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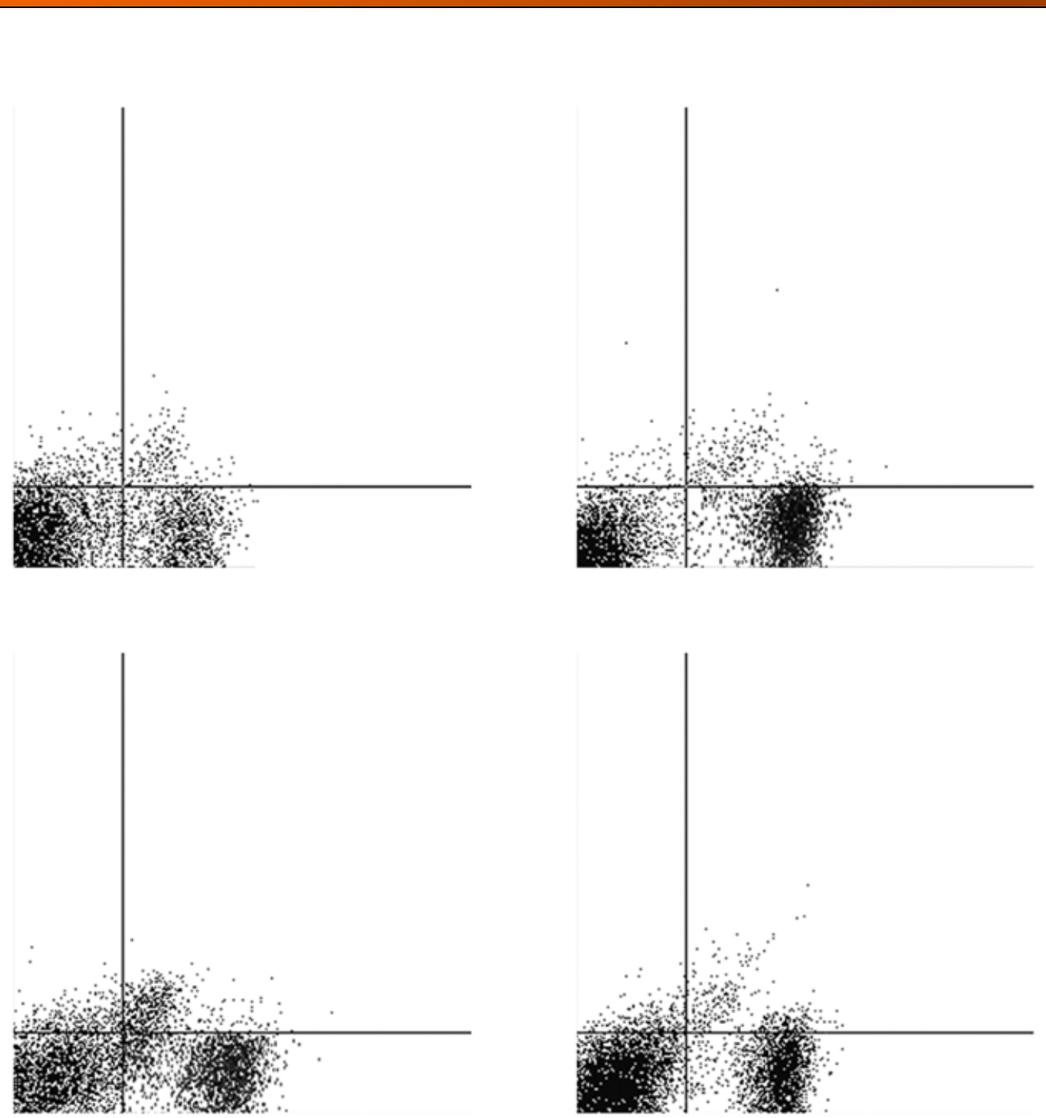

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EDITORIAL

Appendiceal mass: Is interval appendicectomy "something of the past"?

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Abstract

The need for interval appendicectomy (I.A) after successful conservative management of appendiceal mass has recently been questioned. Furthermore, emergency appendicectomy for appendiceal mass is increasingly performed with equal success and safety to that performed in non-mass forming acute appendicitis. There is an increasing volume of evidence -although mostly retrospective- that if traditional conservative management is adopted, there is no need for routine I.A except for a small number of patients who continue to develop recurrent symptoms. On the other hand, the routine adoption of emergency laparoscopic appendicectomy (LA) in patients presenting with appendiceal mass obviates the need for a second admission and an operation for I.A with a considerable complication rate. It also abolishes misdiagnoses and deals promptly with any unexpected ileo-cecal pathology. Moreover, it may prove to be more cost-effective than conservative treatment even without I.A due to a much shorter hospital stay and a shorter period of intravenous antibiotic administration. If emergency LA is to become the standard of care for appendiceal mass, I.A will certainly become 'something' of the past.

INTRODUCTION

Acute appendicitis is the most common surgical emergency which may be complicated by the development of an appendiceal mass in 2%-10% of cases[1]. This mass results from a walled-off appendiceal perforation and represents a wide pathological spectrum ranging from an inflammatory mass that consists of the inflamed appendix, some adjacent viscera and the greater omentum (a phlegmon) to a periappendiceal abscess[2]. Ultrasonography has been advocated as the diagnostic modality of choice, revealing the diagnosis in 70% of cases, however, contrast-enhanced computerized tomography (CT) scanning is far superior[1]. The standard treatment which was introduced by Ochsner in 1901 advocating a conservative regimen (nil by mouth, intravenous antibiotics, bed rest and watchful observation) has proved popular over the years and has been shown to be safe and effective[1]. It allows the acute inflammatory process to subside in more than 80% of cases before interval appendicectomy (I.A) is performed some 8-12 wk later. However, some management issues of appendiceal mass such as the need for I.A after successful conservative treatment, and emergency appendicectomy for a 'hot' appendix

mass have recently surfaced with no general consensus or agreement on the appropriate line of management.

THE CONTROVERSY OVER INTERVAL APPENDICECTOMY

A recent questionnaire study of 67 surgeons in the Mid Trent region of England showed no agreed consensus on the management of appendiceal mass[3]. One of the controversial management issues is the need for I.A after successful conservative treatment. A survey of 663 surgeons in North America revealed that I.A is routinely performed by 86% of the surveyed surgeons[4]. The most cited reason is the risk of recurrent appendicitis which is reported to occur in 21%-37% of cases[4,5]. Another questionnaire survey of 90 consultant general surgeons in England (response rate: 78%) revealed that 53% of surgeons perform I.A routinely some 6-8 wk after resolution of the mass; mainly because of concerns about symptom recurrence[6]. However, the study from Mid Trent region, U.K showed that more than 75% of surveyed surgeons do so[3]. Moreover, the specialist registrars are less likely to offer patients routine I.A after successful conservative management than their consultants ($P < 0.05$)[3] which may reflect a change in the attitude of younger surgeons towards I.A.

The argument of recurrent appendicitis has been questioned as it occurs in less than 20% of cases and the risk becomes minimal after the first 2 years of the initial episode[3,7]. Hence, more than 80% of patients with appendiceal mass can be spared the morbidity of a surgical intervention that has questionable validity. Moreover, a recent large retrospective population-based cohort study of 1012 patients treated initially with conservative therapy showed that only 39 (5%) patients developed recurrent symptoms after a median follow-up of 4 years with male sex having a slight influence on recurrence[4]. Hence, it may be concluded that I.A after initial successful conservative treatment is not justified[4].

THE ARGUMENTS AGAINST I.A

A prospective non-randomized study of 48 I.A specimens, showed 37 (77%) appendices to have a patent lumen, while only 11 (23%) showed fibrosis and obliteration of appendicular lumen and symptom recurrence approaching 40%[5]. This fact has led some authors to advocate routine I.A. However, this means subjecting many patients to unnecessary I.A which necessitates a second admission and is not entirely free of complications; the reported complication rate of I.A is 12%-23%[1,8,9]. It seems that the driving force behind I.A after successful conservative treatment is the fear of symptom recurrence. Many other studies, however, have confirmed a low recurrence which is highest during the first 2 years of the initial inflammation[3,7]. A recent prospective randomized controlled trial (RCT) showed that patients treated conservatively without I.A had the shortest hospital stay and duration of work-days lost, and only 10% of patients developed recurrent appendicitis during a median follow-up period of more than 33.5 mo[10]. This overwhelming evidence from a well conducted RCT and the fact that the histological examination of 30% of the I.A specimens were found to be normal with no evidence of previous inflammation[1] argues strongly against routine I.A after the successful conservative treatment of an appendix mass.

Moreover, 83% of patients presenting with appendix mass did not require any intervention over a mean follow-up of 15.5 mo[11]. Therefore, I.A should not be the rule in every patient presenting with appendiceal mass. Karaca *et al*[12] demonstrated complete disappearance of the mass on repeat ultrasonography and normal appendix on barium enema in 10 out of 11 children with appendiceal mass who were treated conservatively with triple antibiotics for a week. None of these patients developed recurrent appendicitis during the follow-up period of 1-7 years, confirming that conservative treatment is feasible with no need for I.A[12]. However, a week of intravenous triple antibiotics in hospital[12,13] and repeated ultrasonography[12] is certainly not cost-effective. This cost needs to be compared with the cost of emergency laparoscopic appendicectomy (LA) for appendix mass. In term of costs, routine I.A is indeed not cost-effective as it involves another admission and an operation which is not free of complications; it increases the cost per patient by 38% compared with a more selective approach (follow-up and appendectomy only if recurrence occurs)[14].

Furthermore, only very few (20%) patients benefit from prevention of recurrent symptoms if I.A is performed after 6-12 wk and the complication rates for appendicectomy performed before or after recurrence of symptoms were equal at 10%[15].

HIDDEN PATHOLOGY

If I.A is not performed after successful conservative treatment, the fear of missing hidden pathologies such as cecal cancer, Crohn's disease and ileo-cecal tuberculosis masquerading as an appendiceal mass becomes an important issue. In a recent retrospective review of 106 patients, 17 (10.3%) patients had their diagnosis changed during follow-up; 5 patients (3%) were found to have colon cancer[15]. It is therefore essential to perform some follow up investigations to exclude the presence of such hidden pathologies. It is advocated to perform barium enema or colonoscopy after the acute episode has subsided in patients who have been treated conservatively[15], especially if aged more than 40 years[7,12]. However, there is no general consensus as to the right time to perform such an investigation. Timing is important as incompletely resolved appendix mass may mimic cecal carcinoma on barium enema and may give false positive results. A CT scan or CT colonography augmented -when indicated- by colonoscopy is far superior in excluding cecal pathology. It is believed that such investigations can be performed safely 4-6 wk after the acute episode[16].

IS I.A "SOMETHING" OF THE PAST?

Is I.A 'something' of the past? The short answer is no, as

delayed appendicectomy is needed for patients with recurrent symptoms and those with a patent or chronically inflamed appendix[17]. The problem of how to determine the patency of the appendix and chronicity of the inflammation still remains in patients presenting with appendiceal mass who have settled on conservative treatment[16]. This may be done by performing barium enema on all patients treated conservatively and only those with patent appendices may be offered LA. However, this may prove impractical, costly and may increase the workload of any radiology department. Contrast-enhanced CT scanning is another modality that may help in this regard as it may strongly suggest the presence of underlying neoplasm in the majority of patients with secondary appendicitis[18].

EMERGENCY SURGERY FOR APPENDIX MASS IN THE LAPAROSCOPIC ERA

Fear of the increased risk of intraabdominal abscesses[19] after performing LA in complicated appendicitis has recently been dismissed[20]. The successful adoption of laparoscopic I.A after successful conservative treatment is reported without perioperative morbidity[21,22] and the percentage of I.As which are performed laparoscopically has increased in recent years from 30% to 85%[22]. The operating time and complication rates did not differ from those of open I.A, but the hospital stay was much shorter in favor of the interval laparoscopic method[20,22-25].

Is there a role for LA in the emergency intervention for appendiceal mass? The answer is yes. Senapati *et al*[21] reported experience with emergency LA in patients with appendiceal mass in comparison with LA for non-mass-forming appendicitis. It was found that early emergency LA for appendiceal mass is feasible and safe; moreover, its operative time and hospital stay are comparable to those of LA performed for non-mass forming appendicitis[]. However, the proper timing for emergency surgery needs further substantiation.

Another major advantage of emergency surgery is that it obviates the need for a second hospital admission, avoids misdiagnoses and promptly deals with any unexpected ileocecal pathology that masquerades as an appendiceal mass. Furthermore, LA can be offered safely and successfully in the interval setting after successful conservative treatment for those with recurrent symptoms [20,22-25].

THE NEED FOR RCTS

The majority of -if not all- studies on I.A after conservative treatment of appendiceal mass are retrospective. The need for prospective randomized controlled multi-institutional trials is essential to scientifically compare emergency surgery for appendiceal mass with conservative management without I.A[26]. Such trials are needed to establish the safety of emergency open *vs* laparoscopic appendicectomy for appendix mass and to establish the safety of omitting I.A in those treated conservatively with successful outcomes. Such studies should look into various cost issues and the possible differences -if any- in the management of appendiceal masses in various age groups (pediatric *vs* adults) and different sexes (males *vs* females)[26]. The question of "golden hours" for emergency LA for 'hot' appendix masses -similar to that identified for emergency laparoscopic cholecystectomy for acute cholecystitis- needs to be answered. The possibility of increased infertility in females with appendiceal masses treated conservatively should also be studied to determine if emergency surgery is more beneficial in affected females in order to make a stronger argument for emergency management, at least, in females.

CONCLUSION

Based on the above, it seems that I.A can be safely omitted after exclusion of other ilo-cecal pathologies. This avoids a second hospital admission and a surgical procedure which is associated with a 10%-20% complication rate. I.A will still be reserved for patients with recurrent symptoms and can be performed safely by laparoscopic means. Emergency laparoscopic appendicectomy is emerging as a new safe treatment modality for the appendiceal mass, and may prove to be more cost-effective than conservative treatment even without I.A as it is associated with a much shorter hospital stay and obviates the need for long intravenous antibiotic therapy. It further obviates the need for I.A; the centre of controversy. If emergency LA becomes the standard of care, I.A will certainly become 'something' of the past.

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EDITORIAL

Effect of ageing on colonic mucosal regeneration

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Abstract

The physiologic and pathologic cellular and molecular changes occurring with age in the human colon affect both the inflammatory process leading to mucosal injury and the regenerative capacity of the epithelium. On the one hand, age-related telomere shortening and inflamm-ageing may lead to the development of colonic inflammation, which results in epithelial damage. On the other hand, the altered migration and function of regenerative stem cells, the age-related methylation of mucosal healing-associated genes, together with the alterations of growth factor signaling with age, may be involved in delayed mucosal regeneration. The connections of these alterations to the process of ageing are not fully known. The understanding and custom-tailored modification of these mechanisms are of great clinical importance with regard to disease prevention and modern therapeutic strategies. Here, we aim to summarize the age-related microscopic and molecular changes of the human colon, as well as their role in altered mucosal healing.

Key words: Colon; Mucosal repair; Ageing; Colorectal cancer; Telomere shortening; DNA methylation

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INTRODUCTION

With the possible exception of stem cells and tumor cells, ageing is a nearly universal process that, through functional decline, leads to cell death and, eventually, death of the organism. The age-related molecular changes taking place in the human colon have not been exactly determined; moreover, the results of animal tests and human studies are sometimes contradictory.

Researchers have identified a variety of changes in colonic and rectal function associated with ageing. The total number of colonic myenteric neurons decreases with age in rats and in children, particularly during the first 4 years of life[1,2]. While noting an increase in the surface area of myenteric ganglia with age, Hanani *et al*[3] found that the proportion of ganglia with cavities and other structural abnormalities increases with age. Furthermore, a positive association between age and collagen content within myenteric ganglia has also been identified[2]. These changes in colonic innervation may have an impact on colonic motility[4-9].

As far as colonic epithelium is concerned, its renewal takes 4-5 d in humans[10]. The regulated balance of epithelial proliferation and apoptosis allows normal epithelial regeneration. Any deviation in epithelial cell kinetics may result in a loss of not only structural but also functional integrity. The imbalance of colonic epithelial renewal may lead to either ulcer or carcinoma development of the colonic mucosa.

The effect of ageing on colonic epithelial regeneration is not fully understood. In rat colon, crypt epithelial proliferation and apoptosis were found to be the most active in the 3rd week of life[11], which was thought to

be in connection with the development of the gastrointestinal tract. The results of Xiao *et al*[12] are, however, contradictory; the number of proliferative epithelial cells was higher, while the rate of apoptosis was lower in older rats. The epithelial expression of the anti-apoptotic Bcl protein and of the pro-apoptotic Bak protein was also in accord with ageing: the former was high, while the latter was low in older rats. This phenomenon may explain the survival of genetically defective cells, hence the increasing incidence of colorectal cancer in the elderly. The age-related rise in cell proliferation is thought to be in part the result of enhanced transition from G1 to S phase as well as stimulated progression through the S phase of the cell cycle[13,14]. Inconsistencies in results may be due to different sampling locations and different ages of the animals, together with the effects of errors in sample proceedings, immunohistochemical methods and data evaluation[15].

Inflammatory bowel diseases (IBD), including ulcerative colitis (UC) and Crohn's disease, are one of the major causes of epithelial destruction in the colon. It was recently demonstrated that the perceived differences seen in clinical practice between adults and children with UC are largely due to a decrease in histologic features of colitis in children less than 10 years of age[16]. As children approach adulthood, the degree of inflammation and microscopic architectural distortion seen becomes similar to that found in adults. Interestingly, in rectal biopsies of UC, no differences were found amongst all age groups.

Although the understanding of age-related physiological and pathological processes is of great clinical importance, data about the effect of ageing on the induction of mucosal inflammation and the age-related alterations of mucosal healing are scarce in scientific literature.

THE EFFECT OF AGEING ON INFLAMMATION INDUCTION

Telomere shortening and telomerase activity

Telomeres protect the ends of the chromosomes from end-to-end fusions, degradation and recombination. Telomere length decreases with age in most human tissues, including colon[17], and it has been hypothesized that short telomeres might partially explain the connection between cancer and ageing[18]. Telomeres shorten approximately 100 base pairs in each cell division because of their incomplete replication[19] and also as a consequence of oxidative damage[20]. When telomeres become critically short, and in the absence of efficient DNA repair mechanisms, anaphase bridges are formed during mitosis as a result of end-to-end chromosome fusions, which initiates breakage-fusion-bridge cycles. These cycles facilitate the accumulation of genetic changes and chromosomal instability; hence cells need to acquire mechanisms of telomere maintenance, which usually consist of telomerase reactivation. Telomerase prevents further accumulation of chromosomal instability and confers unlimited replicative potential to the cell[21]. It has been previously demonstrated that in UC, colorectal cancer progression is associated with shorter colonocyte telomeres, chromosomal instability and anaphase bridges[22]. It has also been observed that age-related telomere shortening is accelerated in UC[23]. It seems that there is a minimal viable length of colonocyte telomeres, consistent with data from both human cell lines[24] and a telomerase-deficient mouse model[25]. After reaching this critical length, colonocytes defective for DNA damage checkpoints can continue to proliferate but with increased chromosomal instability. Together with alterations in p53 and p16, which are frequent in the non-neoplastic epithelium of UC[10,26,27], this could be a common pathway of tumor progression in this chronic inflammatory disease.

Interestingly, decreased telomerase activity was observed not just in the non-neoplastic epithelium of severely active UC, but also in the non-affected normal mucosa[28,29]. This result suggests that telomerase deficiency may contribute to the pathogenesis of the disease. Moreover, the elevated epithelial telomerase expression found in mildly active UC[30] may help survival and immortalization of the genetically defective epithelial cells in long-standing, chronic inflammation, and thus create the basis for subsequent pathological cell proliferation.

Alterations of immune response

Ageing is associated with a progressive dysregulation of immune response. During ageing, adaptive immunity significantly declines, a phenomenon called immunosenescence, whereas innate immunity seems to be activated, which induces a characteristic pro-inflammatory profile. The latter is called inflamm-ageing[31,32].

Recently, a new subset of CD4+ T cells has been identified. The Th17 cells are distinct from the Th1 and Th2 cells, and secrete interleukin 17 (IL-17) and IL-22[33,34]. IL-17 has been shown to be a primary mediator in several autoimmune and inflammatory diseases, including IBD[35]. Ouyang *et al*[36] demonstrated that the induction of Th17 cytokines is significantly elevated in both aged humans and mice. In addition, they found that memory T cells are an important cell type for the induction of IL-17, and that the transfer of CD4+CD45Rbhi cells from aged mice induced more severe colitis in recombination-activating gene 1-deficient mice compared to cells from young mice. Their results suggest that ageing promotes an intrinsic predisposition towards the pathological Th17 immune response, which may explain the second peak (between 50-80 years of age) in the incidence of IBD which occurs in humans.

