8 ± 21.7 IU/L at wk 12 (P = 0.786) and 33.1 ± 18.9 IU/L at wk 48 (P = 0.760).

The mean pretreatment serum HCV-RNA levels were 7.9 ± 4.8 × 10^5 copy/mL and 8.1 ± 4.5 × 10^5 copy/mL in group A and group B, respectively (Table 1).

The viral load was statistically similar between the groups (P = 0.890). All patients treated with PEG-IFN showed at least a 2-log decline from baseline HCV-RNA level. But HCV-RNA became undetectable in 82.4% of the patients at wk 12 of therapy. Virological EOR and SVR occurred in 82.4% and 64.7% of the patients (Table 2). Virological EOR and SVR 0% of the control group.

Therapy with PEG-IFN was associated with a higher rate of virological response than the control group (P < 0.001). All of the subjects had genotype 1. In the treatment group, three patients had genosubtype 1a and 19 had genosubtype 1b. In group B one subject had genotype 1a, and 13 had genotype 1b. There was no significant difference between the groups with respect to genotype distribution (P = 0.560).

Most adverse events were mild to moderate in severity, and all adverse events were typical of those previously reported for PEG-IFN. The drug was suitably tolerated by patients. Flu-like syndrome, throbocytopenia, leucopenia, and anemia were the most frequent side-effects and were experienced in nine patients (53%). These side-effects included flu-like syndrome in eight (47%), fatigue in six (35%), anemia in four (23.5%), thrombocytopenia in three (17.6%), and leucopenia in three of them (17.6%). We had to stop the treatment in five patients (22.7%) in fourth month at the begin of the treatment due to complications (two of anemia, two of weakness, one of gastrointestinal bleeding). The side-effects led to discontinuation of the treatment in five patients. Seventeen of 22 patients finished the treatment in spite of side-effects due to PEG-IFN. No patient had a serious infection during the treatment period.

DISCUSSION

In patients with normal renal function, pegylation increases the size of the molecule, delays its clearance, and enhances the therapeutic effect of standard IFN. It is possible to hypothesize that, in patients with renal failure; the clearance of PEG-IFN would be even more delayed, resulting in higher serum levels of the drug and in a longer half-life time.

The results of this study confirm the efficacy and safety of PEG-IFN therapy in hemodialysis patients with chronic hepatitis C. Treatment for 48 wk with PEG-IFN resulted in sustained virologic responses in 64.7% of patients. HCV infection increases the risk of death in patients on chronic hemodialysis, along with hepatocellular carcinoma and liver cirrhosis[16]. Many controlled and uncontrolled trials have focused on the treatment of chronic hepatitis C patients on chronic haemodialysis with IFN therapy[17], because treatment with PEG-IFN is rarely recommended.

Fabrizi *et al* have found a mean SVR of 37% in chronic hepatitis C patients on dialysis after IFN therapy. Sustained biochemical and virological response rates in patients under classical IFN therapy were reported as 0%-67% and 15.8%-64%, respectively[6]. Sporea *et al* have found, in treatment of these patients with standard IFN the sustained biochemical response of 46.1% and sustained virological response of 38.4% respectively 6 mo after interferon treatment[17]. The promising results at the standard IFN therapy in chronic haemodialysis patients with chronic hepatitis C, have shown that viral clearance occurs in 27%-64% of patients after 12 mo of treatment with standard IFN[18,19].

Patients with end-stage renal disease and chronic hepatitis C might have severe chronic hepatitis despite normal serum liver enzyme activity[20]. In our study, serum ALT levels were normal in 36.4% of the patients at the beginning of the study. Similarly, Perez *et al* reported normal ALT levels in 49% of patients at the beginning of treatment[20]. In the treatment group in our study, serum ALT levels became normal in 71.4% of the patients by the end of the therapy, whereas 16.7% of the patients in the control group had a biochemical response of end of therapy. In contrast, the side-effects of IFN treatment are very important for the patients with ESRD. In several situations, the IFN treatment could not be continued in those patients. Liver biopsy was avoided because of the risk of bleeding in these patients[21].

Recently, a pharmacokinetic study was carried out with

PEG-IFN in subjects with various degrees of stable renal failure who were not yet dialysis dependent. Adsorption and distribution of PEG-IFN were similar in subjects with stable chronic renal impairment versus individuals with normal renal function[22]. The dose of 135 μg of PEG-IFN in patients with ESRD gave similar serum concentrations to a dose of 180 μg in patients with normal renal function. On the trials of PEG-IFN in patients with end-stage renal disease have been designed using weekly doses of 135 μg (as opposed to 180 μg)[7].

HCV genotype 1 is very common in Turkey. Similarly, all of the patients in the present study had genotype 1. Although the response to IFN treatment is not excellent in genotype 1, our results were outstanding[23]. Most of the patients in our study were infected with genotype 1b (86.4% group A, 92.9% group B).

Recently, Kokoglu *et al* reported the results of a controlled study in which PEG-IFN 135 μg/wk for 48 wk was used in hemodialysis patients with HCV infection. They found 83.4% virological EOR and 71.4% biochemical EOR[7]. Sporea *et al* reported on a 50% SVR in hemodialysis patients receiving PEG-IFN 180 μg/wk[24]. In another study, virological response was 40% with PEG-IFN. The difference in virological response could be related to the duration of treatment in the last study (24 wk) and the molecular weight of PEG-IFN (17 kDa)[25]. We found in our study 82.4% virological EOR and 71.4% biochemical EOR.

PEG-IFNs are likely to become a valuable addition for HCV therapy in ESRD when combined with reduced ribavirin doses. However, the pharmacokinetics and tolerability of PEG-IFN and ribavirin combination therapy need to be studied in prospective studies[26]. To date, PEG-IFN and ribavirin combination therapy is the treatment of choice for patients with HCV infection. Ribavirin is metabolized by the kidneys and its clearance reduces in patients with ESRD. High ribavirin serum levels markedly increase the risk of hemolytic anemia and the use of ribavirin in uremic patients, who are often already anemic, could cause severe and life-threatening anemia. Thus, a combination therapy with ribavirin is not an option for treatment of chronic HCV infection in hemodialysis patients[27].

We found in the present study 64.7% SVR and we can expect that in these subjects the use of PEG-IFN would lead to a higher rate of SVR than that observed with standard IFN, probably with a higher rate of adverse effects[11]. PEG-IFN therapy had a successful efficacy in the present study but tolerability was not perfectly. We had to stop the treatment in five patients (22.7%) due to complications (two of anemia, two of weakness, one of gastrointestinal bleeding).

In conclusion, the results of the present study show PEG-IFN (40 kDa) administered at a dose of 135 μg weekly for 48 mo was efficacious but not perfectly tolerable in dialysis patients with HCV infection.

COMMENTS

Background

Our study showed that PEG-IFN (40 kDa) administered at a dose of 135 μg weekly for 48 mo was efficacious but not perfectly tolerable in dialysis patients with chronic hepatitis C.

Research frontiers

The present study was carried out in South-east Anatolien/Diyarbakir, Turkey, in hemodialysis patients with chronic hepatitis C.

Related publications

Yu JW, Wang GQ, Sun LJ, Li XG, Li SC. Predictive value of rapid virological response and early virological response on sustained virological response in hepatitis C virus (HCV) patients treated with pegylated interferon α-2a and ribavirin. *J Gastroenterol Hepatol*. 2007 Jun; 22(6): 832-836. Sporea I, Popescu A, Sirli R, Golea O, Totolici C, Danila M, Vernic C. Pegylated-interferon α 2a treatment for chronic hepatitis C in patients on chronic hemodialysis. *World J Gastroenterol*. 2006 Jul 14; 12(26): 4191-4194.

Innovations and breakthroughs

Many studies published that PEG-IFN treatment is efficacious and tolerable in hemodialysis patients with chronic hepatitis C. In our study we found similar efficacy, but this treatment was not perfectly tolerable in dialysis patients with HCV infection.

Peer review

Ayaz *et al* investigated the effects of PEG-IFN for treatment of HCV infection in hemodialysis patients. Eleven of 17 patients had a SVR at wk 24 after end-of-treatment. In 5 patients treatment had to be stopped because of side effects. The currently available data on this topic are scarce and therefore this is an important study which should be published.

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RAPID COMMUNICATION

Role of echo Doppler ultrasonography in the evaluation of postprandial hyperemia in cirrhotic patients

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Abstract

AIM: To assess the role of echo-Doppler ultrasonography in postprandial hyperemia in cirrhotic patients by comparing the results with the hepatic vein catheterization technique.

METHODS: Patients with cirrhosis, admitted to the portal hemodynamic laboratory were included into the study. After an overnight fast, echo-Doppler ultrasonography (basal and 30 min after a standard meal) and hemodynamic studies by hepatic vein catheterization (basal, 15 min and 30 min after a standard meal) were performed. Ensure Plus (Abbot Laboratories, North Chicago, IL) was used as the standard liquid meal. Correlation analysis of the echo-Doppler and hepatic vein catheterization measurements were done for the basal and postprandial periods.

RESULTS: Eleven patients with cirrhosis (5 Child A, 4 Child B, 2 Child C) were enrolled into the study. After the standard meal, 8 of the 11 patients showed postprandial hyperemia with increase in portal blood flow, portal blood velocity and hepatic venous pressure gradient. Hepatic venous pressure gradient in the postprandial period correlated positively with postprandial portal blood velocity (r = 0.8, P < 0.05) and correlated inversely with postprandial superior mesenteric artery pulsatility index (r = -1, P < 0.01).

CONCLUSION: Postprandial hyperemia can be efficiently measured by echo-Doppler ultrasonography and the results are comparable to those obtained with the hemodynamic studies.

Key words: Postprandial hyperemia; Echo-Doppler; Hemodynamic study; Cirrhosis; Portal hypertension; Portal pressure

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INTRODUCTION

It is well known that portal pressure increases during the postprandial period. It is caused by an augmentation of blood flow into the splanchnic area, a phenomenon known as "postprandial hyperemia"[1,2]. In portal hypertensive patients, postprandial hyperemia may increase the variceal wall tension and trigger acute variceal bleeding. Therefore, an assessment of postprandial hyperemia (PPH) in cirrhotic patients may provide a better understanding of the pathophysiology of variceal bleeding, and consequently impact our treatment strategy.

Hepatic venous pressure gradient measurement by hepatic vein catheterization is the gold standard technique for the assessment of portal pressure changes in postprandial period in patients with cirrhosis[3]. However, this procedure is costly, is uncomfortable for the patients and is not available in every hepatology unit. By contrast, echo-Doppler ultrasonography (USG) is inexpensive, easy for the patients and is available in most hospitals. Sabba *et al* studied the sensitivity of echo-Doppler USG in detecting hemodynamic changes caused by postprandial hyperemia in an observer-blinded study. They found that echo-Doppler USG was highly sensitive for the evaluation of postprandial hyperemia[4]. Additionally, several studies have been done to investigate the effects of different drugs on postprandial hyperemia by using echo-Doppler USG[5-8]. However, to the best of our knowledge, there is no study which compares the hepatic venous catheterization with echo-Doppler USG in the assessment of PPH in patients

with cirrhosis. Therefore, the aim of present study was to investigate the sensitivity of echo-Doppler USG in the assessment of PPH by comparing results with the gold standard test, hepatic vein catheterization.

MATERIALS AND METHODS

Patients

All cirrhotic patients who were admitted to the portal hemodynamic laboratory of Marmara University School of Medicine, Department of Gastroenterology from November 2003 to March 2004, for the hemodynamic measurements before initiation of primary or secondary variceal bleeding prophylaxis were evaluated for the enrollment into the study. The exclusion criteria were age less than 18 or greater than 80 years, presence of hepatocellular carcinoma, coronary heart disease, hypertension, diabetes mellitus, portal vein thrombosis, refractory ascites, chronic lung disease, hepatic encephalopathy, hepatorenal syndrome and refusal to participate in the study.

The study was approved by the ethical committee of Marmara University School of Medicine and all patients gave written informed consent prior to participating in the study.

Study design

Diuretics and beta-blocker drugs were stopped 3 d before the study. After an overnight fast, baseline echo-Doppler (ATL, Ultramark 8 echo-Doppler duplex system) study was performed and the systolic (SBP), diastolic blood pressure (DBP) and heart rate (HR) were obtained. After these procedures, the patients were prepared for hepatic vein catheterization to obtain baseline hemodynamic measurements. Right hepatic vein catheterization was performed percutaneously through the jugular vein and the pressures were recorded in both the wedged and free position, using a balloon catheter (Medi Tech, Cooper Scientific Corp, Watertown, MA). The wedged position was controlled by the absence of reflux after the injection of 2 mL of contrast medium. Pressures were measured by a strain gauge transducer previously calibrated, and the findings were recorded on paper. Each pressure reading was recorded twice and the mean of the two values was used for the final result.

After the baseline measurements were obtained, a standard mixed liquid meal was given which consisted of 237 mL, of balanced dietary supplement (Ensure Plus, Abbot Laboratories, North Chicago, IL), containing 355 kcal as 14.7 g protein, 53.3 g carbohydrate and 32 g lipid. The meal was consumed over a period of 10 min. The mixed liquid meal was chosen because of ease of consumption, and standard formula and calorie intake. Hemodynamic measurements were repeated at 15, 30 and 60 min after the meal. At 30th min after the meal, echo-Doppler USG measurements were repeated simultaneously with the hemodynamic study. Because of technical difficulties (transport of the patients to the Doppler room), echo-Doppler USG could not be performed at the 15th and 60th min of postprandial period.

Parameters

Mean arterial pressure (MAP) = (systolic + diastolic × 2)/3.

Hemodynamic studies

Hepatic venous pressure gradient (HVPG) = Wedge hepatic venous pressure (WHVP) - Free hepatic venous pressure (FHVP).

Echo-Doppler USG

Portal Blood Flow (PBF) = Portal vein cross-sectional area (A) mm^2 × Portal blood velocity (PBV) cm/s.

An area was obtained from the cross-section of the vessel visualized by B-mode after defining the major and minor diameters of the vessel. Velocity measurements were performed in quiet suspended inspiration and averaged over a few seconds. These measurements were obtained from the longitudinal section of the vessel by positioning the sample volume cursor over the vessel at a known angle of insonation, defined as an angle between the Doppler beam and the long axis of the vessel. The Doppler sample volume probe was positioned over the middle of the vessel and was manipulated to cover about 90% of the vessel section in order to detect the maximum Doppler frequency shift, related to the maximum velocity (4).

According to the Doppler equation:

$$\Delta f = \frac{2 \text{ foV max cos Q}}{c}$$

c: speed of the sound in the tissue

Q: angle between the ultrasound beam and the director of the blood flow (angle of insonation)

$$Vm = 0.57 \times Vmax$$

Additionally, pulsatility index (PI) for the superior mesenteric artery (SMA) was measured according to the formula:

PI = Vmax - Vmin/Vmean

To decrease the inter-observer variability, all measurements were repeated 3 times by 2 different investigators and the mean value was used for statistical analysis.

Statistical analysis

Baseline results for the measurements are given as mean ± SE of absolute values. The values over time (Basal, 15 and 30 min after the test meal) were compared by ANOVA and the Student's *t* test. Spearman correlation tests were done between the measurements of echo-Doppler USG and hemodynamic studies.

RESULTS

One hundred patients attending the hepatology outpatient clinic of the Marmara University Hospital were screened. However, only 11 patients were included in the study; the remainder were excluded due to factors such as clinical status, high Child-Pugh score, hematologic parameters, patients not providing informed consent and other exclusion criteria. The characteristics of the 11 patients are shown in Table 1. Eight out of the 11 patients showed postprandial hyperemia as judged by

Table 1 Demographic characteristics of the study patients

Age	48 ± 11.2
Male/Female	8/3
Alcoholic etiology (yes/no)	2/9
Child-Pugh Score	7.6 ± 2.3
Child-Pugh Class (A/B/C)	5/4/2
History of variceal bleeding	
Yes	6
No	5
History of encephalopathy	
Yes	4
No	7
History of ascites	
Yes	6
No	5
History of SBP	
Yes	2
No	9
ALT (IU/L)	52.27 ± 24.2
Total protein (g/L)	6.97 ± 0.9
Albumin (g/d)	3.52 ± 0.5
PT (s)	17.3 ± 2.9
Total bilirubin (mg/dL)	1.81 ± 0.5

ALT: Alanine aminotransferase; PT: Prothrombin time.

Table 2 Hemodynamic and echo-Doppler measurements of the study patients

	Baseline	Postprandial (15 min)	Postprandial (30 min)	*P*
Hemodynamic measurements				
FHVP (mmHg)	14.2 ±3.6	15.2 ± 4.1	15.9 ± 5.9	NS
WHVP (mmHg)	30.5 ± 7.6	38.7 ± 11.8[b]	37.1 ± 10.4	< 0.05
HVPG (mmHg)	16.5 ± 6.7	23.7 ± 11.1[d]	21.2 ± 9.5	< 0.05[a]
Echo-Doppler measurements				
PBF (L/min)	1.5 ± 0.9		2.4 ± 1.1	< 0.05
PBV (cm/s)	23.5 ± 11.6		32.9 ± 12.3	< 0.01
SMA-V (cm/s)	120.2 ± 23.3		158.1 ± 59.9	< 0.05
SMA-PI	2.8 ± 0.5		1.7 ± 0.2	< 0.05
Systemic measurements				
MAP (mmHg)	85 ± 12.9		80 ± 9.6	< 0.05
Pulse Rate (min)	89.70 ± 21.2		85.3 ±13.3	< 0.05

[b]$P < 0.01$ postprandial 15 min *vs* basal hepatic venous pressure gradient (HVPG); [a]ANOVA statistics of basal, 15 min and 30 min measurements of hepatic vein catheterization; [d]$P < 0.01$ postprandial 15 min HVPG *vs* basal HVPG. FHVP: Free hepatic venous pressure; WHVP: Wedge hepatic venous pressure; PBF: Portal Blood Flow; PBV: Portal blood velocity; SMA: Superior Mesenteric Artery; PI: Pulsatility index; V: Velocity; MAP: Mean arterial pressure.

both echo-Doppler and hemodynamic studies, shown in Table 2. The maximum postprandial hyperemia was obtained at the 15 min. measurement by hepatic vein catheterization. Three patients did not show any increase in the hemodynamic or echo-Doppler parameters during the postprandial period (data not shown).

When compared with the baseline parameters, there was a statistically significant difference in WHVP, HVPG, PBF, PBV, SMA-V, SAMA-PI, MAP and pulse rate at the 15th and 30th min of the postprandial period, with the portal vein velocity showing the highest increase ($P < 0.01$) (Table 2).

Spearman correlation tests were performed in order to evaluate the correlation between the echo-Doppler and hemodynamic parameters. Postprandial PBV showed a positive correlation with post-prandial HVPG values ($r = 0.8$, $P < 0.05$) (Figure 1), whereas SMA-PI showed an inverse correlation with the HVPG ($r = -1$, $P < 0.01$) (Figure 2). The percent increase in the echo-Doppler and hemodynamic measurements did not show any correlation between the echo-Doppler and hemodynamic tests.

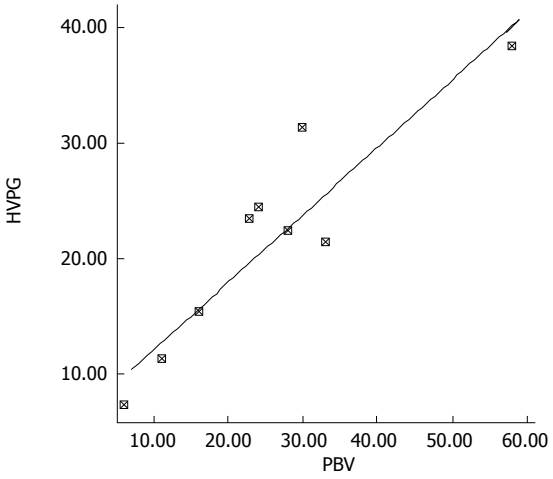

Figure 1 Positive correlation between the portal blood velocity (PBV) and hepatic venous pressure gradient (HVPG) ($r = 0.8$, $P < 0.05$).

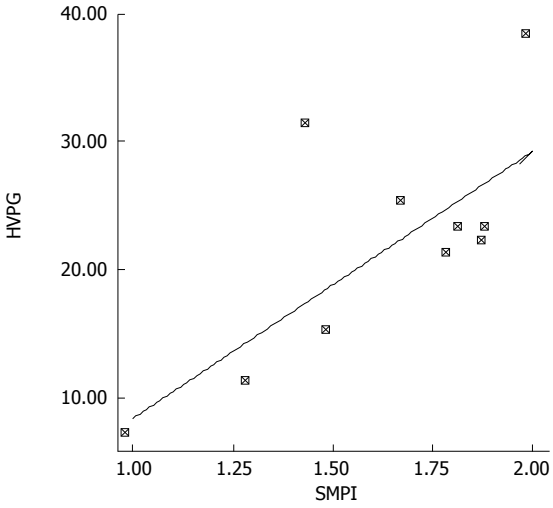

Figure 2 Inverse correlation between the superior mesenteric artery pulsatility index (SMA-PI) and hepatic venous pressure gradient (HVPG) ($r = -1$, $P < 0.01$).

DISCUSSION

The development of a non-invasive test for monitoring portal hemodynamics has been the subject of debate ever since the importance of portal pressure in cirrhotic patients was established. The increase in portal pressure in the postprandial period may initiate variceal bleeding in cirrhotic patients[9]. Therefore, it is important to identify patients who experience increased postprandial hyperemic response, and treat these patients appropriately. Echo-Doppler ultrasonography is a non-invasive test which is widely used to determine portal blood flow[4,7,8,10] In an observer-blinded study, it was shown that echo-

Doppler USG is highly sensitive in detecting postprandial hemodynamic changes[4]. Since hepatic venous pressure gradient measurement is the gold standard for the evaluation of portal hemodynamic changes, the aim of the present study was to compare the results obtained with this technique with echo-Doppler ultrasonography.

We observed that patients who showed postprandial increase in HVPG also demonstrated an increase in the parameters by echo-Doppler ultrasonography. Initially, we did not find any correlation between the basal measurements obtained by echo-Doppler and hemodynamic studies. However, postprandial portal blood flow increased significantly in patients who showed significant postprandial HVPG increase. Moreover, correlation analysis showed a strong correlation between postprandial PBV and hepatic venous pressure gradient (Figure 1). The SMA index showed a negative correlation with the HVPG (Figure 2). However, we did not find any correlation between portal blood flow and hemodynamic measurements. Portal blood velocity and SMA-PI are both velocity measurements which were obtained by direct measurements. However, measurement of portal blood flow requires assessment of cross sectional portal vein area which depends on the position of the probe as well as that of the patient, which may result in marked variability of portal blood flow readings. It is difficult to explain the lack of correlation in the basal measurements obtained by echo-Doppler and hemodynamic studies, although there was a good correlation during the postprandial period. This may be due to individual differences in the basal parameters obtained by echo-Doppler studies, which is why the echo-Doppler studies cannot replace the gold standard hemodynamic studies in the primary and secondary prophylaxis of variceal bleeding.

Hepatic venous pressure gradient measurement is the gold standard for the evaluation of portal hemodynamics. To the best of our knowledge, the present study is the only one which has compared hepatic vein catheter and echo-Doppler measurements. Assessment of patients with significant increase in postprandial portal pressure allows the use of several treatment options. It was recently observed that administration of low dose isosorbide mononitrate attenuates postprandial hyperemia[10]. By contrast, propranolol is ineffective in blunting postprandial hyperemia but has some effect in reducing postprandial portal blood flow[11]. Somatostatin analogs have a favorable effect in reducing postprandial hyperemia in cirrhotic patients[12-14]. Most of these studies are performed by echo-Doppler measurement[8,12,13].

Another unexpected finding in the present study was the absence of postprandial hyperemia in 3 patients (data not shown). These 3 patients showed more collateral vessels in the splanchnic area on echo-Doppler studies, compared to the other patients. In a recent study, it was shown that the extent of collateral circulation influences postprandial increase in portal pressure in patients with cirrhosis[15]. Other factors such as circadian rhythm, posture, age and gender did not have any effect in patients who failed to show a significant increase in postprandial pressure[16-20]. We believe that an increase in collateral circulation prevents postprandial rise in portal pressure despite increased blood flow in the splanchnic area.

In conclusion, if properly performed, echo-Doppler ultrasonography is a good test to measure postprandial portal hemodynamics. Future studies are needed to assess the usefulness of echo-Doppler measurements in primary and secondary prophylaxis of variceal bleeding.

COMMENTS

Background

Echo-Doppler USG is widely used for evaluation of the mechanisms of postprandial hyperemia in healthy people and cirrhotic patients, although the findings have not been validated.

Research frontiers

This is the first study which investigated the validity of echo-Doppler USG in postprandial hyperemia by comparing the results with the gold standard test, hepatic vein catheterization.

Innovations and breakthroughs

Postprandial pressure and portal blood flow were evaluated in eleven patients enrolled from a cohort of cirrhotic patients, by hepatic vein catheterization and echo-Doppler USG at 15 min and 30 min after a standard liquid meal. Although, basal parameters did not show any correlation, hepatic venous pressure gradient and portal blood velocity showed a positive corelation in postprandial measurements.

Applications

Although there were some limitations in the present study, it can be stated that the evaluation of postprandial hemodynamic alterations can be performed safely by echo-Doppler USG, and the measurements obtained by echo-Doppler study reflect accurately the postprandial parameters in cirrhotic patients.

Peer review

This is an experimental work conducted in humans, comparing hepatic vein catheterization with echodoppler measurement of post prandial hyperemia in cirrhotics. The authors conclude that the increased portal pressure observed in the post prandial period can be reliably measured by echo-doppler. The work is novel and may be clinically relevant.

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RAPID COMMUNICATION

Is obesity associated with gastropharyngeal reflux disease?

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Abstract

AIM: To examine the association between obesity and gastropharyngeal reflux disease (GPRD) as well as gastroesophageal reflux disease (GERD)

METHODS: We conducted a cross-sectional study of consecutive patients undergoing ambulatory 24-h dual-probe pH monitoring from July 2003 to December 2006. The association between body mass index (BMI) and parameters about gastroesophageal or gastropharyngeal reflux was examined in univariate and multivariate analyses.

RESULTS: A total of 769 patients (307 men and 462 women; mean age 50.7 years) were finally enrolled. Most variables showing gastroesophageal reflux was higher in the obese patients than the patients with normal BMI. There was no difference in all the variables showing gastropharyngeal reflux according to the BMI. After adjustment for age, sex, alcohol intake and smoking, obese patients demonstrated an about 2-fold increase in risk of GERD compared with patients with normal BMI (OR, 1.9; 95 CI, 1.3-2.9), but overweight patients did not demonstrate increased risk of GERD (OR, 1.2; 95 CI, 0.8-1.7). Both obese patients and overweight patients did not demonstrated increased risk of GPRD compared with patients with normal BMI (OR, 1.1; 95 CI, 0.8-1.7; and OR, 0.9; 95 CI, 0.6-1.3, respectively).

CONCLUSION: Obesity is not associated with GPRD reflux while it is associated with GERD.

Key words: Obesity; Body mass index; Gastroesophageal reflux; Gastropharyngeal reflux

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INTRODUCTION

The worldwide prevalence of being overweight and obesity has been increasing at an alarming rate over the last decade, indiscriminately affecting populations of both higher and lower middle income countries[1]. The rise in obesity coincides with rising prevalence of gastroesophageal reflux disease[2,3], and gastroesophageal reflux disease is a common disorder that has been linked to obesity.

Obesity is a postulated risk factor for gastroesophageal reflux disease, although individual studies have conflicting results[4-8]. Some studies suggest that an increased body mass index (BMI) is associated with increased esophageal acid exposure[9] and with an increased risk of hospitalization for esophagitis[10]. In contrast, other studies, including one of the largest population-based studies to date, have found no association between BMI and gastroesophageal reflux disease[11-13]. Potential explanations for the disparate results include a true lack of an association between BMI and gastroesophageal reflux disease, differences in definitions or methodology, dissimilar study populations, or a lack of power to detect an effect in some studies. Additionally, many studies assessing the relationship between gastroesophageal reflux disease and obesity are symptom-based and lack objective tests to confirm this association.

Gastropharyngeal reflux, also called laryngopharyngeal reflux, is a term used to describe esophageal acid reflux into the laryngeal and pharyngeal areas. It causes extraesophageal manifestations (e.g., chronic cough, hoarseness, asthma, globus sensation, chronic sinusitis, or other pulmonary or otorhinolaryngologic diseases). Currently, the best way to demonstrate gastropharyngeal reflux is ambulatory 24-h dual probe pH monitoring[14]. Up to present, there are few reports on the association between BMI and gastropharyngeal reflux disease[15].

Therefore, we conducted this cross-sectional study to

examine the association of BMI and gastropharyngeal reflux disease as well as gastroesophageal reflux disease by using the ambulatory 24-h dual probe pH monitoring.

MATERIALS AND METHODS

Study design

We conducted a cross-sectional study of consecutive patients who underwent ambulatory 24-h dual-probe pH monitoring from July 2003 to December 2006 at the motility laboratory in Pusan National University Hospital (Busan, Korea). The indications for ambulatory 24-h dual-probe pH monitoring were globus sensation (sensation of a lump, something sticking in the throat), hoarseness, chronic cough, halitosis, throat clearing and laryngeal pathology such as vocal polyp. We did not enroll patients who had history of gastric surgery, were diagnosed as scleroderma or achalasia, or were on anti-reflux medications at the time of the study.

This study was reviewed and approved by the Institutional Review Board at Pusan National University Hospital.

Evaluation of body mass index

Body mass index (BMI) was calculated as body weight (kg) divided by the square of standing height (m). The BMI was categorized into 3 levels according to the WHO for the Western Pacific region[16]: normal weight-BMI < 23 kg/m^2, overweight-BMI $\geqslant$ 23 kg/m^2 and $\leqslant$ 25 kg/m^2, obese-BMI > 25 kg/m^2.