Ageing also results in alterations in the function of Toll-like receptors (TLRs), which have an important role in the pathogenesis of IBD[37]. Recent studies have begun to elucidate the consequences of ageing on TLR function in human cohorts and add to existing findings established in animal models. In general, these studies show that human TLR function is impaired in the context of ageing, and in addition there is evidence for inappropriate persistence of TLR activation in specific systems[38-41].

It has been shown in a mouse model that ageing and TLR2 deficiency have significant effects on the levels of pro-inflammatory cytokines, such as IL-10 and IFN-γ,

which could potentially provide a microenvironment that favors the development of more severe colitis following epithelial damage[42]. It has also been reported that the level of trefoil factor 3 (TFF3), an important colonic protective and repair factor, decreases over time in mice, and is negatively regulated by TLR2 signaling[42]. Cytokines such as TNF and IL-1β negatively regulate TFF3 expression in an epithelial cell line by activation of NF-κB, which has been demonstrated to negatively regulate the transcription of TFF3[43,44]. These interactions among ageing-associated changes, TLR deficiency and TFF3 regulation may be of particular relevance in understanding the development of chronic intestinal inflammation and mucosal injury in the elderly.

Several TLR polymorphisms are also involved in the course of IBD[45-47], although it seems likely that large-scale population studies will be needed to clarify the role of key TLR polymorphisms in ageing-related alterations of IBD pathogenesis.

THE EFFECT OF AGEING ON COLONIC MUCOSAL HEALING

Stem cells

The luminal border of the colonic wall is lined by an epithelial monolayer which has several functions, such as water and electrolyte absorption, and it is also a barrier against luminal pathogens[48,49]. Due to the high turnover of shedding epithelial cells, their continuous replacement from the local stem cell pool is required even in healthy colon. Stem cells are located at the basal part of crypts; their progeny migrate towards the luminal surface and undergo terminal differentiation to secretory (Paneth, enteroendocrine and goblet cells) and absorptive (epithelial) cells[48-50]. In the case of tissue injury, such as in IBD or graft-versus-host disease (GVHD), the capacity of intestinal stem cells is not sufficient for the perfect tissue repair, hence the homing of bone marrow-derived multipotent cells is also essential for mucosal regeneration[48,51,52]. It is well known that circulating hematopoietic stem cells (HSCs) play an important role not just in hematopoietic homeostasis, but in the regeneration of solid-organ tissue, which has been certified by several studies[53]. Stem cells are long-lived cells; therefore, they can sustain several genetic and epigenetic changes during cellular senescence. There is evidence both for and against stem cell ageing, and publications are not in agreement with regard to quality and quantity alterations of stem cells in the course of ageing[54].

HSCs in older mice have decreased per-cell repopulating activity, self-renewal and homing abilities, myeloid skewing of differentiation, and increased apoptosis related to stress[55]. It was recently reported that the cyclin-dependent kinase inhibitor p16INK4a, the level of which was previously noted to increase in other cell types with age, accumulates and modulates specific age-associated HSC functions[56]. Notably, in the absence of p16INK4a, HSC repopulating defects and apoptosis were mitigated, improving the stress tolerance of cells and the survival of animals in successive transplants, in a stem-cell-autonomous tissue regeneration model. As p16INK4a is involved in colorectal carcinogenesis[57], it may be supposed to have a specific role in the regulation of the behavior of migrating stem cells of the human colon as well.

Stem cells are in close connection with their niche by means of mechanical and/or chemical processes. Based on the results of bone marrow transplantation studies, one can assume that stem cell function and life span depend on the recipient's age, since increased post-transplant autoimmunity has been observed in the case of older recipients[54]. The age-related decrease of regenerative stem cell capacity, however, needs to be further studied.

The balance of cell proliferation and death ensures adequate epithelial regeneration. Although colonic epithelial stem cells can be distinguished from other epithelial cells only by morphology, several cellular markers may help the identification of both normal and cancer stem cells of the intestinal tract[58-60].

The effect of ageing on colonic epithelial regeneration and crypt-base stem cell function has come to the frontline of discovery recently. In a mouse model, a few apoptotic cells were seen around the stem cell position and this frequency did not alter with age. However, the apoptotic index within crypts was nearly twice as high in older mice after low dose gamma-irradiation and the number of surviving crypts decreased significantly faster after increasing the dose of irradiation; moreover, in the post-irradiation period the crypt regeneration was much slower in older animals[61]. It was shown that clonogenic cells are more radiosensitive in old mice, and that the growth of surviving crypts after injury was delayed in old mice even though the number of resident clonogenic cells were higher in older colonic crypts[62].

Methylation

In non-cancerous colonic mucosa, repeated injuries are likely to induce adaptive methylation changes that enable efficient wound healing and act against cancer development. The methylation-variable sites that are located in promoter or noncoding neutral regions have demonstrated gradual methylation changes associated with the ageing or long-term adaptation process[63]. The presence of transitional-CpG sites between the unmethylated promoters and nearby densely methylated retroelements has been proposed, in order to describe the complexity of variable methylation in gene control regions[64,65]. The transitional-CpG sites at the margin of the CpG islands and at the non-island CpG sites around the transcription start sites have been found to be either under- or over-methylated in a tissue-specific manner as well as to be methylated to various degrees in the same tissue type[65]. This result suggests that the methylation-variable sites nearest to the transcription start sites may serve as epigenetic markers for adaptive DNA methylation. The methylation-variable site of a strongly expressed tissue-specific gene can influence the expression of the nearby gene as well as its related genes. Therefore, the methylation-variable sites of the key colon-specific genes are expected to participate in both the dis-

crete mucosal adaptation and the interactive changes of methylation patterns that lead to mucosal alterations. In the stomach, recent evidence suggests that mucosal injury induces adaptive changes in DNA methylation[66]. The ulcer-healing genes, such as trefoil factor 1 and 2, (TFF1, -2), cadherin 1 (CDH1), and peroxisome proliferator-activated receptor gamma (PPARG), were found to be concurrently methylated with other genes depending on the presence or absence of CpG islands in the normal mucosa of healthy individuals, while both the TFF2 and PPARG genes were frequently undermethylated in gastric ulcer patients.

Age-related methylation loci, such as estrogen receptor 1 (ESR1) and myogenic differentiation 1 (MYOD1), have also been highlighted[67-69]. These loci showed age-dependent methylation in normal colon mucosa, and this type of methylation is considered to serve as a functional link between ageing and cancer, possibly by deregulating the growth and differentiation of normal colonic epithelial cells and predisposing them to tumorous transformation. In addition, methylation of ESR1 in colon epithelium occurs more frequently in patients with UC who have neoplasia than in UC patients without neoplasia[70].

Differences in methylation levels of age-related methylation loci between the proximal and distal colon have also been described[71]. This phenomenon may have an impact not just in colorectal carcinogenesis, but in the understanding of the pathogenesis of UC.

Growth factor receptors

Gastrointestinal mucosal cell proliferation is known to be under the regulation of a number of nutritional and hormonal factors. It was reported that an age-related rise in gastric and colonic mucosal cell proliferation is accompanied by a marked rise in expression and activation of several tyrosine kinases, including epithelial growth factor receptor (EGFR)[72-74]. The age-related rise in EGFR activation in the mucosa of the gastrointestinal tract is not fully understood, but some recent data suggest this could be partly the result of loss of EGFR-related peptide (ERRP), a "negative regulator"[75]. It was recently reported that in Fischer-344 rats, ageing is associated with increased activation of EGFR in the colonic mucosa, as evidenced by a 30%-35% increase in the levels of tyrosine phosphorylated EGFR in the proximal and distal colon of aged (20-22 mo old) compared to young (4-6 mo old) rats. In contrast, the levels of ERRP in both regions of the colon of aged rats were decreased by 50%-60%, compared to their younger counterparts[76]. Expression of ERRP was also found to be high in benign human colon, stomach and pancreas, but low in the respective invasive adenocarcinomas[77,78].

Age-related decrease of EGFR signaling in cells of ectodermal origin has additionally been described[79]. This may cause delayed mucosal healing in the case of older individuals.

Hepatocyte-derived growth factor (HGF) is mainly produced by mesenchymal cells and acts on cells of epithelial origin which express the HGF receptor C-met (HGFR). The HGF-HGFR system is important in gut homeostasis, and has a crucial role in gastrointestinal wound healing[30]. Furthermore, this system has morphogenetic effects, and it also regulates the formation of epithelial tubular and gland structures[30,80]. It was demonstrated that the production of HGF by fibroblasts increased sharply after about 70% completion of their lifespan in culture, which is regulated at the transcriptional level[80]. The expression of HGFR may decrease with ageing as well[81], which may have consequences on tissue repair.

Revealing age-related alterations in the expression of growth factor receptors involved in colonic mucosal repair, and the better understanding of alterations in receptor signaling, may result in new therapeutic targets of colonic mucosal damage.

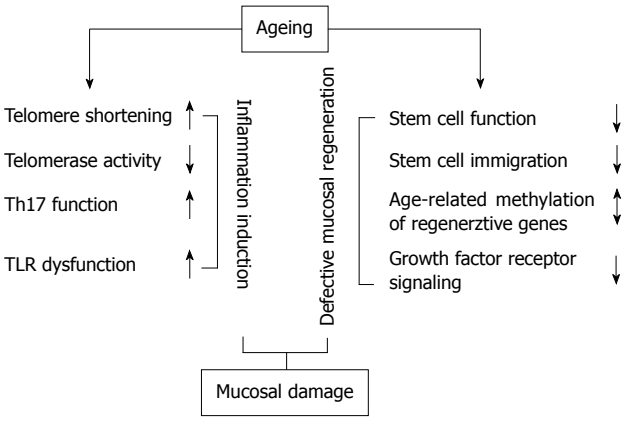

Figure 1 The effect of ageing on factors resulting in colonic mucosal damage.

CONCLUSION

There are numerous signs of ageing in the human gastrointestinal tract, including the colon (Figure 1). Beyond macro- and microscopic alterations, some of these can be detected at the genetic, gene expression and/or epigenetic level. The connection between ageing and colonic mucosal regeneration has been reported by several studies, and their results may provide an insight into physiologic and pathologic mucosal healing in the context of ageing. An understanding of the influence of these age-related mechanisms may help to develop new therapeutic strategies for chronic inflammatory bowel diseases accompanied by mucosal damage.

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REVIEW

Squamous cell carcinoma of the anus-an opportunistic cancer in HIV-positive male homosexuals

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Abstract

Squamous cell carcinoma of the anus (SCCA) is a common cancer in the human immunodeficiency virus (HIV)-infected population, and its incidence continues to increase in male homosexuals. Combined chemoradiation with mitomycin C and 5-fluorouracil was poorly tolerated by severely immunocompromised patients in the early 1990s. In the era of highly active antiretroviral therapy (HAART), however, recent data indicate that: (1) most HIV patients with anal cancer can tolerate standard chemotherapy regimens; and (2) this approach is associated with survival rates similar to those of HIV-negative patients. However, HIV-positive patients with SCCA are much younger, more likely to develop local tumor recurrence, and ultimately die from anal cancer than immune competent patients. Taken together, these findings suggest that anal cancer is an often fatal neoplasia in middle-aged HIV-positive male homosexuals. In this population, SCCA is an opportunistic disease resulting in patients with suboptimal immune function from persistent infection and prolonged exposition to oncogenic human papillomaviruses (HPVs). Large-scale cancer-prevention strategies (routine anuscopy and anal papanicolaou testing) should be implemented in this population. In addition, definitive eradication of oncogenic HPVs within the anogenital mucosa of high-risk individuals might require a proactive approach with repeated vaccination.

Key words: Anal cancer; Chemoradiation; Highly active antiretroviral therapy; Human immunodeficiency virus; Human papillomaviruse; Outcome

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Gervaz P, Calmy A, Durmishi Y, Allal AS, Morel P. Squamous cell carcinoma of the anus-an opportunistic cancer in HIV-positive male homosexuals. *World J Gastroenterol* 2011; 17(25): 2987-2991 Available from: URL: http://www.wjgnet.com/1007-9327/full/v17/i25/2987.htm DOI: http://dx.doi.org/10.3748/wjg.v17.i25.2987

INTRODUCTION

As human immunodeficiency virus (HIV)-infected individuals continue to benefit from highly active antiretroviral therapy (HAART), their risk of dying from neoplasia, including non-AIDS-defining cancers (NADC) is increased[1]. The incidence of squamous cell carcinoma of the anus (SCCA) is not only higher in the HIV-positive population, but continues to increase in the United States[2] (Figure 1). In Australia, anal cancer is now the third most common cancer in the HIV-infected population[3]. SCCA is a sexually transmitted disease clinically related to infection with oncogenic human papillomaviruses (HPV 16-18)[4,5]. Long before the AIDS epidemics, the pivotal role of immune suppression in anal carcinogenesis was highlighted by the high incidence of these tumors in solid organ transplant

patients, irrespective of sexual practice[6,7]. In a large French HIV cohort study, the risk of anal cancer increased with the time during which the CD4 count was < 200 cells/microL and viral load was > 100 000 copies/mL[8]. Thus, both compromised immune function and HPV infection play a role in the development of anal intra-epithelial neoplasia (AIN), the precursor lesion of invasive SCCA.

On a therapeutic standpoint, SCCA has served as a paradigm for the successful application of chemoradiation to solid tumors[9]. Since 1974, it is admitted that: (1) A majority of anal cancers can be cured with chemoradiation therapy (CRT), using 5-fluorouracil (5-FU) and mitomycin C (MMC); and (2) Surgical excision should be restricted to patients who fail to respond to CRT[10,11]. While treatment protocols have remained virtually unchanged during the past three decades, the patients who benefit from this approach nowadays are very different from those who were treated in the 70 s and 80 s. In the 1990s, CRT was poorly tolerated by HIV-positive patients[12,13]. Today, in the Western world, up to 50% of patients with SCCA are relatively young (40-60 years) male homosexuals under HAART[14]. The aim of this paper is to review the clinical data pertaining to clinical outcome of anal cancer in HIV-positive individuals before and after the introduction of HAART.

MANAGEMENT AND OUTCOME OF SCCA IN HIV-NEGATIVE PATIENTS

Combined chemoradiation with MMC and 5-FU is poorly tolerated by immunocompromised patients, and is associated with considerable toxicity in immune competent patients. Many HIV-negative patients with SCCA require radiotherapy breaks and/or chemotherapy dose reduction. In the Memorial Sloan-Kettering Cancer Center series, > 40% (all HIV negative) of patients needed chemotherapy dose reduction of at least one agent, and 77% had at least one radiotherapy break[15]. Data from four prospective randomized trials in HIV-negative patients[16-19] also indicate: (1) a male: female ratio of 1:2; (2) a median age > 60 years; (3) a local failure rate of 30%; and (4) a 3-year overall survival rate of 70%-75% (Table 1).

A closer analysis of data reveals, however, that HIV-negative individuals with SCCA represent a relatively old population of patients who rarely succumb to anal cancer. In the UKCCCR trial[16], 54% of deaths in the chemoradiation group were due to co-morbid conditions or second malignancies, and thus were not related to SCCA. In the RTOG trial[18], out of 146 HIV-negative patients who were treated with MMC-based chemoradiation, there were 32 deaths, but only 15 (46%) were attributed to anal cancer progression. In the MD Anderson Cancer Center series, out of 167 (161 HIV-negative) patients, there were 42 deaths, and only 21 (50%) were due to anal cancer[20]. In summary, 5-year overall survival of HIV-negative patients with SCCA who undergo MMC-based chemoradiation is close to 70%, but > 50% of deaths are unrelated to anal cancer. In accordance with the initial experience of Norman Nigro reported 30 years ago[21], these data indicate that SCCA in this population has limited metastatic potential and is ultimately responsible for the death of < 20% of patients.

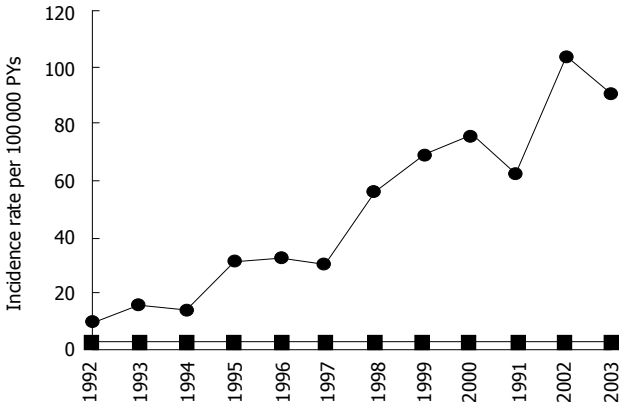

Figure 1 Annual incidence rates of anal cancer among HIV-infected persons (circles) and the general population (squares), USA 1992-2003.

MANAGEMENT AND OUTCOME OF SCCA IN HIV-POSITIVE PATIENTS IN THE HIV ERA (1982-1995)

In the pre-HAART era, HIV-positive individuals demonstrated poor tolerance to MMC-based chemoradiation protocols for anal cancer. Nonetheless, it was recommended that HIV-positive patients with CD4+ > 200/mm^3 should be treated with the standard chemoradiation regimen, whenever possible[22]. In at least seven small series[23-29], clinicians were struck by the fact that HIV-positive and HIV-negative SCCA patients differed by age (40-45 years *vs* 60-65 years), male gender (90%-95% *vs* 35%-40%), and homosexuality. Thus, the experience of treating HIV-positive patients with anal cancer prior to the development of HAART was essentially witnessing the emergence of a high-risk population (Table 2).

Many of these young homosexuals would eventually die of AIDS, with or without evidence of residual anal cancer - but the latter was rarely considered the primary cause of death at a time when median survival with a diagnosis of AIDS was only 17 mo[30]. In the series from Kaiser Permanente Medical Center in Los Angeles[25], after a median follow-up of 38 mo, half of patients were alive and disease-free, while the other half had died from complications of AIDS. Results in terms of local recurrence were disappointing, but many patients did not receive standard chemotherapy for fear of significant hematologic toxicity. Nonetheless, acute toxicity was quite frequent (> 50%), and local tumor recurrence rates were elevated (40%-50%). In addition, Kim *et al*[23] were the first to note that: (1) HIV-positive patients were more likely to die from SCCA than HIV-negative patients, who often succumbed to other, cancer-unrelated causes; and (2) the median time to cancer-related death in HIV-positive individuals was 1.4 years *vs* 5.3 years for HIV-negative patients. Since AIN progresses more quickly towards SCCA in HIV-positive patients, it was logical to hypothesize that

Table 1 Clinical characteristics and outcome of human immunodeficiency virus-negative patients with squamous cell carcinoma of the anus

Author	Trial	Yr	*n*	Male (%)	Age (range)	Local failure (%)	Overall survival
Northover *et al*[16]	UKCCCR	1987-1991	577	45	64 (26-88)	39	65% at 3 yr
Bartelink *et al*[17]	EORTC	1987-1994	103	29	60	29	69% at 3 yr
Flam *et al*[18]	RTOG 87-04	1987-1991	291	30	62 (29-85)	24	
Ajani *et al*[19]	RTOG 98-11	1998-2005	644	31	55 (25-88)	25	84% at 3 yr

Table 2 Clinical characteristics and outcome of human immunodeficiency virus-positive patients with squamous cell carcinoma of the anus before the era of highly active antiretroviral therapy

Author	Yr	*n*	Male (%)	Age (range)	Toxicity 3-4 (%)	Local failure (%)	Overall survival
Kim *et al*[23]	1985-1998	13	92	42	80	61	34% at 5 yr
Holland *et al*[24]	1980-1993	7	100	41	100	43	29% at 2 yr
Peddada *et al*[25]	1987-1995	8	100	48 (37-70)	100	12	50% at 3 yr
Hoffman *et al*[26]	1991-1997	17			64	25	
Cleator *et al*[27]	1989-1999	12	100	43 (30-53)	50	25	60% at 2 yr
Place *et al*[28]	1980-1999	14	100	42 (28-58)	50	57	20% at 5 yr
Efron *et al*[29]	1988-1999	6	100	40 (29-46)		67	

Table 3 Clinical characteristics and outcome of human immunodeficiency virus-positive patients with squamous cell carcinoma of the anus during the era of highly active antiretroviral therapy

Author	Yr	*n*	Male (%)	Age (range)	Local failure (%)	Overall survival
Stadler *et al*[37]	1998-2002	8	100	44 (34-61)	50	67% at 2 yr
Blazy *et al*[38]	1997-2001	9	100	36 (35-49)	11	100% at 2 yr
Bower *et al*[39]	1996-2003	26	100	42 (28-56)	23	47% at 5 yr
Chiao *et al*[40]	1998-2004	175	99.5	49 (43-55)		66% at 4 yr
Wexler *et al*[41]	1997-2005	32	94	45 (31-68)	16	65% at 5 yr
Oehler-Jänne *et al*[42]	1997-2006	40	93	48 (34-75)	62	61% at 5 yr
Abramowitz *et al*[43]	1998-2004	44	100	45	32	85% at 3 yr
Seo *et al*[44]	1999-2007	14	93	45 (34-59)		92% at 3 yr
Barriger *et al*[45]	1995-2008	17	100	44 (29-53)	59	50% at 5 yr
Hogg *et al*[46]	1996-2006	21	100	45	48	73% at 3 yr
Fraunholz *et al*[47]	1997-2008	21	90	45 (31-68)	41	67% at 5 yr

the molecular biology of anal cancer might differ between the two groups[31,32].