Esophageal manometry

All antisecretory and prokinetic medications were discontinued at least 7 d before testing. Esophageal manometry was performed, after an overnight fast, using an eight-lumen catheter (Synetics Medical Co., Stockholm, Sweden) with side holes 3, 4, 5, 6, 8, 13, 18, and 23 cm from the catheter tip and a water-perfused, low-compliance perfusion system (Synetics Medical Co., Stockholm, Sweden), according to a standard protocol. Briefly, the manometry protocol included the following: first, a station pull-through was performed through the lower esophageal sphincter (LES) to determine the end-expiratory resting pressure, LES length, and location relative to the nares. Then the catheter was positioned with the most-distal side-hole 2 cm below the upper margin of the LES. Ten 5-mL water swallows were given to evaluate peristalsis; only esophageal body contractions measured at 3, 8, and 13 cm above the LES were recorded for data analysis. Then the catheter was pulled through the upper esophageal sphincter (UES) in the same manner (station pull-through) to determine the resting UES pressure, length, and location relative to the nares. Esophageal manometric abnormalities were classified as achalasia, diffuse esophageal spasm, nutcracker esophagus, isolated hypertensive LES, ineffective esophageal motility, or nonspecific esophageal motility disorder[17].

Ambulatory 24-h dual-probe pH monitoring

Ambulatory 24-h dual-probe pH monitoring was performed immediately after esophageal manometry, with using a single-use monocrystalline antimony dual-site pH probe (Zinetics 24, Medtronic Inc., Minneapolis, USA) with electrodes placed at the tip and 15 cm proximal to the tip. A cutaneous reference electrode placed on the upper chest was also used. All the electrodes were calibrated in buffer solutions of pH 7 initially and then pH 1. The pH catheter was introduced transnasally into the stomach and withdrawn back into the esophagus until the electrodes were 5 cm above the proximal margin of the LES. The subjects were encouraged to eat regular meals with restriction for intake of drink or food with a pH below 4. All the subjects recorded their meal times (start and end), body position (supine and upright), and any symptoms in a diary. The data was collected using a portable data logger (Digitrapper Mark Ⅲ, Synetics Medical Co., Stockholm, Sweden) with a sampling rate of 4 s, and was transferred to a computer for analysis using "Polygram for Windows release" 2.04 (Synetics Medical Co., Stockholm, Sweden). For both sites, a decrease in pH below 4, which was not induced by eating or drinking, was considered the beginning of a reflux episode, and the following rise to pH above 4 was considered the end of such an episode. To be accepted as a gastropharyngeal reflux event, the decrease at the proximal probe had to be abrupt and simultaneous with the decrease in the esophagus, or to be preceded by a decrease in pH of a similar or larger magnitude in the distal probe. Thus, acid episodes induced by oral intake, aero-digestive tract residue and secretions, proximal probe movement, or loss of mucosal contact in which the proximal pH decline may precede the esophageal pH drop were not included as gastropharyngeal reflux episodes.

The variables assessed for gastroesophageal reflux in the distal probe were the total percentage of time the pH was < 4, the percentage of time the pH was < 4 in the supine and upright positions, the number of episodes the pH was < 4, the number of episodes the pH was < 4 for $\geqslant$ 5 min, the duration of the longest episode the pH was < 4 and the DeMeester composite score[18].

The variables assessed for gastropharyngeal reflux in the proximal probe were the total percentage of time the pH was < 4, the percentage of time the pH was < 4 in the supine and upright positions, and the number of episodes the pH was < 4.

For the diagnosis of gastroesophageal reflux disease (GERD) in the distal probe, two different aspects were analyzed[19,20]: (1) total reflux time: the total proportion of the recorded time with pH < 4; a value of > 4 was considered abnormal; (2) number of reflux episodes: the total number of pH episodes with pH < 4 during the recording; a value of > 35 episodes was considered abnormal.

For the diagnosis of gastropharyngeal reflux disease (GPRD) in the proximal probe, we considered more than 0.1 for the total, 0.2 for the upright, and 0 for the supine time of pH < 4 to be pathological. For the number of reflux episodes, more than 4 reflux episodes were considered pathological[21,22].

Assessment by endoscopy

The presence or absence of reflux esophagitis,

Table 1 Patient profiles and the endoscopic findings according to the body mass index

	Body mass index (kg/m^2)			*P* value
	< 23 (*n* = 344)	23-25 (*n* = 231)	>25 (*n* = 194)	
Age (yr, mean ± SEM)	48.4 ± 0.7	51.5 ± 0.7	53.8 ± 0.8	< 0.001
Gender (%)				0.077
Men	123 (35.8)	104 (45.0)	80 (41.2)	
Women	221 (64.2)	127 (55.0)	114 (58.8)	
Alcohol intake	66 (19.2)	50 (21.6)	55 (28.4)	0.048
Smoking	42 (12.2)	31 (13.4)	33 (17.0)	0.295
Indication for pH monitoring (%)				0.210
Globus	156 (45.3)	100 (43.3)	86 (44.3)	
Hoarseness	76 (22.1)	60 (26.0)	54 (27.8)	
Coughing	54 (15.7)	46 (19.9)	26 (16.4)	
Others[1]	58 (16.9)	25 (10.8)	28 (14.4)	
Endoscopic findings (%)				
Reflux esophagitis[2]	32/291 (11.0)	19/204 (9.3)	32/166 (19.3)	0.009
Hiatal Hernia	15/291 (5.2)	19/204 (9.3)	12/166 (7.2)	0.199
Endoscopically suspected esophageal metaplasia	18/291 (6.2)	18/204 (8.8)	16/166 (9.6)	0.348

[1]Other indications were halitosis, throat clearing and laryngeal pathology such as vocal polyp; [2]Los Angeles classification grade.

endoscopically suspected esophageal metaplasia and hiatal hernia were determined by two endoscopists (G.H. Kim, G.A. Song), who were blind to the information of the ambulatory 24-h pH monitoring.

Reflux esophagitis

If esophagitis was present, it was graded according to the Los Angeles classification[23].

Hiatal hernia

Hiatal hernia was defined as a circular extension of the gastric mucosa above the diaphragmatic hiatus greater than 2 cm in the axial length.

Endoscopically suspected esophageal metaplasia (ESEM)

The presence or absence of ESEM[24] was examined in the lower portion of the esophagus, including the esophagogastric junction, during inflation of the esophagus before inserting the endoscope into the stomach. The esophagogastric junction was defined as the oral side end of the fold, which exists continuously from the gastric lumen[25], as well as the end of the anal side of the fine longitudinal vessel, because the veins in the lower part of the esophagus were distributed uniformly, running parallel and longitudinally in the lamina propria[26,27]. The squamo-columnar junction was defined by a clear change in the color of the mucosa. ESEM was defined as the area between the squamo-columnar junction and the esophagogastric junction.

Statistical analysis

Data were expressed as mean ± SE unless otherwise noted. The differences in gender, alcohol intake, smoking, indication for pH monitoring, reflux esophagitis, hiatal hernia, ESEM, manometric diagnosis, GERD and GPRD according to the BMI were assessed using the χ^2 test. The one-way ANOVA was used to assess statistical significance for age, parameters of esophageal manometry and parameters of ambulatory pH monitoring according to the BMI and post-hoc analysis was performed using the Tukey's HSD. Multiple logistic regression analyses were used to examine association of the two primary outcomes (GERD, GPRD) with the main predictor variable, BMI. GERD and GPRD was adjusted for age, sex, alcohol intake and smoking. For all models, the number of covariates examined was determined by the number of outcome events with 10 events required for one covariate[28]. Patients with normal BMI constituted the reference group in comparisons between BMI levels. Odds ratios (ORs) and their 95 confidence intervals (CIs) were used to assess the association between BMI and GERD or GPRD, defined by ambulatory pH monitoring. A $P < 0.05$ was considered statistically significant. Statistical calculations were performed using the SPSS version 12.0 for Windows software (SPSS Inc., Chicago, IL, USA).

RESULTS

A total of 769 patients were enrolled in the study: 307 men and 462 women, and their mean age was 50.7 years. Of them, 661 patients underwent the upper endoscopy. Obese patients were more likely to be older and alcohol drinker. There was no difference in the hiatal hernia and ESEM according to the BMI. Reflux esophagitis was higher in the obese patients than the patients with normal BMI and the overweight patients ($P < 0.05$) (Table 1).

There was no difference in the proximal esophageal amplitude, LES pressure and LES length according to the BMI. But the distal esophageal amplitude was higher in the obese patients than the patients with normal BMI and the overweight patients ($P < 0.05$). There was no significant difference in esophageal motility abnormalities according to the BMI (Table 2).

There was no difference in all the variables showing gastropharyngeal reflux in the proximal probe according to the BMI. The total and upright time of pH below 4, the number of reflux episodes, and the DeMeester composite score was higher in the obese patients than the patients with normal BMI ($P < 0.05$) (Table 3).

Table 2 Results of esophageal manometry according to the body mass index (mean ± SE)

	Body mass index (kg/m^2)			*P* value
	< 23 (*n* = 344)	23-25 (*n* = 231)	> 25 (*n* = 194)	
Proximal esophageal amplitude (mmHg)	59.6 ± 1.7	60.1 ± 2.0	62.1 ± 2.4	0.650
Distal esophageal amplitude (mmHg)	75.1 ± 2.0	79.5 ± 2.9	90.2 ± 3.1[a]	< 0.001
LES pressure (mmHg)	20.9 ± 0.4	20.3 ± 0.5	20.6 ± 0.6	0.711
LES length (cm)	3.3 ± 0.0	3.3 ± 0.0	3.3 ± 0.0	0.636
Peristalsis (%)	96.5 ± 0.6	95.2 ± 0.8	96.1 ± 0.7	0.640
Manometric diagnosis				0.440
Normal	143	80	84	
Diffuse esophageal spasms	4	1	1	
Nutcracker esophagus	16	15	17	
Hypertensive LES	2	2	2	
Ineffective esophageal motility	103	74	52	
Nonspecific esophageal motility disorder	76	59	38	

Post hoc analysis using Tukey's HSD: [a]$P < 0.05$ *vs* BMI < 23 and BMI 23-25.

Table 3 Results of ambulatory 24-h dual probe pH monitoring according to the body mass index (mean ± SE)

	Body mass index (kg/m^2)			*P* value
	< 23 (*n* = 344)	23-25 (*n* = 231)	> 25 (*n* = 194)	
Proximal probe				
Time pH < 4 (total)	0.7 ± 0.2	0.5 ± 0.1	0.5 ± 0.1	0.502
Time pH < 4 (upright)	0.8 ± 0.1	0.7 ± 0.1	0.8 ± 0.1	0.809
Time pH < 4 (supine)	0.6 ± 0.3	0.2 ± 0.1	0.1 ± 0.0	0.245
No. of reflux episodes	11.1 ± 1.8	8.4 ± 1.0	9.5 ± 1.1	0.452
Distal probe				
Time pH < 4 (total)	2.7 ± 0.2	3.7 ± 0.5	3.8 ± 0.3[a]	0.038
Time pH < 4 (upright)	3.9 ± 0.3	5.8 ± 0.9	5.6 ± 0.5[a]	0.021
Time pH < 4 (supine)	1.4 ± 0.3	2.2 ± 0.6	2.1 ± 0.3	0.211
No. of reflux episodes	39.1 ± 2.3	46.1 ± 3.1	59.4 ± 4.9[a,b]	< 0.001
No. of reflux episodes ⩾ 5 min	1.6 ± 0.2	1.6 ± 0.2	2.0 ± 0.3	0.477
Longest reflux episode (min)	8.6 ± 1.3	11.3 ± 2.4	11.1 ± 0.9	0.385
DeMeester composite score	11.0 ± 0.9	14.5 ± 1.7	15.7 ± 1.2[a]	0.016

Post hoc analysis using Tukey's HSD: [a]$P < 0.05$ *vs* BMI < 23; [b]$P < 0.05$ *vs* BMI 23-25.

Table 4 Association of body mass index with gastroesophageal reflux disease and gastropharyngeal reflux disease, defined by ambulatory pH monitoring

	Body mass index (kg/m^2)			*P* value
	< 23 (*n* = 344)	23-25 (*n* = 231)	> 25 (*n* = 194)	
Gastroesophageal reflux disease (%)				0.001
Absent	203 (59.0)	121 (52.4)	82 (42.3)	
Present	141 (41.0)	110 (47.6)	112 (57.7)	
Gastropharyngeal reflux disease (%)				0.162
Absent	143 (41.6)	103 (44.6)	69 (35.6)	
Present	201 (58.4)	128 (55.4)	125 (64.4)	

The frequency of GERD defined by the ambulatory pH monitoring was higher in obese patients, not in overweight patients than in the patients with normal BMI. There was no significant difference in the frequency of GPRD defined by the ambulatory pH monitoring according to the BMI (Table 4).

After adjustment for age, sex, alcohol intake and smoking, obese patients demonstrated an about 2-fold increase in risk of GERD defined by the ambulatory pH monitoring compared with patients with normal BMI (OR, 1.9; 95 CI, 1.3-2.9), but overweight patients did not demonstrate increased risk of GERD (OR, 1.2; 95 CI, 0.8-1.7). Both obese patients and overweight patients did not demonstrated increased risk of GPRD defined by the ambulatory pH monitoring compared with patients with normal BMI (OR, 1.1; 95 CI, 0.8-1.7; and OR, 0.9; 95 CI, 0.6-1.3, respectively) (Table 5).

DISCUSSION

In present study, we evaluated GERD and GPRD by objective mean (ambulatory 24-h dual-probe pH monitoring) in a large group of 769 patients. Obese patients demonstrated an about 2-fold increase in risk

Table 5 Multivariable analysis: unadjusted and adjusted analyses for relationships of the body mass index with gastroesophageal reflux disease and gastropharyngeal reflux disease, defined by ambulatory pH monitoring

	Body mass index (kg/m^2)		
	< 23	23-25	> 25
Gastroesophageal reflux disease			
Unadjusted OR (95 CI)	1	1.3 (0.9-1.8)	2.0 (1.4-2.8)
Adjusted OR (95 CI)[1]	1	1.2 (0.8-1.7)	1.9 (1.3-2.9)
Gastropharyngeal reflux disease			
Unadjusted OR (95 CI)	1	0.9 (0.6-1.2)	1.3 (0.9-1.9)
Adjusted OR (95 CI)[1]	1	0.9 (0.6-1.3)	1.1 (0.8-1.7)

[1]Adjusted for age, sex, alcohol intake and smoking.

of GERD defined by the ambulatory pH monitoring compared with patients with normal BMI, but overweight patients did not demonstrate increased risk of GERD. Also, most variables showing gastroesophageal reflux was higher in the obese patients than in the patients with normal BMI, but not in overweight patients than in the patients with normal BMI. These results were in accord with the previous report that all measures of esophageal acid exposure were observed only for obesity, but not for overweight when compared to normal BMI[29]. In addition, we analyzed the endoscopic findings according to the BMI. Reflux esophagitis was higher in the obese patients than in the patients with normal BMI and the overweight patients.

The mechanism by which obesity promotes GERD remains unclear. One potential mechanism is related to mechanical factors whereby an increase in abdominal fat leads to an increase in intragastric pressure[9,30,31], and increased frequency of transient lower esophageal sphincter relaxation[32]. Obese patients may have an increased risk for hiatal hernia, which has a role in initiating and promoting gastroesophageal reflux[33,34]. On the other reports[35,36], there was not statistically significant association between BMI and hiatal hernia, similar to our results.

We also assessed the degree of gastropharyngeal reflux according to the BMI. There was no difference in all the variables showing gastropharyngeal reflux in the proximal probe according to the BMI. Also, obese patients did not demonstrated increased risk of GPRD defined by the ambulatory pH monitoring compared with patients with normal BMI. These results were consistent with the only previous report[15] about the association of BMI and GPRD. In that report, the authors showed that obesity was not associated with the pharyngeal reflux events but had a significant association with esophageal reflux events. But they simply compared the mean pharyngeal and esophageal reflux numbers between 195 non-obese patients and 90 obese patients. Even though we demonstrated the similar result that GPRD was not associated with BMI, we included much more patents, categorized them into 3 levels (normal, overweight, obesity) and performed the multiple logistic regression analysis after adjustment for age, sex, alcohol intake and smoking. Also, our results were consistent with a prospective study[37] that obesity was not risk factors for the occurrence of extraesophageal disorders after multivariate analysis, although the presence of extraesophageal disorders was assessed by only a questionnaire.

The prevalence of esophageal motility abnormalities according to the BMI is not yet unknown. In present study, there was no significant difference in esophageal motility abnormalities according to the BMI. Hong *et al*[38] showed the increased distal esophageal amplitude in 33 of morbidly obese patients and they suggested that this might be due to the presence of a high intraabdominal-thoracic pressure gradient in morbidly obese patients. This would cause a functional outflow obstruction of the esophagus, creating a high-pressure zone within the esophagus. In response to this chronic high-pressure zone, the distal esophagus would have to produce high amplitude contractions for passage of oral contents into the stomach. Similarly, in present study, the distal esophageal amplitude was higher in the obese patients than in the patients with normal BMI and the overweight patients. There was no difference in the LES pressure and LES length according to the BMI, which is consistent with previous reports[9,39].

Much more controversy exits about the location of the proximal probe. The recording of the pH in the hypopharynx is technically difficult. Acid exposure in the hypopharynx can easily be missed because of the relatively large space within the hypopharynx[21]. On the contrary, placement of the proximal probe in or below the upper esophageal sphincter allows a more permanent contact with the mucosa during the 24-h period resulting in fewer artifacts[21,22]. We used the dual-site pH probe with electrodes placed at the tip and 15 cm proximal to the tip, and we could not choose the exact location of proximal probe. But in most cases (72.7, 559/769), the proximal probe was located in the upper esophageal sphincter. So, for the diagnosis of GPRD, we used the criteria proposed by Smit *et al*[21,22].

There were some limitations in this study. First, ambulatory pH monitoring can be subjected to measurement errors related to placement of the probe and instrument calibration. However, all procedures were conducted using a similar technique with single-use pH probe. Another limitation is the absence of systematic collection of GERD symptoms. Because our main focus was to examine objective evidence of GERD (i.e., ambulatory pH monitoring) according to the BMI, we did not administer structure questionnaire. Nevertheless, this study had several advantages including the prospective design including the measurement of weight, height, alcohol intake and smoking, the consecutive enrollment to reduce the impact of selection bias and the large sample size. In addition, numerous patients (661/769) underwent the upper endoscopy and we could analyze the endoscopic findings according to the BMI.

Why is obesity not associated with GPRD despite of the association with GERD? First, acid refluxed into esophagus is usually cleared by gravity and peristaltic contractions. In obese patients, the esophageal peristaltic contraction is not impaired compared with patients with normal BMI. On the contrary, the distal esophageal amplitude is increased. This fact would play some role in preventing the refluxed acid extending to the upper level. Second, the amount of acid refluxed into esophagus is

increased in obese patients, so it is easily assumed that the amount of acid refluxed into the upper level would be increased by a secondary phenomenon. The refluxed acid is neutralized by esophageal submucosal secretions and swallowed salivary secretions, so it becomes non-acid reflux material. Therefore, even though this non-acid refluxate would reach to the upper level, the proximal pH probe cannot detect it. To solve this problem, a prospective study using a combined multichannel intraluminal impedance and pH measurement which is able to detect both acid and non-acid reflux, as well as the proximal extent of the refluxate, will be needed.

In summary, this is the largest study to evaluate GERD and GRPD simultaneously according to the BMI by using the ambulatory 24-h dual-probe pH monitoring. Obesity is associated with GERD but is not associated with GPRD. Further studies using a combined multichannel intraluminal impedance and pH measurement will be needed.

COMMENTS

Background

Obesity is a postulated risk factor for gastroesophageal reflux disease, although individual studies have conflicting results. Some studies suggest that an increased body mass index (BMI) is associated with increased esophageal acid exposure and with an increased risk of hospitalization for esophagitis. In contrast, other studies have found no association between BMI and gastroesophageal reflux disease. Gastropharyngeal reflux is a term used to describe esophageal acid reflux into the laryngeal and pharyngeal areas. It causes extraesophageal manifestations such as chronic cough, hoarseness, asthma, globus sensation, chronic sinusitis, or other otorhinolaryngologic diseases. This study was to examine the association of BMI and gastropharyngeal reflux disease as well as gastroesophageal reflux disease.

Research frontiers

The research front in this area is focused on the association of BMI and gastropharyngeal reflux disease as well as gastroesophageal reflux disease. There have been many debates about the association of obesity with gastroesophageal reflux disease. Up to present, there are few reports on the association between BMI and gastropharyngeal reflux disease. This study has examined the association of BMI and gastropharyngeal reflux disease as well as gastroesophageal reflux disease by using the ambulatory 24-h dual-probe pH monitoring. Obesity was associated with increased risk of gastroesophageal reflux disease but was not associated with increased risk of gastropharyngeal reflux disease.

Innovations and breakthroughs

There are few reports on the association between BMI and gastropharyngeal reflux disease. Most previous studies about the association of obesity with gastroesophageal reflux disease are symptom-based and lack objective tests such as ambulatory 24-h pH monitoring. This study is the largest study to evaluate gastroesophageal reflux disease and gastropharyngeal reflux disease simultaneously according to the BMI by using the ambulatory 24-h dual-probe pH monitoring.

Applications

Obesity is associated with GERD but is not associated with GPRD. Further studies using a combined multichannel intraluminal impedance and pH measurement will be needed.

Terminology

Esophageal acid reflux into the laryngeal and pharyngeal areas causes extraesophageal manifestations such as chronic cough, hoarseness, asthma, globus sensation, chronic sinusitis, or other otorhinolaryngologic diseases. This condition is called as gastropharyngeal reflux disease.

Peer review

This manuscript describes a well-designed GPRD. The classification of obesity is much higher in the United States and Europe, so this may be an issue that warrants attention. Nonetheless, this is important research.

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RAPID COMMUNICATION

Referral for anorectal function evaluation is indicated in 65% and beneficial in 92% of patients

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Abstract

AIM: To determine the indicated referrals to a tertiary centre for patients with anorectal symptoms, the effect of the advised treatment and the discomfort of the tests.

METHODS: In a retrospective study, patients referred for anorectal function evaluation (AFE) between May 2004 and October 2006 were sent a questionnaire, as were the doctors who referred them. AFE consisted of anal manometry, rectal compliance measurement and anal endosonography. An indicated referral was defined as needing AFE to establish a diagnosis with clinical consequence (fecal incontinence without diarrhea, 3rd degree anal sphincter rupture, congenital anorectal disorder, inflammatory bowel disease with anorectal complaints and preoperative in patients for re-anastomosis or enterostoma, anal fissure, fistula or constipation). Anal ultrasound is always indicated in patients with fistula, anal manometry and rectal compliance when impaired continence reserve is suspected. The therapeutic effect was noted as improvement, no improvement but reassurance, and deterioration.

RESULTS: From the 216 patients referred, 167 (78%) returned the questionnaire. The referrals were indicated in 65%. Of these, 80% followed the proposed advice. Improvement was achieved in 35% and a reassurance in 57% of the patients, no difference existed between patient groups. On a VAS scale (1 to 10) symptoms improved from 4.0 to 7.2. Most patients reported no or little discomfort with AFE.

CONCLUSION: Referral for AFE was indicated in 65%. Beneficial effect was seen in 92%: 35% improved and 57% was reassured. Advice was followed in 80%. Better instruction about indication for AFE referral is warranted.

INTRODUCTION

Anorectal function evaluation (AFE) consists of several tests. Institutions differ in their selection of tests[1]. At our tertiary centre, anal manometry, rectal compliance measurement and anorectal endosonography are performed as part of our standard procedure[2]. Defecography and colon transit time are performed on strict indications. Neurophysiological tests of the pelvic floor are performed only for research purposes. Anal manometry establishes anal pressures while rectal compliance measures sensitivity and the volume of the rectum. Anal endosonography visualizes possible defects or atrophy of the anal sphincter complex. AFE is often requested in patients with anorectal symptoms including fecal incontinence, anal soiling, fistulas, anorectal tumours, anal pain, constipation *etc*. AFE is available in a limited number of hospitals, mainly academic centres and some large peripheral clinics.

A clinical referral (no research purposes) is indicated when disease can be demonstrated or excluded on the basic of AFE and when it has further therapeutical consequences. Which patients benefit most from anorectal function tests (by reduction of symptoms or reassurance) is unclear. Literature concerning this issue is scarce. Most studies that mention anorectal function tests in relation to anorectal pathology limit themselves to pre- and post-treatment results. Therefore, it often remains unclear whether AFE leads to relevant findings or subsequent change of therapy[1-10]. A large multi-centre Dutch study referred to the value of AFE for outcome of physiotherapy in patients with fecal incontinence[1]. One conclusion was that AFE had no predictive value for outcome of physiotherapy. Further, referral for AFE largely depended on availability of these tests in the referring hospital.

The aim of this study was to determine the indicated referrals to our tertiary center for patients with anorectal symptoms, the effect of the advice on their complaints and the perceived discomfort for the patients during AFE.

MATERIALS AND METHODS

Patients

All patients who were first clinical referrals for AFE between May 2004 and October 2006 were selected from our database. The database contained the complete medical history and extensive data of anorectal symptoms and anorectal test results. Deceased patients were excluded. All patients were sent a questionnaire. Additional data about follow-up in the outpatient clinic, hospital admittance, diagnostic and therapeutic procedures performed in our hospital could be retrieved from the (electronic) patient hospital files.

The Medical Ethical Commission of the VU University Medical Centre granted permission.

The referring doctors

The doctors who referred patients in the study period were also sent a questionnaire.

Anorectal function evaluation (AFE)

This consisted of anal manometry, rectal compliance measurement and anorectal endosonography according to our methods previously described elsewhere[11].

Indicated referral

A referral is indicated when disease can be demonstrated or excluded on the basic of AFE and when it has further therapeutical consequences. These are patients with fecal incontinence without diarrhea, 3rd degree sphincter rupture with or without fecal incontinence, congenital disorders, patients with inflammatory bowel disease with anorectal complaints and preoperative in patients for re-anastomosis or enterostoma, anal fissure or constipation. In patients with fistula, an ultrasound is always indicated but anal manometry and rectal compliance measurement only on indication regarding fecal incontinence. Test results in all these patients influence management. In patients with constipation, AFE was considered indicated in suspected Hirschsprungs' disease and surgery. AFE was not considered indicated in patients with fissures treated conservatively, soiling (defined as anal discharge without overt fecal incontinence), anal pain and hemorrhoids, since results do not change management.

Questionnaires

The questionnaire for patients[2] contained questions about the actual received therapy and changes in their symptoms by the received treatment, stated in a Visual Analogue Score (VAS) (score 1-10, 1 = very bad, 10 = very good) and also stated as (1) improved, (2) no change but reassurance or acceptance of situation without further need for seeking other medical advice and (3) worse and/or no reassurance. Discomfort and pain during the examination was scored with VAS (score 1-10, 1 = very uncomfortable/painful, 10 = no discomfort/pain).

The questionnaire for the referring doctor[2] consisted of questions about implementing the advice (yes, no), the quality of the advice (good, neutral, poor) and the willingness to refer again (yes, no).

Treatment advice strategy

The patients with symptoms of fecal incontinence were divided into five diagnostic subtypes: incontinence due to a sphincter defect, neurogenic incontinence, combined incontinence (sphincter defect and neurogenic), incontinence due to small rectal capacity and incontinence due to diarrhea. Patients with incontinence due to diarrhea were advised to have the cause of their diarrhea treated by the referring doctor.

All patients with fecal incontinence were prescribed fibres and physiotherapy. When unsuccessful additional therapy was advised depending of the cause. Patients with a sphincter defect > 25% were offered a sphincter repair. In patients with a small rectal compliance an enterostoma was proposed (< 60 mL) or strongly recommended (between 60 and 100 mL)[12].

Patients with a known 3rd degree sphincter rupture and as a result fecal incontinence were advised as other patients with fecal incontinence and the strong advice for a cesarean section with a next childbirth. If they were not incontinent, depending on the size of the rupture, the possibility of a cesarean section for next childbirth was discussed.

Advising re-anastomosis or enterostoma depended on the total impression of the anorectal function measured with anal pressures, rectal compliance and sphincter defects or atrophy.

In patients with a fistula the extension of the fistula tract(s) with anal ultrasound determined the type of surgery in our hospital (fistulotomy in simple and curettage with mucosal advancement plasty in complicated fistulas).

Patients where AFE was not indicated also received an advice. In patients with constipation a fibre-enriched diet, additional fibres and laxatives were advised. When unsuccessful and not previously attempted, pelvic floor physiotherapy was advised. When constipation coexisted with complaints of prolapse, a defecography was advised. When surgery was considered (rectocele correction or colectomy) besides an AFE, also a colon transit time was performed. Patients with fissures were treated conservatively; when treatment failed they were referred to the surgeon and AFE was indicated. Hemorrhoids and mucosal prolapse were treated with rubber band ligation. A hemorrhoidectomy was advised only in refractory cases. Local causes of anal pain were treated according to their causes. When no local abnormalities were seen in patients with anal pain, fibres and referral to the anaesthesiologist was advised.

Statistical analysis

The results were described as mean with standard deviation. The χ^2 test for independence and for trend, the Kruskal-Wallis test and the Wilcoxon matched-pair test were used when appropriate (GraphPad InStat Software, San Diego, Ca, USA).

RESULTS

Response questionnaires

There were 216 first referrals for AFE, 181 (84%) females, mean age 51 years, (SD 15, range 15-82). Two patients had died. A total of 167 patients [137 females (82%), mean age 51 years, SD 15, range 16-82] returned an adequate (almost all questions answered) questionnaire (78%).

Indicated referrals

Table 1 shows the indicated referrals. Of the 167 referrals, 109 (65%) were indicated. The most frequent referral was fecal incontinence, from which 93% was indicated (7% had diarrhea).

Non-indicated referrals

Two of the 31 patients with constipation had signs of anismus during physical examination and anal manometry. Of the five patients with soiling, four had a mucosal prolapse and/or hemorrhoids. The fifth patient had an anal fissure on inspection, not previously found. In two patients with anal pain a fissure was found, one treated conservatively and one eventually much later with surgery. AFE revealed no abnormalities in all patients besides high rest pressure in the patients with fissures. AFE did not influence therapeutic advice.

Effect of treatment

Symptoms improved in 54 patients (35%). In 88 patients (57%) symptoms were unchanged but patients were reassured. Despite treatment, 12 patients (8%) deteriorated. The whole group improved one point on the VAS scale (5.1-6.1) ($P < 0.0001$), for those improved (35%) this was even 3.2 points (4.0-7.2). Both indicated and non indicated referred patients improved equally.

The causes of fecal incontinence were: sphincter defect (14), neurogenic (37), combined incontinence (10), incontinence due to diarrhea (5) and incontinence due to small rectal capacity (< 100 mL) (5). Within these groups, the largest improvement was seen in the combined incontinence group (1.8 point) ($P = 0.01$). Patients with a small rectal capacity had no improvement at all.

The actual therapies received by the patients according to the reason for referral are mentioned in Table 2. Some patients received several therapies. The most frequent advice was medication, mainly fibres.

Of all referred patients, only 17% were operated. No difference between effectiveness of conservative and surgical treatment could be observed on patient symptoms ($P = 0.09$).

AFE induced little stress, indicated by an average pain score of 7 (SD 2.7) and a discomfort score of 7.2 (SD 2.8). Two patients with fistulas experienced the examination as unpleasant and painful due to the hydrogen peroxide injection in their fistula tract during anal ultrasound. Thirty five females (26%) preferred to be examined by a female doctor while the remaining 102 (75%) had no preference. Twenty six males (93%) had no preference and the remaining two (7%) preferred a male and a female doctor, respectively ($P < 0.0001$). Dutch ethnic minorities did not influence these data.

Table 1 Indicated referrals in the main groups of patients

	All *n* (% of referrals)	Indicated *n* (% of that group)
Incontinence	71 (43)	66 (93)[1]
Constipation	31 (19)	6 (19)[2]
3rd sphincter rupture	21 (13)	21 (100)
Pain	9 (5)	0 (0)
Re-anastomosis/enterostoma	8 (5)	8 (100)
Soiling	5 (3)	0 (0)
IBD	4 (2)	4 (100)
Hemorrhoids	3 (2)	0 (0)
Anal atresia	2 (1)	2 (100)
Fistulas	2 (1)	2 (100)
Fissure	1 (1)	0 (0)
Other	9 (5)	0 (0)
Total	167 (100)	109 (65[3])

[1]Five patients with diarrhea not indicated; [2]Only patients suspected of Hirschsprung/surgery indicated, IBD-inflammatory bowel disease; [3]Percentage of all referrals.

Questionnaires referring doctors

Of the 214 questionnaires, 102 (48%) responses were obtained. The advice was nearly always implemented (96%). The quality of the advice was considered good in 76% and neutral in 24%. All doctors except one (98%) were willing to refer again.

Agreement between proposed and followed advice.

The proposed and followed therapies are shown in Table 3. Therapies could also be a combination of medication, physiotherapy or surgery. Dietary advice was always followed (100%), while surgical advice was generally followed (89%). Less accepted advice included medication (71%) and physiotherapy (73%) ($P = 0.005$, 99% CI).

DISCUSSION

The 78% response to the questionnaires of the patients was good. In our previous study we reported a similar result[2]. Only 65% of the referrals were indicated. In 35% the diagnosis could have been established by clinical examination or added nothing. This is a signal that more communication and education is warranted, especially in times with restrictions and limited resources. However, many of the referred patients suffered from chronic symptoms, bringing both patient and doctor to despair. The possibility of referring the patients to another centre may come as a welcome alternative. The symptoms of the whole group improved an average of one point from 5.1 to 6.1 on a ten-point scale. Actual improvement took place in 35% of the referred patients; they improved an average of 3.2 points. The moderate improvement might be explained by the fact that it concerned patients with chronic disorders, already treated conservatively for a long time. Success was not related to a specific symptom, diagnosis or treatment, only the five patients with fecal incontinence due to small rectal capacity did not improve. Deterioration in 8% of the patients was mainly due to the fluctuating course of the chronic complaints combined with their reluctance to follow the advice. In 80% of patients, advice was followed. Medication and

Table 2 Reason for referral and effect of treatment on patients

2004-2006		Treatment according to the patients						Symptoms change after treatment				
								VAS		Category		
Reason for referral	Patients	Treatment[1]	Diet *n* (%)	Medication *n* (%)	Surgery *n* (%)	Physiotherapy *n* (%)	Expectative *n* (%)	Before	After	Improved	Reassured	Worse
Incontinence[2]	71	100	8	32	12[3]	26	22	5	5.7	20	39	7
Constipation	31	35	3	12	3[4]	7	10	5	6.2	11	17	1
3rd sphincter rupture	21	22	2	1		2	17	7.1	7.2	2	15	1
Anal pain	9	8		1	1[5]		6	3.5	5.1	3	5	
Surg/Stoma	8	8			6		2	6.3	7	2	4	
Soiling	5	7	2	3	2			3.4	4.8	3	1	1
IBD	4	4		2			2	4.8	6.5	2	2	
Hemorrhoids	3	6	1	3	2[6]			4.3	6.3	2	1	
Anal atresia	2	2		1			1	6	7.5	2		
Fistula	2	2					2	4	5.5	1	1	
Fissure	1	2		1	15			2	8	1		
Pouchitis	1	1					1	8	7			1
Other	9	12	1	1	8	1	1	5.1	6.7	5	3	1
Average (SD)								5.1[b] (2.4)	6.1[b] (2.3)			
Total (%)	167	209	17 (8)	57 (27)	35 (17)	36 (16)	64 (31)			54 (35)	88 (57)	12 (8)

[1]Treatment as answered by patients, several treatments per patient possible; [2]Including all subgroups; [3]Sphincter repair; [4]Rectocele repair; [5]Fissurectomy; [6]Hemorroidectomy. [b]$P < 0.0001$.

Table 3 A comparison between the proposed therapeutic advice and followed therapy. A therapy can consist of more components

	All therapies (%)	Diet (%)	Medication (%)	Physiother (%)	Expectative (%)	Surgery (%)
Followed	130 (80)	7 (100)	54 (71)	32 (73)	31 (100)	36 (90)
Not followed	32 (20)	0	22 (29)	12 (27)	0	4 (10)
Total	162 (100)	7 (100)	76 (100)	44 (100)	31 (100)	40 (100)

physiotherapy were the least applied therapies (Table 3). Some disagreement between advised and followed therapy could be explained by the fact that patients considered fibres a diet instead of medication. Physiotherapy was advised in 44 patients (26%) and effectuated in 32 (73%). Ten years ago this was only respectively 18% and 67%[2]. Increasing interest in pelvic floor disorders and special training for physiotherapists has certainly contributed to the change in attitude towards physiotherapy[1,13]. Although therapeutic advice was given after AFE, actual improvement in symptoms is not necessarily caused by AFE. A placebo effect due to the referral to a specialized centre and the knowledge present in a 3rd referral centre may play a role. This is comparable with biofeedback studies for fecal incontinence, were the added value of the biofeedback was very difficult to separate from the received specialized care and treatment[13,14].

The examination was generally well tolerated, except in two patients with fistulas who reported the examination as painful. This was caused by local injection of hydrogen peroxide into the external fistula opening in order to visualize the fistula tract. It was remarkable that ten years ago only 13% of the females[2] and now 26% of the females preferred a female doctor. The larger number of referred Dutch ethnic minorities could not explain this.

Although the questionnaire was retrospective and has not been officially validated (we had used it before[2]), it has proven to be very useful. Questions about for instance surgery or 3-6 mo of physiotherapy could not easily be misunderstood. In patients treated in our own hospital follow up data were also obtained from the (electronic) patient files and no discrepancies were found with the answers provided by these patients.

Our treatment advice strategy is derived from clinical practice and the literature. In patients with fecal incontinence, regulating defecation and thickening of the fecal mass has proven to be effective and should always be tried first[15-17]. Biofeedback aimed at improving rectal sensation, recto-anal coordination and training external anal sphincter contraction is the next step and has a success rate varying from 40%-85% and is closely related to patient motivation[18]. Diarrhea should be properly diagnosed and treated before referring the patient for AFE since this overwhelming factor makes it impossible to establish the (possible) importance of anorectal causes. A rectal capacity between 60 and 100 mL will lead to fecal incontinence in 50% and < 60 mL in 100% of patients[12]; they will often need an enterostoma. Patients with fecal incontinence with a significant sphincter defect (> 25%) without severe neuropathy leading to atrophy can be identified as suitable candidates for a sphincter repair[1,19-21].

In our group of incontinent patients, only 12 (18%) ultimately underwent sphincter repair. Two patients were later referred for sacral neuromodulation elsewhere and eleven patients[22] were treated with SECCA® (radio frequent energy application to the external sphincter[23,24]).

Women who experienced a 3rd degree sphincter rupture are indicated for AFE, even without complaints. There in always some damage to the external anal sphincter and appropriate advice concerning defecation regulation, physiotherapy and possible future cesarean section can be discussed.

Most patients with constipation were referred for assessment of anismus/hyper tonic pelvic floor or rectocele. Generally AFE is not needed in these patients. Both anismus and a rectocele can be diagnosed by proper rectal examination[6,25,26]. When prolapse complaints dominate a defecography is indicated to demonstrate a possible enterocele as this can be corrected surgically. In patients with constipation, correction is not indicated in accidentally found intussusception since the obstructed defection will not improve[26-29]. AFE is indicated when (partial) colectomy is considered to be informed about the continence reserve. For patients with fistulas anal endosonography demonstrates the fistula tracts and anal manometry will establish the continence reserve[21,30-33]. In patients with soiling (anal secretion), medical history, good physical and rectal examination and an additional proctoscopy have proven be sufficient to establish a diagnosis[10], without the need for AFE, as was shown again in our patients. For patients with pain, AFE does not contribute[34]. Suspected discrete abnormalities e.g. an occult abscess, could not be demonstrated in our study as well. Sometimes a fissure is found in these patients, diagnosed on the basis of the medical history and rectal examination. In patients with a fissure, high pressures are usually found using manometry, but this does not alter therapy[35]. Only in those who where conservative measures have failed and will undergo surgery AFE seems indicated. In patients with haemorrhoids, anal manometry can also reveal high pressures and anal endosonography can demonstrate a thickened mucosa; however, these findings have no influence on therapy[36,37]. AFE is indicated in patients with an enterostoma when re-anastomosis is considered. In some rare disorders like anal atresia AFE can also be indicated to document anorectal problems and help choose a specific therapy.

In conclusion, feferral for AFE was indicated in 65%, communication and education to colleagues seems warranted. Indications are fecal incontinence without diarrhea, 3rd degree sphincter rupture, pre-operative for stoma or re-anastomosis, fistula, fissures or constipation. Anal ultrasound is always indicated in patients with fistula, anal manometry and rectal compliance when impaired continence reserve is suspected. Generally, in patients with constipation and soiling the medical history, physical examination and additional proctoscopy is sufficient and AFE is not necessary.

In 80% of patients, advice was followed. After AFE 92% benefited (35% of the patients improved and 57% was reassured). AFE is well tolerated. Women preferred a female doctor in 26% of cases.

COMMENTS

Background

Anorectal disorders like fecal incontinence, peri-anal fistula, pre-operative decisions for stoma are distressing and isolating conditions, with a large impact on quality of life. With restricted resources it is important to make a good selection of referrals for anorectal function evaluation, those patients who benefit most. In this study we established the indicated referrals our tertiary referral centre for patients with anorectal symptoms, the effect of the advised treatment and the discomfort of the tests.

Research frontiers

A clinical referral is indicated when disease can be demonstrated or excluded on the basic of anorectal function evaluation and when it has further therapeutical consequences. Which patients benefit the most from anorectal function tests (by reduction of symptoms or reassurance) is unclear.

Innovations and breakthroughs

Most studies mention anorectal function tests in relation to anorectal pathology and limit themselves to pre- and post-treatment results. Therefore it often remains unclear whether anorectal function evaluation leads to relevant findings or subsequent change of therapy. Literature concerning this issue is scarce. The aim of our study was to determine the indicated referrals to our tertiary center for patients with anorectal symptoms, the effect of advice on their complaints and the perceived discomfort for the patients during anorectal function evaluation.

Applications

It is very important to understand the usefulness of the anorectal function evaluation to provide referrals of those patients, which could benefit the most. Indications for anorectal function evaluation are fecal incontinence without diarrhea, 3rd degree sphincter rupture, pre-operative for stoma or re-anastomosis, fistula, fissures or constipation. Anal ultrasound is always indicated in patients with fistula, anal manometry. Rectal compliance is indicated when impaired continence reserve is suspected. Generally, in patients with constipation and soiling the medical history, physical examination and additional proctoscopy is sufficient and anorectal function evaluation is not necessary.

Terminology

Anorectal function evaluation consists of several tests: (1) anal manometry: establishes anal pressures; (2) rectal compliance: measures sensitivity and the volume of the rectum; (3) anal endosonography: visualizes possible defects or atrophy of the anal sphincter complex.

Peer review

The manuscript presents referral patterns for anal function investigation. Although the study has all limitations of a retrospective study, the authors provide valuable information from their experience as tertiary care center for patients with anorectal symptoms.

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RAPID COMMUNICATION

Changing spectrum of Budd-Chiari syndrome in India with special reference to non-surgical treatment

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Abstract

AIM: To evaluate patterns of obstruction, etiological spectrum and non-surgical treatment in patients with Budd-Chiari syndrome in India.

METHODS: Forty-nine consecutive cases of Budd-Chiari syndrome (BCS) were prospectively evaluated. All patients with refractory ascites or deteriorating liver function were, depending on morphology of inferior vena cava (IVC) and/or hepatic vein (HV) obstruction, triaged for radiological intervention, in addition to anticoagulation therapy. Asymptomatic patients, patients with diuretic-responsive ascites and stable liver function, and patients unwilling for surgical intervention were treated symptomatically with anticoagulation.

RESULTS: Mean duration of symptoms was 41.5 ± 11.2 (range = 1-240) mo. HV thrombosis (HVT) was present in 29 (59.1%), IVC thrombosis in eight (16.3%), membranous obstruction of IVC in two (4%) and both IVC-HV thrombosis in 10 (20.4%) cases. Of 35 cases tested for hypercoagulability, 27 (77.1%) were positive for one or more hypercoagulable states. Radiological intervention was technically successful in 37/38 (97.3%): IVC stenting in seven (18.9%), IVC balloon angioplasty in two (5.4%), combined IVC-HV stenting in two (5.4%), HV stenting in 11 (29.7%), transjugular intrahepatic portosystemic shunt (TIPS) in 13 (35.1%) and combined TIPS-IVC stenting in two (5.4%). Complications encountered in follow-up: death in five, re-stenosis of the stent in five (17.1%), hepatic encephalopathy in two and hepatocellular carcinoma in one patient. Of nine patients treated medically, two showed complete resolution of HVT.

CONCLUSION: In our series, HVT was the predominant cause of BCS. In the last five years with the availability of sophisticated tests for hypercoagulability, etiologies were defined in 85.7% of cases. Non-surgical management was successful in most cases.

Key words: Budd-Chiari syndrome; Interventional radiology; Ascites; Hepatic vein thrombosis; Percutaneous transluminal angioplasty; Stent; Transjugular intrahepatic portosystemic shunt; Thrombophilia

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INTRODUCTION

Budd-Chiari syndrome (BCS) refers to hepatic venous outflow tract obstruction (HVOTO) starting from the level of small hepatic veins (HV) through large HV and inferior vena cava (IVC) to the junction of the IVC and right atrium[1-3]. This includes both hepatic vein thrombosis and IVC thrombosis or obliterative hepatocavopathy[4,5].

Clinical studies on BCS in India date back to the 1970s[6-10]; following which, there are many case reports and case studies from India, concentrating on clinical spectrum, on underlying etiology, on diagnostic modalities and/or on various treatment strategies[11-47]. In contrast to the western world, in the Indian series, there is a striking predominance of IVC obstruction (mainly from membrane or web) or combined IVC-HV obstruction rather than HV obstruction alone[12-15]. Tumors, pregnancy, oral contraceptive pills (OCP) and infections were proposed as predominant underlying etiologies, hypercoagulable states were uncommon, and idiopathic cases were the most common[9,12-21,42]. In the series of cases during the last 10 years, various hypercoagulable states are being increasingly described as etiologies of BCS[24-26,28-30,46,47].

Obstruction of the IVC, either thrombotic or non-thrombotic, was considered to be a major cause of BCS in Asia[5]. Two-thirds of IVC obstruction cases leading to BCS are due to membranous obstruction[11]. Datta *et al*[6] reported 40 cases of membranous obstruction of IVC (MOVC) while Victor *et al*[11] reported 17 cases

of membranous obstruction. Though MOVC and membranous obstruction of HV was initially thought to be congenital, Okuda demonstrated these lesions to be an after-effect of thrombosis in the IVC or HV which were organised over a period of time[5]. Similar cases of have been reported from Nepal[53]. Originally, it was perceived that MOVC and membranous obstruction of HV are important causes of BCS which is different from the West. Over the years, many prothrombotic states leading to BCS have been described in India[28,47]. In an elegant review, Valla has described the level of HVOTO, the nature of obstructive lesions, presentation, course of disease and causes that are comparable in Indian and western literature[4]. A few differences in etiologies were noticed between India and the West i.e. peripartum occurrence of BCS was common in India while oral contraceptive use was commonly implicated in the West.

The majority of patients with BCS present with a chronic course, while only a small number of patients present with acute or fulminant forms[6-22]. BCS is mostly encountered in the adult population and considered uncommon in children; when seen in children, the clinical presentation is similar[13]. Anti-thrombotic drugs and anticoagulants form the mainstay in the treatment of acute and chronic BCS. Percutaneous balloon angioplasty for membranous obstruction of the IVC and hepatic vein has also been used successfully in the treatment of BCS. Although TIPS has gained popularity in the treatment of BCS, it has rarely been performed in India, with only one case being reported[41]. Furthermore, there are only a few reports on the use of covered TIPS in BCS. In this report, we evaluated patterns of obstruction, aetiological spectrum and non-surgical treatment of BCS in patients.

MATERIALS AND METHODS

In last seven years (during the study period of 1999 to 2005), all the consecutive cases of BCS were prospectively evaluated. Diagnosis of BCS was based on angiographic evidence of HVOTO (i.e. obstruction of IVC and/or HV) and/or histological evidence of BCS (sinusoidal dilation with centrilobular congestion with variable amount of pericentral hepatocyte necrosis and pericentral fibrosis).

The protocol for evaluation of BCS in our unit is shown in Figure 1. BCS was suspected in the following situations: patients with ascites of high serum-ascitic fluid albumin gradient (> 1.1) and with low cell count (< 250 cells/cm^2); patients with ascites and/or back veins in the presence of hepatomegaly and/or right upper quadrant pain; patients with refractory ascites, patients with acute liver failure with hepatomegaly and/or ascites; patients with a known hypercoagulable state who showed evidence of liver involvement (on clinical examination, biochemistry or imaging); and patients with unexplained chronic liver disease (when work-up for alcoholic, drug-induced, viral, metabolic or autoimmune liver disease were negative). All such patients (suspected BCS) were subjected to ultrasonography of the abdomen and Doppler studies with special emphasis on IVC, HV and splanchnic venous system. If HVOTO was confirmed, patients were subjected to inferior vena cavogram and hepatic venogram

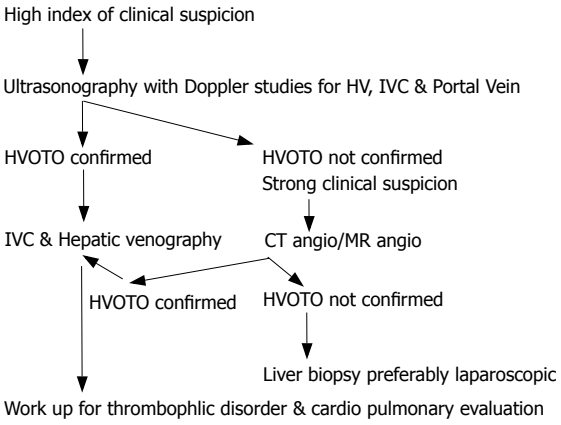

Figure 1 Evaluation of patients with Budd Chiari syndrome.

(*via* transfemoral, transjugular and/or transhepatic route) to define the site and morphology of the obstruction. However, if ultrasonography/Doppler was negative or ambiguous for HVOTO, computerised tomography (CT) angiography or magnetic resonance (MR) angiography (if CT angiography was contraindicated) was performed. If CT angiography or MR angiography showed HVOTO, these patients were subjected to catheter venography. If computerised tomography with angiography or magnetic resonance angiography was negative, these patients were subjected to liver biopsy (either *via* percutaneous or transjugular route depending on clinical status) to define BCS and rule out other diseases like veno-occlusive disease. All patients with suspected BCS were subjected to chest x-ray, electrocardiogram and 2-dimensional echo of the heart to rule out cardiac etiology. Liver histology was performed whenever possible to confirm the diagnosis and to define the presence of cirrhosis. Upper gastrointestinal endoscopy was performed in all cases of BCS to determine the presence of varices.

All the patients with BCS were subjected to tests available for hypercoagulable state before starting any treatment. In the initial two years of the study period, tests for protein C, protein S and antithrombin Ⅲ levels in addition to work-up for myeloproliferative disease and paroxysmal nocturnal haemoglobinuria (PNH) were performed. During the last five years of the study period, tests for protein C, protein S and antithrombin Ⅲ levels (corrections for liver dysfunction were done)[48], serum homocysteine levels, factor V Leiden, prothrombin gene 20210 and MTHFR gene mutations, lupus anticoagulant, anticardiolipin and antiphospholipid antibodies, tests for PNH (Sucrose lysis and Ham tests), complete blood counts and bone marrow histopathology/cytogenetic studies (whenever feasible) for myeloproliferative disorder were performed. Imaging (ultrasonography, computerised tomography and/or magnetic resonance imaging of abdomen), serological markers (including tumor markers and tests for amoebiasis or echinococcus) and/or histology were done to identify underlying etiology for BCS as and when required. All the female patients were subjected to a urine test for pregnancy and were questioned regarding use of oral contraceptive pills.

All patients with refractory ascites (RA) or deteriorating

Table 1 Clinical parameters in 49 patients with BCS

Clinical features	% of Patients
Ascites	86
Distended abdominal wall veins & back veins	28
Jaundice	20
Splenomegaly	20
Pedal edema	12
Upper GI bleeding	8
Infertility	6
Fever	8
Hepatic encephalopathy	4
Hydrothorax	6
Vericocity	4
Asymptomatic	6
Previous episode of thrombosis	8
Family history of thrombotic event	2
Previous antituberculous treatment	12
Mean duration of symptoms prior to diagnosis	41.5 + 11.2 mo (range 1-240 mo)
Types of presentations	
Asymptomatic	6
Fulminant	8
Acute	41
Chronic	42

Patients were classified as per definitions in reference number 4

Table 2 Laboratory parameters in 49 patients with BCS

Parameter	Value	Range
Sr. Bilirubin mg%	1.49 ± 1.2	0.2-5.6
Sr. ALT Iu/mL	53.4 ± 13.2	17-424
Sr. AST Iu/Ml	61.6 ± 15.6	16-724
Sr. AlkPO4 Iu/mL	159.6 ± 35	91-313
Sr. GGPT	109.6 ± 1.2	18-417
Sr. Albumin g/Dl	3.44 ± 1.2	1-4.8
Increase in prothrombin time	3.5 ± 1.1	1-10 s

All the patients were negative for serology of Hepatitis B, C & HIV.

liver function (presence of hepatic encephalopathy (HE) or jaundice, serum bilirubin > 2 mg/dL, serum albumin < 3 gm/dL and/or international standardised ratio of prothrombin time (INR) > 2), depending on morphology of IVC and/or HV obstruction, were triaged for either IVC stenting, HV stenting, TIPS or in combination: (1) For supra-hepatic IVC block with patent HV: IVC balloon angioplasty with self-expandable metallic stent (SEMS) placement; (2) For membranous obstruction of inferior vena cava (MOVC): IVC balloon angioplasty; (3) For juxta-hepatic IVC block with patent HV: IVC balloon angioplasty with SEMS placement; (4) For juxta-hepatic IVC block and short-segment HV block (< 3 cm) involving all three HV or two major HV: IVC balloon angioplasty with SEMS placement and HV balloon angioplasty with SEMS placement; (5) For juxta-hepatic IVC block and long-segment HV block (> 3 cm): IVC balloon angioplasty with SEMS followed by TIPS placement; (6) For short-segment HV block (< 3 cm) involving all three or two major HV: HV balloon angioplasty with SEMS placement; (7) For long-segment HV block (> 3 cm) involving all three or two major HV: TIPS placement. In addition to symptomatic therapy, all these patients were started on anticoagulation following radiological intervention with the aim of keeping INR 2-3. Asymptomatic patients, patients with diuretic-responsive ascites, patients with stable liver function or patients who were not willing to undergo radiological intervention were subjected to anticoagulation to keep INR 2-3 in addition to symptomatic therapy (including low-salt diet, diuretic therapy and/or beta-blockers as needed). Peritoneo-venous shunting was offered to patients with RA who were not candidates for any intervention.

Follow-up was done on the 3rd d, at the end of the 1st mo, every 3rd mo for the 1st year and every 6th mo thereafter. During each follow-up visit, clinical, biochemical and ultrasonography/Doppler evaluations were done. Patients with TIPS or SEMS dysfunction were managed by repeat radiological interventions.

For patients undergoing TIPS, the following outcome measures were used: (a) technical success: creation of a channel by stent between hepatic vein and portal vein to reduce the portosystemic pressure gradient (PPG) to less than 12 mmHg; (b) TIPS dysfunction: 50% decrease in portal vein blood flow velocity on Doppler, angiographic evidence of stent stenosis (> 50% reduction in diameter) or increase in PPG > 12 mmHg in presence of recurrent ascites/hepatic hydrothorax or gastrointestinal bleeding; and (c) primary patency: duration of continuous TIPS patency without re-intervention. Comparison between uncovered TIPS and covered TIPS was done using chi square test. Institutional review board permission was obtained.

RESULTS

During the study period, 49 patients (mean age = 34.3 ± 6.5 years; age range = 1-57 years; male:female ratio = 24:25) were included in the study. In our series there were only two children below the age of 18 years. One child at the age of one year presented with rapidly accumulating ascites, the other child at the age of 11 had refractory ascites for 5 mo.

Clinical presentation and biochemical features of these patients are shown in Tables 1 and 2.

Ultrasonography with Doppler studies was diagnostic in 80% of patients. The remaining 20% of patients required CT/MR angiography for diagnosis. Liver histology was done in 28 (57.1%) cases, of which 14 (50% of 28 cases) were cirrhotic.

Morphology of hepatic venous outflow tract obstruction was short segment hepatic vein thrombosis in 13 patients, long segment in 16 patients, supra hepatic IVC in three, juxta hepatic IVC in five, IVC plus short segment hepatic vein in six and long segment hepatic vein in four. Associated portal vein thrombosis was seen in two (4%) and superior mesenteric vein thrombosis was seen in one (2%) patient. Features of caudate lobe hypertrophy and extrinsic compression of IVC (on imaging and/or IHV) was seen in 30 (61.2%) cases. One MOVC case had past imaging (on CTA) evidence of IVCT, which was done to rule out intraabdominal malignancy as a cause of femoral vein thrombosis and was negative for the same. At that time, IVCT patient was asymptomatic. Four years later this patient presented with symptomatic MOVC and was found to have factor V Leiden mutation.

Etiological spectrum in 35 patients who had undergone

all tests for thrombophilia work up was Polycythmia vera 3 (8.5%), Protein C deficiency two (6%), Protein S deficiency one (3%), Hyper homocysteinemia two (6%), Anti thrombin Ⅲ deficiency three (9%), Anti cardiolipin antibodies five (14%), Factor V laden mutation four (11.4), Lupus anti-coagulant two (6%), PNH one (3%), Multiple abnormalities four (11%), Renal Cell Carcinoma one (3%), IVC leiomyoma one (3%), Acute myeloid leukemia one (3%), Idiopathic six (19%). None of the patients was pregnant or taking OCP.

Radiological interventions done in 38 patients were as follows: IVC stenting eight (20%), IVC balloon angiography two (5%), Combined IVC Hepatic Vein stenting two (5%), Hepatic vein stenting 11 (30%), TIPS 15 (40%), and IVC stenting with TIPS two (5%).

In one patient with supra-hepatic IVC block with refractory ascites and deteriorating liver function, attempted IVC stenting failed; he was then lost to follow-up. In the radiological intervention group, technical success was achieved in 97.3% (37/38 cases). One (2.7%) patient had a technical complication of post-TIPS (uncovered TIPS) peritoneal haemorrhage, to which the patient succumbed. Another patient (HV stenting) with acute myeloid leukemia succumbed within 1 mo of follow-up. Complete resolution of ascites or improvement in liver function was seen within 3 mo of follow-up in the remaining 35 cases. During further follow-up (mean period = 24.5 ± 12.5 mo, range = 3-84 mo) of these 35 cases, mortality was 8.5% (3/35 cases): causes of death were liver-related in one case [resistant HE (covered TIPS-related)] and non-liver related in two cases [one with intracranial bleeding (anticoagulation therapy related, one in IVC-HV stenting group) and one with metastatic renal cell carcinoma (one with IVC stenting)]. Details of other complications encountered during follow-up are as follows: (a) Episodes of HE responsive to medical treatment were seen in two post-TIPS (both covered TIPS) cases (5.7%). (b) Re-occlusion of stent was seen in six (17.1%) cases (one at 1-mo, two at 3-mo, two at 6-mo and one at 1-year follow-up) of which one had IVC stent, two had HV stent, two had uncovered TIPS and one had covered TIPS, radiological re-intervention (balloon dilatation) was possible in all cases. During further follow-up, in the event of stent occlusion, one patient with uncovered TIPS was offered covered TIPS and one patient with HV stent underwent balloon angioplasty again. (c) Hepatocellular carcinoma (HCC) was encountered at 2-year follow-up in one (2.8%) patient with MOVC, who had undergone IVC balloon angioplasty and was treated three times with trans-arterial chemo-embolisation during further five years follow-up. During follow-up of these 35 cases, only two patients (of HV stenting) were lost to follow-up after 6 mo.