MANAGEMENT AND OUTCOME OF SCCA IN HIV-POSITIVE PATIENTS IN THE HAART ERA (1996-)

HAART does neither prevent the development of AIN, nor the progression of AIN towards SCCA[33,34]. The rising incidence of anal cancer in the HIV-positive population during 1996-2004 is well documented[35]. HAART certainly had a positive impact on patients' ability to tolerate chemo-radiation treatment; accordingly, many radiologists strongly caution against scaling back treatment of anal cancer in HIV-positive individuals[36]. This is also motivated by the recent recognition that SCCA is the greatest threat to these patients' lives. We have summarized, in Table 3, the results of eleven studies published since 2004, which evaluated the outcome of HIV-positive patients with SCCA in the HAART era[37-47]. With two exceptions[40,42], these small series are underpowered, and inadequate to detect survival differences between HIV-positive and HIV-negative individuals.

In the Veterans Affairs study[40], the authors concluded that in the HAART era, survival of SCCA is equivalent between HIV-positive and HIV-negative patients (overall 4-year survival 66% *vs* 62%). However, the age distribution of both groups was quite different; among HIV-positive individuals, patients aged 45-49 represented the largest percentage, whereas among HIV-negative individuals the largest percentage of patients was greater than age 75 (Figure 2). In other words, two populations with an age difference greater than 20 years have the same survival, which strongly suggests that SSCA-related mortality was higher in the HIV-positive group. This hypothesis is supported by the multicenter series reported by Oehler-Jänne *et al*[42]: five-year overall survival was similar in both groups (61% *vs* 65%), but HIV-positive individuals had a 4-fold higher risk of locoregional tumor recurrence (62% *vs* 13%), and the majority of them, unlike HIV-negative individuals with SCCA, died of anal cancer. In summary, and in the

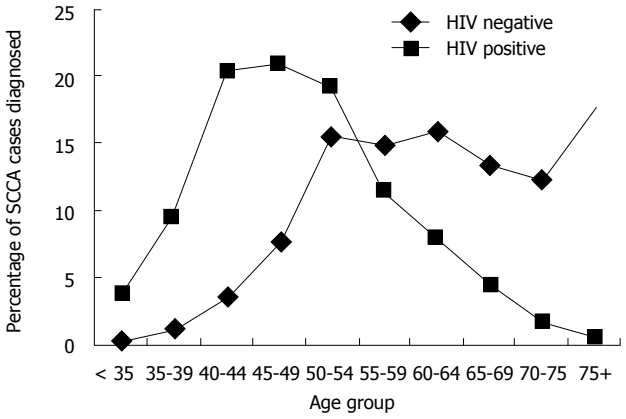

Figure 2 Percentage of anal cancer diagnosed among us veterans by age group (1998-2004). HIV: Human immunodeficiency virus.

HAART era, HIV-individuals with SCCA carry a 50% risk of local relapse and a 33% risk of dying from anal cancer.

CONCLUSION

In some countries, anal cancer is now the third most common cancer in HIV-infected individuals and its incidence continues to increase, despite (or because of) the use of HAART. It is a disease of relatively young male homosexuals, who should be considered candidates for chemoradiation, using standard doses of MMC and 5-FU, as well as pelvic irradiation. There is, however, evidence that HIV-positive patients experience a higher rate of locoregional tumor recurrence and are more likely to die from anal cancer than their HIV-negative counterparts; this explains why both HIV-positive and HIV-negative groups have similar survival, despite a > 20 years difference in age. HIV-positive male homosexuals under HAART are protected from opportunistic infections, but have an increased risk of developing, and eventually succumbing to anal cancer.

SCCA was not a frequent cause of death in HIV-positive patients before 1997-1998, and this affirmation stands true in 2010 for elderly HIV-negative patients. In contrast, for middle-aged male homosexuals under HAART, SCCA is an often fatal, opportunistic cancer which results from the combination of two factors: (1) persistent immune deficiency; and (2) persistent infection with oncogenic HPVs in the anal canal. Cancer-prevention strategies should be implemented in this population: male homosexuals should undergo routine anuscopy and an anal Papanicolaou test to detect and treat precursor lesions of SCCA. This approach, if successful, might hopefully mimic in male homosexuals, the dramatic improvement observed for cancer of the uterine cervix in women. Complete eradication of oncogenic HPVs in the anogenital mucosa might also require a proactive vaccination program for high-risk individuals[48,49]. This approach could also serve an important public health purpose, reducing the pool of susceptible individuals and contributing to the control of re-emerging HPV infection.

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ORIGINAL ARTICLE

Down-regulation of STAT3 expression by vector-based small interfering RNA inhibits pancreatic cancer growth

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Abstract

AIM: To evaluate the effect of RNA interference (RNAi) mediated silence of signal transduction and activation of transcription (STAT)3 on the growth of human pancreatic cancer cells both *in vitro* and *in vivo*.

METHODS: STAT3 specific shRNA was used to silence the expression of STAT3 in pancreatic cancer cell line SW1990. The anti-growth effects of RNAi against STAT3 were studied *in vitro* and in experimental cancer xenografts in nude mice. The potential pathways involved in STAT3 signaling were detected using reverse transcription polymerase chain reaction and western blotting.

RESULTS: The expression of the STAT3 was inhibited using RNAi in SW1990 cells. RNAi against STAT3 inhibited cell proliferation, induced cell apoptosis and significantly reduced the levels of CyclinD1 and Bcl-xL when compared with parental and control vector-transfected cells. *In vivo* experiments showed that RNAi against STAT3 inhibited the tumorigenicity of SW1990 cells and significantly suppressed tumor growth when it was directly injected into tumors.

CONCLUSION: STAT3 signaling pathway plays an important role in the progression of pancreatic cancer, and silence of *STAT3* gene using RNAi technique may be a novel therapeutic option for treatment of pancreatic cancer.

Key words: Signal transduction and activation of transcription 3; RNA interference; Pancreatic cancer; Growth

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INTRODUCTION

Pancreatic cancer is one of the most lethal solid malignancies and its overall 5-year survival is less than 5%. It represents one of the leading causes of cancer deaths in industrialized countries despite advances in medical and surgical modalities[1,2]. Up till now, surgical resection still remains the only treatment for pancreatic cancer[3,4]. However, because of the aggressiveness of this disease, most patients

have had local or metastatic spread by the time of diagnosis, and surgical resection is possible in only a few patients. Even among patients undergoing a potentially curative resection, the long-term prognosis remains poor due to early recurrence and metastasis[5]. Unfortunately, effective systemic therapy capable of reversing its aggressiveness is unavailable and the specific molecular regulatory pathways involved in pancreatic cancer initiation and progression have not been fully identified[6,7]. Targeting the currently known signaling pathways, however, may lead to effective treatment for pancreatic cancer.

STAT3, a member of the signal transduction and activation of transcription (STAT) family, is a key cytoplasmic transcription factor activated by tyrosine kinase growth and cytokine receptors. Once tyrosine is phosphorylated, two STAT3 monomers form a dimer through reciprocal phosphotyrosine-SH2 interactions, and translocate to the nucleus where they bind to STAT3-specific DNA-response elements of target genes, and induce gene transcription[8,9]. Elevated activity of STAT3 has been found frequently in a wide variety of human tumors including pancreatic cancer[10-13] and STAT3 participates in the occurrence and development of cancers by promoting cell proliferation, inhibiting cell apoptosis, inducing immune escape, and promoting angiogenesis and metastasis[14,15].

STAT3 signaling pathway may represent a new molecular target for novel therapeutic approaches for human cancers. Several reports showed that blocking of STAT3 expression in human cancer cells suppresses proliferation *in vitro* and tumorigenicity *in vivo*. Antisense oligonucleotides and decoy oligonucleotides[16,17], tyrosine kinase inhibitors[18,19], dominant negative STAT3 protein[20], drug-like non-peptide small molecules[21], and RNA interference (RNAi)[22,23] can target STAT3 signaling pathways. Among them, RNAi is the most popular one.

RNAi is a phenomenon of gene silencing, resulting from specific degradation of homologous mRNA mediated by small interfering RNA (siRNA) produced through degradation of double-stranded RNA (dsRNA)[24,25]. Gene silencing involving RNAi requires the processing of long double-stranded RNA (dsRNA) into 19- to 21-nt RNAs, which is called small interfering RNA (siRNA). This process is mediated by Dicer, a type of endonuclease. Subsequently, the siRNA molecules are incorporated into the RNA-induced silencing complex (RISC). The active complexes recognize and cleave the homologous mRNAs, thus selectively inhibiting the expression of the target gene[26,27]. Currently, a prompt and highly-effective method has been developed in RNAi technique to inhibit the expression of specific genes, and has been widely applied in the research of viral diseases, genetic diseases and malignant tumors[28].

The present study was designed to evaluate the use of RNAi to knockdown STAT3 expression and activation, and their effects on human pancreatic cancer cell growth both *in vitro* and *in vivo*. The phenotypic growth changes resulting from the reduction of STAT3 expression were observed both *in vitro* and *in vivo*. We found that knockdown of STAT3 gene by RNAi significantly suppressed the expression of CyclinD1 and Bcl-xL, both of which were accompanied with marked inhibition of tumor cell growth *in vitro* and *in vivo*. Our results demonstrate that STAT3 signaling pathway plays an important role in the progression of pancreatic cancer and that knockdown of STAT3 gene using RNAi technique may be a novel therapeutic option for treatment of pancreatic cancer.

MATERIALS AND METHODS

Cell lines and culture conditions

Human pancreatic cancer cell line SW1990 and PANC-1 were purchased from American Type Culture Collection (Manassas, USA). They were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, 100 units/ml penicillin, and 100 μg/mL streptomycin in a humidified incubator with an atmosphere of 5% CO_2 and 95% air at 37℃.

In our previous studies, three coding regions corresponding to nucleotides 1819-1837, 1025-1043 and 237-255 of STAT3 sequence in the GenBank (NM003150) were selected to form siRNA target sequences. Three primer pairs were synthesized: one pair encoding nucleotides sites 1819-1837 (CTGCTAAGATTCAGTGAAA) followed by a 9 base "loop" (TTCAAGAGA) and the inverted repeat (STAT3-siRNA-1), the second one encoding nucleotides sites 1025-1043 (GCGTCCAGTTCACTACTAA) also followed by the loop and the inverted repeat (STAT3-siRNA-2), and the third one encoding nucleotides 237-255 (TCAGCACAATCTACGAAGA) again followed by the loop and the inverted repeat (STAT3-siRNA-3). We then constructed three STAT3 specific shRNA expression vectors (pRNAT-STAT3-siRNA-Ⅰ, Ⅱ, Ⅲ) and found that pRNAT-STAT3 siRNA-Ⅱ had the most obvious gene silencing effect. We also constructed scrambled siRNA expression vector as a negative control (pRNAT-Con). We established stable SW1990 pRNAT-Con transfectants (SW1990-Con) and SW1990 STAT3-RNAi transfectants (SW1990-RNAi) and found that stable transfection of pRNAT-STAT3-siRNA-Ⅱ vector silenced STAT3 expression. The stably transfected cells were used for subsequent studies[29].

Immunohistochemical detection of STAT3 in pancreatic tissues

Primary pancreatic tumors were found in 71 patients suffering from pancreatic cancer. Informed consent was obtained for the use of tissues in this study from all the patients, who underwent surgical treatment at Affiliated First People's Hospital of Shanghai Jiao Tong University. Ten normal pancreatic tissues were collected through regular multi-organ donor procedures. Paraffin wax samples from the 71 cases of primary pancreatic tumors and 10 with normal pancreatic tissues were cut into 4-μm-thick slices. These slices were dewaxed and the endogenous peroxidase activity was quenched after incubation in methanol con-

taining 3% hydrogen peroxide for 10 min. The histologic sections were incubated with a rabbit anti-human STAT3 polyclonal antibody (Cell Signal, USA) or rabbit polyclonal IgG controls (Vector Laboratories, USA) in blocking buffer overnight at 4℃. The sections were then rinsed in PBS (containing 0.5% bovine serum albumin and 0.1% Tween-20) and incubated for 30 min with biotinylated goat anti-rabbit IgG (ABC staining kit, Santa Cruz, USA) diluted according to the manufacturer's protocol. Next, a solution of avidin-conjugated horseradish peroxidase (ABC staining kit) was applied for 30 min, according to the manufacturer's instructions. Peroxidase activity was developed in 0.5% (vol/vol) 3,3'-diaminobenzidine hydrochloride (DAB, Sigma, USA) in PBS containing 0.03% (vol/vol) hydrogen peroxide for 2 min. Sections were counterstained with Harris' hematoxylin and mounted in gelatin (Sigma, USA). The criteria for immunohistochemical assay are as follows: positive cells contained brown particle staining in the nucleus or cytoplasm. Samples with < 5% positive cells were designated as negative (-); samples stained slightly (5%-25% positive) were designated as (+); samples stained moderately (25%-50% positive) as (++), and stained deeply (> 50 % positive) as (+++).

Cell proliferation assay and anchorage-independent growth assay

To quantify cell proliferation, SW1990 cells and stably transfected cells (SW1990-Con, SW1990-RNAi) were seeded in a 96-well plate at a concentration of 5×10^3 /well (100 μL/well). Eight parallel wells were assigned to each group. Then 20 μL/well of 5 mg/mL MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) was added at 24 h, 48 h and 72 h after seeding and the cells were nurtured for another 4 h. The supernatant was removed and the product converted from MTT was dissolved by adding 150 μL/well dimethyl sulfoxide (DMSO) and shaking for 10 min. Optical density (OD) readings were obtained at 490 nm. The cell growth rate was represented by the relative ratio of OD490 at 24, 48 and 72 h, to OD490 at 0 h, respectively. The growth curve was drawn according to the cell growth rate. A soft agar colony formation assay was used to assess the anchorage-independent growth ability of cells. Specifically, SW1990, SW1990-Con and SW1990-RNAi cells were plated on a 0.6% agarose base in six-well plates (1.0×10^3 per well) in 1 mL DMEM medium containing 10% fetal bovine serum and 0.3% agarose. Colonies > 100 μm were counted 14 d after plating.

Cell apoptosis assay

Cell apoptosis was assessed by apoptosis kit (Roche, USA), according to the manufacturer's protocol. Briefly, SW1990 cells, and stably transfected cells SW1990-Con and SW1990-RNAi were collected to make single cell suspensions (5×10^6 cells). And 20 μL fluorescence-tagged Annexin-V and 20 μL pyridine iodinate (PI) were added into 1 mL incubation buffer to prepare the marking liquor. Cells were washed once by PBS and centrifuged at 500 × *g* at 4℃ for 5 min. The supernatant was discarded. The cell deposition was resuspended with 100 μL marking liquor and placed in dark at normal temperature for 10-15 min. Flow cytometric analysis showed that Annexin-V^+/PI cells were early apoptotic cells, while Annexin-V^+/PI^+ cells were late apoptotic and dead cells.

Cell cycle assay

SW1990 cells and stably transfected cells, SW1990-Con and SW1990-RNAi , were collected and fixed. After incubation in RNase A for 30 min at 37℃, the cells were stained with PI. Flow cytometric analysis was done using a FACScan instrument (Becton Dickinson, Mountain view, CA) and CellQuest software

Animals

Male athymic BALB/c nude mice were obtained from the Animal Center of Chinese Academy of Sciences (Shanghai, China) and housed in laminar flow cabinets under specific pathogen-free conditions. The mice were used when they were 6-8 wk old. The use of animals in this study complies with the Guide for the Care and Use of Laboratory Animals (NIH publication No. 86-23, revised 1985) and the current Chinese regulations and standards on the use of laboratory animals.

In vivo tumorigenicity assay

Male athymic BALB/c nude mice (6-8 wk old) were housed in laminar flow cabinets under specific pathogen-free conditions. SW1990, SW1990-Con and SW1990-RNAi cells were injected into the right flank of mice with a total volume of 100 μL (1.0×10^7 cells). The tumor-bearing mice were sacrificed 35 d after inoculation and the tumors were taken and weighed.

Gene therapy studies

SW1990 cells were injected into the right flank of BALB/c nude mice with a total volume of 100 μL (1.0×10^7 cells). Tumors were allowed to grow *in vivo* for 2 weeks, reaching an average size of 5 mm in diameter. The animals were divided randomly into three groups (six mice per group): (1) PBS buffer alone (mock), (2) pRNAT-Con (20 μg/mouse), and (3) pRNAT-STAT3-siRNA-Ⅱ (20 μg/mouse). The samples were diluted in 50 μL PBS buffer and injected percutaneously into the tumor using a syringe with a 27-gauge needle. Immediately after injection, tumors were pulsed with an electroporation generator. This process was repeated on day 21. Tumor sizes were measured every 5 d. Tumor masses (in cubic millimeter) were calculated as $a \times b^2 \times 0.52$ (a represents the length, b represents the width)[30]. The tumor-bearing mice were sacrificed on day 35, and the tumors treated with either pRNAT-Con or pRNAT-STAT3-siRNA-Ⅱ were taken, weighed and sectioned for STAT3 immunostaining with rabbit anti-human STAT3 polyclonal antibody (Cell Signal, USA) as before.

Reverse transcription polymerase chain reaction (RT-PCR)

Total RNA extraction from tumor cells was performed with Trizol Reagent (Life Technologies, USA). Two μg of total RNA was reverse-transcribed with the First Strand

cDNA Synthesis Kit (Promega, USA) to synthesize cDNA samples. Subsequently, 2 μL cDNA product was subjected to PCR amplification with Taq DNA polymerase (Sangon, China) on a thermal cycler using the following primers. The oligo-nucleotide primers for STAT3 were constructed using a software "Primer Premier 5.0". The oligo-nucleotide primers for Bcl-xL, Cyclin D1 and β-actin were constructed based on the published sequence. The PCR primers used to detect each factor were as follows: Bcl-xL, sense strand 5'-CCCAGAAAGGATACAGCTGG-3', antisense strand 5'- GCGATCCGACTC ACCAATAC-3', with a product length of 448 bp[31]; Cyclin D1, sense strand 5'- GAGACCATCCCCCTGACGGC-3', antisense strand 5'-TCTTCCTCCTCCTCGGCGGC-3', with a product length of 485 bp[31]; β-actin, sense strand 5'-ATCTGGCACCACACCTTCTACAATGAGCTGCG-3', antisense strand 5'-CGTCATACTCCTGCTTGCTGATCCACATCTGC-3', with a product length of 838 bp[32]. The PCR conditions were: one cycle of denaturing at 94℃ for 5 min, followed by 30 cycles of 94℃ for 1 min, 60℃ for 1 min and 72℃ for 1 min, before a final extension at 72℃ for 10 min. The PCR products were loaded onto 2% agarose gels and visualized with ethidium bromide under UV light. This experiment was performed three times and a representative data was shown.