On evaluating the patient subset with TIPS treatment, technical success was achieved in all 15 (100%) patients (five uncovered TIPS and 10 covered TIPS), procedural complication (uncovered TIPS) was seen in one (6.6%) patient, total mortality (one in uncovered TIPS and one in covered TIPS) was 13.3% (two patients), re-occlusion rate was 23% (one with covered and two with uncovered TIPS) in 13 survivors, primary patency rate at 6 mo for uncovered TIPS was 50% and for covered TIPS was 88.9%, and occurrence of new-onset HE was in 20% (3 patients of covered TIPS). There was a statistically significant difference ($P < 0.05$) in primary patency rate between covered and uncovered TIPS.

Eleven (22.4%) patients [all patients had stable liver functions, three cases were asymptomatic cases and three cases had RA; five patients with HVT (two short-segment and three long-segment) and six patients with IVCT-HVT (two long-segment HVT and four with short-segment HVT)] were treated with anticoagulation in addition to symptomatic therapy (mean duration of follow-up = 28.4 ± 10.8 mo, range = 1-74 mo). Two patients with RA, who were not willing to undergo radiological intervention, underwent placement of PVS. Of these, one patient had blockage of PV shunt and died of sepsis after five years; the other patient was all right at four years follow-up. Of the remaining nine cases, one patient with RA (unwilling to undergo any intervention) died of progressive liver failure after 3 mo; two patients (one at 6-mo and one at 1-year follow-up) with HVT showed complete resolution of ascites, normalisation of liver function tests and re-canalisation of HV on USG-D (repeat IHV was not done); three asymptomatic patients remained asymptomatic until 6-mo follow-up, but were lost to follow-up then; three patients were lost to follow-up after 1 mo.

Overall, in 42 cases who completed at least 6 mo of follow-up, mortality was seen in 4/42 cases (9.5%), and evidence of HCC in 1/42 (2.3%) cases.

DISCUSSION

Our study is distinct from previous Indian reports in many aspects. It showed predominance of HV thrombosis and identification of etiologies in more than three out of four of cases, the majority being hypercoagulable states. To the best of our knowledge, IVC leiomyoma and acute myeloid leukemia are being reported as etiologies for the first time in India. Also, cases showing transition from IVC thrombosis to MOVC and development of hepatocellular carcinoma in MOVC are well documented. For the first time in India, the experience of using covered TIPS in BCS is presented.

In our series, HV thrombosis represented the majority of cases (59.1%), which were followed in decreasing order by combined IVCT/ HV thrombosis (20.4%), IVC thrombosis (16.3%) and MOVC (4%). All previous Indian series, except two[29,32], describe the predominance of IVC up to 79.2%[7,9,11-13,15,30,39,40,42] or IVC-HV obstruction up to 57.7%[14,24,25,35]; HV involvement was described in 0%-32% of cases in these series[7,9,11-15,24,25,30,35,39,40,42]. Of the two distinct previous series, one (total 53 cases) showed similar frequency of HV (35.8%), IVC (33.9%) and combined IVC-HV involvement (30.1%)[29]; whereas the other series involving only chronic BCS showed predominance of HV (45.9%) followed by combined IVC-HV (29.7%) and IVC involvement (24.3%)[32]. In the western countries, hepatic vein thrombosis remains responsible for majority of BCS cases, IVC obstruction is rarely found. In Asian and African countries, more common is IVC involvement. In Japan, over span of last 30 years, there is change in the spectrum i.e. marked decrease in cases of IVC

obstruction[5,49-52]. Is similar trend is following in India or this is just reflection of selection bias at tertiary care centre? For definite answer, reports from all over the country will be needed in support.

Previously, MOVC was thought to be the predominant cause of IVC involvement (20.4%-58.6%) and of BCS in India[6,7,11-15,17,29,35,39], as well as in Asian countries[49,50,53]. In two recent Indian studies, MOVC was present in 0% and 17.2%, while IVC involvement was seen in 56.2% and 62% cases, respectively[24,40]. In our series, IVC involvement in the majority of cases was due to IVC thrombosis. Previously, MOVC and IVC thrombosis were considered to be idiopathic in origin by most Indian and Asian workers[5,6,11,13-15,17,35,49,50,53,54]; but this view has been challenged recently in a few reports[22,29,30,42,55-58]. In cases of IVC thrombosis in our study, the etiology was identified in 50%. In our series, one of two MOVC cases had a hypercoagulable state and in that case, we were able to demonstrate evolution of IVC thrombosis to MOVC. Transition from IVC thrombosis to MOVC is well described in worldwide literature[5,59,60]. The other patient with MOVC in our study developed hepatocellular carcinoma, without any other predisposing factors, such as: hepatitis B, hepatitis C or alcohol. Reports of hepatocellular carcinoma developing in MOVC are rare in Indian literature[7,12-15]. However, such an association is commonly described in most other countries[5,49,50,53,61-64].

In our study, with the application of all commercially available hypercoagulability tests, an underlying etiology could be defined in 85.7% of cases, the majority having a hypercoagulable state. This was in contrast to the initial study period (before 2001), during which few tests were performed, where etiology was evident only in 28.5% cases. None of our patients were pregnant, taking oral contraceptive pills, in a post-partum state, had abdominal trauma, abdominal surgery, amoebic liver abscess, pyogenic liver abscess, hydatid cyst, hepatic tuberculosis or filariasis; these were the predominant causes in the previous Indian studies[9,12-20,23,42]. Previous Indian series (before the year 2001) have shown identification of underlying etiology in 12%-50%, of which hypercoagulable states were uncommon[9,12-15,42]. In the last decade, there are many reports identifying one or another hypercoagulable state[21,22,24-28,46,47]. Recently, application of more tests has defined hypercoagulability in up to 59% of cases[29,30]. In addition to tests performed in our study, studying haemopoietic stem cell defects in bone marrow may further identify occult MPD, as previously shown in two Indian studies[26,46] and in many western studies[4,64]. As in our series, multiple co-existing etiologies have recently been described throughout the world[29,65]. Our work was in accordance with the majority of western reports, where identification of etiology is more than 75%[2], and at a few centres more than 90%[64-66]. Changes in the etiological spectrum probably represents the effect of availability and application of tests for hypercoagulability.

Renal cell carcinoma, which was present in three cases in our study, was rarely reported in the past[15]. Likewise, IVC leiomyoma and acute myeloid leukemia as underlying etiologies are described for the first time in Indian literature. Other malignancies described in the past include hepatocellular carcinoma, adrenal tumor, Wilm's tumor, cholangiocarcinoma and chronic lymphoid leukemia[9,13-15,39,42].

In a few examples, medical treatment only was shown to be effective in a subset of patients with stable liver function, diuretic-responsive ascites and asymptomatic presentation[2,67,68].

Few reports have shown the beneficial results of surgery in selected BCS patients with low operative mortality up to 5%, high long-term assisted patency rate of more than 90% and five-year survival rates more than 75%[2,69-72]. Poor results of surgery in patients with liver dysfunction; anatomical difficulties causing success rates as low as 30%; higher post-operative complications and mortality rates (more than 20%); high re-stenosis rates (around 30%) requiring surgical revisions in a minority (around 10%); and poor survival rates as low as 57% at five years have been described[1,3,4,70,73,74]. Surgical treatment has also failed to show a favorable effect in two multivariate analyses[68,75], but was successful in one other study[76]. In most Indian reports, while surgery for IVC involvement has shown fair results[34,36]; surgical treatments for HV occlusion have shown poor results[12,38]. These were the driving force in deciding not to offer surgical treatment to our patients.

Radiological interventions for both IVC and HV achieved technical success rates of more than 90% and long-term patency rates of more than 80%; also radiological re-interventions are usually successful[1,69,77-80]. In our series, radiological intervention protocols according to morphology of the obstruction yielded good results. Previous Indian series have shown the efficacy of balloon angioplasty of IVC with or without stent placement [11-13,37,39,40,43-45], with a re-stenosis rate ranging from 16.6% to 28.5%[11,39]. Balloon angioplasty of HV thrombosis has shown variable results throughout the world, restricting its use to short-segment HV thrombosis only[4,77]. In India, experience with HV balloon angioplasty is rare[12,37]. Patients with combined IVC-HV blockages, which are considered difficult to treat, were treated with balloon angioplasty followed by surgical portosystemic shunting in previous Indian studies, but post-operative complications and mortality were prominent[12]. In a recent study from China, a two-stage approach was recommended, the first stage being IVC stenting followed by another session of HV stenting[77]. In our work, we successfully performed balloon angioplasty with stent placement in IVC followed by either TIPS or HV balloon angioplasty with stent placement in a single session.

In the last decade, the use of TIPS in BCS is increasingly described in the world literature[81-94]. In cases with severe liver dysfunction requiring liver transplantation, TIPS used as an interim bridge to transplantation, can improve the situation dramatically[4]. In most studies, TIPS was used as rescue therapy in patients with failed or re-occluded radiological or surgical interventions or was used as a bridge to liver transplantation. Difficulty with TIPS may be associated splanchnic venous thrombosis, which can be successfully tackled by radiological interventions in the same session[89]. Technical success for TIPS ranges from 75% to 100% in various works[81,84,88,90,93]. The problem

of TIPS dysfunction (present in 40% to 75% if followed up for more than two years[80,82,84,90,91,93]) necessitates re-intervention in up to 70% of cases[1,87-89,91], giving a revision rate of 1.4 revisions per patient[91]. TIPS related complications occur in less than 20% of patients[91]. TIPS provided a survival rate of 85% at four years in one series and 74% at five years in another series[82,91]. Covered TIPS in BCS has shown lower TIPS dysfunction rate (33% *vs* 87% in uncovered group) and higher primary patency rate at 1-year (67% *vs* 19% in uncovered group) in one recent series comprising nine patients in a covered TIPS group[94]. Another series using covered TIPS in eight patients showed there was no need for revisions during almost one year of follow-up[91]. In our study, TIPS was used as a primary treatment in the subset of the patients with long-segment HV thrombosis with or without IVCT. During the study period (mean follow-up of more than two years), the technical success rate was 100%; procedural complication rate was 6.6% (post-TIPS peritoneal haemorrhage); total mortality rate was 13.3%, re-occlusion rate was 23%; primary patency rate for uncovered TIPS was 50% and for covered TIPS was 88.9%; and new-onset hepatic encephalopathy rate was 20%. All the re-occlusions were tackled radiologically. Our study, although with a small number of patients, showed promising results with TIPS as the primary therapy in BCS patients. Covered TIPS was better than uncovered TIPS in terms of long-term patency.

COMMENTS

Background

Budd-Chiari syndromes (BCS) has been commonly described in India. It was always considered that the spectrum of BCS in India is different from that of the west.

Research Frontiers

In this study we discussed the changing spectrum of BCS in India which is almost comparable to the west.

Innovation and Breakthroughs

Radiological interventions including TIPS with a covered stent has been useful in managing these patients with good long term efficacy.

Application

This study will help to improve the management of patients with BCS in India.

Terminology

BCS is an uncommon condition induced by thrombotic or nonthrombotic obstruction to hepatic venous outflow. It was first described by Budd in 1845 and Chiari later added the first pathologic description in 1899.

Peer review

It's an excellent overview of BCS in India, showing a change in the aetiology towards hypercoagulopathic diseases. They authors could as well demonstrate the effectiveness of TIPS, especially when using covered stents.

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RAPID COMMUNICATION

Child-Pugh-Turcott versus Meld score for predicting survival in a retrospective cohort of black African cirrhotic patients

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Abstract

AIM: To compare the performance of the Child-Pugh-Turcott (CPT) score to that of the model for end-stage liver disease (MELD) score in predicting survival of a retrospective cohort of 172 Black African patients with cirrhosis on a short and mid-term basis.

METHODS: Univariate and multivariate (Cox model) analyses were used to identify factors related to mortality. Relationship between the two scores was appreciated by calculating the correlation coefficient. The Kaplan Meier method and the log rank test were used to elaborate and compare survival respectively. The Areas Under the Curves were used to compare the performance between scores at 3, 6 and 12 mo.

RESULTS: The study population comprised 172 patients, of which 68.9% were male. The mean age of the patient was 47.5 ± 13 years. Hepatitis B virus infection was the cause of cirrhosis in 70% of the cases. The overall mortality was 31.4% over 11 years of follow up. Independent factors significantly associated with mortality were: CPT score (HR = 3.3, 95% CI [1.7-6.2]) (P < 0.001) (stage C *vs* stage A-B); Serum creatine (HR = 2.5, 95% CI [1.4-4.3]) (P = 0.001) (Serum creatine > 1.5 mg/dL versus serum creatine < 1.5 mg/dL); MELD score (HR = 2.9, 95% CI [1.63-5.21]) (P < 0.001) (MELD > 21 *vs* MELD < 21). The area under the curves (AUC) that predict survival was 0.72 and 0.75 at 3 mo (P = 0.68), 0.64 and 0.62 at 6 mo (P = 0.67), 0.69 and 0.64 at 12 mo (P = 0.38) respectively for the CPT score and the MELD score.

CONCLUSION: The CPT score displays the same prognostic significance as does the MELD score in black African patients with cirrhosis. Moreover, its handling appears less cumbersome in clinical practice as compared to the latter.

Key words: Model for end-stage liver disease score; Child score; Cirrhosis; Black African; Survival

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INTRODUCTION

The Child-Pugh-Turcotte (CPT) score has usually been used to assess the prognosis of patients with cirrhosis[1], since it is related to the severity of the liver disease.

The detrimental effect of renal failure on survival of patients with cirrhosis is widely acknowledged[2-6]. The prevalence of renal failure in cirrhotic patients varied between 7%-65%[7-9]. About 20% of the cirrhotic patients presenting with ascites were likely to display an acute pre-renal failure. This value has been reported to exceed 50% when end-stage liver disease is present[10].

The model for end-stage liver disease (MELD) score has recently been developed to better rationalize liver graft allocation. Indeed liver graft allocation has been so far performed according to the severity of the liver failure as determined by the CPT score plus the time spent on the waiting list. Since the first transplanted have almost been the first on the waiting list and knowing that they did not necessary display the most severe disease, such an approach has been subjected to debate[11-17]. The MELD score has been elaborated using three independent prognostic factors: creatine, International Normalized Ratio (INR) and bilirubin. In a study in 311 patients on the waiting list, the MELD score has been found adequate to predict mortality[12-20]. The MELD score has been thus far used in the USA for liver graft allocation in place of the CPT score.

Several studies have been performed to compare the performance of the two scores in predicting survival in patients with cirrhosis[21-33]. However such studies have yielded conflicting results[23, 24, 28, 30-33].

The aim of the present study was to compare the performance of the MELD score to that of the CPT score to predict survival in a retrospective cohort of 172 black African patients with cirrhosis.

MATERIALS AND METHODS

Type of study

A retrospective analysis of a cohort of black African patients with cirrhosis has been undertaken. Patients have been recruited from January 1, 1991 to December 31, 2001 (11 years) at the hepatology unit of the University hospital of Yopougon (Abidjan), one of the three tertiaries hospitals of the capital, admitting and hospitalising at least 650 patients a year for a total capacity of 20 beds.

Patients

In our daily practice, all the patients who were diagnosed as having cirrhosis were systematically hospitalised, because most of them were seen at a very late stage of the disease. Of 307 patients recruited over this study period, 135 have been excluded because of missing data. 172 patients have, therefore, been considered for analysis.

Methods

Data of the patients such as, age, gender, motives of admission, aetiology of cirrhosis, serum creatine, CPT score and the MELD score, date of first symptoms of decompensated cirrhosis, date of first admission, date of last visit for the patients lost to follow up or still alive, the date of death for deceased patients have been all collected using a standardized collection sheet.

Definitions

The CPT score has been widely described elsewhere. It has been elaborated by using five (5) parameters: hepatic encephalopathy, ascite, prothrombin in percentage, serum albumin in g/L and bilirubin in mg/L.

The MELD score: It has been elaborated by using the Malinchoc formula which comprises 3 parameters: INR, bilirubin (mg/dL) and creatine (mg/dL). This formula is as follow: [9.57 × Loge creatine (mg/dL) + 3.78 × Loge bilirubin (mg/dL) + 11.20 × Loge INR + 6.43].

Time of follow up: It corresponds to the time between the date of first admission for cirrhosis and the date of last visit or the date of death for deceased patients.

Time of survival: It corresponds to the time between the date of the first symptoms of decompensated cirrhosis as reported by the patient and the date of last visit for the patients lost to follow up or still alive or the date of death for the deceased patients.

Statistical analysis

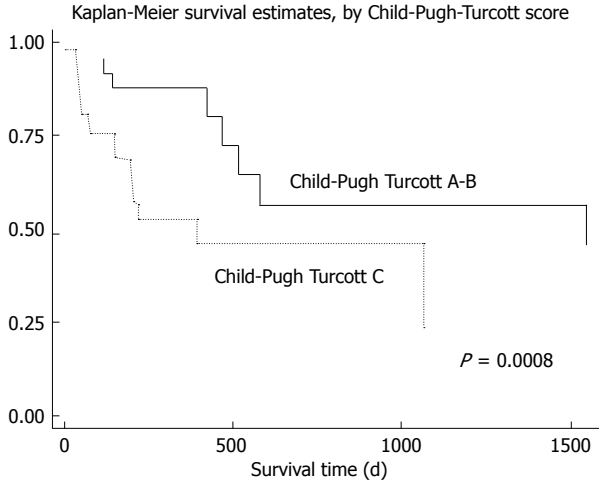
By univariate analysis, relationship between mortality and the following parameters has been assessed: age, gender, aetiology of cirrhosis, serum creatine, CPT score and the MELD score. The Student t-test or the Mann-Whitney test was used for quantitative parameters and the chi square test or the Fischer exact test for qualitative parameters. A multivariate analysis has been performed using a Cox model in a forward selection manner. Two models have been elaborated subsequently: the first has included all the parameters used in the univariate analysis with the exception of serum creatine. The second model has included all the parameters used in the univariate analysis at the exception of the MELD score. Survival has been elaborated using the Kaplan Meier method. The log rank test has been used for comparison of survival between groups. Correlation between the two scores has been evaluated. The ROC curve has been used to compare performance between scores.

Table 1 Characteristics of the study population

Male (%)	120/172 (69.8)
Mean age (yr)	47.5 ± 13.54
Child-Pugh-Turcott score (%)	
A	20 (11.6)
B	54 (31.4)
C	98 (57)
Serum creatine (mg/dL) (%)	
⩽ 1.5	132 (76.7)
> 1.5	40 (23.3)
MELD score (mean ± SD)	20.9 ± 10.87
MELD: Median (extreme)	19.5 [3-66]
Etiology (%)	
Virus B	78 (45.3)
Virus C	17 (10)
Both alcohol and virus B or C	41 (23.8)
Unknown	36 (20.9)

RESULTS

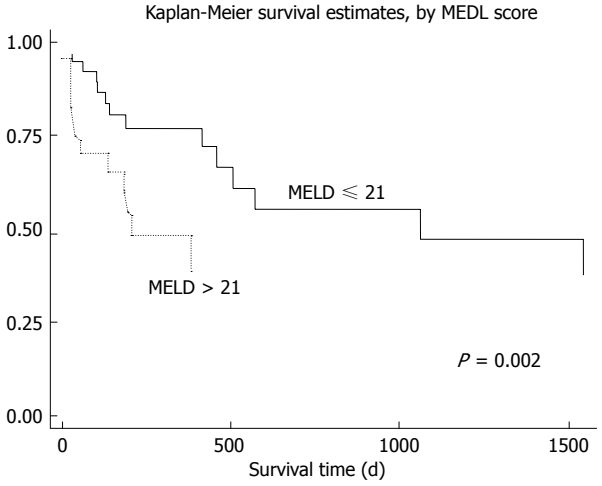
The different motives of admission of patients with cirrhosis were: ascites and oedema of the lower limbs (68.6%), jaundice (25.2%), haemorrhage of the digestive tract (15.1%) and hepatic encephalopathy. The study population comprised 172 patients, of which 69.8% were male. The mean age of the patients was 47.5 ± 13.54 years. 57% of the patients were with a CPT score stage C, 23.3% had renal failure and the mean MELD score was 20.9 ± 10.87. Hepatitis B was the main cause of cirrhosis in 45.3% of the case (Table 1). Over the study period (11 years), 54 out of 172 patients (31.4%) have died, of which 22 during the first three months and 32 the first 6 mo. The median and the mean duration of follow up were 206 and 226.6 + 41.6 d, respectively. By univariate analysis, the following parameters have appeared to significantly influence mortality: age > 48 years old ($P < 0.023$); male gender ($P < 0.003$); CPT score stage C ($P < 0.016$); serum creatine > 1.5 mg/dL ($P < 0.001$) and MELD score over 21 ($P < 0.03$). Indeed, the mean CPT score has appeared significantly less elevated in living cirrhotics as compared to those who have died during the study period (10.2 ± 0.28 *vs* 11.3 ± 0.33, respectively; $P = 0.02$), as it was with the MELD score (19.4 ± 0.83 *vs* 24.2 ± 1.85, $P = 0.007$, respectively). Moreover mortality increased proportionally to the level



Probability of survival (95% CI):

	Child-Pugh-Turcott A-B (%)	Child-Pugh-Turcott C (%)
-3 mo	100	75.1 (64.5-82.9)
-6 mo	87.3 (73.9-94.1)	68.6 (57-77.7)
-12 mo	87.3 (73.9-94.1)	52.1 (38.8-63.9)
-24 mo	56.0 (35.9-72)	45.6 (31.4-58.8)

Figure 1 Survival according to the CPT score.



Probability of survival (95% CI):

	MELD ⩽ 21[1] (%)	MELD > 21[1] (%)
-3 mo	93.3 (85.6-97.0)	71.2 (57.2-81.4)
-6 mo	81.7 (70.9-88.8)	66.1 (51.3-77.4)
-12 mo	78.0 (66.2-86.1)	49.0 (33.1-63.1)
-24 mo	56.3 (40.4–69.4)	39.2 (22.3-55.7)

Figure 2 Survival according to the MELD score. [1]Through value of the MELD score significantly associated with mortality.

Table 2 Relationship between mortality and parameters

Parameters	Mortality		
	Univariate analysis		Multivariate analysis
	Death (%)	*P*	Hazard ratio (95% CI); *P*
Age (yr)[1]			
⩽ 48	23.90	0.023	
> 48	40.00		
Gender			
Female	15.40	0.003	
Male	38.30		1.8 [0.96 - 3.24]; *P* = 0.07
Child-Pugh-Turcott			
A-B	21.60	0.016	
C	38.80		3.3 [1.7 - 6.2]; *P* < 0.001
Serum creatine (mg/dL)			
⩽ 1.5	24.20	< 0.001	
> 1.5	55.00		2.5 [1.4 - 4.3]; *P* = 0.001
MELD[2]			
⩽ 21	25.50	0.030	
> 21	41.90		2.9 [1.63 - 5.21]; *P* < 0.001
Aetiology			
Virus B or C	32.90	0.670	
Both alcohol and virus[3]	40.20		
Unknown	36.80		

[1]Median age; [2]Through value of the MELD score significantly associated with mortality; [3]Aetiology both virus and alcohol.

Table 3 Stepwise evaluation to find out the optimal cutoff point for both CPT and MELD score (%)

	Sensitivity	Specificity	Positive predictive value	Negative predictive value
CPT score				
8	96.30	20.30	35.60	92.30
9	74.00	33.00	33.60	73.60
10[1]	70.40	49.20	38.80	78.40
11	66.70	52.60	39.10	77.50
12	59.30	57.60	39.00	75.60
MELD score				
19	55.60	52.50	34.90	72.10
20	55.60	57.60	37.50	73.90
21[2]	48.20	69.50	41.20	74.60
22	44.00	69.50	40.00	73.20
23	44.40	76.30	46.20	75.00

[1]Optimal cutoff point for CPT (Child-Pugh-Turcott) score; [2]Optimal cutoff point for MELD (Model for End-stage Liver Disease) score.

of the CPT score (0% for stage A, 29.6% for stage B and 38,8% for stage C; $P = 0.001$), as it did with the MELD score (26.5% for MELD < 30, 42.9% for MELD between 30 and 40, 75% for MELD > 40; $P = 0.007$) (Table 2). By multivariate analysis, parameters such as CPT score stage C (HR=3.3 95% CI [1.7-6.2]) ($P < 0.001$); serum creatine > 1.5 mg/dL (HR = 2.5 95% CI [1.4-4.3]) ($P = 0.001$) and MELD score over 21 (HR = 2.9 95% CI [1.63-5.21]) ($P < 0.001$) have appeared to significantly influence mortality (Table 2). The overall survival at 3, 6 and 12 mo was 85.4% (95% CI: 78.5-90.1), 76.1% (95% CI: 67.7-82.6) and 67% (95% CI: 57.3-75), respectively. The median and the mean survival time were 142.6 and 318 + 45.1 d, respectively. When the CPT score was considered, survival at 1 year was just 52.1% for CPT score stage C as compared to CPT stage A-B which was over 85% (Figure 1). A MELD score over 21 was synonymous of 50% of survival at 1 year as compared to a MELD score less than 21 for which survival was about 80% (Figure 2). A cut off point of 10, corresponding to the very least value of the CPT score stage C, has been found optimal to predict sensitivity (74%), specificity (49.2%), positive predictive value (38.8%) and negative predictive value (78.4%). A cut off point of 21 for the MELD score has been found optimal to predict sensitivity (48.2%), specificity (69.5%), positive predictive value (41.9%) and negative predictive value (74.6%) (Table 3). However, when the performance of the two scores was

Table 4 Comparison between The Area Under the Curve (AUC) of the CPT score and the MELD score in predicting survival at 3, 6 mo

Survival	Pronostic score	AUC (95% CI)	*P*
At 3 mo	CPT	0.72 (0.64-0.80)	0.68
	MELD	0.75 (0.62-0.88)	
At 6 mo	CPT	0.64 (0.54-0.74)	0.67
	MELD	0.62 (0.49-0.74)	
At 12 mo	CPT	0.69 (0.60-0.78)	0.38
	MELD	0.64 (0.53-0.75)	

CPT: Child-Pugh-Turcott; MELD: Model for End-stage liver disease.

compared at 3, 6 and 12 mo, no significant difference could be found between them. Moreover, the correlation coefficient between the two scores was 0.57 ($P < 0.001$) (Table 4 and Figure 3).

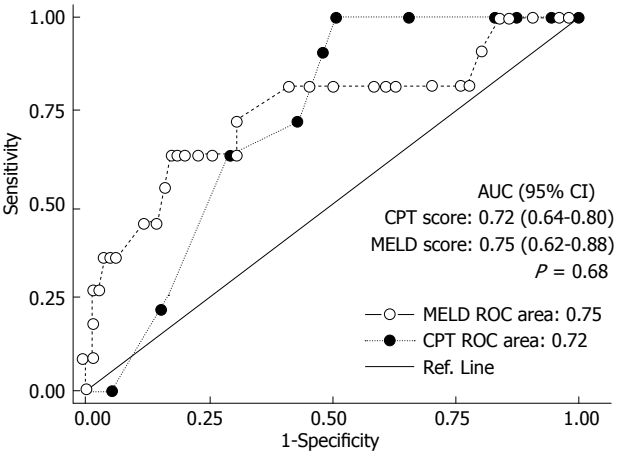


Figure 3 Comparison between AUROC of the CPT score and of the MELD score in predicting survival at 3 mo. CPT: Child-Pugh-Turcott; MELD: Model for End-stage liver disease; Ref. Line: Reference line.

DISCUSSION

About 60% of our patients have displayed a CPT score stage C that amounts to severe liver disease. Hepatitis B has been the main cause of cirrhosis in our study population. The main factors that have appeared to influence mortality were a CPT score stage C (with an optimal cutoff point of 10), renal failure that was present in 1/4 of the patients, and a MELD score over 21. The performances of the two scores were comparable.

Renal failure occurring in the setting of cirrhosis has widely been reported to represent an independent risk factor for mortality[2-6]. However, the CPT[1] score that has been used so far to appreciate prognosis in patients with cirrhosis does not take into account the renal function. This has led, therefore, to the elaboration of the MELD score which formula encompasses serum creatine for the evaluation of survival in patients with cirrhosis[11]. The performance of the two scores has been compared in several studies with conflicting results: some studies[11,18,22,25-27,29] have showed superiority of the MELD score over the CPT score in predicting survival in patients on the transplantation waiting list or in patients awaiting Transjugular Intrahepatic Portosystemic Shunt (TIPS), or even in patients with acute alcoholic hepatitis, while others have found the two score to be comparable[13,14,18,20,21]. Indeed, in the study by Zhang *et al*[27], the area under the curve (AUC) was significantly more with the MELD score than the CPT score: 0.95, 0.85 and 0.83 for the MELD and 0.70, 0.66 and 0.61 for the CPT score ($P < 0.05$) at 3, 6 and 12 mo, respectively. In the study by Papatheodoridis *et al*[28] analysing a cohort of 102 decompensated cirrhotics, the AUC as determined by the MELD score was comparable to that of the CPT score in predicting survival at 3, 6, 12 mo: 0.79, 0.77, 0.78 and 0.79 for the MELD score and 0.73, 0.71, 0.68 and 0.70 for the CPT score. And a recent review by Cholongitas *et al*[31] has highlighted the lack of a clear cut superiority of the MELD score over the CPT score in predicting mortality of cirrhotic patients before and after liver transplantation.

The present study has showed that the two scores were also comparable as the AUC was: 0.75, 0.62, and 0.64 for the MELD score and 0.72, 0.64, 0.69 for the CPT score at 3, 6, and 12 mo, respectively. Moreover, the through value of the MELD score in our study confirms the fact that this value varies from one study to another as it was at 14, 17 and 18 in the Angermayr[24], Cestron[32] and Ferral[25] study, respectively. This could be attributed to the fact that most of our patients were admitted a very late stage of the disease, explaining an elevated mean and median MELD score. The retrospective nature of our study has led to the exclusion of many patients for missing data. Should these patients have been recruited, different findings as the one reported might have been found. However, the present findings could easily be extrapolated to any of our patients since the epidemiological profil of the 172 recruited patients represent the one currently encountered in our daily practice. Our study, which represents the only of its kind to be performed in black African patients with cirrhosis, appears to be of a great interest. Indeed, our population characteristics are quite different from what are currently reported in western countries since our patients are much younger with a diagnosis of cirrhosis made at a very late stage of the disease and hepatitis B being the main cause of cirrhosis. Moreover, the high prevalence of hepatitis B virus in an endemic country, like the Ivory Coast, is often responsible for contamination at birth and in infancy, leading to chronic hepatitis in 30%-50% (when contamination take place in infancy) to 90% (when contamination take place at birth) of the case[34-40]. In addition, the prognosis of our patients is further aggravated by the fact that most of them are diagnosed as having cirrhosis at a very late stage of the disease for different reasons; lack of insurance coverage, delay in hospital visit because of financial problems or cultural considerations. Consequently, few interventions could be offered to these patients as liver transplantation or endoscopic and/or radiological treatments are not currently available in our country. Inversely, Hepatitis C virus and alcohol remain the main causes of cirrhosis in western countries[40-42]. Contamination by hepatitis C virus occurs in adult age and chronic hepatitis related to alcohol represents a disease of adult patient.

Patients with cirrhosis are diagnosed at a very late

stage of the disease. Consequently, mortality is elevated. Renal and liver failures represent independent risk factors of mortality. In the present study, the performance of the MELD score has appeared comparable to that of the CPT score which remains the primary tool to assess patients in the daily practice. The improvement in the MELD score predicting survival ability over the CPT score may necessitate the incorporation in its determination of several others factors such as age, and population characteristics to allow the universal utilizability of this equation. The present study has indeed suggested the impact of the population characteristics on the MELD score level. The use of the CPT score in association with serum creatine as proposed by Angermayr[24] could also be currently performed in daily practice in order to also improve its predicting survival ability.

COMMENTS

Background

Renal failure occurring in the setting of cirrhosis is an independent risk factor of mortality. The Child-Pugh-Turcotte (CPT) score has usually been used to assess the prognosis of patients with cirrhosis. Formulas devoted to include the renal function in the determination of the CPT score have not gained wide acceptance. The Model for End-Stage Liver Disease (MELD) score which encompasses renal function and other factors known to influence mortality in cirrhotic patients has also been found adequate to predict mortality in patients awaiting liver transplantation. The MELD score has thus far been used in the USA for liver graft allocation instead of the CPT score.

Research frontiers

The superiority of one score over the other is subjected to debate as studies comparing the two scores have yielded conflicting results.

Innovations and breakthroughs

Studies performed so far were mostly in western populations, with few in Asian. The present study represents the first of its kind in black Africans. In Africa the main cause of cirrhosis is hepatitis B virus, inversely to hepatitis C and alcohol in western countries. Affecting patients are much younger. Cirrhosis is diagnosed at a very late stage and very little can be done to prolong life of the patients, explaining very high mortality related to it. No difference has been found between the performance of the two scores. However, the optimal cut-off point of the MELD score that is determined from our study appears much higher than any of what that has been published to date.

Applications

The improvement in the MELD score predicting survival ability may necessitate the incorporation in its formulation of several other factors including population characteristics to allow the universal applicability of this equation. Consequently, the setting up of an international collaborative study appears urgent than ever.

Terminology

Cirrhosis: End-stage liver disease. Renal failure: Kidney dysfunction amounting to serum creatine value more than 1.5 mg/dL. CPT score: Five parameters are used to calculate the CPT score. These are hepatic encephalopathy, ascites, and prothrombin in percentage, serum albumin in g/L and bilirubin in mg/L. Each parameter is affected a number going from 1 to 3 depending on its degree of impairment, so that the very least score amounts to 3 and the very most to 15. MELD score: The MELD score is determined through the following equation: [9.57 × Loge creatine (mg/dL) + 3.78 × Loge bilirubin (mg/dL) + 11.20 × Loge INR + 6.43]. Its value appears to vary from to one study to another, depending probably to the population characteristics. Optimal cut-off point: The value of the equation that yields the best sensitivity, specificity, positive predictive value and the negative predictive value.

Peer review

The authors conducted a retrospective study to compare the ability of the CPT and MELD scores to predict mortality in a cohort of black patients with cirrhosis. The key findings of the study are that the CPT and MELD scores had similar predictive ability. Based on the fact that the CPT is easier to compute than the MELD score, the authors recommend the CPT over the MELD score in blacks with cirrhosis. Although not entirely novel, the study does provide interesting and important findings.

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RAPID COMMUNICATION

Expression of pituitary homeobox 1 gene in human gastric carcinogenesis and its clinicopathological significance

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Abstract

AIM: To investigate the effect of pituitary homeobox 1 (PITX1) expression in cases of human gastric cancer on cancer differentiation and progression, and carcinogenesis.

METHODS: Using polyclonal PITX1 antibodies, we studied the expression of PITX1 in normal gastric mucosa, atypical hyperplasia, intestinal metaplasia, and cancer tissue samples from 83 gastric cancer patients by immunohistochemistry. Moreover, semi-reverse transcription polymerase chain reaction (semi-RT-PCR) was performed to detect the mRNA level of PITX1 in three gastric cancer cell lines and a normal gastric epithelial cell line. Subsequently, somatic mutations of the PITX1 gene in 71 gastric cancer patients were analyzed by a combination of denaturing high performance liquid chromatography (DHPLC) and DNA sequencing.

RESULTS: Immunohistochemistry showed that PITX1 was strongly or moderately expressed in the parietal cells of normal gastric mucosa (100%), while 55 (66.3%) out of 83 samples of gastric cancers showed decreased PITX1 expression. Moreover, PITX1 expression was reduced in 20 out of 28 cases (71.5%) of intestinal metaplasia, but in only 1 out of 9 cases (11%) of atypical hyperplasia. More importantly, PITX1 expression was significantly associated with the differentiation, position and invasion depth of gastric cancers (r = -0.316, P < 0.01; r = 0.213, P < 0.05; r = -0.259, P < 0.05, respectively). Similarly, levels of PITX1 mRNA were significantly decreased in 2 gastric cancer cell lines, BGC-823 and SGC-7901, compared with the normal gastric epithelial cell line GES-1 (0.306 ± 0.060 *vs* 0.722 ± 0.102, P < 0.05; 0.356 ± 0.081 *vs* 0.722 ± 0.102, P < 0.05, respectively). Nevertheless, no somatic mutation of PITX1 gene was found in 71 samples of gastric cancer by DHPLC analysis followed by sequencing.

CONCLUSION: Down-regulation of PITX1 may be a frequent molecular event in gastric carcinogenesis. Aberrant levels of PITX1 expression may be closely correlated with the progression and differentiation of gastric cancer.

Key words: Pituitary homeobox 1; Gastric cancer; Immunohistochemistry; Reverse transcription polymerase chain reaction; Denaturing high performance liquid chromatography

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INTRODUCTION

Cancer develops as a result of multiple genetic and epigenetic alterations[1,2]. Functional imbalances between oncogenes and tumor suppressor genes have been identified in most cancers, including gastric cancer. Better knowledge of the changes in gene expression that occur during gastric carcinogenesis may lead to improvements in diagnosis, treatment and prevention[3]. Recently, PITX1, first identified as a *bicoid*-related transcription factor involved in proopiomelanocortin (POMC) gene expression[4], was reported to be a tumor suppressor in many parenchyma tumors.

The evidence that *PITX1* gene is a significant tumor suppressor gene in human cancer remains largely circumstantial, but is clearly worth further study. As little is known about the tumor-inhibitory roles of PITX1 in gastric cancer, we focused on the expression of PITX1 in gastric cancer tissues and gastric cancer cell lines; the

potential roles of PITX1 in cancer differentiation and carcinogenesis of the stomach will be discussed.

MATERIALS AND METHODS

Tissue materials

For immunohistochemical analysis, we used archival formalin-fixed, paraffin-embedded tissues from 83 patients with gastric cancer. The average age of patients was 61 ± 1.5 years and the male/female ratio was 63/20. Histological classification of gastric cancers was performed according to the general rules established by the Japanese Gastric Cancer Association and staging was performed according to the TNM classification of Malignant Tumors (5th edition) in 1997[5].

Tissues from 71 cases of gastric cancer were used for DNA mutation analysis. Tissue fragments were frozen and stored at -80℃ for DNA extraction. DNA was isolated using a standard proteinase K digestion and phenol-choloroform extraction procedure.

All of the above tissues were obtained from the patients who underwent surgery for gastric cancer at the First Affiliated Hospital of China Medical University between December 2001 and November 2004.

Cell lines and cell culture

Three human gastric cancer cell lines, including the poorly-differentiated MGC-803 and BGC-823 lines and the well-differentiated SGC-7901 line, as well as the normal gastric epithelial cell line GES-1, were cultured by the Research Center for Medical Genomics of China Medical University. Among the three gastric cell lines, SGC-7901 was originally obtained from metastatic lymph nodes of gastric cancer while the other two were obtained from gastric primary loci. All cell lines were grown in RPMI 1640 medium containing 10% heat-inactivated fetal calf serum at 37℃, in 99% humidity under 5% CO_2, subcultured every 3 d, and checked routinely to avoid microbial contamination. Those cells growing logarithmically were harvested for total RNA isolation and RT-PCR.

Immunohistochemistry

An S-P Kit (Zhongshan Golden Bridge Biotech Co., Ltd) was used for immunohistochemical analysis. In brief, paraffin sections were deparaffinized in xylene and gradually rehydrated in alcohol. Antigen retrieval was performed by microwave treatment in 0.01 mol/L sodium citrate buffer (pH = 6.0) for 15 min, cooled to room temperature for 1 h, and then washed with phosphate-buffered saline (PBS). After endogenous peroxidase activity was blocked by incubation with 3% methanol for 10 min and washed with PBS, sections were incubated with normal rabbit serum for 1 h to block non-specific staining. Subsequently, sections were treated with polyclonal PITX1 antibodies (Santa Cruz, CA, USA) diluted to 1:300 with PBS, overnight at 4℃ in a humidified chamber. Sections then were incubated with biotinylated anti-rabbit IgG for 10 min and then with peroxidase-labeled streptavidin for 10 min. Staining was completed with a 10-min incubation with 3,3'-diaminobenzidine. Sections were counterstained with 0.1% haematoxylin. Intracytoplasmic and/or intranuclear buffy granular staining in gastric mucous cells was regarded as positive. Ten visual fields were randomly selected from each section, and at least 100 cells were counted in each field.

Omission of primary antibodies acted as a negative control, while normal gastric tissue was regarded as a positive control. Immunohistochemical results were assessed as 'negative' if fewer than 50% of cancer cells were stained. When at least 50% of cancer cells were stained, immunostaining was considered to be 'positive'.

Semi-RT-PCR

Total RNA was isolated from the cell lines using Trizol (Invitrogen, CA, USA), and 1 μg of total RNA was converted to cDNA using a First Strand cDNA Synthesis Kit (Takara, Japan). Then, PCR reactions were performed in volumes of 20 μL. A 1 μL aliquot of the cDNA was used for PCR amplification with the primers 5'-ATGGA CGCCTTCAAGGGGGGC-3' (forward) and 5'-TGCA ACTGCTGGCTTGTGAAG-3' (reverse), resulting in a DNA product of 305 bp. PCR conditions were 95℃ for 1 min, 55℃ for 30 s and 72℃ for 45 s, for 30 cycles. Under the same PCR conditions, *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*) transcripts (227 bp) were amplified as an external control for RT-PCR analysis from the same cDNA samples, using the following primer pair: 5'-GACC ACAGTCCATGCCATCAC-3' (forward) and 5'-GTCCAC CACCCTGTTGCTGTA-3' (reverse).

PCR products were separated by 1.5% agarose gel electrophoresis, stained with ethidium bromide, and visualized under UV light. Electrophoresis strips were analyzed by Fluor-S software. By calculating the ratio of the IDVs (integrated density value) for PITX1 transcripts to those for *GAPDH* transcripts, we compared the PITX1 mRNA levels between gastric cancer cell lines and the normal gastric epithelial cell line.

DHPLC analysis and DNA sequencing

DNA was amplified in a final reaction volume of 20 μL using 100 ng of genomic DNA, 10 × buffer, 1.5 mmol/L $MgCl_2$, 200 μmol/L dNTPs, 0.27 μmol/L (100 ng) primers and 1.25 units of rTaq polymerase (Takara, Japan).

In order to form the presumed heteroduplex, unpurified PCR products were pretreated by heating at 94℃ followed by cooling down to 25℃ at a rate of 0.03℃/s. DHPLC was carried out using the Transgenomic WAVEMAKE DNA fragment analysis system (Transgenomic, Ohama, USA). PCR products showing aberrant chromatograms on DHPLC were subjected to direct DNA sequencing.

Statistical analysis

The software SSPS for Windows version 11.5 was used for statistical analysis. Correlations between clinicopathological parameters and PITX1 expression were analyzed by χ^2-test, and comparisons of the mRNA levels in different cell lines were performed using *t*-tests. *P* values of less than 0.05 were considered to be statistically significant.

RESULTS

Expression of PITX1 protein in human gastric cancer samples

As shown in Table 1, all parietal cells (100%) of normal

Table 1 Comparison of the expression of PITX1 in normal tissue and tumor tissue from patients with gastric cancer

	PITX1		
	n	Negative (%)	Positive (%)
Normal tissue	83	0 (0)	100 (100)
Tumor tissue	83	55 (66.3)[b]	28 (33.7)[b]

[b]$P < 0.001$ *vs* normal tissue.

gastric mucosa showed strong or moderate PITX1 staining, while 55 (66.3%) out of 83 gastric cancer samples showed decreased staining for PITX1 ($P < 0.001$). In addition, the expression of PITX1 in intestinal metaplasia tissue samples was reduced in 20 out of 28 cases (71.5%), but in only 1 out of 9 (11%) cases of atypical hyperplasia.

The correlations between clinicopathologic parameters and PITX1 expression in 83 patients are shown in Table 2. PITX1 expression did not differ significantly by age, gender, size, clinical staging and lymph node metastasis. However, significant differences in PITX1 expression were found in tumors with different degrees of differentiation, position or depth of invasion. That is, reduced expression of PITX1 was significantly linked with tumors with poor differentiation, located in the upper and middle parts of the stomach, or with T3/T4 invasion depth ($P < 0.01$, $P < 0.05$, and $P < 0.05$ respectively). Representative results of PITX1 expression in gastric cancer samples are depicted in Figure 1.

Table 2 Relationship between the expression of PITX1 in gastric cancer and biological behavior

	PITX1		
	n	Negative (%)	Positive(%)
Gender			
Male	53	42 (66.7)	21 (33.3)
Female	20	13 (65.0)	7 (35.0)
Age (yr)			
< 60	32	22 (68.7)	10 (31.3)
⩾ 60	51	33 (64.7)	7 (35.3)
Position			
Upper (cardia & fundus)	16	12 (75)	4 (25)
Middle (body)	15	14 (93.3)	1 (6.7)
Lower (antrum)	53	30 (56.6)[a]	23 (43.4)[a]
Depth of invasion			
T1/T2	29	15 (48.4)	16 (51.6)
T3/T4	54	40 (74.1)[c]	14 (25.9)[c]
Clinical stage			
Early stage	17	8 (47.1)	9 (52.9)
Advanced stage	66	47 (71.2)	19 (28.8)
Differentiation type			
Well[1]	38	19 (50)	19 (50)
Poorly[2]	45	36 (80)[b]	9 (20)[b]
Lymph-node metastasis			
Yes	47	33 (70.2)	14 (29.8)
No	36	22 (61.1)	14 (38.9)

[a]$P < 0.05$ *vs* upper and middle part of stomach; [c]$P < 0.05$ *vs* gastric cancer in T1/T2; [b]$P < 0.01$ *vs* well differentiation type. [1]Well-differentiated tumors, for example, papillary adenocarcinoma and tubular adenocarcinoma. [2]Poorly-differentiated tumors, including undifferentiated tumors, signet-cell carcinoma and mucous adenocarcinoma.

Expression analysis of PITX1 mRNA in gastric cancer cell lines and human gastric cancer

RT-PCR was repeated 12 times to analyze the differences in PITX1 mRNA expression between the three gastric cancer cell lines and the normal GES-1 cell line (Figure 2). The average IDV ratios of the gastric cancer cell lines BGC-823 and SGC-7901 were significantly lower than that in the normal gastric cell line GES-1 ($P < 0.05$); there was no statistically significant decrease in IDV ratio in the MGC-803 cell line (Table 3).

PITX1 gene mutation analysis

All of the DHPLC chromatographies in coding area from 71 tumor tissues showed a normal single peak shape except that the deletion of a repeated sequence "gggcgc" in 3' UTR in one sample was found through DHPLC screening and sequencing.

DISCUSSION

Carcinogenesis refers to changes of multiple genes, and is closely related to the corresponding transcription factors (TFs) and their mutual regulation. As a family of TFs, homeobox genes not only play important roles in embryonic development and differentiation, but also control the differentiation and proliferation of mature tissues[6,7]. Class I human homeobox-containing genes (*HOX* genes) have been reported to act as a network of transcriptional regulators involved in the process of cell to cell communication during normal morphogenesis, the alteration of which may contribute to the evolution of cancer[8]. Moreover, a recent study concluded that *HOX* genes may play a critical role in the genesis and progression of gastric cancer[9].

PITX1 and PITX2 are members of the PITX (pituitary homeobox) family, a family of homeobox genes; they have been widely researched and contain a highly conserved homeodomain (HD) with a lysine residue at amino acid position 50, which is responsible for recognition of the TAA (T/G) CC motif in the promoter regions of several target genes[10]. Besides sharing similar DNA binding specificities, PITX1 and PITX2 are co-expressed in the stomodeum and have overlapping expression patterns in stomodeal derivatives[11-13]. Specifically, PITX1 is responsible for the morphology of muscles, tendons and the bones of hind limbs, and is involved in the development of anterior structures as well as pituitary and craniofacial morphogenesis[14,15].

Although PITX1 has essential roles in human development, the possible involvement of PITX1 in human cancer has been suspected since an elegant study by Gidekel *et al*[16] showed that a homeobox gene normally required for the differentiation of a particular tissue can play a causal role in the carcinogenesis of the same tissue if expressed at the wrong time, at the wrong levels, or in the wrong contexts. For instance, PITX1 was found in adrenocorticotropic hormone (ACTH or corticotrophin) -positive tumors as well as in ACTH-negative carcinoids[17]. Expression of PITX1 mRNA might be a potential biomarker of malignancy in patients with

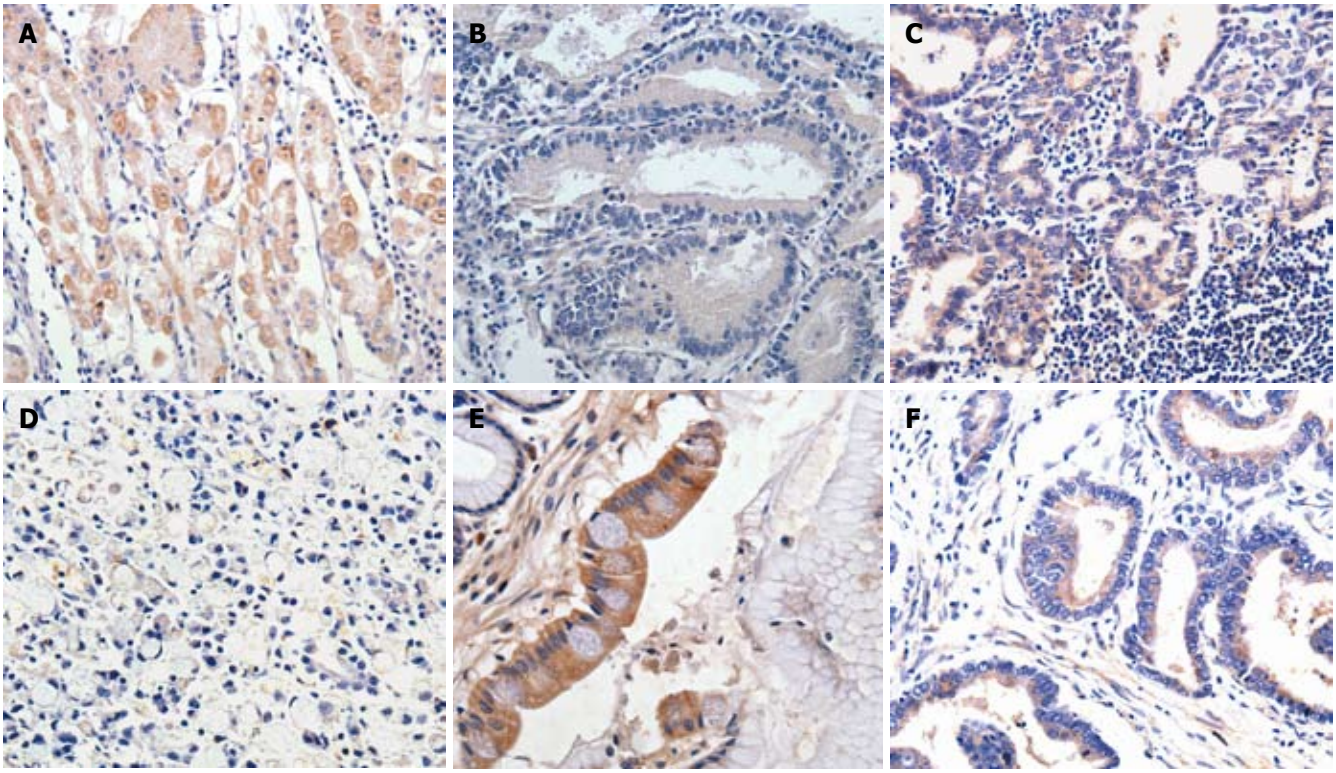

Figure 1 PITX1 expression in normal gastric mucosa and gastric cancer tissue samples (x 400). **A**: Normal gastric mucosa, PITX1 positive; **B**: Well-differentiated gastric carcinoma, PITX1 positive; **C**: Gastric carcinoma with lymph node metastasis, PITX1 positive; **D**: Signet-ring-cell carcinoma (poor-differentiated), PITX1 negative; **E**: Intestinal metaplasia, PITX1 negative; **F**: Atypical hyperplasia, PITX1 positive.

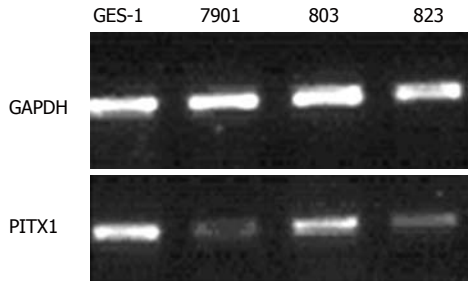

Figure 2 PITX1 expression in three cancer cell lines (SGC-7901, MGC-803 and BGC-823) and the normal gastric epithelial cell line GES-1 (GAPHD serves as the external control).

Table 3 Relationship between the expression of PITX1 in gastric cancer cell lines and the normal gastric cell line

	IDV
MGC-803	0.636 ± 0.092
BGC-823	0.306 ± 0.060[a]
SGC-7901	0.356 ± 0.081[a]
GES- 1	0.722 ± 0.102

[a]$P < 0.05$ *vs* GES-1 cell line.

Barrett's esophagus[18]. Moreover, PITX1 expression is also decreased in lung, prostate and bladder tumor tissues compared with normal control tissues[19-22].

Considering that the expression of the homeobox genes, such as *BARX1* and *PITX1*, is confined to the stomach mesenchyme during gastrointestinal development[23], we studied PITX1 expression in human gastric cancer tissues. In our study, PITX1 expression declined in 63.3% cases of gastric cancer, compared with adjacent normal tissues ($P < 0.001$). Together with former reports on the role of PITX1 in various cancers, our results hinted the negative regulation of PITX1 during carcinogenesis and the development of gastric cancer.

In addition, we found that PITX1 mRNA expression in gastric cancer cell lines was in accordance with its protein expression in immunohistochemistrical results, which agrees with the research of Chen *et al*[19] in lung cancer. Given the insufficient evidence of gastric cancer cell lines, a further study on PITX1 mRNA expression in gastric cancer tissues is necessary to confirm the results of immunohistochemistry and RT-PCR in cell lines.

The low expression of PITX1 suggested that *PITX1* may have a tumor suppressive function, inspiring us to examine its potential role in carcinogenesis. Kolfschoten *et al*[22], reported that *PITX1* regulates the RAS (rat sarcoma) signaling pathway and, therefore, tumorigenesis. As a transcriptional activator, PITX1 might activate RASAL1 to inhibit RAS activity, because PITX1 consensus binding sites, TAA(T/G)CC, were found in the 2 kb upstream promoter region of *RASAL1*, a member of the RAS-GAP family of genes (GTP-activating factors-negative regulators of RAS). Another novel explanation for *PITX1* tumorigenesis came from a study on the role of PITX1 in the transcription of the p53 tumor suppressor gene in human mammary carcinoma (MCF-7) cells, implying that p53 is a direct transcriptional target of PITX1[24].

It has been long recognized that the inactivation of tumor suppressor genes results from mutation, loss of heterozygosity (LOH), aberrant methylation and haploinsufficiency[25,26], among which aberrant promoter hypermethylation is well-known to participate in homeobox gene silencing[27,28]; however, PITX1 promoter hypermethylation has not been found in studies of lung cancer[19]. Therefore, we examined the *PITX1* gene for the presence of mutations in order to explore the mechanism of inactivation of this candidate tumor suppressor gene. We failed to find any mutation in the coding area of *PITX1*, although we did identify the deletion of a repeated sequence in 3'UTR of only one gastric cancer sample. Hence, the absence of detectable mutations or epigenetic silencing suggest that haploinsufficiency is behind the loss of *PITX1* expression[29]. Indeed, it was recently shown that the striking phenotype observed in patients with Rieger's syndrome resulted from decreased PITX2 expression due to deletion mutations in one allele of the gene[30]. As a close relative of PITX2, is PITX1 also subject to haploinsufficiency in gastric carcinogenesis? Further studies are required to investigate the mechanism of PITX1 down-regulation in gastric cancers.

Our study showed that the levels of PITX1 protein are decreased in gastric cancer tissues and that PITX1 mRNA levels are decreased in gastric cancer cell lines, suggesting that *PITX1* is a candidate tumor suppressor gene in gastric cancers, and that its expression is related to the differentiation, position and depth of invasion of gastric cancers.

Knowledge of transcription factors is crucial for understanding the molecular basis of neoplasia. PITX1, a pituitary transcription factor, mediates a plethora of embryonic functions, and its down-regulation in parenchyma tumors may be associated with carcinogenesis. Our study is the first time to explore the link between PITX1 and gastric cancer. Down-regulation of PITX1 both in gastric cancer and gastric cancer cell lines may be involved in the development of gastric cancer and may be a frequent molecular event in gastric carcinogenesis; thus, PITX1 represents a candidate tumor suppressor gene. Therefore, further study should investigate potential mechanisms for modulating PITX1 expression during carcinogenesis and the progression of gastric cancer.

COMMENTS

Background

Gastric cancer is one of the most common types of cancer worldwide. A more complete molecular genetic understanding of gastric cancer will allow the development of specific therapies. Recently, studies on tumor suppressor genes have been highly valued in gastric cancer research owing to their widespread involvement in diverse cellular activities.

Research frontiers

A family of homeobox genes, the PITX family, contains a highly-conserved homeodomain responsible for recognition of the TAA(T/G)CC motif in the promoter regions of several target genes. By virtue of its DNA-binding specificity, PITX1, an important member of this family, has been reported to be involved in many human cancers, such as Barrett's esophagus and lung cancer. The carcinogenic mechanism of PITX1 has been explored in terms of regulation of the RAS signaling pathway and direct activation of p53 transcription.

Innovations and breakthroughs

The study observed a change in the expression levels of PITX1 that parallels the progression of gastric carcinogenesis; we discussed the possible carcinogenic mechanisms used by PITX1 and its clinicopathological significance in gastric cancer. This is the first study to explore the link between PITX1 and gastric cancer.

Applications

The present study suggests that down-regulation of PITX1 may be a frequent molecular event in gastric carcinogenesis. However, the exact function and mechanism of PITX1 in gastric cancer remain to be elucidated.

Terminology

Homeobox genes contain a 180-base-pair segment (the homeobox) that encodes a protein domain (homeodomain) that is involved in binding to and, thus, regulating the expression of DNA; members of the Ras family of small G proteins have been implicated as key intermediates mediating signals from upstream tyrosine kinases to downstream cascades of serine/threonine kinases, which then activate the nuclear factors that control gene expression and protein synthesis.

Peer review

An excellent paper - well written, nicely researched. The results support the hypothesis but further research across multiple centers is needed to establish a positive correlation.