Western blotting

Whole-cell protein extracts and nuclear protein extracts from tumor cells were prepared with RIPA Lysis Buffer (Santa Cruz, USA) and Nuclear Extract Kit (Active Motif, USA), according to the manufacturer's instructions, respectively. Protein concentrations were determined using a Bio-Rad assay kit (Bio-Rad, USA). Lysates containing 100 μg protein were mixed with loading-buffer with 5% β-mercaptoethanol, and heated for 5 min at 100℃. Samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto nitrocellulose membranes by semi-dry blotting. Membranes were incubated in blocking buffer (1 × TBS, 0.1% Tween 20, and 5% non-fat dry milk) for 1 h at room temperature, followed by hybridization with anti-p-STAT3 [tyr-705] antibody (Cell signal, USA, 1:1000 dilution), anti-STAT3 antibody (Cell signal, USA, 1:1000 dilution), anti-Bcl-xL antibody (Cell signal, USA, 1:1000 dilution), anti-CyclinD1 antibody (Cell signal, USA, 1:000 dilution) or anti-β-actin antibody (Labvision, USA, 1:100 dilution) at 4℃ overnight. After three washes in TBS/0.1% Tween 20, the membranes were hybridizated with a horseradish peroxidase-conjugated secondary antibody rabbit IgG (Santa Cruz, USA, 1:5000 dilution) for 1 h at room temperature. After three washes in TBS/0.1% Tween 20, signals were detected by chemiluminescence using the Western blotting Luminol Reagent (Santa Cruz, USA). The same experiment was performed three times and a representative data was shown.

Table 1 Immunochemical analyses of signal transduction and activation of transcription 3 and p-signal transduction and activation of transcription 3 expression in normal pancreatic specimens and pancreatic tumor specimens

Specimens		*n*	-	+	++	+++
STAT3	Normal specimens	10	9	1	0	0
	Cancer specimens	71	19	14	23	15
p-STAT3	Normal specimens	10	10	0	0	0
	Cancer specimens	71	21	11	26	13

STAT3: Signal transduction and activation of transcription 3.

RESULTS

STAT3 and p-STAT3 are overexpressed in pancreatic cancer cell lines and pancreatic cancer tissues

To determine whether STAT3 and p-STAT3 are overexpressed in pancreatic cancer tissues, we compared the level of STAT3 and p-STAT3 expression in normal pancreatic tissues with that in the pancreatic cancer tissue and pancreatic cancer cell lines (PANC-1 and SW1990) using immunohistochemical and Western blot analyses with an anti-STAT3 antibody and anti-p-STAT3 antibody. Both approaches showed that STAT3 and p-STAT3 were overexpressed in cancer tissues and pancreatic cancer cell lines (Figure 1). STAT3 and p-STAT3 protein levels were measured by Western blotting. Quantitative evaluation of the relative expression of STAT3 revealed that this protein was overexpressed by an average of 2.8-fold in the 71 primary pancreatic tumors compared with normal pancreatic tissues. As summarized in Table 1, the STAT3 levels were significantly different ($P < 0.05$) between the pancreatic tumor specimens and normal pancreatic specimens. Immunohistochemical analyses also showed that pancreatic cancer specimens had a high density staining for p-STAT3.

RNAi targeting STAT3 inhibits SW1990 cell proliferation and anchorage-independent growth ability

To determine whether inhibition of STAT3 affects cell proliferation and metabolic activity of parental SW1990 cells, SW1990-Con cells and SW1990-RNAi cells were determined at 24, 48 and 72 h by the MTT assay. The cell proliferation was reduced significantly after treatment with pRNAT-STAT3-siRNA-Ⅱ ($P < 0.05$) as compared with that of parental SW1990 or SW1990-Con cells (Figure 2A). Furthermore, pRNAT-STAT3-siRNA-II reduced SW1990 cell colony formation by 72.6% ($P < 0.05$, Figure 2B).

RNAi targeting STAT3 arrests SW1990 cells at G_0/G_1 phase and increases SW1990 cells apoptosis

To analyze the mechanisms by which pRNAT-STAT3-siRNA-Ⅱ inhibits cell proliferation, the cell cycle and cell apoptosis of SW1990 cells as well as stably transfected cells SW1990-Con and SW1990-RNAi, were analyzed by flow cytometry. As shown in Table 2, the percentage of cells at G_0/G_1 phase was increased from 38.76% (parental

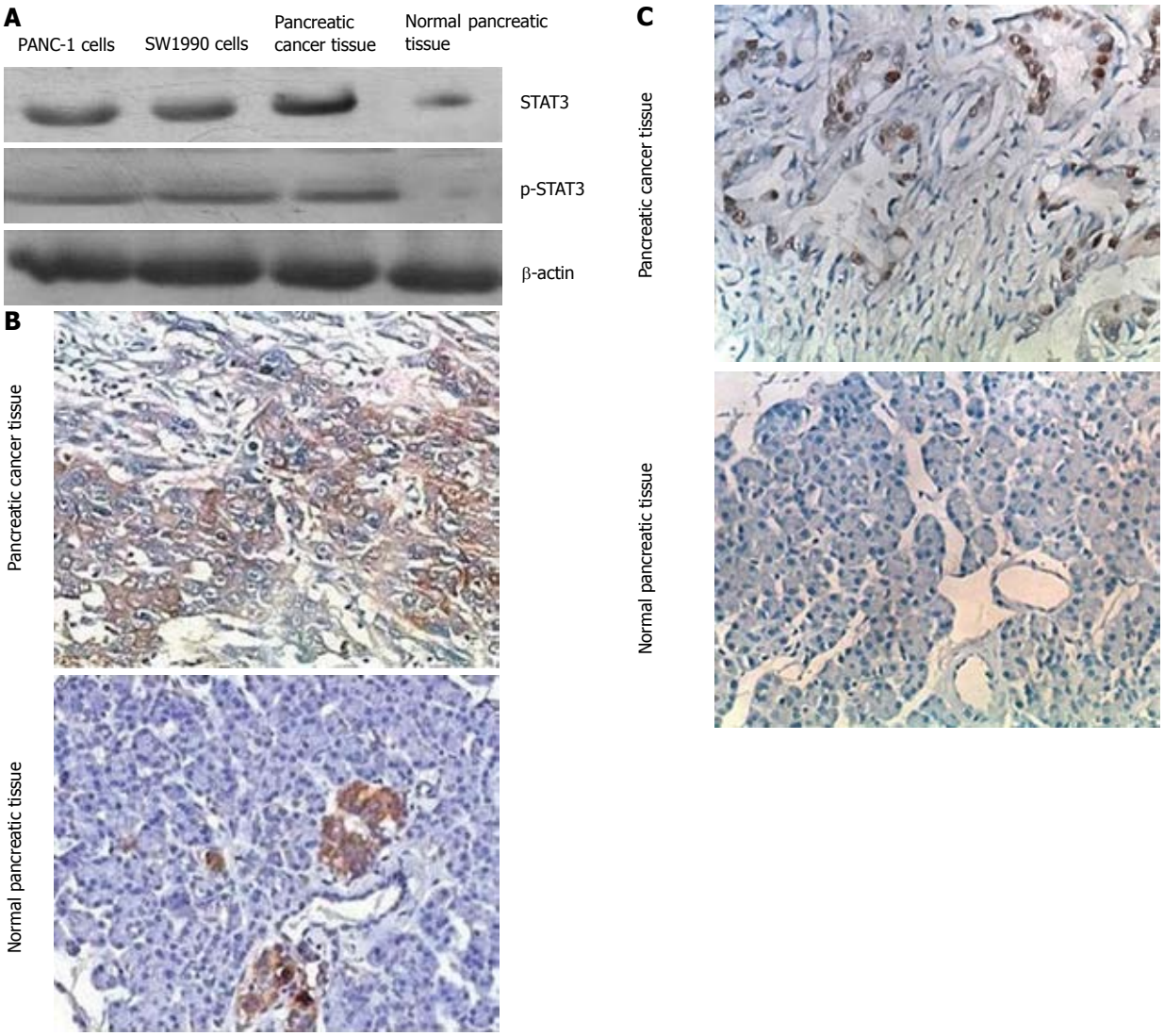

Figure 1 Signal transduction and activation of transcription 3 expression in human pancreatic cancer cells. A: Western blotting analysis of STAT3 and p-STAT3 expression in PANC-1, SW1990, pancreatic cancer, and normal pancreatic tissue with 100 μg total protein for each sample; B: Immunohistochemical analysis of STAT3 expression: pancreatic cancer tissue shows a high-density staining for STAT3 while normal pancreatic tissues show a low-density staining; C: Immunohistochemical analysis of p-STAT3 expression: pancreatic cancer tissue shows a high-density staining for p-STAT3 while normal pancreatic tissues does not show any staining. STAT3: Signal transduction and activation of transcription 3.

Table 2 Effects of silence of signal transduction and activation of transcription 3 gene on cell cycle of pancreatic cancer cells (mean ± SD, %)

Group	G_0/G_1	S	G_2/M
SW1990	38.76 ± 4.64	29.47 ± 3.52	31.76 ± 4.05
SW1990-Con	40.12 ± 5.12	26.53 ± 3.15	33.34 ± 4.39
SW1990-RNAi	65.39 ± 5.83[a]	9.88 ± 2.98[a]	24.73 ± 2.97

[a]$P < 0.05$ *vs* control. SW1990-Con: SW1990 pRNAT-Con transfectants; SW1990-RNAi: SW1990 STAT3-RNAi transfectants.

SW1990) to 65.39% (SW1990-RNAi) and the S-phase cells were decreased from 29.47% (parental SW1990) to 9.88% (SW1990-RNAi). Figure 3 also indicates that the difference was not statistically significant in the rates of the early apoptotic cells and the late apoptotic cells between SW1990 cells and SW1990-Con cells, while these rates in SW1990-RNAi cells increased significantly ($P < 0.05$) as compared with parental SW1990 or SW1990-Con cells. These data showed that silencing of STAT3 can arrest cells at G_0/G_1 phase and increase cell apoptosis.

RNAi targeting STAT3 inhibits tumorigenicity in vivo

The tumorigenicity of SW1990 cells was examined after silencing of STAT3 *in vivo*. All mice developed tumors from parental SW1990 cells or pRNAT-Con-infected SW1990 cells (control) without significant difference in tumor weight. In contrast, only three of six mice developed tumors from pRNAT-STAT3-siRNA-Ⅱ-infected SW1990 cells and the tumors were significantly smaller than those of the control mice (Figure 4). These results suggest that STAT3 plays an important role in tumorigenicity.

RNAi targeting STAT3 inhibits tumor growth in vivo

We further investigated the possibility of using STAT3

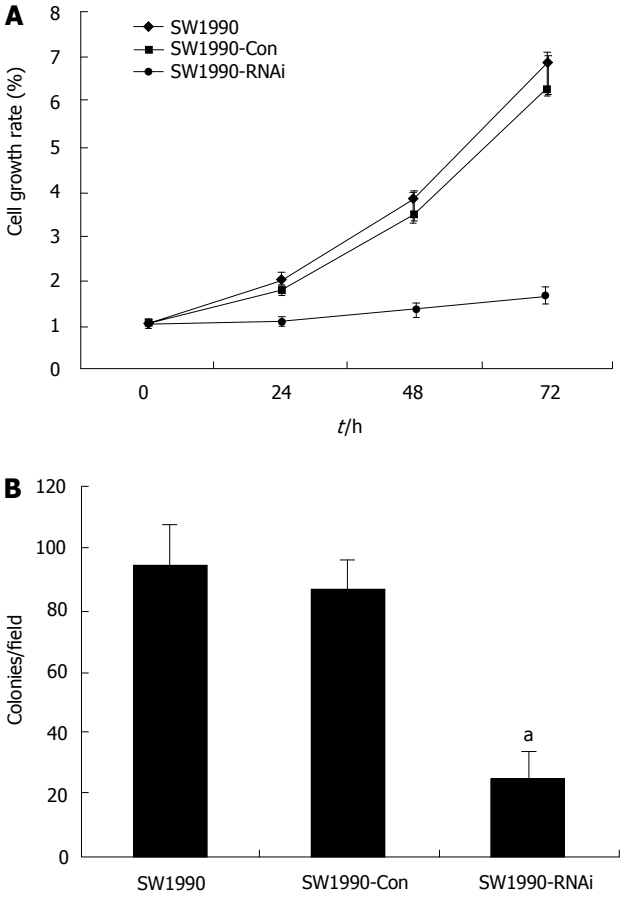

Figure 2 Effects of silence of signal transduction and activation of transcription 3 gene on cell proliferation and anchorage-independent growth of pancreatic cancer cells. A: Cell growth curve of pancreatic cancer cells. SW1990 cells and stably-transfected cells (SW1990-Con and SW1990-RNAi) were subjected to MTT assay as described in materials and methods. Cell proliferation of SW1990-RNAi cells was significantly reduced compared with parental SW1990 and SW1990-Con cells; B: Cell colony formation in soft agar of pancreatic cancer cells. SW1990 cells and stably-transfected cells (SW1990-Con and SW1990-RNAi) were subjected to colony formation assay as described in materials and methods. The anchorage-independent growth ability of SW1990-RNAi cells was significantly reduced compared with parental SW1990 and SW1990-Con cells. Columns: mean (n = 3); Bars: SD. $^aP < 0.05$ *vs* control. SW1990-Con: SW1990 pRNAT-Con transfectants; SW1990-RNAi: SW1990 STAT3-RNAi transfectants.

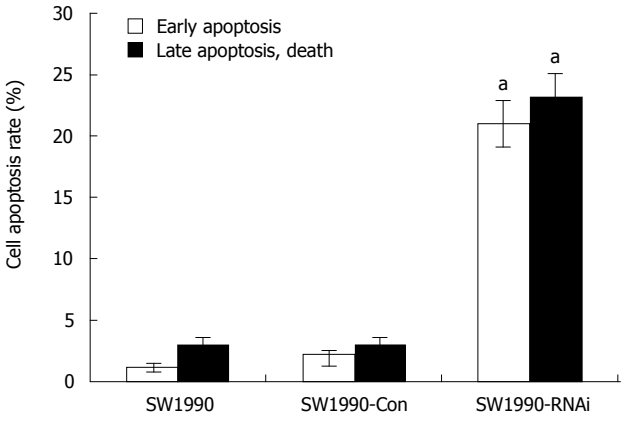

Figure 3 Effects of silence of signal transduction and activation of transcription 3 gene on apoptosis of pancreatic cancer cells. SW1990 cells and stably-transfected cells (SW1990-Con and SW1990-RNAi) were collected to analyze the cell apoptosis with flow cytometry. More apoptotic cells were detected in SW1990-RNAi cells than in parental SW1990 and SW1990-Con cells. Columns: mean (n = 3); bars: SD. $^aP < 0.05$ *vs* control. SW1990-Con: SW1990 pRNAT-Con transfectants; SW1990-RNAi: SW1990 STAT3-RNAi transfectants.

as a target gene for pancreatic cancer therapy in the nude mouse tumor xenograft model. Mice were transplanted s.c. with 1.0×10^7 SW1990 cells in the right flank. By day 14, palpable tumors had grown at the sites of injection. These mice were divided into three groups with six mice in each group and injected intratumorally with either PBS buffer alone (mock), pRNAT-Con, or pRNAT-STAT3-siRNA-Ⅱ. This process was repeated on day 21. Animals were sacrificed on day 35. As shown in Figure 5A, the mean tumor size of mice treated with PBS buffer control (mock) was 1349.36 ± 164.41 mm^3 on day 35; the mean tumor size in mice treated with pRNAT-Con was 1288.59 ± 129.26 mm^3 and that of the group treated with pRNAT-STAT3-siRNA-Ⅱ was 335.81 ± 55.74 mm^3. The difference was not statistically significant in tumor size between the mock group and pRNAT-Con group ($P > 0.05$). The group treated with pRNAT-STAT3-siRNA-Ⅱ showed marked tumor growth suppression compared with the pRNAT-Con ($P < 0.05$). STAT3 expression was significantly reduced after RNAT-STAT3-siRNA-Ⅱ treatment (Figure 5B and C). These results suggested that silencing STAT3 has a therapeutic potential for pancreatic cancer.

RNAi targeting STAT3 suppresses Bcl-xL and CyclinD1 expression in SW1990 cells

STAT3 activation contributes to oncogenesis through regulation of its target genes. To determine the effect of STAT3 downregulation on growth-related target gene expression, we assayed the expression of CyclinD1 and Bcl-xL by RT-PCR, both of which were directly involved in tumor cell proliferation and apoptosis. As shown in Figure 6A, the expression of CyclinD1 and Bcl-xL mRNAs in SW1990 cells was significantly inhibited after STAT3 silencing. The densitometric analyses revealed that the relative CyclinD1 expression in SW1990-RNAi cells was reduced to 52% compared with that of the parental SW1990 cells. And Bcl-xL relative expression in SW1990-RNAi cells was reduced to 39% of that of parental SW1990 cells. A similar inhibitory effect on protein levels is shown in Figure 6B, which demonstrated that the expression of CyclinD1 and Bcl-xL proteins in SW1990 cells was also significantly inhibited after STAT3 silencing. These results indicated that silencing of STAT3 gene suppressed CyclinD1 and Bcl-xL expression.

DISCUSSION

Pancreatic adenocarcinoma remains a widespread disease and difficult to be treated. Surgical resection can only cure a few cases, and most patients are not suitable for the surgical resection, and conventional chemotherapy and radiation

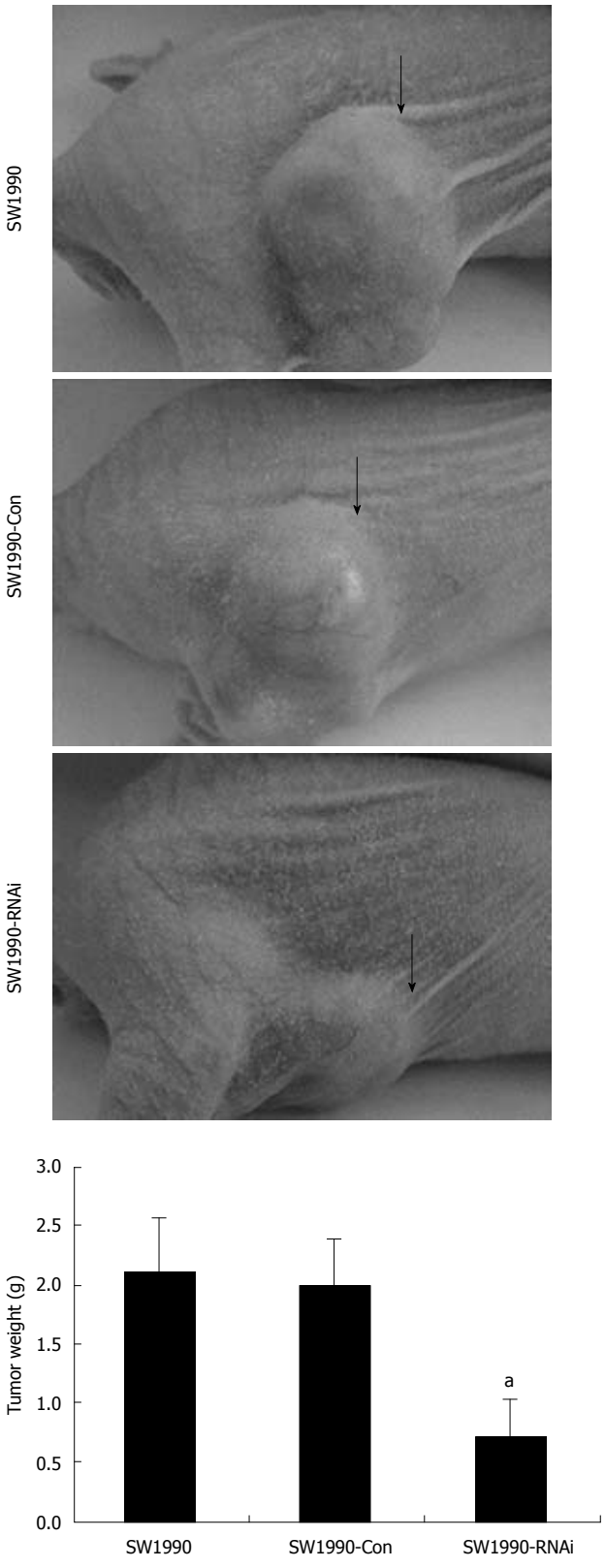

Figure 4 Effects of silence of signal transduction and activation of transcription 3 gene on tumorigenicity *in vivo*. *In vivo* assay was done as described in materials and methods. Six weeks after injection, tumors were harvested and analyzed. Tumors from pRNAT-STAT3-siRNA-Ⅱ–infected SW1990 cells were significantly smaller than those of control mice. Columns: mean (n = 6); Bars: SD. $^{a}P < 0.05$ *vs* control. SW1990-Con: SW1990 pRNAT-Con transfectants; SW1990-RNAi: SW1990 STAT3-RNAi transfectants.