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RAPID COMMUNICATION

Detection of hMSH2 and hMLH1 mutations in Chinese hereditary non-polyposis colorectal cancer kindreds

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Abstract

AIM: To establish and validate the mutation testing for identification and characterization of hereditary non-polyposis colorectal cancer (HNPCC) in suspected Chinese patients.

METHODS: Five independent Chinese kindreds with HNPCC fulfilling the classical Amsterdam criteria were collected. Genomic DNA was extracted after informed consent was obtained. The coding region of hMSH2 and hMLH1 genes was detected by polymerase chain reaction (PCR) and denaturing high-performance liquid chromatography (DHPLC). Mutations identified in the proband by DHPLC were directly sequenced using a 377 DNA sequencer, analyzed with a basic local alignment tool (BLAST), and tested in the corresponding family members by direct DNA sequencing.

RESULTS: Mutations were identified in two Chinese HNPCC kindreds. One was the missense mutation of hMSH2 c.1808A→G resulting in Asp 603 Gly identified in the proband of the fifth HNPCC (HNPCC5) kindred. In the HNP5 kindred, three family members were found to have this mutation and two of them had colorectal cancer. The other mutation of hMLH1 c.1882A→G was identified in the HNP2 kindred's proband, which might be the nonsense mutation analyzed by BLAST.

CONCLUSION: Pedigree investigation and mutation testing of hMSH2 and hMLH1 are the practical methods to identify high-risk HNPCC patients in China.

INTRODUCTION

Hereditary non-polyposis colorectal cancer (HNPCC) syndrome is characterized by autosome-dominantly inherited predisposition to early colorectal carcinoma and extracolonic epithelia-derived tumors most often located in the gastrointestinal and urogenital tracts[1]. The mean age of HNPCC and sporadic colorectal cancer (CRC) patients at diagnosis is 42 years and 65 years, respectively. HNPCC, accounting for approximately 5%-15% of all CRCs, is categorized as Lynch I or Lynch Ⅱ syndrome (according to revised Amsterdam criteria)[2]. Germline mutations of the mismatch repair (MMR) genes identified in HNPCC kindreds, including *MSH2*, *MLH1*, *MSH6*, *PMS1* (promotion of mutS homology 1), *PMS2*, and *MLH3*, have been proved as the major cause for HNPCC by linkage analysis. Mutations in two of these MMR genes, *MSH2* and *MLH1*, account for the majority of the kindreds with HNPCC[3]. Thus, pedigree investigation and MMR gene testing, as the basis for efficient prevention and treatment of HNPCC, are most often used in early diagnosis of at-risk family members and in confirmation of the diagnosis of HNPCC.

CRC is the third life-threatening cancer in China. However, HNPCC pedigree and its predisposition gene have not been extensively studied. We have established a CRC database since 1994 and the follow-up rate is above 90%. We have recently paid attention to hereditary colorectal cancer in South China and found that about 3% of CRC patients have multiple CRCs to which the young are vulnerable[4,5]. Thus, we collected those CRC families and finally obtained eleven independent Chinese kindreds with HNPCC by deep pedigree investigation until January,

2004. Five of them fulfilled the classical Amsterdam criteria. To identify high-risk populations with HNPCC, we tested hMSH2 and hMLH1 mutations in these classical kindreds.

MATERIALS AND METHODS

Patients

The clinical diagnosis of classical HNPCC was established and verified at the Department of Gastrointestinopan creatic surgery, the First Affiliated Hospital of the Sun Yat-Sen University[2]. Five independent Chinese kindreds with HNPCC fulfilling the classical Amsterdam criteria were collected. The study was approved by the Ethical Committee of Sun Yat-Sen University.

DNA isolation

Peripheral blood samples were collected from both patients and their family members in each Chinese kindred after informed consent was obtained for genetic analysis. DNA was extracted directly from leukocytes following the standard procedures.

All exons of *MLH1* and *MSH2* were amplified for mutation testing. Samples used were amplified in 20 mL reaction volume containing approximately 100 ng DNA in 50 mmol/L KCl, 10 mmol/L Tris-HCl, 1.5 mmol/L $MgCl_2$, 200 mmol/L each dNTP, 0.01% gelatin, 1 U Taq-polymerase, and 20-40 pmol each primer. The PCR amplification conditions were as follows: denaturation at 95℃ for 5 min, followed by 30 cycles of denaturation at 95℃ for 30 s, annealing at fragment-specific annealing temperature for 1 min, extension at 72℃ for 1 min, and a final extension at 72℃ for 7 min. The primers, annealing temperature, and size of the PCR products for each of the investigated hMLH1 and hMSH2 exons are described elsewhere[6-8]. The primers were synthesized by Shanghai Bio-Chemical Corporation.

DHPLC analysis

The amplified PCR fragments were screened for sequence variants by denaturing high pressure liquid chromatography (DHPLC) on a WAVE DNA fragment analysis system (Transgenomic, San Jose, CA, USA). The running conditions for each amplicon (available upon request) were determined by the Wavemaker 3.4.4 software (Transgenomic, San Jose, CA, USA) based on the DNA sequence. Five mL of each PCR product (containing 50-100 ng of DNA) was denaturated at 95℃ for 3 min and then gradually reannealed by decreasing the sample temperature from 95℃ to 65℃ over 30 min. PCR products were then separated at a flow rate of 0.9 mL/min with a linear acetonitrile gradient. Generally, analysis took approximately 10 min including column regeneration and re-equilibration to starting conditions. The column mobile phase consisted of a mixture of 0.1 mol/L triethylamine acetate (pH 7.0) with (buffer B) or without (buffer A) 25% acetonitrile. Samples displaying variant elution peaks in each run were chosen for sequence analysis. For some amplicons displaying variations in elution profiles of control samples, all samples were sequenced.

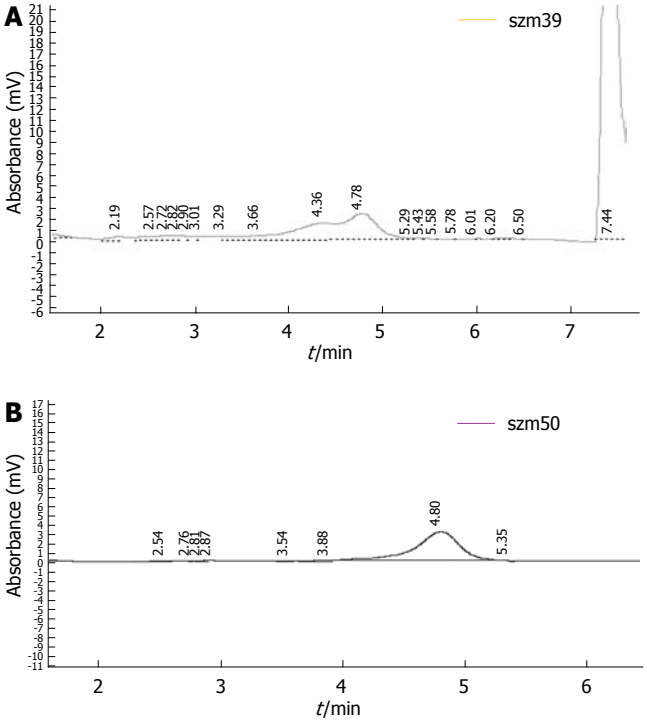

Figure 1 DHPLC showing variant elution peaks (**A**) and normal elution peaks (**B**) in exon 12 of hMSH2 gene.

DNA sequencing

DHPLC variants were confirmed by direct sequencing of independently amplified PCR products of the amplicons, in both sense and antisense direction, using the same primers. Sequencing was performed with Big Dye™ terminator cycle sequencing ready reaction kit (Applied Biosystems, Inc., Foster City, CA) on an ABI Prism™ 377 DNA sequencer following the standard conditions recommended by the manufacturer. Sequences obtained were aligned and compared to published wild-type sequences by Sequencher 3.1.1 analysis software.

Mutations identified in the kindred's proband were tested among their family members by direct DNA sequencing.

RESULTS

Among the 5 probands of the five Chinese HNPCC kindreds, 180 PCR products were screened by DHPLC. Two PCR products found with variant elution peaks by DHPLC were identified to have mutations. One was hMSH2 c.1808A→G resulting in Asp 603 Gly identified in the HNP5 kindred's proband and tested among the family members (Figures 1-3). The other was hMLH1 c.1882A →G identified in the HNP2 kindred's proband (Figures 4 and 5), which was not tested among the family members, because it might be the nonsense mutation analyzed by BLAST.

As shown in Figure 3, in the HNPCC5 kindred, there were four colorectal carcinoma patients in two successive generations, and three of these were diagnosed before the age of 45 years. The proband developed endometrial carcinoma at the age of 61 years, bladder carcinoma at the age of 66 years and colorectal carcinoma at the age of 72

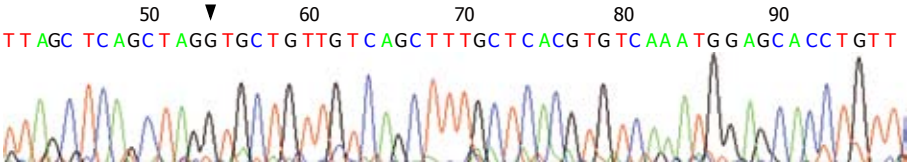

Figure 2 HPLC variants confirmed by direct sequencing displaying a single nucleotide substitution of c.1808A→G in exon 12 of hMSH2 gene.

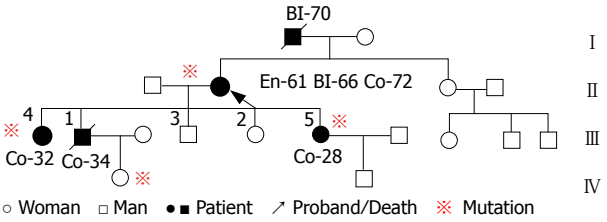

Figure 3 Pedigree tree of HNPCC 5 kindred. Co: Colon; Bl: Bladder; En: Endometrium.

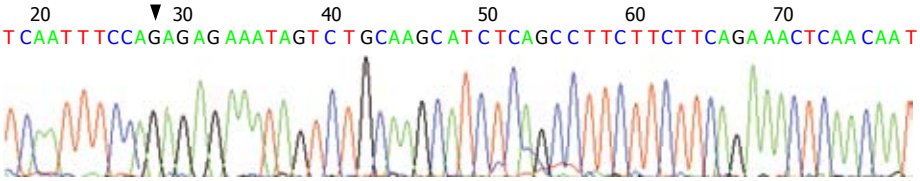

Figure 4 DHPLC variants confirmed by direct sequencing revealing a single nucleotide substitution of c.1882A→G in exon 16 of hMLH1 gene.

years, while his father developed bladder carcinoma at the age of 70 years. In addition, patient Ⅲ-1 had colorectal carcinoma at the age of 34 years and died of synchronous hepatic metastasis.

In the HNP5 kindred, each PCR product from nine proband's family members was tested by direct DNA sequencing. Only three of them were found to have the missense mutation in hMSH2 at position A1808G. The missense mutation sequence variant was found in exon 12 of hMSH2 gene. It was a single nucleotide substitution of c.1808A→G (Figure 2), which resulted in Asp 603 Gly of hMSH2 (NCBI Ref. Seq. NM 000251 and NP 000242 for mRNA and protein, respectively). Proband and patient Ⅲ-4 and -5 had this mutation and developed colorectal carcinoma. Patient Ⅲ-1's daughter had no colorectal disease even though she had this mutation, and was still under follow-up. No mutation was found in the others.

DISCUSSION

CRC is one of the most common cancers and its clinical selection criteria for cancer families were first established in Amsterdam in 1990 by the International Collaborative Group on HNPCC and modified in 1999[2]. Detection of constitutional mutations in genes associated with predisposition to cancer is practical in molecular diagnosis. Data suggest that molecular testing is much more efficient, if analyses are focused on a limited number of alterations[9-13]. Detection of germline mutation carriers is an efficient method to define high-risk CRC patients. Identification of germline mutations of either hMSH2 or hMLH1 could be performed in 50%-70% of families meeting the Amsterdam criteria for HNPCC, whereas the families not complying with these criteria show a much lower frequency of the MMR gene mutations[14-17]. Thus, hMSH2 or hMLH1 gene testing is most often used in early diagnosis of at-risk family members with HNPCC.

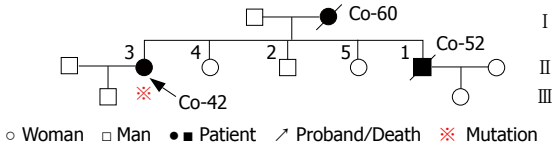

Figure 5 Pedigree tree of HNPCC 2 kindred. Co: Colon.

To identify a population-specific panel of mutations, it is crucial to describe them in all ethnic groups.

Even though CRC is common in China, few studies on HNPCC pedigree and its predisposition gene are available[4,17]. In our study, HNPCC kindreds were collected and hMSH2 and hMLH1 gene mutations were tested in order to find high-risk CRC populations. Two mutations were found among the five Chinese HNPCC kindreds. In the 5HNPCC kindreds, missense mutation was found in four members, three of them developed colorectal carcinoma. Although it has not been confirmed as a germline mutation yet, it may be an important factor for CRC development. Thus, the patient Ⅲ-1's daughter with this mutation should be closely followed up.

Approximately 20% of patients with colorectal cancer have a genetic component and HNPCC is the most common autosome dominant hereditary syndrome predisposing to colorectal cancer[18,19]. Various methods have been described to screen for HNPCC and directly test for mismatch repair gene mutations[20-27]. For patients with available tumor specimens, MSI and IHC are widely performed. Ruszkiewicz *et al*[28] reported that immunohistochemistry is an alternative method for assessment of MSI status, which is fast and relatively inexpensive compared with MSI testing. Some reports[26,29,30] suggest that detection of MSI and IHC for hMSH2/hMLH1 proteins is a reliable pre-screening test for hMLH1/hMSH2 germline mutations in families suspected of having HNPCC. Because China is a developing country with a large population and the incidence rate of CRC

increases, a screening protocol specific for the Chinese population is necessary to detect the high-risk populations with HNPCC. hMSH2 and hMLH1 gene mutation testing is a practical method for detecting HNPCC in Chinese population. Meanwhile, further research should be performed in Chinese HNPCC kindreds with germline mutations.

COMMENTS

Background

Approximately 20% of colorectal cancer patients have a genetic component and hereditary non-polyposis colorectal cancer (HNPCC) is the most common autosome dominant hereditary syndrome predisposing to colorectal cancer. Various methods have been described to screen for HNPCC and directly test for mismatch repair gene mutations. Even though CRC is common in China, few studies on HNPCC pedigree and its predisposition gene are available.

Research frontiers

Data suggest that molecular testing is much more efficient when analyses are focused on a limited number of alterations. Detection of germline mutation carriers is an efficient method to define high-risk CRC patients. Germline mutations of either hMSH2 or hMLH1 can be identified in 50%-70% of families meeting the Amsterdam criteria for HNPCC. Thus, hMSH2 or hMLH1 gene testing is most often used in early diagnosis of at-risk family members with HNPCC.

Innovations and breakthroughs

Five independent Chinese kindreds with HNPCC fulfilling the classical Amsterdam criteria were collected. Mutations were identified in two Chinese HNPCC kindreds. One was the missense mutation of hMSH2 c.1808A→G resulting in Asp 603 Gly, the other was the mutation of hMLH1 c.1882A→G .

Applications

Molecular pathological tests should be performed in order to identify individuals and at-risk family members with HNPCC. Close follow-up and intensive surveillance should be performed.

Terminology

Denaturing high-performance liquid chromatography (DHPLC) is a method for separating DNA duplexes that differ in the identity of one or more base pairs. The method is believed to be most efficient at the site of mutation. Basic local alignment search tool (BLAST) is powerful to compare novel sequences with previously characterized genes. Both functional and evolutionary information can be obtained from well designed queries and alignments. BLAST 2.0 can rapidly search for nucleotide and protein in their databases.

Peer review

The results of this clinical study indicate that mutation testing for hMSH2 and hMLH1 by PCR and direct sequencing is a preferable method to identify high-risk HNPCC patients.

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RAPID COMMUNICATION

Assessment of duodenal circular drainage in treatment of superior mesenteric artery syndrome

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Abstract

AIM: To assess the clinical value of duodenal circular drainage for superior mesenteric artery syndrome (SMAS).

METHODS: Forty-seven cases of SMAS were treated with duodenal circular drainage from 1959 to 2001. Clinical data were analyzed retrospectively.

RESULTS: In this group, good effects were achieved in 39 cases treated with duodenal circular drainage after 2-15 years of follow-up. The other eight cases were first treated with anterior repositioning of the duodenum (two cases), duodenojejunostomy (five cases), subtotal gastrectomy and billroth Ⅱ gastrojejunostomy (one case), but vomiting was not relieved until duodenal circular drainage was performed again. A follow-up study of 8-10 years revealed satisfactory results in these eight patients.

CONCLUSION: In SMAS, if the reversed peristalsis is strong and continuous, and vomiting occurs frequently, the symptom can not be relieved even if the obstruction of duodenum is removed surgically. The key treatment is the relief of reversed peristalsis. The duodenal circular drainage can resolve the drainage direction of duodenal content, thus relieving the symptom of vomiting.

Key words: Duodenum; Superior mesenteric artery syndrome; Circular drainage operation

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INTRODUCTION

The pathogenesis of superior mesenteric artery syndrome (SAMS) is so complicated[1-3] that there are different operations for treating SAMS[4-7], such as ligament of Treitz amputation, gastrojejunostomy, subtotal gastrectomy and Billroth Ⅱ gastrojejunostomy, duodenojejunostomy, anterior reposition of the duodenum, duodenal circular drainage operation, *etc*[8-10]. The last one of all these procedures is suitable for the patients who have failed in other operations or have stronger and continuous reversed peristalsis[11]. Among a total of 108 patients with SAMS who received operations, 47 patents were treated with duodenal circular drainage from 1959 to 2001 in the Second Affiliated Hospital of Harbin Medical University.

MATERIALS AND METHODS

Patients

This study included 28 men and 19 women, aged from 16 to 50 years (average 29), including 38 patients aged 16-35 years. The clinical course of the diseases lasted 2-11 years, averaging 3.5 years.

Clinical characteristics

The onset of SAMS was slow in all 47 patients. The symptoms were upper abdomen expanding, dull and bursting pain. The pain may present in upper abdomen, upper left or right of umbilicus and right hypochondrium, which was not relieved after body position change. The vomiting could be triggered by eating and drinking. Body position change (knee-chest position) did not make any difference. The vomit contained bill-stained material and no dejection smell. Six patients were misdiagnosed as having obstruction of pylorus (five patients were misdiagnosed by other hospitals) and 14 patients had ejecting vomiting. Three patients were misdiagnosed as having gastric cancer because of severe dehydration and disorder of electrolyte induced by a high frequency of vomiting.

All patients suffered from severe dehydration, disorder of electrolyte and loss of weight. The patients weighed from 45-53 kg in 28 men and 40-42 kg in 19 women. The patients' upper abdomen was distended and lower abdomen was plainness. There were no abdominal muscle guarding and obviously tenderness, but shaping of the stomach and succussion splash occasionally. There was no change of borhorygmus.

Laboratory examination

Obvious expansion in stomach, and descending and

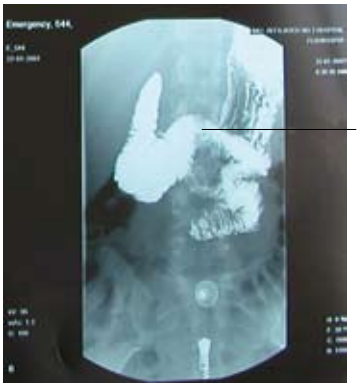

Figure 1 Barium X ray study in duodenal circular drainage operation.

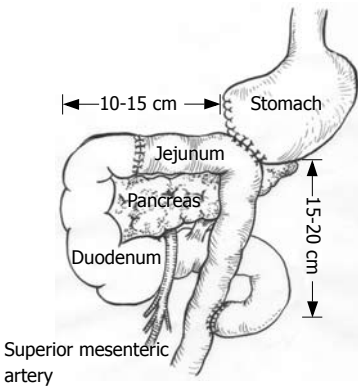

Figure 2 Duodenal circular drainage operation.

transverse portion of duodenum were found in all the patients in barium X-ray study. The barium was delayed in the ascending portion of duodenum which looked like pen trace (Figure 1). The reversed peristalsis of duodenum was much stronger than direct peristalsis. It looked like the movement of pendulum, and barium could reverse to stomach. If the patients were put in prone position, a smaller portion of barium could go through the obstructive site and enter into jejunum. Five patients had histories of peptic ulcer at the same time. Duodenum expanded obviously, reaching 7.5 cm-8 cm in diameter, in 26 patients with SMAS.

Duodenum air charge test in operation

A total of 108 SMAS patients received duodenum air charge test in operation. After interdiction duodenum with an elastic tourniquet, about 200-400 mL air was injected into the proximal duodenum. The diameter of duodenum expanded to 5.5 cm-6.0 cm was considered as light expansion, 6.5 cm-7.0 cm as moderate expansion, and 8 cm or broader as severe expansion. In our group, the duodenum expanded to 8 cm in 47 patients.

Operation

All the 47 patients received duodenal circular drainage operation finally. Two patients received anterior reposition of the duodenum and gastrojejunostomy and five patients underwent duodenojejunostomy before receiving this operation. Vomiting was not alleviated after these procedures in these patients, and they received duodenal circular drainage operation 3-6 mo after the first surgery. One patient suffered from duodenal partial obstruction after subtotal gastrectomy and Billroth Ⅱ gastrojejunostomy, which induced obstructive jaundice and suppurative cholangitis because the bilious and pancreatic liquid can not be drained easily. The reason was that when duodenal nub was closed, a bowel segment was formed between duodenal nub and the position compressed by the duodenum. The patient was cured after duodenal circular drainage operation two mo after the first operation.

Operational methods

Under epidural anesthesia or general anesthesia with tracheal intubation, midline incisions were made in upper abdomen in all patients. We performed duodenum air charge test first. If the duodenum was distended and widened obviously to 8 cm or wider, distal stomach should be resected, and if the patients had gastric ulcer, subtotal gastrectomy should be performed. Afterwards the jejunum should be cut off 10 cm-15 cm from ligament of Treitz (sometimes 20 cm according to blood vessel supply). Distal jejunum was drawn through mesentery of transverse colon and was retrocolically anastomosed end to end with duodenal cap. Half length stomach (about 5 cm) was anastomosed to jejunum end to side 10 cm-15 cm from the jejunum-duodenal cap anastomosis. Finally proximal jejunum was anastomosed with distal jejunum end to side 15 cm-20 cm below the gastrojejunoanastomosed stoma. The incision was stitched after washing of abdominal cavity (Figure 2).

RESULTS

All 47 received gastroenteric X-ray test (60-100 mL of 76% meglumine diatrizoate injection or 200 mL barium was taken orally). Contrast agent could pass the gastroenteroanastomosed stoma and jejunal-jejunostosed stoma successfully. No stricture of anastomotic stoma was found in our group. The contrast agent was refluxed to the end to end duodenal cap anastomosis in 6 patients. Meglumine diatrizoate injection (76%) was administered in 2 cases after 10 min. Barium was influxed to the jejunum, and some was refluxed to the end to end duodenal cap anastomosis, and partly refluxed to stomach. Six patients had bile refluxed gastritis 20-25 d after operation. The symptom disappeared after one month treatment with pectous bismuth and domperidone. The contrast agent was administered to the jejunum and no reflux was found in barium X-ray study in other patients. Reverse peristalsis disappeared in all patients finally.

All patients were released from symptoms in our group 10-15 d after operation. All patients had a good status in a follow-up study of 2-15 years. The ratio of follow-up was 100%, including 47 cases followed within two years. No abdominal expanding, pain and vomiting occurred. The weight increased by 8 kg in 25 patients, 5 kg in 15 patients and 7 kg in 3 patients. Gastroenteric barium X-ray studies showed no stricture in two anastomosed stomas and no contrast agent reflux was observed in these cases (Figure 3). Thirty-eight (80.9%) patients were followed in the third year and no abdominal pain or expanding and vomiting were found and appetite all became normal, with weights

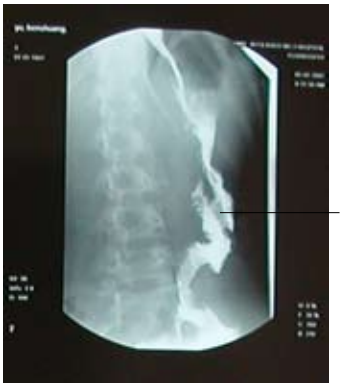

Figure 3 Barium X ray study after duodenal circular drainage operation.

of 60-64 kg in male patients and 46-51 kg in female. In 21 patients who received ultrasonic examination, no obvious expansion was found in duodenum, the diameter of duodenum being 3 cm-5 cm. Eighteen (38.3%) patients have been followed up to the present. Eight patients had received other operations before the duodenal circular drainage operation. A follow-up study of 8-10 years revealed good effects in these patients. No abdominal pain, expansion, and vomiting were found in all the 18 patients and all with normal appetite. Gastroenteric barium X-ray study showed no stricture and ulcer in two anastomosed stomas and no contrast agent reflux was observed in these cases. The weight was 60-64 kg in male patients and 45-51 kg in female. No obvious expansion was found in duodenum in the 18 patients, the diameter of duodenum being 4-5 cm[12-14].

DISCUSSION

Theoretic basis and indications for duodenal circular drainage operation

The movements of duodenum are complicated and the most important movements are reversed peristalsis and direct peristalsis[15-18]. The direct peristalsis is greater than reversed peristalsis under normal circumstances. Complete or partial obstruction could be induced when the ascending portion of duodenum was compressed by superior mesenteric artery[19-22]. The reversed peristalsis is greater than the direct peristalsis, which is the most important reason inducing opening of pyloric duct[23,24]. The patients may suffer from frequent vomiting in such situation[25-27]. If reversed peristalsis exists continually and chronically, the reversed peristalsis will be hardly eliminated. Releasing from obstruction by operation can not alleviate clinical symptoms because the strongly reversed peristalsis still exists. Therefore, emphasis should laid on eliminating the reversed peristalsis to alleviate frequent vomiting. Duodenojejunostomy is commonly used but can not stop the vomiting while duodenal circular drainage operation can manage the drainage direction of duodenal contents. When duodenum takes peristalsis directly, the duodenal contents could only partly enter into jejunum through anastomosis of proximal and distal jejunum. When the strongly reversed peristalsis with the duodenal contents could come into jejunum completely through anastomosis of distal jejunum and duodenal cap, the vomiting is eliminated completely[28-30].

Indications for duodenal circular drainage are as follows: (a) the history of the disease is up to 2 years, especially 5 years; (b) frequent vomiting, no obvious abdominal pain but marked abdominal distention after eating. The pain may be alleviated after vomiting; (c) the vomiting can not be alleviated by changing body position (prostrate position after eating), it may result from chronic reversed peristalsis; (d) the descending and transverse portion of duodenum is markedly expanded and show strong and frequent reversed peristalsis in barium X-ray test, pyloric duct is opened, the stomach is expanded and atonic; (e) the cavity of duodenum was expanded to 8 cm or more in air charge test, and the pressure of duodenum is 15-17 cm H_2O; and (f) the patients are not cured after duodenojejunostomy and anterior reposition of the duodenum. Duodenal circular drainage should conform to the indications above. If the patients do not meet with these indications, duodenal circular drainage can induce disorder of duodenal function.

Assessment of duodenal circular drainage

Duodenal circular drainage can eliminate frequent vomiting induced by chronic reversed peristalsis in SMAS. The ulcer focus and most of gastric body can be resected (gastrojejunostomy similar to Billroth Ⅱ) if the patients suffer from the ulcer intercurrently. The operation also can eliminate blind end between anastomosis and obstructive site after duodenojejunostomy and can not induce blind loop syndrome. No ulcer or stricture of anastomosis occurred in our group.

There are still several disadvantages of duodenal circular drainage operation. The operation is complicated as compared with duodenojejunostomy, and the patients may suffer from more damages as a result of the operation; small stomach syndrome and anemia can be induced by resection of most gastric body; the mucous membrane of alimentary tract can not be nourished by gastrin after resection of gastric antrum; and bilious reflux gastritis may occur after resection of sphincter of pylorus. A few patients had bilious reflux gastritis in our group and recovered within one month.

COMMENTS

Background

Superior mesenteric artery syndrome (SMAS) is an uncommon disease which can result in postprandial epigastric pain, nausea, vomiting, anorexia and weight loss. The pathogenesis of SMAS is ascribed to compression of the third part of the duodenum in the angle between the aorta and the superior mesenteric artery. If conservative management fails, surgical options should be applied.

Research frontiers

Conservative therapy with nutritional supplementation is the initial treatment, and surgery is reserved for those who do not respond to medical therapy. The main character of the SMAS is that the reversed peristalsis is greater than the direct peristalsis, which is the most important reason inducing the symptoms. If reversed peristalsis exists continually and chronically, the reversed peristalsis will be hardly eliminated. Only releasing from obstruction by operation can not alleviate clinical symptoms because the strong reversed peristalsis still exists. Therefore, emphasis should be laid on eliminating reversed peristalsis to alleviate frequent vomiting.

Related publications

Surgical options include open or laparoscopic duodenojejunostomy or ligament of Treitz amputation, gastrojejunostomy, subtotal gastrectomy and Billroth Ⅱ gastrojejunostomy, duodenojejunostomy, anterior reposition of the duodenum, duodenal circular drainage operation, and so on. SMAS is associated with a wide range of predisposing conditions and surgical procedures, more studies are needed to better define the optimal treatment for this disease.

Innovations and breakthroughs

We and many other authors had performed other operations for SMAS. However, some patients showed bad results, especially those with stronger reversed peristalsis. So we designed the operation of duodenal circular drainage. In practice, the operation achieved good results in most patients, All patients showed good status during a follow-up study of 2-15 years.

Applications

Forty-seven cases of SMAS were treated with duodenal circular drainage operation from 1959 to 2001 and had good effects after 2-15 years of follow-up.

Terminology

Duodenal circular drainage: A kind of operation we designed to treat SMAS. The distal jejunum is retrocolically anastomosed with duodenal cap end to end. Half length stomach is anastomosed to jejunum end to side 10 cm-15 cm from the jejunum-duodenal cap anastomosis. Proximal jejunum was anastomosed with distal jejunum end to side 15 cm-20 cm below the gastrojejunoanastomosed stoma.

Peer review

The manuscript evaluates the clinical value of duodenal circular drainage operation for superior mesenteric artery syndrome. The method is simple and correct. Discussion is well organized and conclusions are valuable.

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RAPID COMMUNICATION

Association between the presence of *H pylori* in the liver and hepatocellular carcinoma: A meta-analysis

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Abstract

AIM: To evaluate the arguments for and against the possible roles of *H pylori* in hepatocellular carcinoma (HCC).

METHODS: We performed a systematic review of all relevant studies published in the literature. A total of 103 clinical trials and reports were identified, but only 10 trials qualified under our selection criteria. A meta-analysis was carried out by a biostatistician according to the Cochrane Reviewers' Handbook recommended by The Cochrane Collaboration.

RESULTS: Nine case-control studies and one retrospective cross sectional study were included in the final analysis. Overall the prevalence of *H pylori* infection was 53.3% (129 of 242) in cases and 10.4% (29 of 280) in controls, and the summary odds ratio for the association of *H pylori* infection with the risk for HCC (using the fixed-effects model, which accounted for the homogeneity across the 10 studies) was determined to be 13.63 (95% CI, 7.90-23.49).

CONCLUSION: Our analysis showed a positive association between *H pylori* infection and the risk of HCC, with an indication of possible publication bias and possible confounders due to study designs that showed results of less pronounced associations.

Key words: *H pylori*; Hepatocellular carcinoma; Meta-analysis; Publication bias

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INTRODUCTION

The profound impact of hepatocellular carcinoma (HCC) on human health is known worldwide[1]. Persistent hepatitis B virus (HBV) and hepatitis C virus (HCV) infections and aflatoxins are the main causes of HCC[2]. The real risk factors for HCC may be far more numerous than the known causes. The infectious agent *Helicobacter hepaticus* (*H hepaticus*), for example, first described by Ward *et al* in 1994, has recently received new attention for its role in causing chronic active hepatitis and associated liver tumors[3].

Since *H pylori* was first cultivated from a human gastric biopsy specimen in 1982, it has become apparent that *H pylori* infection is correlated with gastric cancer and mucosa-associated lymphoid tissue lymphoma. More recently, researchers have reported that *Helicobacter spp.* have been identified in liver tissue resected from patients with HCC[4]. Experimental infection by *Helicobacter hepaticus* in mice causes chronic hepatitis and HCC. It is highly noteworthy that *H pylori* was found in liver tissues resected from patients with HCC[5-7].

The question of whether *H pylori* could play a role in the development of HCC remains controversial. Many conflicting reports have been published to date; thus, we performed a systematic rev iew of all of the relevant studies published in the literature to evaluate the arguments for and against the possible roles of *H pylori* in HCC.

MATERIALS AND METHODS

Selection criteria

We searched different databases, including the *Cochrane Controlled Trials Register on The Cochrane Library* Issue 1, 2007, MEDLINE (January, 1989-March, 2007), EMBASE.com (January, 1989-March, 2007) and the China Biological Medicine Database (CBMdisc) (January, 1989-March,

Table 1 Characteristics of 10 studies investigating the association between the presence of *H pylori* infection in liver and hepatocellular carcinoma

Reference	Country, year of publication	*H pylori* positivity/total subjects		Type of controls (*n*)	Age range (mean), yr	
		Cases	Controls		Cases	Controls
Pellicano *et al*[6]	Italy, 2004	17/20	2/6	Metastatic cancer (*n* = 6)	NA	NA
Ito *et al*[7]	Japan, 2004	13/15	0/17	Cirrhotic liver tissue specimens (*n* = 10), normal liver tissue specimens (*n* = 7)	36-73 (59.2)	NA
Coppola *et al*[8]	Italy, 2003	0/21	0/34	Metastatic liver carcinoma (*n* = 7), chronic hepatitis (*n* = 27)	NA	NA
Dore *et al*[9]	Italy, 2002	6/11	5/30	Chronic viral hepatitis without (*n* = 18) or with (*n* = 12) cirrhosis	19-78 (54.9)	49-78 (65.2)
Avenaud *et al*[10]	France, 2000	8/8	1/8	Patients without primary liver carcinoma (*n* = 8)	NA	NA
Nilsson *et al*[11]	Sweden, 2001	12/16	0/20	Metastatic liver carcinoma (*n* = 20)	NA	NA
Zhang *et al*[12]	China, 2004	16/48	2/37	Liver cirrhosis (*n* = 12), pericacinomatous tissues (*n* = 10), benign tumor of liver (*n* = 9), chronic hepatitis (*n* = 6)	25-67 (46.5)	35-65 (42.5)
Huang *et al*[13]	China, 2004	16/38	0/30	Liver cirrhosis (*n* = 15), benign tumor of liver (*n* = 15)	NA	NA
Li N *et al*[14]	China, 2006	22/34	0/20	Liver external injury (*n* = 5) giant hemangioma (*n* = 5), macrosis hepatic cyst (*n* = 3), ntrahepatic bile duct stone (*n* = 7)	28-71 (52)	30-68 (48)
Rocha *et al*[15]	France, 2005	19/31	19/78	Non-cirrhotic chronic hepatitis C (*n* = 24), HCV Positive cirrhosis without HCC (*n* = 29), HCV Positive cirrhosis and HCC (*n* = 25)	NA	NA

2007), for the terms 'hepatocellular carcinoma' and '*H pylori*'. The reference lists of pertinent reviews and retrieved articles were also checked for the identification of additional studies. We also performed a full manual search from the bibliographies of selected papers. Studies were identified by two researchers, independently; the two lists were compared and discrepancies were resolved. We also contacted the authors of studies containing relevant information, who did not report the results necessary for this analysis. Unpublished data were also accepted if an abstract was available and further information was obtained from the author.

In the meta-analysis, the following inclusive selection criteria were set and reviewed by two independent investigators: (1) each trial is an independent case-controlled study; (2) the purpose of all studies and statistical methods is similar; (3) the numbers of cases, controls and positive rate of *helicobacter* (the presence of *H pylori* DNA sequences in human liver or other standard methods for definition of *H pylori* infection in the liver) are concrete; (4) the study groups have definite HCC by pathology, histology and operation; (5) only studies that selected HCC patients as cases and subjects with other liver diseases or normal liver tissue specimens as controls are included.

The following exclusive selection criteria were set: (1) incomplete raw data; (2) repetitive reports (if more than one version of the same study was retrieved, only the most recent is used); (3) the positive rate of *helicobacter* is not detected by the presence of *H pylori* DNA sequences or other standard methods for detection of *H pylori* infection in liver (that is, all subjects are tested for the presence in serum of IgG antibodies against *H pylori*).

A total of 103 clinical trials and reports were identified and only 10 trials[6-15] qualified on the basis of our selection criteria. The studies were independently evaluated by two researchers. Discrepancies in the evaluations of some studies were resolved by discussion between the reviewers. The main features of the trials included in the meta-analysis are shown in Table 1.

Data extraction and outcomes

Data extracted included year of publication, country of origin, number of cases and controls, characteristics of controls, age of participants, prevalence of *H pylori* infection in cases and controls, and reported odds ratios (OR). All available studies were reviewed by two investigators independently. Reference 18 is a retrospective cross-sectional study; the others are case-controlled studies.

Statistical analysis

The meta-analysis was carried out by a biostatistician (Chen AJ) according to the Cochrane Reviewers' Handbook recommended by The Cochrane Collaboration. First, a pooled OR was calculated using the fixed-effect model. The heterogeneity of the studies was examined using the DL Q statistic[16]. Because the results were homogeneous ($P = 0.07$), a fixed-effects model was employed using the DerSimonian and Laird (DL) methods. A pooled OR was presented as a standard plot with 95 percent confidence intervals (CI). Begg and Mazumdar's proposed adjusted rank correlation test[17] and Egger's linear regression approach[10] were used to measure publication bias, which was shown as a funnel plot (Figure 1). Fixed (Figure 2A) and random-effects models (Figure 2B) were also used to perform sensitivity-analysis to assess the reliability of meta-analysis. The statistical package RevMan version 5.0 (provided by The Cochrane Collaboration, Oxford, England) was used for statistical analyses.

RESULTS

Nine case-controlled studies and one retrospective cross-sectional study were were identified and reviewed, as shown in Table 1[6-15].

In the meta-analysis, the overall prevalence of *H pylori* infection was 53.3% (129 of 242) in cases and 10.4% (29 of 280) in controls, and a summary OR for the association of *H pylori* infection with the risk for hepatocellular carcinoma (using the fixed-effects model, which accounted for the homogeneity across the 10 studies) was determined to be 13.63 (95% CI, 7.90-23.49) (Figure 2A). Figure 2A shows the ORs and 95% CIs of each study, and the summary OR determined by meta-analysis. The proportion of the total variation in study estimates, because of heterogeneity, was 44.0% (heterogeneity test statistics χ^2 = 14.28, on 8 df, P = 0.07, I^2 = 44.0%). A random-effects model was also used to perform sensitivity-analysis to assess the reliability of meta-analysis, as shown in Figure 2B.

Graphical and statistical evaluation of publication bias

Publication bias was assessed for all pooled ORs with confidence intervals using Begg's test[17,18]. This bias is shown as a funnel plot in Figure 1.

The funnel plot method was used to assess the possible presence of publication bias[19,20]. It consists of plotting each study's OR on a logarithmic scale (horizontal axis) against its SE (vertical axis), with an informal visual examination of the funnel plot graph to check for funnel plot asymmetry as an indication of potential publication bias. Studies of smaller size normally have a wider distribution of results than larger studies, because of a higher degree of random variation, and the ORs scatter more widely at the bottom of such a graph, with the spread narrowing with increasing precision among larger studies. In the absence of a publication bias, the graph appears as a symmetrical inverted funnel, as the risk estimates should be symmetrically distributed around the midpoint value (that is, the summary OR). Publication bias may occur if smaller studies showing no significant results remain unpublished, leading to an asymmetrical appearance of the funnel plot with a gap at the bottom of the graph.

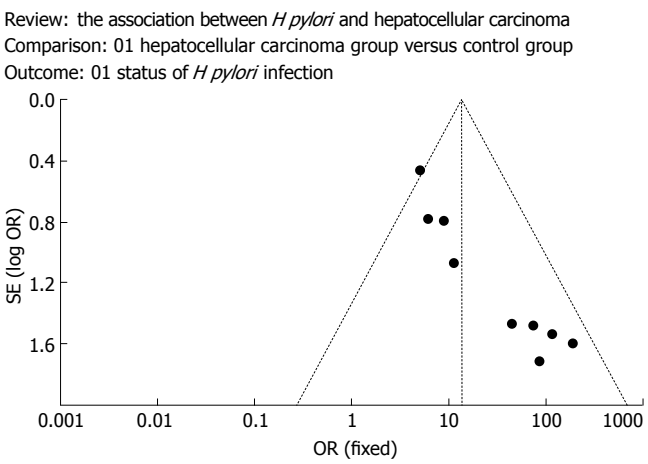

Figure 1 Funnel plot to explore publication bias. The graphical funnel plot of the 10 published studies appears to be asymmetrical.

DISCUSSION

H pylori infection is a classical model with which to study cancer development as a consequence of chronic inflammation. The estimated total of infection-attributed malignancies per year is 1.9 million cases or 17.8% of the global cancer burden[21]. Among the principal carcinogenic agents, *H pylori* is a leading factor, being responsible for 5.5% of all cancers. *H pylori* was classified as a type I carcinogen by the International Agency for Research on Cancer in 1994[22]. A striking finding indicated by Ward *et al* is that bacterial infection of the liver in healthy A/JCr male mice is capable of inducing a strong inflammatory change in the parenchyma (for example, hepatitis) leading to HCC.

We analyzed the published evidence investigating the association between *H pylori* infection and HCC. Studies concerning this possible association have been undertaken since the early 2000s. To our knowledge, this is the first published meta-analysis investigating this association. The summary OR for the association of evidence of *H pylori* infection and the risk for HCC was estimated to be 13.63 with a 95% CI from 7.90 to 23.49, with a confound of study design (lower for studies of prospective design and higher for retrospective case-controlled studies).

In our study, only publications in English or Chinese were used for evaluation. 'Meta-analytical' research on 29 meta-analyses investigating language bias has provided evidence that the OR estimated in meta-analyses from non-English publications are on average 0.8-fold (95% CI, 0.7-1.0) the OR estimates from English-written publications[23]. Therefore, even if we had not searched for non-English publications, this might have introduced only a small bias in the overall findings, which, in our opinion, would not have altered our main conclusions.

Several other points should be considered when interpreting the results of our study.

First, the positive rate of *helicobacter* of the most studies was detected by the presence of *H pylori* DNA sequences. Polymerase chain reaction (PCR) amplification using two sets of primers located in the 16S ribosomal DNA (rDNA) was used to detect the presence of bacteria, but this is not a 'gold standard' method for detecting *H pylori* infection in the liver. Histology with standard stains and culturing maybe more precise than 16S rDNA; however, false negatives are likely. Research in this area has been limited by the lack of a gold standard for the diagnosis of these organisms in the liver. Most published data to date have been based on molecular techniques that detect the DNA of *Helicobacter* species in liver tissues, rather than evidence of viable organisms in the liver.

Secondly, Reference 18 is a retrospective cross-sectional study, whereas the other studies are case-controlled studies. Such studies are generally lower in quality for use as prospective design studies and higher in quality as retrospective case-controlled studies. These observational studies are more prone to bias than randomized clinical trial (RCT) studies.

Third, in this analysis, graphical and statistical methods for testing and adjusting for a possible publication bias and a test for potential heterogeneity between studies were performed. A graphical funnel plot of the 10 published studies was asymmetrical, which may suggest the probable

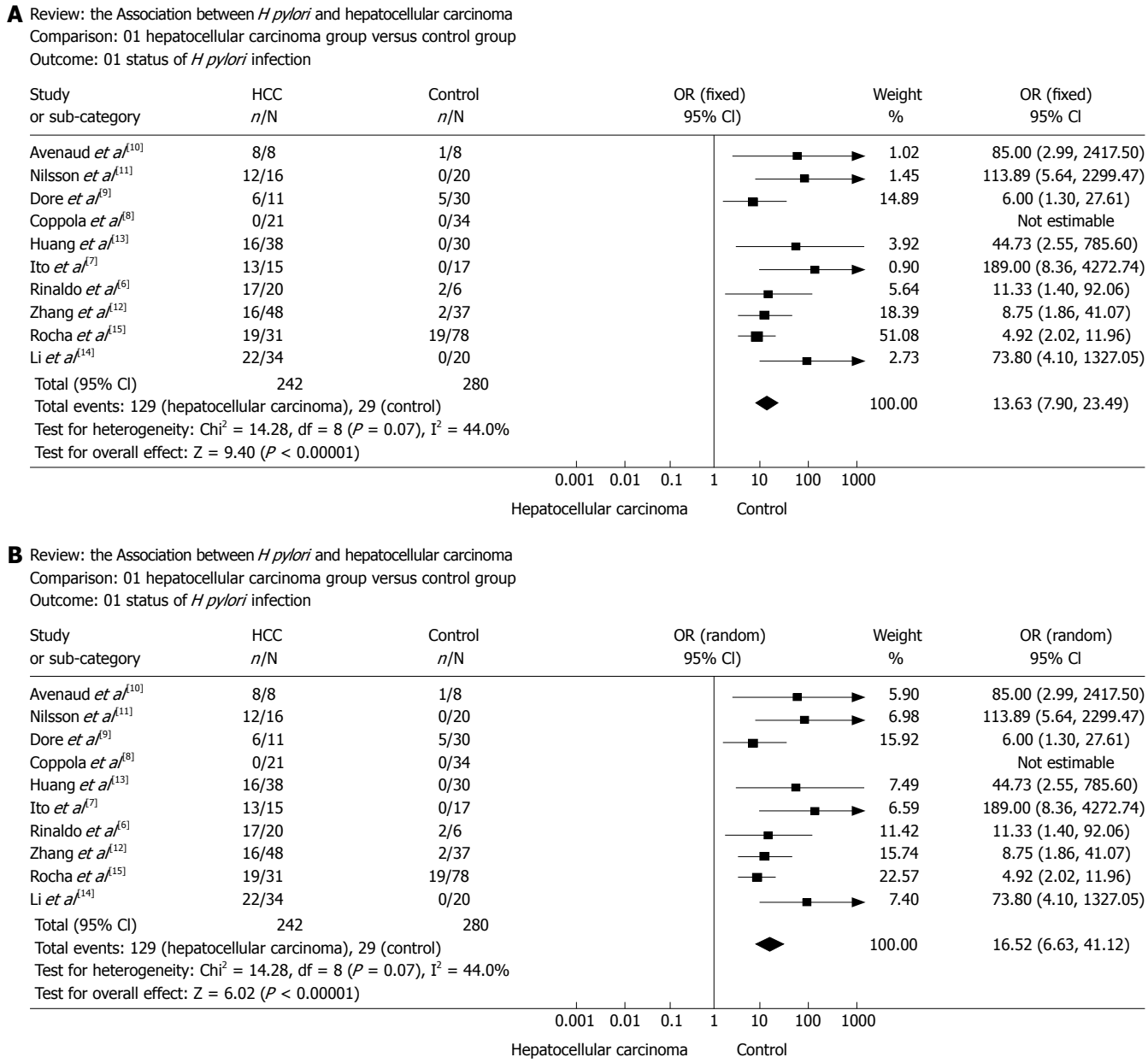

Figure 2 **A**: Forest plot of a meta-analysis of the association between *H pylori* and hepatocellular carcinoma risk (fixed-effects mode). It shows the ORs and 95% CI of each study, and the summary OR determined by meta-analysis. The proportion of total variation in study estimates, because of heterogeneity, was 44.0% (heterogeneity test statistics χ^2 = 14.28, on 8 df, P = 0.07, I^2 = 44.0%); **B**: Sensitivity-analysis: A Forest plot of a meta-analysis of the association between *H pylori* and hepatocellular carcinoma risk (random-effects mode).

existence of publication bias, although this may occur if some studies showing no significant results remained unpublished or if such negative studies are few.

Additionally, because the information used in our research was based on data from observational studies, the characteristics of each study population and the different methodologies of these studies should be taken into account when interpreting the results of our analysis. For example, different inclusion criteria for selection of the participants might have influenced the results of this research. Differences in the age distribution, different countries and different types of control groups (cirrhotic patients, patients with chronic viral hepatitis without or with cirrhosis, patients without primary liver carcinoma, patients with metastatic liver carcinoma, pericacinomatous tissues, benign tumors of the liver, liver external injuries, giant hemangiomas, macrosis hepatic cysts, and intrahepatic bile duct stones) could also be among the potential causes of variation in the studies' estimates.

Most studies did not control for the matching variables in the analysis, and the risk for HCC was not controlled for possible confounders such as HBV or HCV, except in Reference 18.

Only the articles of Dore *et al*[9] and Rocha *et al*[15] demonstrated the association of *Helicobacter* species with hepatitis C cirrhosis and HCC. These two studies included a large series of patients and examined both tumor and cirrhotic liver tissue samples from patients with HCV-positive HCC. *Helicobacter* DNA was found in a small percentage of liver biopsies from controls as well as from patients with chronic hepatitis C (4.2% and 3.5%, respectively). However, the prevalence of *Helicobacter*

species was high in patients with HCV-positive cirrhosis and in those with cirrhosis and HCC (68% and 61%, respectively). In nearly all cancer tissues, *Helicobacter* DNA was detected and identified as *Helicobacter* pullorum or *H pylori*-like organisms. The authors suggested a possible causal role of these bacteria in the progression of chronic hepatitis C and the development of HCC.

Furthermore, although we tried to maximize our efforts to identify all relevant published studies in peer-reviewed journals, it is possible that some escaped our attention.

There is some evidence to suggest that *Helicobacter* infection may be associated with an increased risk of extra-gastric malignancies[24]. The presence of *Helicobacter* species in liver tissues from patients with different liver diseases, including hepatic neoplasias, has been reported by numerous authors. The most intriguing hypothesis is that these bacteria might play a role in the development of HCC[25].

Despite its limitations, the present analysis has some implications: as relatively few studies are available in this field and current evidence remains limited, the necessity to conduct large studies with an adequate methodological quality, properly controlling for possible confounds in order to obtain valid results, should be emphasized; for example, tissue from unaffected liver metastatic carcinoma could be used as a suitable control.

In conclusion, our analysis showed a positive association between *H pylori* infection and the risk of HCC. We obtained from our meta-analysis a summary OR of 13.63 for the association of *H pylori* infection and HCC, with an indication of possible publication bias and confounds of study design, with less pronounced associations in prospective studies than in retrospective studies. Therefore, this risk increase should be interpreted with caution. Better designed and better controlled studies are needed to clarify the strength of this association and the possible causal role of *H pylori* infection in patients with HCC, and further prospective studies are required to prove this hypothesis. Given the importance of this potential association, further verification is warranted.

COMMENTS

Background

The real risk factors for hepatocellular carcinoma (HCC) may be far more than the known causes. A new infectious agent, *Helicobacter hepaticus* (*H hepaticus*), causing chronic active hepatitis and associated liver tumors, has been described by Ward *et al*. The question of whether *H pylori* could play a role in the development of HCC remains controversial. Many conflicting reports have been published to date, so we performed a systematic review of all of the relevant studies published in the literature to evaluate the arguments for and against the possible roles of *H pylori* in HCC.

Research frontiers

Since *H pylori* was first cultivated from human gastric biopsy specimens in 1982, it has become apparent that *H pylori* infection is correlated with gastric cancer and mucosa-associated lymphoid tissue lymphoma. More recently, researchers have reported that *Helicobacter spp*. were identified in liver tissue resected from patients with HCC. Experimental infection by *Helicobacter hepaticus* in mice causes chronic hepatitis and HCC. It is highly noteworthy that *H pylori* was found in liver tissue resected from patients with HCC.

Innovations and breakthroughs

To our knowledge, this is the first published meta-analysis investigating the association between *H pylori* and hepatocellular carcinoma risk.

Applications

Our analysis showed a positive association between *H pylori* infection and the risk of hepatocellular carcinoma, with an indication of possible publication bias and confound of study design, with less pronounced associations in prospective studies than in retrospective studies. Therefore, this risk increase should be interpreted with caution; better designed and better controlled studies are needed to clarify the strength of the association and the possible causal role of *H pylori* infection in hepatocellular carcinoma. Further prospective studies are requested to prove this hypothesis; given the importance of this potential association, further verification is warranted.

Terminology

Meta analysis: In statistics, a meta-analysis combines the result of several studies that address a set of related research hypotheses. *H pylori*: *H pylori* is a bacteria that can cause digestive illnesses, including gastritis and peptic ulcer disease. More recently, researchers have reported that *Helicobacter spp*. were identified in liver tissue resected from patients with hepatocellular carcinoma.

Peer review

Authors evaluated the arguments for and against the possible roles of *H pylori* in HCC through a systematic review of all relevant studies published in the literature. The analysis showed a positive association between *H pylori* infection and the risk of HCC.

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RAPID COMMUNICATION

Small intestinal bacteria overgrowth decreases small intestinal motility in the NASH rats

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Abstract

AIM: To explore the relationship between small intestinal motility and small intestinal bacteria overgrowth (SIBO) in Nonalcoholic steatohepatitis (NASH), and to investigate the effect of SIBO on the pathogenesis of NASH in rats. The effect of cidomycin in alleviating severity of NASH is also studied.

METHODS: Forty eight rats were randomly divided into NASH group (n = 16), cidomycin group (n = 16) and control group (n = 16). Then each group were subdivided into small intestinal motility group (n = 8), bacteria group (n = 8) respectively. A semi-solid colored marker was used for monitoring small intestinal transit. The proximal small intestine was harvested under sterile condition and processed for quantitation for aerobes (*E. coli*) and anaerobes (Lactobacilli). Liver pathologic score was calculated to qualify the severity of hepatitis. Serum ALT, AST levels were detected to evaluate the severity of hepatitis.

RESULTS: Small intestinal transit was inhibited in NASH group (P < 0.01). Rats treated with cidomycin had higher small intestine transit rate than rats in NASH group (P < 0.01). High fat diet resulted in quantitative alterations in the aerobes (*E. coli*) but not in the anoerobics (Lactobacill). There was an increase in the number of *E. coli* in the proximal small intestinal flora in NASH group than in control group (1.70 ± 0.12 log10 (CFU/g) *vs* 1.28 ± 0.07 log10 (CFU/g), P < 0.01). TNF-α concentration was significantly higher in NASH group than in control group (1.13 ± 0.15 mmol/L *vs* 0.57 ± 0.09 mmol/L, P < 0.01). TNF-α concentration was lower in cidomycin group than in NASH group (0.63 ± 0.09 mmol/L *vs* 1.13 ± 0.15 mmol/L, P < 0.01). Treatment with cidomycin showed its effect by significantly lowering serum ALT, AST and TNF-α levels of NASH rats.

CONCLUSION: SIBO may decrease small intestinal movement in NASH rats. SIBO may be an important pathogenesis of Nash. And treatment with cidomycin by mouth can alleviate the severity of NASH.

Key words: Nonalcoholic steatohepatitis; Small intestinal motility; Small intestinal bacteria overgrowth treatment

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INTRODUCTION

With much more prevalence of nonalcoholic fatty liver disease (NAFLD) than previously thought noticed, there has been an explosion on the research of pathogenesis of NAFLD. NAFLD may develop into cirrhosis of liver through NASH, in some cases, to hepatocellular carcinoma[1-5], Insulin resistance and peroxidative injury were thought to place an important role in the pathogenesis of NASH[6-12]. But there are still other mechanisms that can trigger and maintain NASH[13-18]. Small intestinal bacterial overgrowth has been reported to place a role in the pathogenesis of NASH, endotoxin and TNF-α being the possible mediator[19-21]. But contrary to this hypothesis, in another study, antibiotic treatment did not normalize aminotransferase levels in NASH patients[22]. Therefore more study is needed on the influence of SIBO on the pathogenesis of NASH and the curative effect of antibiotic on NASH. Alejandro S[22] found that prolonged Orocecal Transit Time (OCTT) in NAFLD patients coexist with SIBO. It is generally thought that prolonged OCTT is the cause of SIBO. But it is not clear if alteration of intestinal motility is a cause or a consequence of SIBO.

We established an NASH animal model by high fat diet to explore the relationship between intestinal motility and SIBO in NASH. The relationship between SIBO and NASH, and the curative effect of antibiotic on NASH were also studied.

MATERIALS AND METHODS

Materials

Forty eight male SD strain rats were used in this study.

Rats were housed individually in cages at constant room temperature in a 12-h light/dark cycle and had free access to laboratory feed and water. Semi-solid colored marker (carbon-ink 85%, gum acacia 10%, activated charcoal 5%) was made in our laboratory. Bacteria evaluation kit (API 20) was purchased from French Biomerieux CO. TNF-α kit was purchased from Technology Development Center of the General Hospital of PLA.

Establishment of animal mode

Forty eight SD rats were randomly divided into NASH group, cidomycin group and control group. Each group were subdivided into small intestinal motility group (n = 8) and bacteria group (n = 8) respectively. Rats in NASH group and cidomycin group were fed with high fat diet that was made by ordinary diet (88%) plus fat (10%) and cholesterol (2%) (supported by Qinglongshan Nursery) Rats in control group were fed with ordinary diet (supported by Qinglongshan Nursery). Furthermore, rats in cidomycin group were treated with cidomycin (12Mu ig qd) after eight weeks of high fat diet while those in the other two groups were treated with isotonic Na chloride (1 mL/d ig qd). At the end of the twelfth week, when the rats in NASH group and cidomycin group had received high fat diet for 12 wk, all the rats were killed, the serum levels of aminotransferase, TNF-α were tested and the histology of liver specimen was observed by H&E staining.

Measurement of small intestinal transit

Rats in small intestinal motility group was deprived of food for 24 h and water for 12 h prior to measurement of small intestinal transit. Then 1.0 mL semi-solid colored marker was administered into stomach by orogastric gavage. Twenty minutes later, the rat was killed, abdomen was opened and small intestine was dissected. The distance traveled by the marker was calculated. The small intestinal transit was represented by ratio of the distance traveled by the marker to the total length of the small intestine.

Histological evaluation

The liver pathologic score was calculated according to the lecture[23]: Inflammation in portal canal area was denoted by "P", inflammation inside lobules of liver by "L", piecemeal necrosis by "PN" and bridging necrosis by "BN". According to the severity of inflammation and necrosis, every item was scored from one to four. The total score of the liver inflammation was "P+L+2PN+2BN".

Measurement of small intestinal bacteria

Rats in the bacteria group was deprived of food for 24 h and water for 12 h prior to measurement of small intestinal bacteria. Then the rat was killed, the abdomen was opened and the proximal small intestine was harvested under sterile condition. A two-centimeter-long small intestine was dissected from the point about ten centimeters from pylorus, then rinsed with sterile saline thrice, and next, the leftover was sucked by sterile filter paper. After weighing, the leftover and 2 mL sterile saline were placed in a sterile glass homogenizer and homogenized. The homogenate was diluted with sterile saline at the ratio of 1:1, then, 100 μL dilution was plated on SS agar (*E. coli*) and MRS agar (Lactobacilli) respectively. The quantity of *E. coli* was determined after 24 h of aerobic cultivation at 37℃. While the quantity of Lactobacilli was determined after 48 h of anaerobic cultivation at 37℃. The number of Colony forming units (CFU) of bacteria was quantified. The quality of aseptic manipulation was evaluated by inoculating swab of abdominal cavity on sheep-blood agar. The serum TNF-α levels were detected, using radio-immunity method, in our laboratory.