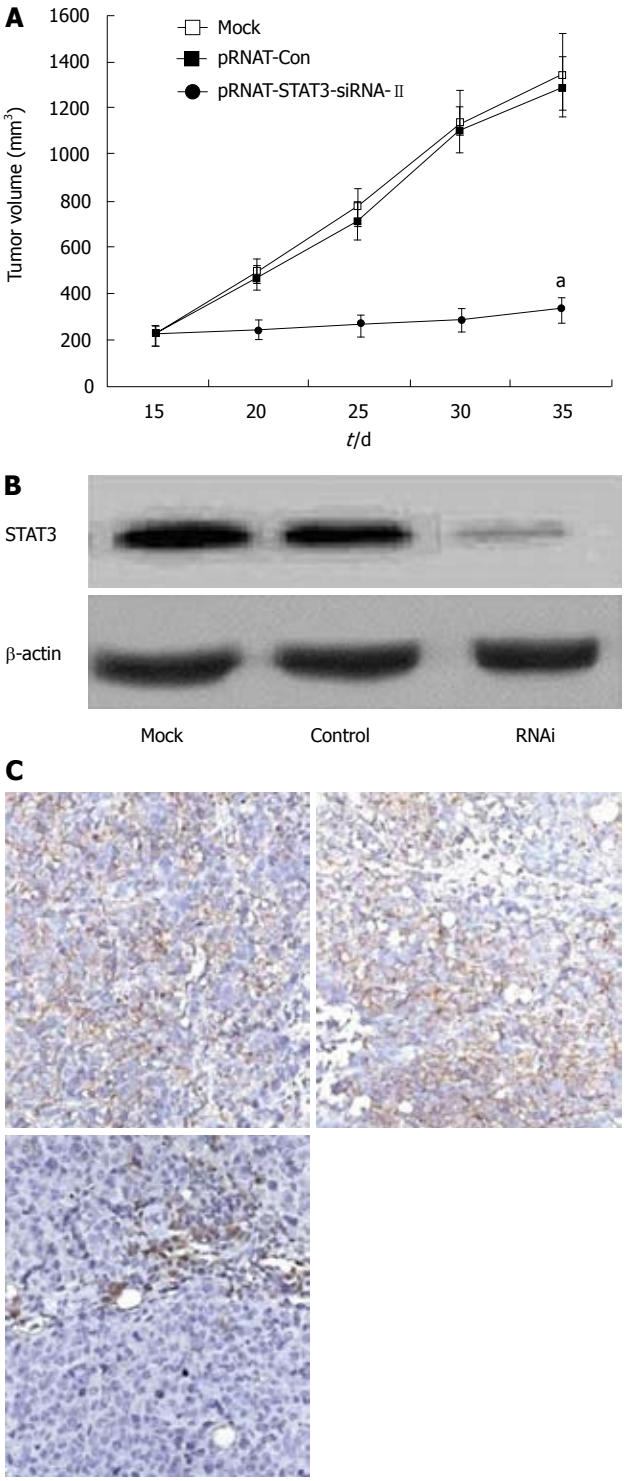

Figure 5 Effects of silence of signal transduction and activation of transcription 3 gene on tumor growth *in vivo*. A: Tumor growth curve after injection with mock, pRNAT-Con, and pRNAT-STAT3-siRNA-Ⅱ cells. Intratumoral electroinjection of pRNAT-STAT3-siRNA-Ⅱ resulted in significant inhibition of tumor growth; Points: mean (n = 6); bars: SD. $^{a}P < 0.05$ *vs* control; B: STAT3 expression of the tumors after injection with mock, pRNAT-Con, and pRNAT-STAT3-siRNA-Ⅱ cells was analyzed by Western blotting. In pRNAT-STAT3-siRNA-Ⅱ–treated tumors, STAT3 expression was significantly reduced. β-actin expression served as a control for equivalent protein loading; C: Tumor sections obtained from mock-, pRNAT-Con-, and pRNAT-STAT3-siRNA-Ⅱ cells –injected tumors were immunostained using anti-STAT3 antibody. In pRNAT-STAT3-siRNA-Ⅱ–treated tumors, STAT3 expression was significantly reduced.

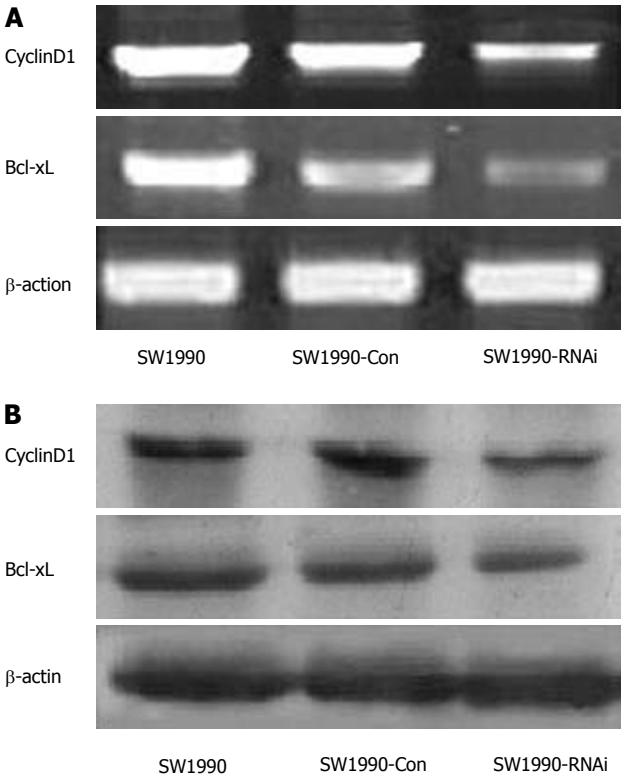

Figure 6 Effects of silence of signal transduction and activation of transcription 3 gene on the expression of CyclinD1 and Bcl-xL. A: Reverse transcription polymerase chain reaction analysis. The RNA samples (2 μg in each) extracted from SW1990 cells, SW1990 cells transfected with a control vector (SW1990-Con), and SW1990 cells transfected with STAT3-RNAi (SW1990-RNAi) were subjected to RT-PCR for CyclinD1, Bcl-xL and β-actin mRNAs. RT-PCR for β-actin was performed in parallel to show an equal amount of total RNA in the sample; B: Western blotting analysis. Whole protein extracts (100 μg in each) were prepared from SW1990 cells, SW1990-Con, and SW1990-RNAi. The expression of CyclinD1 protein was determined by Western blotting with an anti-CyclinD1 antibody. The expression of Bcl-xL protein was determined by Western blotting analysis with an anti-Bcl-xL antibody. The β-actin expression levels were determined as a control for equivalent protein loading. Results shown represent one of the three experiments.

remain largely ineffective. Thus, pancreatic adenocarcinoma represents one of the leading causes of cancer deaths in industrialized countries. With the expectation of increasing therapeutic efficacy, gene therapy is being investigated as a new treatment modality[33]. STAT3 has been considered a very promising target molecule for cancer therapy because it plays a pivotal role in tumorigenesis by cell cycle progression, apoptosis, angiogenesis, metastasis and tumor cell evasion of the immune system[14,15]. Strong evidence has proved that aberrant Stat3 signaling may play an important role in the development and progression of pancreatic adenocarcinoma. We also demonstrated that increasing STAT3 activation in pancreatic adenocarcinoma and blocking Stat3 activation by AG490 (a JAK-specific inhibitor) resulted in suppression of pancreatic cancer growth and invasion *in vitro*[34,35]. Collectively, these findings indicate that targeting STAT3 signaling may represent a novel approach to treat pancreatic adenocarcinoma.

RNAi represents a promising new experimental tool for the analysis of gene function and has become a key gene therapy technique in mammalian systems. Compared with traditional gene therapy, RNAi possesses the advantages of an exquisite precision and high efficacy in down-regulating gene expression[36,37].

In the present study, we used shRNAs targeting STAT3 to silence the expression of STAT3 in human pancreatic cancer cells SW1990. We successfully constructed the recombinant plasmid pRNAT-STAT3-RNAi-II and employed the recombinant plasmid to generate SW1990-RNAi cell line, which showed a significantly decreased STAT3 expression. Attenuation of STAT3 changed the growth behavior of human SW1990 cells both *in vitro* and *in vivo*. MTT assay and soft agar colony formation assay revealed that STAT3 silencing by RNAi inhibited SW1990 cell proliferation and anchorage-independent growth ability. Flow cytometry revealed that RNAi targeting STAT3 arrested SW1990 cells at G_0/G_1 phase and increased SW1990 cell apoptosis. Moreover, *in vivo* study showed that STAT3 silencing inhibited tumorigenecity and tumor growth of SW1990 cells in nude mouse tumor xenograft model.

The inhibitory mechanism in the tumor growth after STAT3 silencing with RNAi is considered as down-regulation of genes related with cell proliferation and apoptosis. CyclinD1 is believed to play a key role in the cell proliferation through promoting cell cycle[38] and overexpression of cyclin D1 was reported to correlate with poor prognosis in pancreatic cancer[39]. Recently, some studies have found that STAT3 signaling directly regulates CyclinD1 expression, tumor proliferation and growth and proved that CyclinD1 is a target gene of STAT3[40]. Our previous study found that inhibition of STAT3 signal with AG490 could inhibit growth of pancreatic cancer cells and decrease CyclinD1 expression[34]. This study also showed that the silencing of STAT3 markedly reduced the mRNA and protein expression of CyclinD1 in SW1990 cells.

Besides persistent proliferation, phenotypes of anti-apoptosis are also required for cancer cells to grow well *in vivo*. Bcl-xL, an anti-apoptotic gene of the BCL-2 family, is associated with poor survival and prognosis of pancreatic cancer patients[41]. Increased expression of Bcl-xL is dependent on the constitutively activated STAT3, and Bcl-xL has been proved to be a target gene of STAT3. Blocking STAT3 signal in human tumor cells has been shown to downregulate Bcl-xL expression and induce tumor-cell apoptosis[42]. As shown in our previous study, inhibition of STAT3 signal with AG490 could retard the growth of pancreatic cancer cells and decrease Bcl-xL expression[34]. This study also found that the silencing of STAT3 with RNAi significantly decreased the mRNA and protein expression of Bcl-xL in SW1990 cells.

In conclusion, the present study indicates that siRNA targeting STAT3 mRNA *via* a plasmid based system effectively sustains the silencing of STAT3 gene expression in SW1990 cells. The impaired STAT3 expression results in reduced SW1990 cell growth both *in vitro* and *in vivo* due to the the downregulation of the expression of CyclinD1 and Bcl-xL. Targeting STAT3 activation by RNAi may be a potential therapeutic strategy in the treatment of pancreatic adenocarcinoma.

COMMENTS

Background

signal transduction and activation of transcription (STAT)3 is a central cytoplasmic transcription factor. Uncontrolled activation of STAT3 plays a critical role in cell survival and proliferation during oncogenesis. The authors evaluated the effect of RNA interference (RNAi) mediated silence of STAT3 on the growth of human pancreatic cancer cells both *in vitro* and *in vivo*.

Research frontiers

Activated STAT3 has been shown to promote tumor cell proliferation, metastasis, and angiogenesis by regulating associated genes. The authors determined whether the STAT3 signaling pathway regulates the growth of pancreatic cancer cells, and found that silencing of STAT3 with RNAi may offer a novel strategy for pancreatic cancer intervention.

Innovations and breakthroughs

The expression of the STAT3 was inhibited using RNAi in SW1990 cells. RNAi against STAT3 inhibited cell proliferation, induced cell apoptosis and significantly reduced the levels of CyclinD1 and Bcl-xL. *In vivo* experiments showed that RNAi against STAT3 inhibited the tumorigenicity of SW1990 cells and significantly suppressed tumor growth.

Applications

The present study indicates that siRNA targeting STAT3 mRNA *via* a plasmid based system effectively sustains the silence of *STAT3* gene expression in SW1990 cells. The impaired STAT3 expression results in reduced SW1990 cell growth both *in vitro* and *in vivo*. Therefore, targeting STAT3 activation by RNAi may be a more effective approach in the treatment of pancreatic cancer.

Terminology

STAT3 is a key cytoplasmic transcription factor activated by tyrosine kinase growth factor and cytokine receptors. Once tyrosine is phosphorylated, two STAT3 monomers form a dimer through reciprocal phosphotyrosine-SH2 interactions, and translocate to the nucleus where they bind to STAT3-specific DNA-response elements of target genes, and induce gene transcription. It has been demonstrated that STAT3 participates in the occurrence and development of cancers.

Peer review

In this study, the authors evaluate the effect of RNAi mediated silence of STAT3 on the growth of human pancreatic cancer cells. They showed that RNAi for STAT3 not only inhibited cell proliferation and induced cell apoptosis *in vitro* but also suppressed pancreatic tumor growth *in vivo*. As the authors stated, the present study suggested the possibility that silence of *STAT3* gene using RNAi may be a novel therapeutic option for treatment of pancreatic cancer. Overall, the manuscript is well written.

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ORIGINAL ARTICLE

Cisplatin pretreatment enhances anti-tumor activity of cytokine-induced killer cells

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Abstract

AIM: To investigate whether cisplatin (DDP) enhances the anti-tumor activity of cytokine- induced killer (CIK) cells in a murine colon adenocarcinoma model.

METHODS: Tumor size and weight served as indicators of therapeutic response. Immunohistochemistry was performed to observe intratumoral lymphocyte infiltration and tumor microvessel density. Changes in the percentage of regulatory T (Treg) cells within the spleens of tumor-bearing mice preconditioned with DDP were monitored using flow cytometry.

RESULTS: A marked T cell-dependent, synergistic anti-tumor effect of the combined therapy was observed (1968 ± 491 mm^3 *vs* 3872 ± 216 mm^3; $P = 0.003$). Preconditioning chemotherapy with DDP augmented the infiltration of CD3+ T lymphocytes into the tumor mass and reduced the percentage of both intratumoral and splenic Treg cells.

CONCLUSION: Preconditioning with DDP markedly enhances the efficacy of adoptively transferred CIK cells, providing a potential clinical modality for the treatment of patients with colorectal cancer.

Key words: Colorectal cancer; Preconditioning chemotherapy; Cytokine-induced killer cells; Regulatory T cells; Immunomodulation

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INTRODUCTION

Colorectal cancer is one of the most common malignancies in the world[1]. Despite advances in surgery, chemotherapy and radiotherapy, the prognosis of the patients with advanced colorectal cancer remains poor[2,3]. Therefore, new and effective treatment modalities, such as immunotherapy, are urgently needed.

Cytokine-induced killer (CIK) cells are *ex vivo*-expanded T lymphocytes that share phenotypic and functional properties with both natural killer (NK) and T cells[4]. CIK therapy is a promising approach for the treatment of a broad array of malignant hematopoietic diseases and solid tumors[4-8]. However, clinical trials in CIK therapy did not show any noticeable improvement in cure rates or long-term survival[6-8], suggesting that the treatment needs to be refined to maximize its efficacy.

Recent advances in molecular immunology have un-

masked the crucial mechanisms that inhibit anti-tumor immune responses *in vivo*. In particular, regulatory T (Treg) cells, which are a distinct lymphocyte lineage that inhibits both adaptive and innate immunity[9], have received a great deal of attention. Treg cells can also hinder the anti-tumor activity of CIK cells[10,11]. Thus, strategies aimed at depleting Treg cells may increase the efficacy of CIK cells.

A number of studies have shown that some chemotherapeutic agents, in addition to their direct cytotoxic effects on tumor cells, possess the ability to modulate anti-tumor immune responses[12,13]. Cisplatin (DDP) is one of the conventional anticancer agents endowed with immunomodulating features. It can sensitize tumor cells to lysis by NKG2D-expressing lymphocytes by up-regulating the expression of the NKG2D ligand (NKG2DL) on tumor cells[14]. It may also increase the vulnerability of tumor cells to Fas ligand (FasL)-positive immune effectors by increasing Fas expression on the targets[15]. However, there have been no studies characterizing the potential suppressive effects of DDP on Treg cells.

In this study, to investigate whether DDP can enhance the anti-tumor activity of CIK cells, we used a combined therapy consisting of pretreatment with DDP followed by adoptive CIK therapy in a murine colon adenocarcinoma model. A marked T cell-dependent synergistic anti-tumor effect was observed. Preconditioning chemotherapy with DDP also increased the infiltration of $CD3^+$ T lymphocytes into the tumor mass and reduced the percentage of both intratumoral and splenic Treg cells, suggesting a potential mechanism underlying the immunostimulatory capacity of DDP. These results provide an immunological rationale for the combined chemoimmunotherapy and suggest a potential clinical modality for the treatment of patients with colorectal cancer.

MATERIALS AND METHODS

Animals

BALB/c wild type (WT) and BALB/c nu/nu male mice were purchased from the Chinese Academy of Military Medical Sciences (Beijing, China), and those at 6-8 wk of age were used for the experiment. All mice were maintained at controlled temperature and humidity, with a 12 h light-dark cycle, and sterile food and water *ad libitum*. The animal studies were conducted in accordance with the Animal Experiment Guidelines of the Ethics Committee of Jingling Hospital.

Tumor cells

Murine CT-26 colon adenocarcinoma cells were obtained from the Shanghai Institute of Biochemistry and Cell Biology (Shanghai, China) and maintained in cRPMI-1640 (Hyclone, Waltham, MA, USA) supplemented with 10% fetal calf serum, 100 U/mL penicillin and 100 μg/mL streptomycin at 37℃ in a humidified atmosphere of 5% CO_2.

Generation of Cytokine-induced killer cells

Murine CIK cells were obtained as previously described[16]. Briefly, spleen single cell suspensions were prepared from BALB/c WT mice and enriched for lymphocytes by Ficoll-Hypaque (Beijing Chemical Reagents Company, Beijing, China) density gradient centrifugation. Cells were then resuspended in cRPMI-1640 medium supplemented with 1000 U/mL interferon γ (PEPROTECH, Rocky Hill, NJ, USA) on the first day of culture. After 24 h, interleukin-2 (PEPROTECH, Rocky Hill, NJ, USA) and an anti-CD3 antibody (eBioscience, San Diego, CA, USA) were added at 500 U/mL and 50 ng/mL, respectively. Thereafter, cRPMI-1640 supplemented with interleukin-2 (300 U/mL) was added every other day for two weeks and to generate CIK cells.

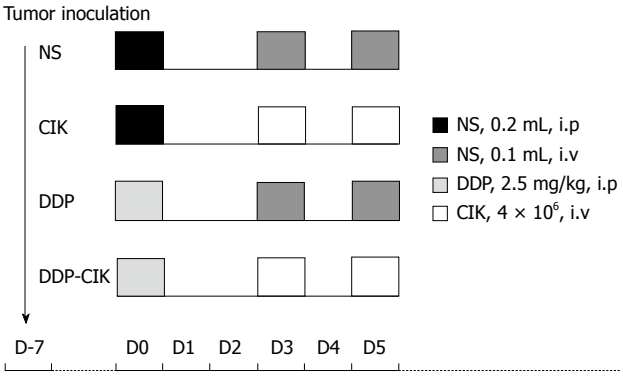

Figure 1 Treatment scheme. BALB/c WT or BALB/c nu/nu mice were injected s.c with 1 × 10^6 CT-26 cells on Day 7 and were treated with the respective regimens according to the treatment scheme. Group cytokine-induced killer cells (CIK) received two i.v infusions of 4 × 10^6 CIK cells at a 1-d interval; Group cisplatin (DDP) was treated with DDP (2.5 mg/kg, i.p); Group DDP-CIK was given preconditioning DDP followed by infusions of CIK cells 3 d later; Group normal saline (NS) was treated with normal saline as control.

In vivo experimental design

CT-26 cells (1 × 10^6/100 mL phosphate buffered saline) were subcutaneously inoculated into the right flank of BALB/c WT and BALB/c nu/nu mice. When tumors became approximately 5 mm in mean diameter, animals were randomly divided into four groups, five in each group, and subjected to the corresponding treatment. The preconditioning chemotherapy used in this study was a single intraperitoneal injection of 2.5 mg/kg DDP; and adoptive immunotherapy consisted of two intravenous transfusions of CIK cells at a 1-d interval (4 × 10^6 cells per dose in a total volume of 100 mL). The treatment scheme of each group is shown in Figure 1 and the detailed grouping was as follows: (1) Group normal saline (NS), treated with normal saline; (2) Group CIK, treated with CIK cells alone; (3) Group DDP, treated with DDP alone; and (4) Group DDP-CIK, preconditioned with DDP followed by transfusion of CIK cells. The tumor size (mm) was measured every other day using a caliper and tumor volume was calculated as: 0.5 × length × $width^2$.