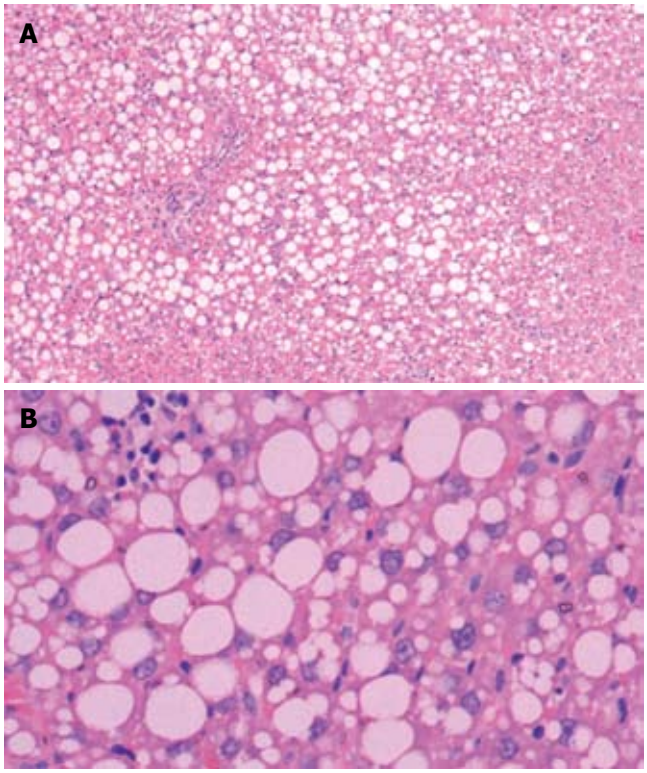

Figure 1 Pathological changes of liver after 12 wk of high fat diet. **A**: Fat drops in more than one thirds of liver cells (HE, × 40); **B**: Lymphocytes in hepatic steatosis background (HE, × 100).

Statistical analysis

Throughout this report, data were expressed as mean ± SD. Experimental results were analyzed by one-factor analysis of variance. $P < 0.05$ was considered statistically significant.

RESULTS

The weight of the model group rise *vs* controls was 455.38 ± 11.48g *vs* 395.38 ± 10.91 g, $P < 0.05$.

Pathologically a NASH model has been successfully made (Figure 1).

TNF-α, liver pathologic score, serum ALT and AST levels are higher in the NASH group than in the other two groups indicating a NASH model has been successfully made ($P < 0.01$, $P < 0.01$, $P < 0.05$). There are no statistical differences between the control group and the cidomycin group in serum TNF-α level. Liver pathologic score, serum ALT and AST levels are higher in cidomycin group than in control group ($P < 0.01$) (Table 1).

Table 1 The concentration of TNF-α, liver pathologic score, serum ALT and AST levels (mean ± SD)

	n	TNF-α (ng/mL)	ALT (U/L)	AST (U/L)	Liver pathologic score
Control group	8	0.57 ± 0.09[b]	39.73 ± 5.10	168.00 ± 16.41	1.00 ± 0.93
NASH group	8	1.13 ± 0.15	86.63 ± 20.91[d]	379.63 ± 61.73[d]	6.00 ± 1.20[d]
Cidomycin group	8	0.63 ± 0.09[b]	70.88 ± 11.93[a,d]	318.75 ± 52.62[a,d]	4.25 ± 1.58[a,d]

[b]$P < 0.01$ *vs* NASH group; [d]$P < 0.01$ *vs* control group; [a]$P < 0.05$ *vs* NASH group.

Table 2 Small intestinal bacteria, small intestinal transit rate (mean ± SD)

	n	*E. coli* [$\log_{10}$(CFU/g)]	*Lactobacilli* [$\log_{10}$(CFU/g)]	Small intestinal motility(fraction)
Control group	8	1.28 ± 0.07	1.67 ± 0.16	0.58 ± 0.06
NASH group	8	1.70 ± 0.12[b]	1.69 ± 0.16	0.39 ± 0.11[b]
Cidomycin group	8	1.17 ± 0.08[a,d]	1.81 ± 0.13	0.51 ± 0.07[d]

[b]$P < 0.01$ *vs* control group; [a]$P < 0.05$ *vs* control group; [d]$P < 0.01$ *vs* NASH group.

The number of *E. coli* in the proximal small intestinal flora are more in the NASH group than in the control group and cidomycin group ($P < 0.01$, $P < 0.01$). Small intestinal motility are weaker in NASH group than in control group and cidomycin group ($P < 0.01$, $P < 0.01$). The number of *E. coli* are less in cidomycin group than in control group ($P < 0.05$). There are no statistic difference in small intestinal motility between control group and cidomycin group. There are no statistical differences in the number of Lactobacilli between every two groups of the three (Table 2).

DISCUSSION

It was found that NASH coexist with SIBO[19,24]. In our study excessive multiplication of *E. coli* and increased serum level of aminopherase coexist in NASH rats, that was consistent with previous studies. We thought that SIBO is an important, though not the only, pathogenesis of NASH by the fact that antibacterial treatment can alleviate the severity of NASH. We also found the level of aminopherase went up and down with serum level of TNF-α. It strongly supported TNF-α to be an important mediator in the promotion of NASH by SIBO. In our study, rats treated with cidomycin had a serum level of aminopherase slightly higher than controls and a serum level of TNF-α similar to controls. The reason or this phenomenon might be that TNF-α is not the only mediator. Generally endotoxemia was thought to be a link between SIBO and elevated TNF-α level[19,20]. But Wigg A J[25] observed that serum levels of TNF-α and endotoxin were not parallel in NASH patients. Given the disadvantage of clinical trials to control experimental conditions, and the disadvantage of C^{14} breath test in identification of bacteria, animal experiments are necessary to find the link between SIBO and NASH. Our study found a significant increase in the number of *E. coli*, a widely known source of endotoxin, in the proximal small intestinal flora in NASH group. It might support endotoxin to be the promoter to TNF-α in NASH.

With respect to the causality between SIBO and small intestinal motility in NASH, ALEJANDRO-S[22] found that delayed intestinal transit coexist with SIBO in patients effected by NASH. We also observed that NASH rats suffered both delayed intestinal motility and an increase in intestinal E. coli. It is widely believed that delayed intestinal motility could cause SIBO. Leveau P *et al*[26] noticed a delay in intestinal transit time, appeared as an early event in acute pancreatitis, preceding intestinal bacterial overgrowth, and suggested that impairment in intestinal motility probably played a role in the development of bacterial overgrowth. Gangarosa[27] demonstrated that intestinal motility served as a normal cleansing mechanism of the intestine, and drugs that decreased this motility might facilitate replication of pathogens and their attachment to or invasion of the intestinal tissue. Nieuwenhuijs *et al*[28,29], clarified the role of the migrating motor complex (MMC) in the regulation of small intestinal microflora by the fact that the disruption of MMC with morphine promotes duodenal bacterial overgrowth. Since MMC can also coordinate with the movement of the pylorus, gut and cholecyst, the alteration of MMC necessarily has an influence on the secretion of bile (the inhibiter of gram-negative bacilli). MMC might influence intestinal bacteria *via* another mechanism. Grzesiuk *et al*[30], demonstrated that intestinal bacteria, particularly those adhering to intestinal epithelial cells, were exposed to electric fields and currents generated by the muscular activity of the small intestine and that the myoelectrical activity of the duodenum, through action on cell membrane, can affect cell division of intestinal bacteria.

Though it is generally believed that insulin resistance can explain the delay of small intestinal motility in NASH, and abnormality of intestinal bacteria may be caused by delayed intestinal motility, in our experiment, treatment with antibiotics improved the abnormality of small intestinal motility in NASH rats, indicating that small intestinal bacterial can also regulate intestinal motility in NASH rats. The interpretation may be founded on the influence microflora has on intestinal smooth muscle cell myoelectricity action. Huseby *et al*[31] demonstrated that after introduction of conventional intestinal microflora the interval between activity fronts of the MMC in proximal jejunum of germ-free rats was reduced. Sjogren *et al*[32] reported that bacterial adherence to the intestinal mucosa appeared to be important in eliciting the abnormal myoelectric responses. Different intestinal bacterial genus may have different influence on intestinal motility. *E. coli* adherence to intestinal mucosa delayed spike bursts[29], reduced the frequency and amplitude of the intestinal contractions and inhibit small intestinal transit[29]. while

Lactobacillus promoted regular spike burst activity, reduced the MMC period and accelerated small intestinal transit[29]. In our study the overgrowth of *E. coli* coexist with the reduction of small intestinal motility, and the elimination of *E. coli* could increase the small intestinal motility, strongly supporting *E. coli* to be an important mediator in the small intestinal motility in NASH rats. In our study Lactobacillus didn't change according to high fat diet or cidomycin treatment, indicating Lactobacillus may not be important in the pathogenesis of NASH.

The results of this study suggest that SIBO may be not only the result of but also a promoter to delayed intestinal motility in NASH. There might be a vicious circle between SIBO and delayed intestinal motility in NASH rats. SIBO can trigger NASH *via* TNF-α, and treatment with cidomycin orally can alleviate the severity of NASH.

COMMENTS

Background

While once considered a benign process, nonalcoholic steatohepatitis (NASH) has been found to lead to cirrhosis and, in some cases, to hepatocellular carcinoma. The mechanisms underlying disease development and progression are awaiting clarification; however the finding that not all patients with steatosis develop hepatic inflammation and hepatocellular damage has led to the hypothesis that different pathogenic factors lead firstly to hepatic steatosis and secondly to hepatic damage ("the second hit"). Insulin resistance and obesity-related inflammation, among other possible genetic, together with oxidative stress, microcirculation disturbance, malnutrition are thought to play a key role in the second stage. People also found that small intestinal bacteria overgrowth (SIBO) may also play a role in the process of NASH. Some people think the medium of TNF-α to be an endotoxin, but all people do not accept this.

Research frontiers

Great effort has been and is still being done to clarify all the possible mechanism in the development of NASH, The source and pathogenesis of mediators of inflammation in the process has been the hotspot. People also want to know if it can act as a target point in the treatment of NASH.

Innovations and breakthroughs

In this article the relationship between the small bowel moment and SIBO is studied. And the cidomycin was first studied for the treatment of NASH.

Applications

This article helps to understand the process of SIBO leading to NASH, and suggests that Cidomycin might be useful in the treatment of NASH. Since there might be a vicious circle between SIBO and delayed intestinal motility, the treatment of accelerator of intestinal motility might also be useful.

Terminology

NASH: A liver disease, which resembles alcoholic liver disease consists of steatosis plus inflammation, necrosis, and fibrosis while lack of history of drinking. SIBO: Small intestinal bacterial overgrowth.

Peer review

This article tries to study the relationship between SIBO and delayed intestinal motility in NASH rats and suggest that cidomycin might be effective in the treatment of NASH. But the conclusion needs further probation in future clinical study before clinical application.

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CASE REPORT

Acute inflammatory demyelinating polyneuropathy associated with pegylated interferon α 2a therapy for chronic hepatitis C virus infection

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Abstract

The combination of pegylated interferon (Peg-IFN) and ribavirin is the standard of care for chronic hepatitis C virus (HCV) infection treatment. In general, common side effects related to this combination therapy are mild and are very well tolerated. However, peripheral neuropathy including demyelinating polyneuropathy related to Peg-IFN is extremely rare. We present the first case of an acute inflammatory demyelinating polyneuropathy (AIDP) associated with Peg-IFN-α 2a (Pegasys) after 16 wk of a combination therapy with Pegasys and ribavirin in a 65-year-old woman with chronic HCV infection. She developed tingling, numbness, and weakness of her upper and lower extremities and was hospitalized for acute neurological deficits. Her clinical course, neurological findings, an electromyogram (EMG), nerve conductions studies (NCS), muscle biopsy, and a sural nerve biopsy were all consistent with AIDP likely related to Pegasys use. The patient recovered completely with the use of intravenous immunoglobulin (IVIG) including physical therapy and neurological rehabilitation. It is very important that gastroenterologists and/or hepatologists recognize this rare neurological complication related to Peg-IFN treatment very early, since it requires a prompt discontinuation of therapy including an immediate referral to a neurologist for the confirmation of diagnosis, management, and the prevention of long-term neurological deficits.

INTRODUCTION

The combination of Peg-IFN and ribavirin has been shown to be an effective treatment for chronic hepatitis C. Overall, the common side effects associated with these two drugs are well known. The common side effects of IFN include flu-like symptoms and psychiatric symptoms such as depression, suicidal ideation, irritability, nervousness, and insomnia[1-5]. Less common side effects include hematopoietic suppression, reversible hair loss, hearing loss, retinopathy, dermatitis, seizures, and the development or exacerbation of autoimmune diseases such as thyroid dysfunction, rheumatoid arthritis, sarcoidosis, systemic lupus erythematosus, vitiligo, type 1 diabetes, and myasthenia gravis[1-5]. Neurological complications, however, are rare. Only some publications reported on IFN-related peripheral neuropathy including sensory neuropathy[6,7]. In addition, rare neurological complications such as vasculitic neuropathy, autonomic neuropathy, Bell's palsy, and chronic inflammatory demyelinating neuropathy (CIDP) related to IFN use have also been documented in the literature[1,5,8,9]. Here we present the case of a severe AIDP that developed at wk 16 of Pegasys therapy.

CASE REPORT

A 65-year-old white female presented to liver clinic at the University of Chicago Medical Center for chronic hepatitis C treatment. The initial physical examination was unremarkable with the exception of spider angiomata in her neck and face, and bilateral palmer erythema. Initial liver profile included aspartate aminotransferase (AST) 56 (8-37) IU/mL, alanine aminotransferase (ALT) 67 (8-35) IU/mL,

total bilirubin 0.6 mg/dL, alkaline phosphatase 77 IU/mL, albumin 4.8 g/dL, and prothrombin time (PT) 13.1 s. Serum viral load was estimated to 2414896 IU/mL with a genotype 1a. A liver biopsy examination revealed a grade I and stage Ⅲ liver disease with a focal bridging fibrosis. The patient participated in a clinical trial for chronic hepatitis C and received Pegasys 180 μg subcutaneously per week and ribavirin 1000 mg/d in divided doses orally. HCV RNA levels decreased to 416 IU/mL after 12 wk of therapy, indicating an early viral response (EVR).

Approximately 13 wk after start of the treatment, the patient presented to the University of Chicago hospital with a 24 h onset of fever, chills, nausea, vomiting, headache, and fatigue. Her chest X-ray and CT scan of chest demonstrated a right upper lobe pneumonia. A cerebrospinal fluid (CSF) analysis revealed the presence of single WBC (monocyte), glucose levels of 74 mg/dL within the normal range, and a protein level of 15 mg/dL. However, her sputum culture grew pneumococci requiring treatment with vancomycin, ceftriaxone, and moxifloxacin for pneumonia. Pegasys and ribavirin treatment thus was temporarily discontinued. The patient was discharged home with oral moxifloxacin medication and recovered from pneumonia. However, shortly after discharge, the patient developed a macular rash on the anterior and posterior chest wall with an extension of rash into the anterior abdominal wall, which was attributed to the use of antibiotics. Two weeks after the onset of pneumonia (wk 15), the patient was restarted with Pegasys and ribavirin. Unfortunately, she reported tingling, numbness, and weakness of her lower extremities 3 wk later (wk 16). Her neurological symptoms progressed rapidly over the course of 1 wk to the point of losing the ability to ambulate, and she became wheelchair bound. In addition, the patient also complained of tingling and numbness in the upper extremities involving the fingers and wrists bilaterally. Pegasys and ribavirin were discontinued due to an acute onset of weakness of upper and lower extremities. The patient was hospitalized again. The neurological examination revealed normal cranial nerves with normal bulk of muscles and tone; but a weak muscle strength (4/5) in the upper extremities as well as in hips, knees, and ankle dorsiflexion and ankle plantarflexion bilaterally. Other muscle groups were even weaker (3/5) including ankles and toes. Tendon reflexes were diminished significantly. Sensory examination revealed decreased light touch, pinprick, and temperature sensation from the feet to mid-calves as well as hands except vibratory sense. A clinical diagnosis of acquired, acute demyelinating sensorimotor polyneuropathy was made. Other laboratory tests included a normal complete blood count, chemistry profile, erythrocyte sedimentation rate, and C-reactive protein. Antinuclear antibodies, rheumatoid factor, human immunodeficiency virus (HIV) test, anti-GM1 (ganglioside) antibodies, serum cryoglobulin level, Epstein-Barr virus serology, and cytomegalovirus serology were tested negative. A second CSF analysis was acellular again with protein (29 mg/dL) and glucose (60 mg/dL) levels within the normal range. In addition, myelin associated glycoprotein antibody was tested negative, and immunoglobulin A level was normal including negative immunoelectrophoresis for monoclonal antibodies in CSF.

Table 1 EMG/Nerve conduction studies

	Distal latency (m/s)	Conduction velocity (m/s)	Amplitude (uV (sensory) mV (motor))
Sensory			
Right sural	NR	NR	NR
Left sural	NR	NR	NR
Motor			
Right peroneal	8.9	27	0.6
Right tibial	9.5	27	1.4
Left tibial	7.9	30	1.1
Right ulnar	6	37	3.9
Right median	7.6	49	2.4
Left median	7.8	48	1.1
F wave	F-Latency		
Right tibial	Unclear		
Right ulnar	42.6		
Right median	54.8		
Left median	Unclear		

NR: No response obtainable.

An EMG, NCS, and Sural nerve biopsy were consistent with an AIDP (Table 1). The patient was treated with a course of IVIG at a dose of 0.4 g/kg per day. Unfortunately, she was only able to complete 4 d of treatment due to the development of a rash and leukopenia from IVIG. The patient was subsequently discharged for physical therapy and neurological rehabilitation where she had some improvement in her ability to write, to use utensils, and to ambulate with a walker.

Even though her neurological symptoms somewhat improved after a short course of IVIG, the patient developed bilateral foot drop, loss of finger grips, and a progressive weakness and numbness in both her hands and feet four weeks later. A neurological examination revealed decreased muscle bulks in the upper and lower extremities distally. Muscle strength was weaker (3/5) in finger abduction, plantar flexion and dorsiflexion bilaterally with bilateral foot drops. Tendon reflexes were 2/4 in the upper extremities but diminished significantly in the lower extremities, including absent reflexes at patella and achilles bilaterally. Remainders of motor examinations were normal. Sensory examination revealed normal sensation in the upper and lower extremities except absence of all modalities of sensation at her toes excluding sense of vibration and decreased pinprick sensation below the knees. Laboratory findings were again negative including anti- Sjogren's syndrome A (SSA) and Sjogren's syndrome B (SSB). Subsequently, a sural nerve biopsy and a gastrocnemius muscle biopsy were obtained and findings were consistent with an AIDP without showing any vasculitic features (Table 1). The patient was treated with 5 d of 2nd course of IVIG and the symptoms of weakness and bilateral legs numbness markedly improved over two months. Six months after the completion of the 2nd course of IVIG, the patient felt very well and neurological symptoms and signs resolved completely without having any residual neuromuscular deficits.

DISCUSSION

Peripheral neuropathy is a rare and uncommon side effect

seen in patients treated with IFN-α. In recent years, a variety of peripheral neuropathies have been reported in patients treated with IFN including sensory neuropathy, autonomic neuropathy, Bell's palsy, and more recently, CIDP[6-12]. As an example, one patient who received multiple cycles of IFN-α developed parasthesias of both legs and was diagnosed with sensorimotor polyneuropathy due to high cumulative effect of IFN-α[6]. IFN-α 2a has also been shown to have caused peripheral sensory neuropathy given for a period of twenty months[7]. In this case, when IFN-α 2a was discontinued for 4 wk, peripheral neuropathy resolved. However, upon reinitiating the IFN-α 2a, the peripheral neuropathy re-appeared suggesting IFN-related neuropathy. Furthermore, Lapinski *et al* reported a case of peripheral polyneuropathy in a patient who received Pegasys for 1 mo followed by pegylated IFN α-2b (Pegintron) for 3 mo that resulted in paresis of hands and legs leading to a CIDP confirmed by an EMG[9].

The more probable etiologies in our patient included infectious processes, toxins and/or drugs, and immune-mediated processes leading to demyelinating neuropathies[13]. Viral infections such as HIV and CMV have been described to cause demyelinating neuropathy[14,15]. HIV infection can lead to peripheral neuropathy that can rapidly progress to either acute or chronic demyelinating neuropathy due to macrophage-mediated immune attachment within the endoneurial parenchyma[16]. Since HIV test was negative in our patient this was not the cause of her neuropathy. A CMV infection in a patient with chronic HCV can present as an acute Guillain-Barre syndrome (GBS)[15]. It has been hypothesized that host immune system mistakenly attacks own nerve cells that expressed similar epitope as of HCV or CMV antigen. Our patient had multiple CSF analysis that failed to detect CMV DNA. Therefore, our patient also did not have CMV-related demyelinating neuropathy.

Toxins and/or drugs are also common etiologies for demyelinating neuropathies, which can be acute or chronic in nature. Common toxins/drugs are barbital, sulfonamides, phenytoin, nitrofurantoin, heavy metals, carbon monoxide, industrial poisons, and certain AIDS drugs (e.g., zalcitabine, didanosine)[13]. Our patient had no exposure to any of these above toxins or drugs other than Pegasys, ribavirin, and moxifloxacin. There are several reported cases of moxifloxacin induced peripheral sensory neuropathy including an evidence of a tendon rupture without showing demyelinating neuropathy[17]. Similarly, ribavirin also has not been associated with any type of reported neuropathy. But Pegasys including IFN-α has been implicated to cause immune mediated CIDP during chronic hepatitis C treatment[5,8] due to cytokine-induced apoptosis in the myelin-producing oligodendrocyte resulting in inhibition of central nervous system remyelination thus causing demyelinating neuropathy[5,18,19]. To the contrary, IFN-α has also been shown to be a successful treatment in patients with CIDP[8]. However, if a patient develops demyelinating neuropathy secondary to IFN use, it should be discontinued immediately since it may cause irreversible nerve damage due to inhibition of remyelination process. Two cases of CIDP have been described either with Pegasys or IFN-α use[5,8]. In both cases, parasthesia and muscle weakness developed 6 wk after therapy and lasted more than 8 wk suggesting CIDP. It has been shown that patients with CIDP respond to prednisone, plasma exchange, and IVIG[5,20,21]. The time course for our patient's symptoms was not long enough to meet the criteria for a CIDP. The criteria for CIDP usually include the clinical deterioration of neurological symptoms for a period of greater than 8 wk as opposed to AIDP and/or GBS, which usually has deterioration over a period of approximately 4 wk or less[21]. Furthermore, immune-mediated AIDP of GBS type has been reported after infection, post-vaccination, or surgery and also accounts for a significant portion of demyelinating polyneuropathies[13]. In our patient, there was an initial high suspicion for AIDP of GBS type due to a pneumonia prior to neurogical symptoms and signs. Her time course fit an acute demyelinating neuropathy given that it developed approximately 3 wk later. A typical presentation of GBS includes a significant motor weakness along with mild to moderate paresthesias. The muscle weakness typically starts in the distal muscles of the lower extremities and weakness ascends rapidly involving upper extremities. In addition, CSF analysis usually reveals an elevated protein level with normal white blood cell count. Even though our patient's presentation was suggestive of a GBS due to a preceding pneumonia, CSF analysis on multiple occasions revealed a normal CSF protein. Furthermore, our patient had significant painful paresthesias that are not commonly seen in GBS. Thus, the clinical course including normal CSF protein and painful paresthesias was not suggestive of a typical GBS, but rather suggestive of an acute immune mediated process related to Pegasys use as AIDP. The pathogenesis of AIDP related to Pegasys use is thought to be an acute immune-mediated progress similar to GBS. The precise and exact immune regulatory function of IFN-α including Peg-IFN is not well understood and it is likely similar to mechanism associated with IFN induced CIDP described above[5,18,19]. In addition, IFN-α has been reported to enhance *in vivo* and *in vitro* autoantibody production and may upregulate transcription of genes associated with class I major histocompatibility complex (MHC) antigens[22]. It is likely that the levels of proinflammatory cytokines may trigger autoimmune phenomena in immunologically predisposed individuals when IFN is administered. Therefore, the immune system mistakenly attacks the host's nerve tissue after recognizing a molecular epitope similar to a foreign antigen and may result in acute inflammatory neuropathy[23]. Immunomodulation with plasma exchange or IVIG usually shortens the disease process and provides the best outcome[23]. Our patient fit this clinical picture of an acute inflammatory neuropathy and showed a quick response to IVIG therapy.

One other possible immune-related process as the etiology of the demyelinating neuropathy would be IgM binding to Myelin-Associated Glycoprotein (MAG). Demyelinating polyneuropathy with monoclonal IgM is often associated with anti-MAG autoantibodies[24]. Our patient was negative for this antibody including negative immunoelectrophoresis suggesting this is not the case.

Vasculitic neuropathy, an autoimmune process may

also occur in association with chronic HCV infection. Generally, patients may develop a chronic symmetrical axonal sensorimotor peripheral polyneuropathy due to a necrotizing vasculitis[25]. Subsequent treatment with IFN-α can worsen vasculitic neuropathy[25]. In addition, there have been multiple reported cases of GBS associated with HCV related to cyroglobulinemia[26,27]. However, our patient had an undetectable cryoglobulin level on multiple occasions, which rules out a possibility of cryoglobulin induced demyelinating neuropathy.

In conclusion, the treatment of chronic HCV infection has come a long way in recent years. Physicians and other medical providers must be aware of possible side effects, including demyelinating neuropathy in patients who are treated with IFN and/or pegylated IFN in addition to HCV/cryoglobulin related neuropathy. As we learned from our case, Pegasys may cause neurological symptoms including AIDP. In addition, it is imperative on the part of the health care providers recognize AIDP very early for the diagnosis and treatment including the prevention of a long-term neurological complication by obtaining a neurology consultation.

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LETTERS TO THE EDITOR

Clinical guidelines: Involvement of peers increases physician adherence

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Abstract

The literature illustrates the important issue of physician adherence to guidelines in their daily practice. In a quantitative study, we asked a random sample of 100 hospital gastroenterologists to evaluate their knowledge of guidelines and awareness of promoters. The degree to which guidelines were considered reliable was not related to the scientific evidence but was significantly associated with the promoter. The French Society of Gastroenterology was considered to be a more reliable promoter than national health agencies and pharmaceutical industries. Gastroenterologists become aware of guidelines mainly through their specialty society (62%). Specialty societies appear to be a more important source of information on guidelines for physicians. National health agencies should involve the specialty societies in the guideline development process to achieve changes in clinical practice.

Key words: Guidelines; Adherence; Quality; Quantitative study

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TO THE EDITOR

Recently, Grassini *et al*[1] have shown that the percentage of inappropriate referrals for colonoscopy in an open-access endoscopy system is still high, despite the number of papers published on the issue and the definition of international guidelines. These results illustrate the important issue of physician adherence to guidelines in their daily practice.

In a study carried out in 2005, we showed how gastroenterologists judge and adhere to guidelines, based on who promotes them. Using a multiple-choice questionnaire, we asked a random sample of 100 hospital gastroenterologists to evaluate their knowledge of guidelines and awareness of promoters, and investigated how they became aware of the guidelines. The overall response rate was 71%. The degree to which guidelines were considered reliable was not related to the scientific evidence on which they were based (consensus conferences were considered more reliable compared to clinical practice guidelines 89.5% *vs* 77.6%, $P < 0.01$), but rather was significantly associated with the promoter. Specifically, the French Society of Gastroenterology was considered to be a more reliable promoter than national health agencies and pharmaceutical industries (67.4 *vs* 11.6 and 0.8%, $P < 0.001$).

Gastroenterologists become aware of guidelines mainly through their specialty society (62%), but also through congresses (31%), hospital colleagues (23%) and medical publications (14%). The main resources used for finding guidelines are the websites of specialty societies.

According to these results, peers and, in particular, specialty societies appear to be a more important source of information on guidelines for physicians. When previous studies have shown that the quality of some guidelines developed by specialty societies might be unsatisfactory, the authors have not criticized the specialty societies' influence for supporting guidelines[2,3]. Only the lack of explicit methodological criteria for production of guidelines was confirmed. Grol has recommended targeting each specific type of public for a best integration of the guidelines and an real impact in clinical practices[4]. Since many medical specialists (such as gastroenterologists, internists and primary care physicians) intervene in the field of gastroenterology, the target audience of the guidelines is very diverse and difficult to reach. National health agencies should integrate the specialty societies into the guideline development process to achieve change in clinical practice.

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Meetings

Events Calendar 2008-2009

Global Collaboration for Gastroenterology

For the first time in the history of gastroenterology, an international conference will take place which joins together the forces of four pre-eminent organisations: Gastro 2009, UEGW/WCOG London. The United European Gastroenterology Federation (UEGF) and the World Gastroenterology Organisation (WGO), together with the World Organisation of Digestive Endoscopy (OMED) and the British Society of Gastroenterology (BSG), are jointly organising a landmark meeting in London from November 21-25, 2009. This collaboration will ensure the perfect balance of basic science and clinical practice, will cover all disciplines in gastroenterology (endoscopy, digestive oncology, nutrition, digestive surgery, hepatology, gastroenterology) and ensure a truly global context; all presented in the exciting setting of the city of London. Attendance is expected to reach record heights as participants are provided with a compact "all-in-one" programme merging the best of several GI meetings. Faculty and participants from all corners of the earth will merge to provide a truly global environment conducive to the exchange of ideas and the forming of friendships and collaborations.

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Instructions to authors

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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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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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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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