Immunohistochemical analysis

Twenty days after the treatment, the tumor mass was excised, fixed in 10% formalin, embedded in paraffin and sectioned at 3 μm for histological and immunohistochemical

studies. Anti-CD3 (1:500, rat monoclonal, Abcom, Cambridge, MA, USA), anti-FoxP3 (1:1000, rabbit polyclonal, Abcom, Cambridge, MA, USA) and anti-CD31 antibodies (1:100, rat polyclonal, Abcom, Cambridge, MA, USA) were used for immunostaining. All procedures were carried out according to the manufacturer's instructions. Images of the sections were acquired using an Olympus BX-60 microscope to determine the $CD3^+$, $FoxP3^+$ or $CD31^+$ cell density. The number of positive cells was counted in 10 independent fields (0.16 mm^2 at ×400 magnification) within each section by two independent observers.

Flow cytometry

Spleen single cell suspensions were prepared from untreated or DDP-pretreated tumor-bearing mice at the indicated time points and enriched for lymphocytes using Ficoll-Hypaque density gradient centrifugation. A mouse regulatory T cell staining kit (eBioscience, San Diego, CA, USA) was used to determine the percentage of Treg cells. All operations were performed according to the manufacturer's instructions. Phenotypic analysis of splenocytes was performed using a FACSCalibur (BD Biosciences, San Jose, CA, USA). Splenic lymphocytes were gated by plotting forward *vs* side scatter and then by the expression of CD4 and CD25. $CD4^+CD25^{hi}$ T cells were further analyzed for expression of FoxP3. Ten thousands gated events were collected and analyzed using CellQuest software (BD Biosciences, San Jose, CA, USA). The following conjugated antibodies were used: PE-conjugated anti-FoxP3, FITC-conjugated anti-CD4, APC-conjugated anti-CD25, and isotype-matched controls (eBioscience, San Diego, CA, USA).

Statistical analysis

Differences between groups were compared using ANOVA, and LSD was used for multiple mean comparisons. A P value < 0.05 was considered significant. Statistical analysis was conducted using SPSS software v13.0 (SPSS Inc., Chicago, Illinois, USA).

RESULTS

DDP pretreatment and CIK therapy synergistically inhibits tumor growth in BALB/c WT mice

To investigate whether DDP pretreatment enhanced the anti-tumor activity of CIK therapy, CT-26 carcinoma-bearing BALB/c WT mice were injected i.p. with DDP and then i.v. with CIK cells. Tumor size change was monitored every other day throughout experiment (Figure 2A, left panel). On Day 19, the tumor mass was isolated (Figure 2B, left panel). Treatment with either DDP or CIK cells alone inhibited tumor growth compared with the NS control (Figure 2, left panel). However, a significantly greater inhibition of tumor growth was observed after the combined therapy in terms of tumor volume (Figure 2C, left panel) and tumor weight (Figure 2D, Left panel) compared with that seen in the single regimen or the NS control.

T cells are required for synergistic anti-tumor effect of the combined therapy

Previous studies showed that an intact immune system is essential for the immunostimulatory anti-tumor effects of chemotherapeutic agents[17,18]. To examine the mechanisms by which DDP treatment increased the efficacy of CIK therapy, the combined treatment protocol (DDP pretreatment plus CIK therapy) was also evaluated in a CT-26 carcinoma-bearing nude mouse model (Figure 2, right panel). With no treatment, the intrinsic tumor growth pattern in nude mice was similar to that in WT mice (Figure 3). In the therapeutic setting, tumor volume was monitored every other day (Figure 2A, right panel) up until Day 19, when the tumor mass was isolated (Figure 2B, right panel). DDP treatment efficiently inhibited tumor growth in WT mice (Figure 2, left panel) but showed only minor inhibitory effects on tumor growth in nude mice (Figure 2, right panel). In addition, CIK therapy alone did not inhibit tumor growth (Figure 2, right panel) when compared with the NS control. Moreover, no synergy between DDP treatment and CIK therapy was observed in the nude mice (Figure 2, right panel).

DDP enhances accumulation of $CD3^+$ T lymphocytes within tumor mass

Since the synergistic anti-tumor effects of the combined therapy rely on the presence of T lymphocytes, we analyzed the intra-tumoral accumulation of lymphocytes. Tumor tissues from all the experimental groups were removed on Day 19 and CD3 was used as a specific marker for counting T lymphocytes (Figure 4A). Tumor tissues from untreated hosts were infiltrated by a small number of $CD3^+$ T lymphocytes, and DDP or CIK treatment alone only slightly increased the density of intratumoral $CD3^+$ T lymphocytes, but this was not significant. In contrast, DDP pretreatment combined with CIK therapy reversed this phenomenon, significantly enhancing the influx of $CD3^+$ T lymphocytes into the tumor parenchyma (Figure 4B). This was consistent with the marked retardation of tumor growth seen in the combined treatment group.

DDP reduces percentage of Treg cells in tumor microenvironment

Because some chemotherapeutic agents selectively eliminate Treg cells[19,20], we tested whether DDP possessed this Treg-reducing immunostimulatory effect. We examined the changes in intra-tumoral Treg cell numbers in mice treated with CIK, DDP or combination therapy on Day 19. Nuclear transcription factor forkhead box protein P3 (FoxP3), the most specific Treg cell marker identified to date, was used to label Treg cells infiltrating the tumor (Figure 4C). The number of Treg cells, as assessed by the density of intra-tumoral $FoxP3^+$ cells, was not significantly different among the four groups (Figure 4D). However, due to the degree of lymphocyte infiltration into the tumor mass, it may be not accurate to determine the actual level of intra-

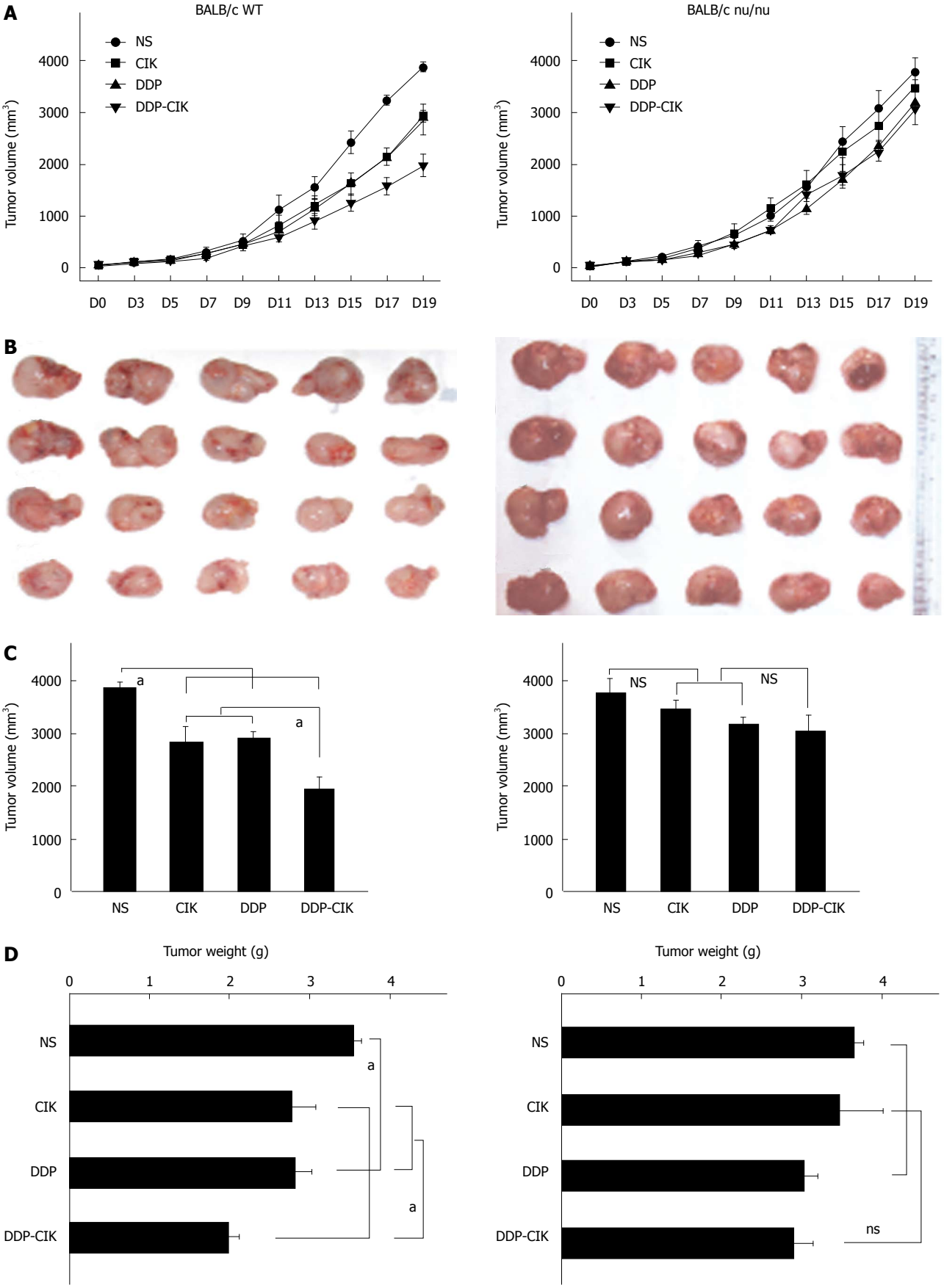

Figure 2 Anti-tumor effect of cisplatin and cytokine-induced killer cells therapy in BALB/c WT and nude mice. BALB/c WT and BALB/C nu/nu mice were inoculated s.c. with 1 × 10^6 CT-26 cells on Day 7 and treated according to the treatment scheme. Tumor size was monitored every other day (A). On Day 19, the tumors were isolated (B), and tumor volume (C) and weight (D) were measured. The results for BALB/c WT mice are shown in the left panel of each figure, while the results for nude mice are shown in the right panel. Points and columns : mean tumor volume or weight (n = 5); Bars: SE. $^aP < 0.05$.

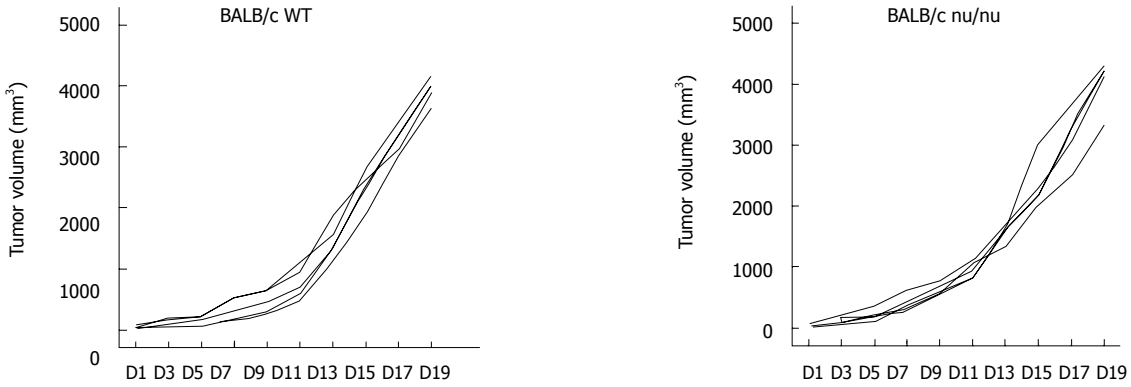

Figure 3 Intrinsic tumor growth pattern of CT-26 carcinomas in untreated BALB/c WT and nude mice. BALB/c WT (wild type) and BALB/c nu/nu mice were inoculated s.c with 1 × 10^6 CT-26 cells on Day 7 and tumor size was monitored every other day. The inherent growth patterns in both strains of mice are shown. Each line represents tumor growth in a single mouse.

tumoral Treg cells using the absolute number of $FoxP3^+$ cells. Therefore, two consecutive sections from each tumor sample were prepared and stained for CD3 and FoxP3, respectively. The percentage of Treg cells, represented as the ratio of $FoxP3^+$ lymphocytes to $CD3^+$ lymphocytes, was calculated to determine the adjusted level of Treg cells within the tumor microenvironment. We found that the percentage of intratumoral Treg cells in Group DDP and Group DDP-CIK was significantly reduced compared with that in Group NS and Group CIK (Figure 4E). This suggests that the systemic administration of DDP locally reduced the percentage of Treg cells in the tumor mass.

DDP reduces percentage of Treg cells in spleens of tumor-bearing mice

To profile the kinetics of Treg accumulation in the spleens of CT-26 carcinoma-bearing mice after DDP treatment, splenic lymphocytes were obtained at various time points and analyzed by flow cytometry. In tumor-free mice, the percentage of Treg cells in spleen was approximately 2% (data not shown). In the absence of intervention, the level of Treg cells in the tumor-bearing hosts increased in line with increasing tumor burden (Figure 5A and B). After DDP administration, the percentage of Treg cells declined at all time points compared with that in untreated mice (Figure 5A and B), suggesting that DDP significantly reduced the percentage of splenic Treg cells. We monitored the duration of Treg depletion induced by DDP, and found that the nadir was around Day 3 after treatment. Treg cell numbers then rebounded and expanded, following the pattern of tumor growth (Figure 5A and B). However, the number of Treg cells in tumor-bearing mice receiving DDP treatment was consistently lower than that in untreated mice throughout the period of observation.

Tumor microvessel density is not affected by DDP treatment

Chemotherapeutic agents have been shown to exert toxic effects on the endothelium of the growing vasculature[21]. Therefore, we tested whether, in addition to its action on the immune system, DDP possessed antiangiogenic effects. To this end, we compared the tumor microvasculature in all the experimental groups by determining microvessel density (MVD). The MVD within the tumor tissues was estimated from tumor sections stained with an antibody to CD31 (Figure 6A) and quantified as described in Materials and Methods. We found that the MVD was comparable among all four groups (Figure 6B), suggesting that DDP had no toxic effect on the tumor microvasculature in this model.

DISCUSSION

Immunosuppression is a significant obstacle to the generation of effective anti-tumor immunity. During tumor progression, tumor cells foster a tolerant and resistant microenvironment by employing various immunosuppressive mechanisms[22]. It is now clear that successful cancer immunotherapy will be achieved only after the removal of immunity-hampering barriers. Previous studies showed that preconditioning a host with immunomodulating chemotherapy can effectively augment the anti-tumor effects of adoptively transferred effectors[23]. In the present study, DDP pretreatment in combination with adoptive CIK therapy was tested in a murine model of colon adenocarcinoma. Our data showed a marked synergy between DDP chemotherapy and CIK immunotherapy in treating established CT-26 carcinomas in immunocompetent mice, suggesting that a single dose of DDP was sufficient to "groom" the immune system, eventually enhancing the efficacy of subsequent CIK therapy.

A number of murine tumor models show that eradication of tumors requires T cells[17,18,24]. In accordance with these studies, the synergic anti-tumor effect in our study was not evident in the BALB/c nu/nu mice which lack T cells, implying that T lymphocytes were the effectors in our model of anti-tumor therapeutic synergy. The natural growth pattern of CT-26 carcinomas in untreated nude mice was similar with that in immune replete WT mice, suggesting that "default" endogenous immunity was not able to prevent the growth of tumors, or to eradicate established tumors. Furthermore, DDP treatment efficiently

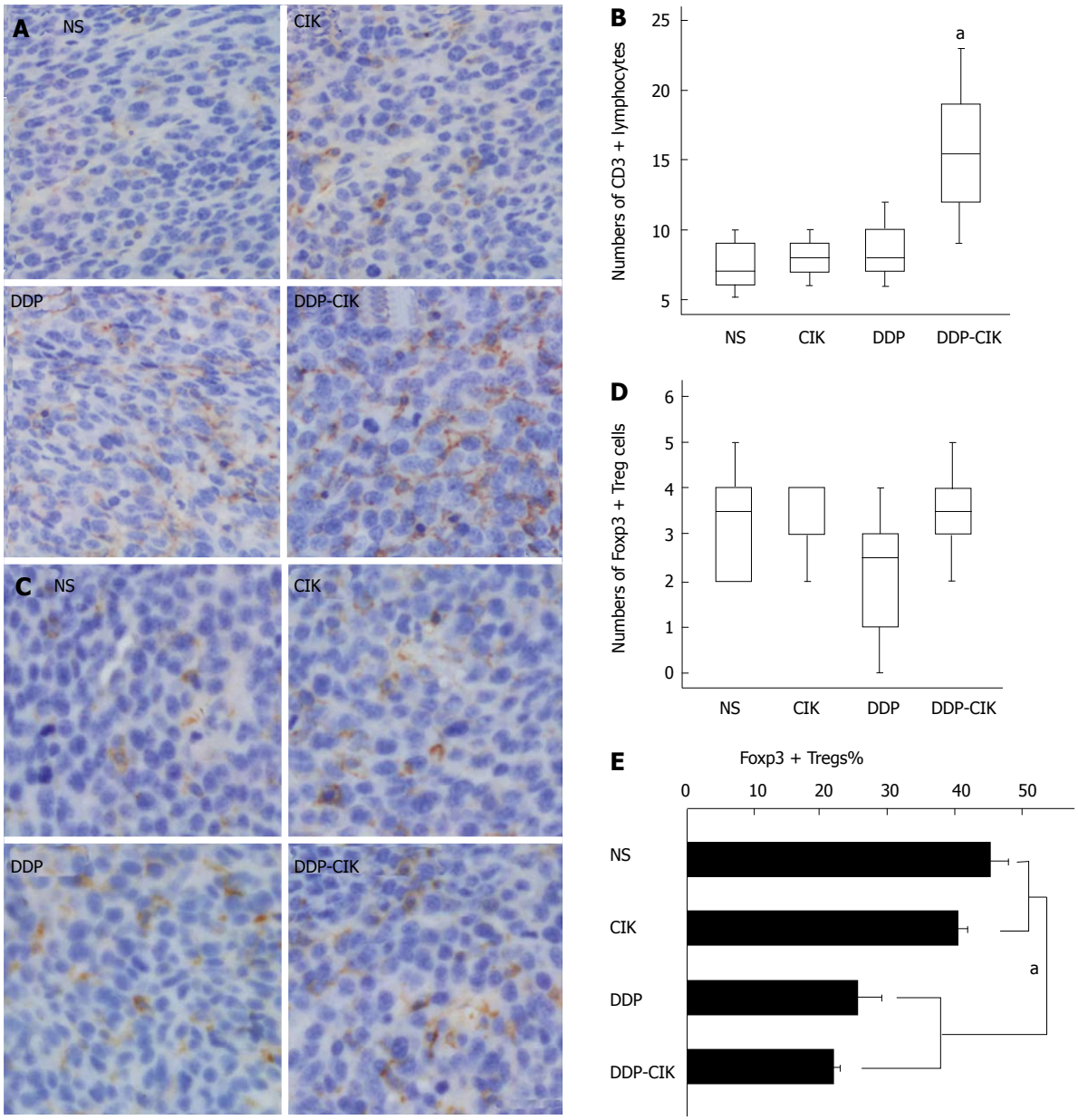

Figure 4 Intratumoral infiltration of lymphocytes after combined therapy. BALB/c WT mice were injected s.c. with 1 × 10^6 CT-26 cells and the treatment protocols were initiated 7 d later. On Day 19, consecutive tumor sections were prepared and analyzed by CD3 (A) and FoxP3 (C) staining. Ten individual fields (0.16 mm^2) surrounding the apoptotic area (× 400 magnification) were chosen to count the number of intratumoral $CD3^+$ T lymphocytes (B), and to determine the absolute number (D), and percentage (E) of FoxP3 + Treg cells. Experimental groups consisted of five mice per group. Representative sections from all groups are shown. Scale bar: 25 μm. Lines: median; Boxes:75% percentile; Bars (B, D) : SD. Columns : Mean Treg percentage; Bars (E): SE. $^aP < 0.05$. NS: Normal saline; CIK: Cytokine-induced killer cells; DDP: Cisplatin.

inhibited the growth of CT-26 carcinomas in WT mice, but failed to induce tumor growth retardation in the absence of T lymphocytes. This is in line with previous findings suggesting that inhibition of tumor growth by chemotherapeutic agents is strictly dependent on T cells[17,18,24]. Based on these results, we proposed that most of the anti-tumor effect seen in DDP could be attributed to its immunostimulating capacity, rather than to any direct cytotoxicity against tumor cells. Similarly, the efficacy of CIK therapy was observed only in immune-replete hosts, implying that endogenous T lymphocytes participate in the fight against tumor cells by assisting, or co-operating with, exogenously infused CIK cells. Therefore, in T lymphocyte-deficient nude mice, neither DDP pretreatment nor CIK therapy could induce tumor shrinkage. Moreover, preconditioning with DDP lost its capacity to enhance the effect of CIK therapy in the nude mouse model, and the synergy seen with combination therapy was abrogated. We speculate that endogenous T lymphocytes comprise both pro-tumor and anti-tumor subpopulations. The anti-tumor subpopulation collaborates with CIK cells to inhibit tumor growth, and is essential for the effect of CIK therapy. Also, the protumor subgroup is suppressed by DDP, whereas the anti-tumor subgroup is stimulated by DDP, leading to DDP-induced

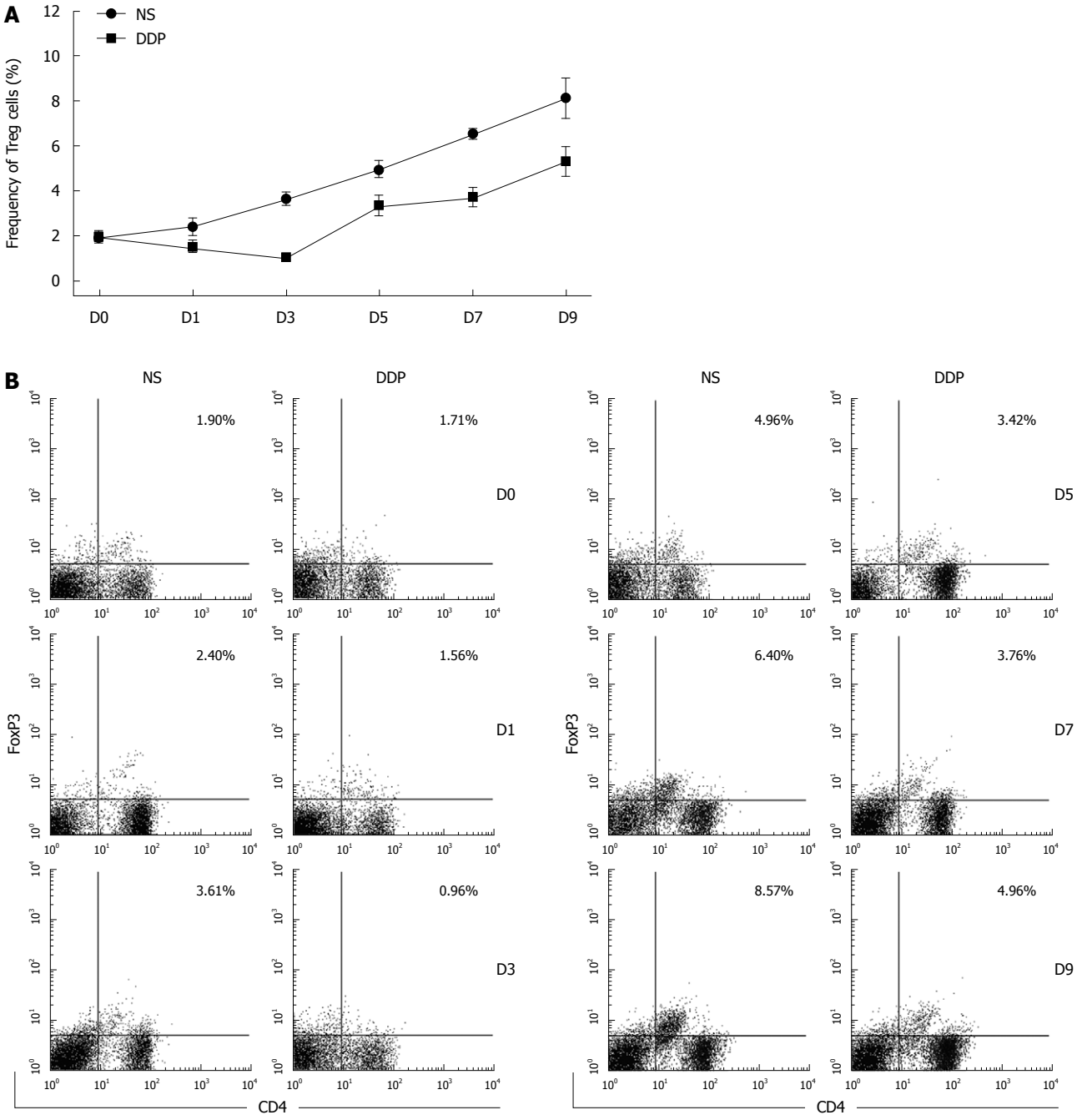

Figure 5 Dynamic changes in the percentage of splenic Tregs after cisplatin (DDP) administration. BALB/c WT mice were injected s.c. with 1 × 10^6 CT-26 cells 7 d before treatment. The tumor-bearing mice were treated with DDP (2.5 mg/kg, i.p., D0) and the spleen single cell suspensions prepared to analyze the Treg percentage by flow cytometry at the indicated post-treatment time points (A). Typical data from a representative experiment are shown (B). Points: mean Treg cell percentage (n = 3); Bars: SD. All experiments were performed twice with similar results. NS: Normal saline; DDP: Cisplatin.

suppression of tumor growth and increased CIK efficacy.

Because of the important effector role played by T lymphocytes in anti-tumor therapeutic synergy, the intratumoral accumulation of T lymphocytes was observed. Our data showed that, compared with the NS control and single therapy alone, the combination therapy significantly augmented the number of $CD3^+$ T lymphocytes infiltrating into the tumor mass, which correlated well with the inhibition of tumor growth.

However, as mentioned above, T lymphocytes comprise both protumor and antitumor subpopulations. Of the protumor subpopulations, Treg cells were mainly involved in tumor-induced immunosuppression. Treg cells are $CD4^+CD25^+$ T lymphocytes with the ability to suppress anti-tumor immune response. These cells accumulate in the peripheral blood, lymph node and tumors in many human cancers and animal tumor models[25]. Elimination of Treg cells *in vivo* using cytotoxic agents or antibodies enhances anti-tumor responses and results in tumor regression[26]. In this study, we determined the percentage of Treg cells in the local tumor microenvironment and found that DDP decreased the percentage of intratumoral Treg

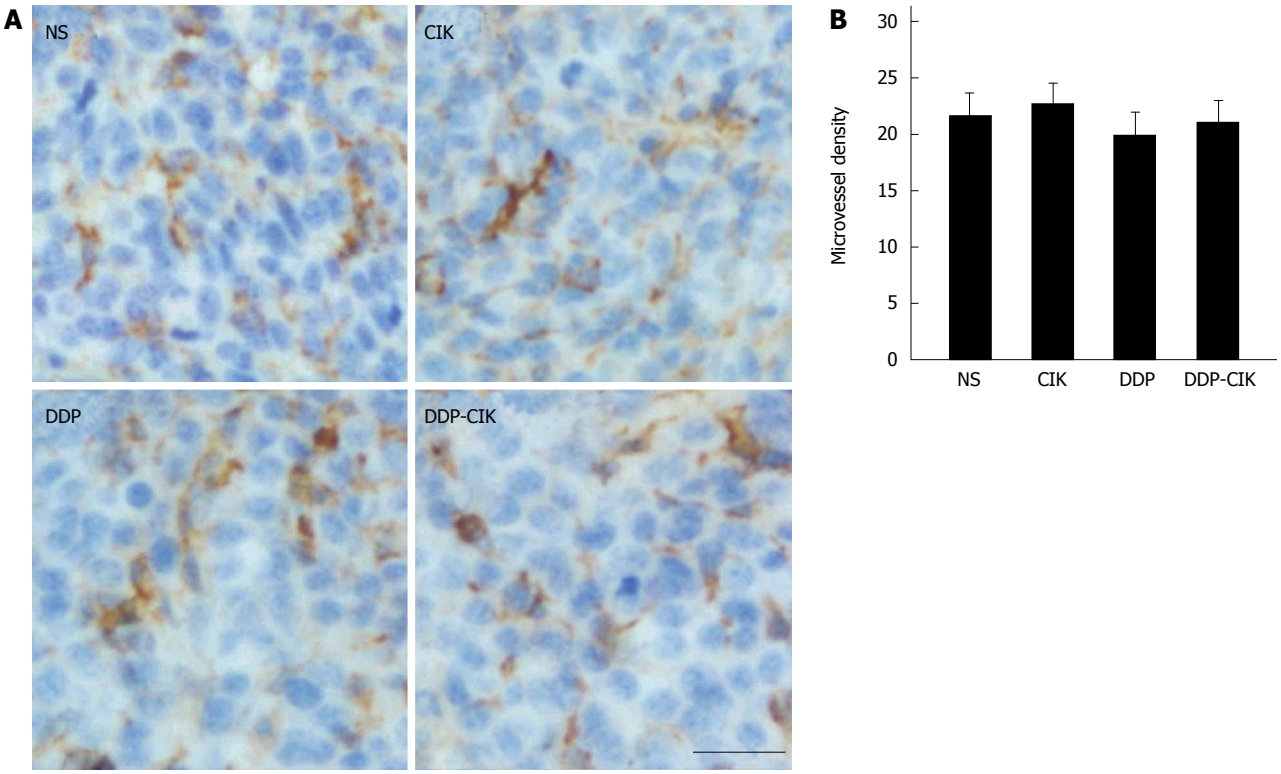

Figure 6 Tumor microvessel density after combination therapy. BALB/c WT mice were injected s.c. with 1 × 10^6 CT-26 cells and the treatment protocols were initiated 7 d later. On day 19, tumor sections were prepared and analyzed by CD31 staining (A). Ten individual fields (0.16 mm^2) at × 400 magnification were chosen to assess the tumor microvessel density (B). The experimental groups consisted of five mice per group. Representative sections from all groups are shown. Scale bars: 25 μm; Columns: mean microvessel number; Bars: SE.

cells. Based on these results, we propose that the DDP-induced reduction in the percentage of Treg cells could enable the development of an anti-tumor immune response, leading to the retardation of tumor growth.

We also examined the longitudinal changes in the percentage of splenic Treg cells in tumor-bearing mice after DDP treatment. It is noteworthy that, of the several cell types within the spleen, Treg cells were preferentially targeted by DDP. We found that the percentage of splenic Treg cells in pretreated tumor-bearing mice was reduced at all time points compared with that in untreated tumor-bearing hosts, implying that, besides its immunomodulating effect, DDP may also ameliorate systemic immunosuppressive factors by depleting splenic Treg cells. Regarding the DDP-induced dynamic changes in the number of Treg cells, the nadir was around Day 3 after treatment. Interestingly, the time points we chose for the transfer of CIK cells in the combined schedule were Days 3 and 5 after treatment, when the percentage of Treg cells was relatively low. Nevertheless, the percentage of Treg cells seemed to rebound and rise quickly on Day 5 after treatment. Therefore, CIK cells infused on Day 5 may have encountered a more "hostile" environment and were unable to exert their anti-tumor effects. Further studies are needed to determine the optimal schedule for combined therapy which triggers the maximal synergistic anti-tumor effects.

In addition to the immunosuppressive factors *in vivo*, angiogenesis, a pivotal process in tumor growth and metastasis, also plays a role in the failure of cellular immunotherapy. Angiogenesis suppresses the expression of the endothelial cell (EC) adhesion molecules involved in leukocyte adhesion to blood vessel walls, and that inhibition of angiogenesis may increase leukocyte-vessel wall interactions and the subsequent infiltration of lymphocytes into the tumor mass[27]. Some chemotherapeutic agents are characterized by their antiangiogenic effect when administered at small doses on a frequent schedule (sometimes referred to as metronomic chemotherapy). Metronomic administration of DDP showed toxic effects on the tumor microvasculature[28]. However, in this study, a single dose of DDP was used as the pretreatment therapy and we did not observe any antiangiogenic effect, ruling out any putative anti-angiogenic effect of DDP in this model.

In conclusion, our data showed that DDP pretreatment acted synergistically with CIK therapy to efficiently inhibit tumor growth in a murine colon adenocarcinoma model. This anti-tumor synergy was T lymphocyte-dependent. Preconditioning with DDP enhanced the infiltration of $CD3^+$ T lymphocytes into the tumors and reduced the percentage of both intratumoral and splenic Treg cells, revealing a potential mechanism underlying the immunostimulatory effects of DDP. In summary, the results of this study provide a potential combination regimen incorporating preconditioning chemotherapy and adoptive CIK therapy for the treatment of colon cancer.

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COMMENTS

Background

Immune suppression constitutes a large obstacle to hinder the generation of effective anti-tumor immunity. It now becomes clear that successful cancer immunotherapy can be achieved only after the removing of immunity-hampering barriers. A number of studies have shown that preconditioning a host with immunomodulating chemotherapy can effectively augment the anti-tumor efficacy of adoptively transferred effectors.

Research frontiers

Regulatory T (Treg) cells, a distinct lymphocyte lineage inhibiting both adaptive and innate immunity, were mainly involved in tumor-induced immunosuppression. Elimination of Treg cells *in vivo* using agents targeting Treg cells such as cytotoxic agents or antibodies have been shown to enhance the anti-tumor responses, resulting in tumor regression.

Innovations and breakthroughs

To our knowledge, this is the first study to evaluate the potential synergy between Cisplatin (DDP) pretreatment and subsequent adoptive cytokine-induced killer cells (CIK) therapy in treatment of colon cancer. A dramatic T cell-dependent synergistic anti-tumor effect of the combination therapy was revealed in the model established in this study. Preconditioning chemotherapy with DDP could augment the infiltration of $CD3^+$ T lymphocytes into the tumor and diminish the percentages of both intratumoral and splenic Treg cells, thus improving the anti-tumor effect of CIK therapy.

Applications

This is a potential combination regimen incorporating the preconditioning chemotherapy to the adoptive CIK therapy for the patients with colorectal cancer.

Terminology

Preconditioning chemotherapy: Chemotherapy administered at a special dose and time point to modulate the host immune environment, thus providing a better ground for the subsequent adoptive cell therapy.

Peer review

How did the authors come upon the timeline for preconditioning and timing of sacrifice? Especially with the curves demonstrated in Figure 3, are we catching this too early? In Figure 3, it still appears that the slope of all of the curves continues to go up. As such, is this an initial impediment to grow more slowly but the end result is no difference? This is especially in light of the clinical trials that demonstrate no improvement in cure rate or long-term survival.

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BRIEF ARTICLE

T1-weighted dual-echo MRI for fat quantification in pediatric nonalcoholic fatty liver disease

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Abstract

AIM: To determine in obese children with nonalcoholic fatty liver disease (NAFLD) the accuracy of magnetic resonance imaging (MRI) in assessing liver fat concentration.

METHODS: A case-control study was performed. Cases were 25 obese children with biopsy-proven NAFLD. Controls were 25 obese children matched for age and gender, without NAFLD at ultrasonography and with normal levels of aminotransferases and insulin. Hepatic fat fraction (HFF) by MRI was obtained using a modification of the Dixon method.

RESULTS: HFF ranged from 2% to 44% [mean, 19.0% (95% CI, 15.1-27.4)] in children with NAFLD, while in the controls this value ranged from 0.08% to 4.69% [2.0% (1.3-2.5), $P < 0.0001$]. HFF was highly correlated with histological steatosis ($r = 0.883$, $P < 0.0001$) in the NAFLD children. According to the histological grade of steatosis, the mean HFF was 8.7% (95% CI, 6.0-11.6) for mild, 21.6% (15.3-27.0) for moderate, and 39.7% (34.4-45.0) for severe fatty liver infiltration. With a cutoff of 4.85%, HFF had a sensitivity of 95.8% for the diagnosis of histological steatosis $\geq$ 5%. All control children had HFF lower than 4.85%; thus, the specificity was 100%. After 12 mo, children with weight loss displayed a significant decrease in HFF.

CONCLUSION: MRI is an accurate methodology for liver fat quantification in pediatric NAFLD.

Key words: Nonalcoholic fatty liver disease; Children; Obesity; Fast-magnetic resonance imaging; Liver fat quantification

Peer reviewer: Akihito Tsubota, Assistant Professor, Institute of Clinical Medicine and Research, Jikei University School of Medicine, 163-1 Kashiwa-shita, Kashiwa, Chiba 277-8567, Japan

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INTRODUCTION

Over the last two decades, the rise in the prevalence rates of overweight and obesity probably explains the emergence of nonalcoholic fatty liver disease (NAFLD) as the

leading cause of liver disease in the pediatric population worldwide[1,2]. NAFLD comprises a disease spectrum ranging from simple steatosis to nonalcoholic steatohepatitis (NASH), progressive to cirrhosis. It is a likely common cause of cryptogenic cirrhosis[3]. There is currently no specific biochemical or serological test for fatty liver and the diagnosis can be established accurately only by liver biopsy. The invasive nature of liver biopsy means that it cannot be used to screen large numbers of subjects at risk, or be performed repeatedly to measure fat changes following treatment. Therefore, availability of an accurate non-invasive tool to assess the presence and severity of liver fat will have important clinical implications in children.

To date, several imaging techniques are used to detect hepatic steatosis: ultrasonography (US), computed tomography (CT), proton magnetic resonance spectroscopy (MRS), and magnetic resonance imaging (MRI)[4-7]. Ultrasonography is a low-cost, widely used technique for the qualitative assessment of steatosis. However, it cannot provide reliable quantitative data, and its sensitivity is reduced in morbidly obese subjects and in those with small amounts of fatty liver infiltration. CT is accurate in the semiquantitative diagnosis of macrovesicular steatosis of 30% or greater; in addition, its use for monitoring treatment response is somewhat limited due to exposure to ionizing radiation. MRS is currently considered the most accurate non-invasive technique for detecting fat quantities as low as 0.5%. However, MRS demonstrates some limitations in that it is too time consuming for routine clinical practice, and requires a skilled operator to correctly perform the examination, process the data, and interpret the results. Because of these limitations, MRS still lacks general availability in current clinical practice for assessment and monitoring of hepatic steatosis. Unlike MRS, MRI is easy to perform and interpret, and, therefore, may be more suitable for widespread use. In adult patients several investigations have demonstrated a good correlation between the severity of hepatic steatosis on MRI and liver biopsy[8-11]. However, to the best of our knowledge, no studies to date have validated MRI with liver histology in the pediatric population. Thus, the purpose of the present study was to determine in a cohort of obese children with biopsy-proven NAFLD the accuracy of MRI for the detection and quantitative assessment of liver steatosis, and to correlate results with clinical, metabolic and histologic findings. We also sought to assess the usefulness of MRI for the evaluation of liver fat changes after a 1-year lifestyle intervention.

MATERIALS AND METHODS

Study design and patients

Twenty-five obese children and adolescents, 16 males and 9 females, aged 7-16 years, with suspected NAFLD ("cases") were recruited for study participation at the Department of Pediatrics, Sapienza University of Rome. Controls were 25 obese children matched for age, gender and pubertal stage, without ultrasound evidence of fatty liver and with normal levels of aminotransferases, as well as of insulin. All participants were of Caucasian ethnicity. The study was approved by the Institutional Review Board, and written consent was obtained from the parents or guardians of the children.

NAFLD diagnosis

NAFLD was suspected if the patients had elevated serum alanine aminotransferase (ALT) either persistently or intermittently, associated with diffusely hyperechogenic liver at ultrasound examination, and hyperinsulinism. Secondary causes of steatosis, including alcohol consumption, total parenteral nutrition, and the use of hepatotoxic medications, were excluded in all cases. In all patients, hepatic virus infections (hepatitis A-E and G, cytomegalovirus, and Epstein-Barr virus), autoimmune hepatitis, metabolic liver disease, α-1-antitrypsin deficiency, cystic fibrosis, Wilson's disease, and celiac disease were ruled out with appropriate tests. The final diagnosis of NAFLD was reached by liver biopsy.

Liver biopsy

The clinical indication for biopsy was either to assess the presence of NASH and degree of fibrosis or other likely independent or competing liver diseases. Percutaneous needle liver biopsy was performed with an 18-gauge needle, under general anaesthesia and ultrasound guidance. In all patients, in order to obtain an adequate sample, biopsy specimens were obtained twice at two different sites in the right hepatic lobe. Liver specimens that were at least 1.5 cm in length and contained at least 10-11 complete portal tracts were considered adequate for histological assessment. Sections were stained with hematoxylin-eosin, periodic acid Schiff, periodic acid Schiff-digested, iron stain, and Masson trichrome reagents. Biopsy specimens were evaluated for the following, using the NASH Clinical Research Network criteria[12]: steatosis [grade 0 (< 5% macrovesicular fat), grade 1 (mild = 5%-33%), grade 2 (moderate = 34%-66%), and grade 3 (severe ≥ 66%)], portal inflammation (0-2), lobular inflammation (0-3), ballooning degeneration (0-2), and fibrosis (stage 0 to 4).

Clinical and laboratory investigations

All study participants underwent physical examination including measurements of weight, standing height, body mass index (BMI), waist circumference (WC), determination of the stage of puberty, as well as systolic blood pressure (BP) and diastolic BP as previously reported in detail[13]. The degree of obesity was quantified by Cole's least mean square method, which normalizes the skewed distribution of BMI and expresses BMI as an SD score[14]. Blood samples were taken from each subject, after an overnight fast, for estimation of glucose, insulin, total and high density lipoprotein (HDL) cholesterol, triglycerides, ALT, aspartate aminotransferase (AST), and γ-glutamyl transferase (GGT).

Insulin concentrations were measured on a COBAS 6000 immunometric analyzer (Roche Diagnostics) by an electrochemiluminescent method. The remaining analytes were measured on a COBAS INTEGRA 800 analyzer (Roche Diagnostics). Insulin resistance (IR) was determined

by a homeostasis model assessment of insulin resistance (HOMA-IR). Scores were calculated as the product of the fasting serum insulin level (μU/mL) and the fasting serum glucose level (mmol/L), divided by 22.5.

MRI technique for hepatic fat quantification

NAFLD patients underwent MRI before liver biopsy and within a short-time interval [mean (SD) 3.1 (2.1) d; range, 1-7]. In controls, MRI was performed within 1 wk of clinical, laboratory and sonographic assessment. Hepatic MRI was performed with a 1.5-T magnet (Magnetom Avanto, Siemens Medical Systems, Erlangen, Germany) equipped with a phased-array surface coil and a spine array coil. Image acquisition was performed in the axial plane during an end-expiratory breath-hold using a sensitivity encoding (SENSE) technique, to reduce the overall acquisition time to approximately 15 s. We used the two-point Dixon method as modified by Fishbein *et al*[15]. The method is based on phase-shift imaging in which hepatic fat fraction (HFF) is calculated from the signal difference between the vectors resulting from in-phase (IP) and out-of-phase (OP) signals.

The multi-breath-hold T1-weighted dual gradient-echo sequence parameters were as follows: repetition time of 174 ms, echo time of 2.1 ms for OP images and 4.9 ms for IP images; flip angle, 70°; section thickness, 5 mm; matrix size, 256 × 182; field of view, 35 cm × 40 cm. Pixel signal intensities (SI) from IP and OP images were obtained from selected regions of interest (ROIs). The SI values both in the liver and the spleen were recorded for the IP and OP images by means of 1 cm^2 circular ROIs. At three different sections (above, at the level of, and below the transverse fissure of the liver), three different ROIs were drawn (2 in the right hepatic lobe; 1 in the left hepatic lobe), totaling nine ROIs. ROI selection within the liver parenchyma was performed taking care to avoid areas with vessels, motion artefacts, and partial volume effects. ROIs were placed at anatomically matched locations on paired images by using a co-registration tool available on the picture archiving and communication system workstation. The SI of the spleen was similarly measured and a mean SI was calculated from three ROIs selected at the corresponding liver levels. The standard deviation of the SI measurements within each ROI was kept to less than 10%. Liver fat was quantified as the percentage of relative SI loss of the liver on OP images, with the following formula: $[(SI_{in}-SI_{out})/2 \times SI_{in}] \times 100$ where SI is average liver signal intensity divided by the average spleen SI, SI_{in} and SI_{out} are signal intensity of IP and OP images, respectively. The SI of the spleen was used as a denominator in the formula to adjust for the lack of an objective SI scale at MRI[16,17]. MR imaging results were interpreted by an experienced radiologist who was blinded to clinical, laboratory, and histologic findings.

To assess reproducibility of MRI technique, measurements were performed again in 8 study subjects who agreed to a longer examination time. Standard deviations of the differences between measurements were less than 2% in HFF.

Follow-up

All 25 NAFLD children were offered the chance to take part in a 12-mo intervention program. This program consisted of physical exercise and nutrition education for the individual and his or her family. Diet was hypocaloric (25-30 calories/kg per day), consisting of carbohydrate (50%-60%), fat (23%-30%), and protein (15%-20%); fatty acid composition was two-thirds saturated, and one-third unsaturated; the ω6/ω3 ratio was 4:1 as recommended by the Italian Recommended Dietary Allowances. The diet regimen was prescribed with a recommendation to engage in a moderate daily exercise program (60 min/d at least 5 d a week).

Follow-up medical examinations (including assessment of changes in anthropometric characteristics) and laboratory measurements (including serum glucose, insulin, ALT, AST, GGT, total cholesterol, HDL cholesterol, and triglycerides) were performed at 6 and 12 mo of the intervention program. MRI was repeated at 12 mo. The NAFLD children were divided into those with and without substantial weight loss during the 1-year intervention. Substantial weight loss was defined as a decrease in the SDS-BMI $\geqslant$ 0.5. This division was used because in previous studies an improvement of cardiovascular risk factors and insulin resistance was only detectable if SDS-BMI decreased $\geqslant$ 0.5[18,19].

Statistical analysis

Statistical analyses were performed using the SPSS package. Data are expressed either as frequencies or as arithmetic means or geometric means with 95% confidence intervals (CI). The measured insulin, total cholesterol, HDL cholesterol, triglycerides and HOMA-IR values were distributed with a long tail to the right (positive skew), but their logarithms were approximately normally distributed. Thus, their mean values with 95% CI are reported as geometric means. Pearson correlations and linear regression analysis were used to analyse the relationship between HFF and the histological degree of steatosis as well as clinical variables. We also performed receiver operating characteristic (ROC) curve analysis to determine the best cut-off values for MRI to predict any grade, moderate, and severe hepatic steatosis. The area under the curve (AUC) was used to assess the accuracy of MRI. Values for sensitivity, specificity, and the optimum discriminative values were also obtained. We considered false-positive and false-negative results to be equally important, and thus values were chosen that maximized sensitivity plus specificity.

Pairwise comparisons were performed using paired *t* test or Wilcoxon's rank sum test, as appropriate. A *P* value of less than 0.05 was considered to be statistically significant.

RESULTS

Clinical and laboratory features of study population

The clinical and laboratory characteristics of cases and controls are summarized in Table 1. Obese children with NAFLD had higher BMI, BMI-SDS, WC, systolic and diastolic BP, triglycerides, insulin, and HOMA-IR values than the control group. Furthermore, compared to controls,

Table 1 Clinical and laboratory characteristics of children with and without nonalcoholic fatty liver disease

Characteristics	NAFLD (n = 25)	No NAFLD (n = 25)
BMI, kg/m^2	28.4 (26.4-30.3)[a]	25.6 (24.4-26.8)
BMI-Standard deviation score	2.20 (2.02-2.30)[a]	2.01 (1.92-2.17)
Waist circumference, cm	96.9 (91.8-102.1)[b]	85.2 (80.3-89.0)
Systolic BP, mmHg	117 (112-122)[b]	107 (105-109)
Diastolic BP, mmHg	70 (67-74)[b]	68 (65-70)
Aspartate aminotransferase, U/L	45 (33-58)[c]	24 (20-28)
Alanine aminotransferase, U/L	73 (55-91)[c]	21 (18-25)
γ-glutamyl transferase, U/L	31 (23-39)[c]	13 (12-14)
Total cholesterol, mg/dL	162 (143-181)	168 (146-190)
HDL cholesterol, mg/dL	42 (38-49)	40 (37-43)
Triglycerides, mg/dL	161 (115-207)[b]	112 (61-134)
Glucose, mmol/L	4.89 (4.69-5.10)	4.88 (4.77-5.02)
Insulin, μU/mL	31.2 (21.9-40.6)[a]	20.1 (16.2-24.1)
HOMA-IR values	4.27 (3.40-5.10)[a]	3.45 (2.97-4.01)
Hepatic fat fraction, %	19.0 (15.1-27.4)[c]	2.0 (1.3-2.5)

Results are expressed as n (%), mean (95% CI), or geometric mean (95% CI) for log-transformed variables. [a]$P < 0.05$, [b]$P < 0.01$, [c]$P < 0.0001$. BMI: Body mass index; NAFLD: Nonalcoholic fatty liver disease; BP: Blood pressure; HDL: High density lipoprotein; HOMA-IR: Homeostasis model assessment of insulin resistance.

Table 2 Features of the liver biopsies of the 25 children with nonalcoholic fatty liver disease

	Grade or stage	n	%
Steatosis	0	-	-
	1	9	36.0
	2	9	36.0
	3	7	28.0
	Total	25	100
Lobular Inflammation	0	-	-
	1	15	60.0
	2	9	36.0
	3	1	4.0
	Total	25	100
Portal inflammation	0	4	16.0
	1	20	80.0
	2	1	4.0
	Total	25	100
Ballooning	0	9	36.0
	1	12	48.0
	2	4	16.0
	Total	25	100
Fibrosis	0	7	28.0
	1	8	32.0
	2	9	36.0
	3	1	4.0
	4	-	-
	Total	25	100

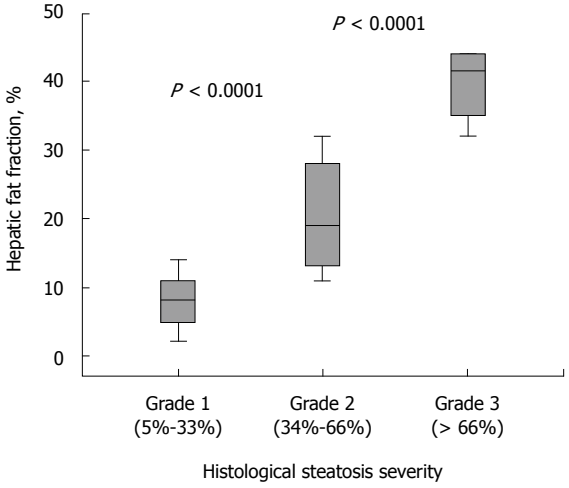

Figure 1 Magnetic resonance imaging hepatic fat fraction according to the histopathological results (grade of steatosis). Boxplots give the median value (black), 25th and 75th percentiles (lower and upper limits of the box), and lower and upper adjacent values (whiskers).

NAFLD children had significantly higher concentrations of ALT and AST, as well as of GGT. HFF ranged from 2% to 44% [mean, 19% (95% CI, 15.1 to 27.4)] in children with NAFLD, while in the control group this value ranged from 0.08% to 4.69% [2.0% (95% CI, 1.3 to 2.5), $P < 0.0001$].

Histological findings in children with NAFLD

All 25 cases fulfilled the histopathological requirements; that is, the length of liver specimens was on average 1.9 ± 0.2 cm, and included 14 ± 2 complete portal tracts. Macrovesicular steatosis was present in all cases and combined with microvesicular in 15% of cases. The amount of steatosis ranged from 10% to 95% with a mean of 42.6% (95% CI, 31.3 to 54.0) steatotic hepatocytes. The distribution of steatosis across the cohort was mild in 36%, moderate in 36%, and severe in 28% of subjects. Lobular inflammation was present in all patients, and it was of mild to moderate grade in 96% of patients. Sixty-four percent of children had ballooning of the hepatocytes that was in most cases of grade 1. Mild, more than mild, and no portal inflammation were found in 80%, 4%, and 16% of biopsies, respectively. Some degree of fibrosis was present in 72% of patients. Thirty-two percent showed stage 1 fibrosis, whereas 36% of biopsy specimens revealed stage 2 fibrosis (Table 2). Stage 3 fibrosis was present in 4%. No patient had hepatic iron deposit.

MRI and histological measurement of steatosis

HFF was highly correlated with histological steatosis over-

all ($r = 0.883$; $P < 0.0001$). According to the histological grade of steatosis, the mean HFF was 8.7% (95% CI, 6.0 to 11.6) for mild, 21.6% (95% CI, 15.3 to 27.0) for moderate, and 39.7% (95% CI, 34.4 to 45.0) for severe fatty liver infiltration. MRI imaging could differentiate between mild and moderate steatosis ($P < 0.001$), and between moderate and severe steatosis ($P < 0.001$) (Figure 1). Linear regression analysis was performed to determine the influence of the stage of fibrosis as well as of the degree of inflammation on the relationship between MRI and histological assessment of steatosis. Fibrosis was found to have no statistically significant influence [unstandardized coefficient, 1.10 (95% CI, 2.25 to 4.45); $P = 0.503$] on the estimates of HFF. Similarly, inflammation had no impact on the accuracy of MRI for the assessment of steatosis.

Accuracy of MRI for the diagnosis of steatosis

The accuracy of MRI for the diagnosis of mild, moderate, and severe steatosis is shown in Table 3. At the diagnostic threshold of 4.85% for HFF, MRI had a 95.8% sensitivity for diagnosing "any" grade of steatosis. There was only one child with histological steatosis of grade 1 who had HFF of 2%. A threshold of 9.0% for HFF was the best cutoff for the diagnosis of moderate to severe steatosis (sensitivity, 100%). A cutoff value of 19.0% for HFF was indicative of severe steatosis (sensitivity, 100%). All control children had HFF lower than 4.85% (specificity, 100%).

Relationship between MRI and variables

Among clinical and laboratory data, HFF was significantly associated with SDS-BMI ($r = 0.486$, $P < 0.01$), WC ($r = 0.406$, $P < 0.01$), triglycerides ($r = 0.374$, $P < 0.05$), insulin ($r = 0.290$, $P < 0.05$), and HOMA-IR values ($r = 0.349$, $P < 0.05$) in the whole study population. When the NAFLD group was analysed separately, HFF remained significantly associated with insulin ($r = 0.425$, $P < 0.05$), and HOMA-IR values ($r = 0.506$, $P < 0.05$).

Follow-up

After 12 mo, eleven NAFLD children demonstrated a substantial weight loss [mean SDS-BMI change: -0.88 (95% CI, -0.57 to -1.19)]. In these children, HFF [mean change: -13.1% (95% CI, -9 to -19); $P < 0.05$)], systolic BP [mean change: -8 mmHg (95% CI, -5 to -14); $P < 0.05$), ALT [mean change: -45 U/L (95% CI, - 37 to -70); $P < 0.05$), AST [mean change: -27 U/L (95% CI, -18 to -37); $P < 0.05$), triglycerides [mean change: -56 mg/dL (95% CI, -32 to -60); $P < 0.05$), and HOMA-IR values [mean change: -3.2 (95% CI, -2.0 to -5.8); $P < 0.05$)] decreased significantly. In contrast, in the 14 NAFLD children without substantial weight loss [mean SDS-BMI change: -0.10 (95% CI, -0.01 to -0.19), there was no significant change in HFF [mean change: -3.0% (95% CI, -0.3 to -5.0); $P = 0.48$] as well as in clinical and laboratory parameters.

DISCUSSION

Liver fat accumulation is becoming a common complication in pediatric obesity[1,2]. Biopsy remains the criterion standard to accurately determine, in a semiquantitative manner, the amount of fatty liver infiltration. Furthermore, liver biopsy is able to evaluate lesions associated with steatosis, such as fibrosis and inflammation, and thus, to evaluate the stage and grade of the disease. However, we cannot perform liver biopsy as a screening method to detect NAFLD in the general pediatric population. Therefore, a reliable, non-invasive method of screening NAFLD would represent a major advance in clinical hepatology.

Several non-invasive imaging techniques have been advocated as diagnostic tests. Standard MRI, MRS, and CT may not be feasible for children because of their long scan time, reliance on compliance of the patient, and ionizing radiation. Signal intensity loss on opposed-phase gradient-echo T1-weighted MR images frequently is regarded as an accurate method of detection and quantification of liver fat[8,20,21]. By using gradient-echo chemical shift imaging (Dixon method), MRI can be performed either as readily available T1-weighted dual echo, triple echo, multiecho, or multi interference. We chose to use the T1-weighted dual-echo MRI because of its simplicity.

In the adult population, a close relationship has been observed between the percentage of steatosis estimated by histology and dual-echo chemical shift imaging[8,11,21]. However, a major point to underline is that HFF appears to be influenced greatly by fat morphology[8]. Lipid is accumulated within the liver as a response to various disease states and may be deposited in a macrovesicular, microvesicular, or mixed steatosis pattern. In a group of 38 patients undergoing liver biopsy for a variety of liver diseases (including hepatitis C, NAFLD, methotrexate monitoring, chronic hepatitis of unknown etiology, cryptogenic cirrhosis, primary biliary cirrhosis, autoimmune hepatitis), Fishbein *et al*[8] showed that fast-MRI correlated better with macrovesicular steatosis ($r = 0.92$, $P < 0.001$) than with mixed steatosis ($r = 0.60$, $P < 0.05$). In NAFLD, a disorder associated with severe macrovesicular steatosis, fat fraction was higher than other liver disorders associated with lesser degrees of fatty infiltration, including hepatitis C. Similarly, in a very recent study including 46 patients undergoing liver resection, van Werven *et al*[21] showed that T1-weighted dual-echo MR imaging was strongly correlated with histopathologic steatosis assessment ($r = 0.85$, $P < 0.001$). In the 23 patients with macrovesicular steatosis greater than 5%, dual-echo MR imaging showed an even stronger correlation with histopathologic examination ($r = 0.92$, $P < 0.001$) than in the overall group.

In the pediatric population, the 2-point Dixon method

Table 3 Diagnostic accuracy of magnetic resonance imaging

Steatotic hepatocytes	⩾ 5%	> 33%	> 66%
Cutoff	4.85	9	19
AUC	0.98 (95% CI, 0.98-1.0)	1	1
Sensitivity (%)	95.8	100	100
Specificity (%)	100	100	100

AUC: Area under the curve.

as modified by Fishbein is an accepted technique for measuring hepatic fat content[22-24]. It can also be helpful in identifying fat regression or progression in children, and it has been found useful in differentiating increased liver echogenicity due to simple steatosis from that related to glycogen storage disease[25,26]. However, no previous studies in children have used the degree of hepatic steatosis at histologic analysis as the reference standard. In normal liver, lipid accounts for approximately 5% total wet weight[22]. Initial studies determining liver fat in children by fast-MRI defined as abnormal a threshold value for HFF greater than 2 SD above mean hepatic fat content of healthy adult volunteers[15] or lean, nondiabetic (mean age 21.6 ± 8.2 years) subjects[27]. Recent studies have validated the modified 2-point Dixon method against MRS in obese and lean adolescents who were at increased risk of having or developing hepatic steatosis, and found a very strong correlation between the two methods (r = 0.954, $P < 0.001$)[23,24]. In a cohort of 28 (mean age, 15.9 ± 5.3 years) obese and lean subjects, Kim *et al*[23] demonstrated that a 2-point Dixon HFF cutoff of 3.6% provided a good sensitivity (80%) and specificity (87%) compared to MRS reference. Our present results obtained in a homogeneous population indicate that the modified 2-point Dixon method may be a good alternative to biopsy for quantifying liver fat content in obese youngsters with NAFLD and for assessing the relation between HFF and metabolic outcomes in these patients. The clinical efficacy of fast-MRI has been previously demonstrated. Burgert *et al*[27] showed that obese children with a high HFF were significantly more insulin resistant, compared with those with a low HFF, and had higher triglycerides and lower adiponectin levels, even after adjustment for BMI-z scores, race/ethnicity, gender, and age. Furthermore, obese children with a high HFF had a significantly greater prevalence of the metabolic syndrome, after controlling for the above confounders. More recently, D'Adamo *et al*[28] suggested that the severity of fatty liver, as determined by the modified 2-point Dixon method, plays a central role in the insulin resistant state in obese adolescents, independently of visceral fat and intramyocellular lipid content. Using the disposition index, an estimate of β-cell function weighted by insulin sensitivity, the authors found this was reduced by 30% in children with fatty liver, thus increasing susceptibility to type 2 diabetes. We also previously showed that the increasing severity of MRI fat accumulation was strongly related to fasting hyperinsulinemia and insulin resistance after correction for confounding variables such as SD score-BMI, sex, age and pubertal status[29]. The present results obtained in obese youths with biopsy-proven NAFLD not only corroborate the clinical efficacy of the modified 2-point Dixon method, but also highlight the potential application of this method for tracking longitudinal changes in liver fat content in patients under targeted lifestyle intervention or medical therapy. In concordance with our longitudinal findings, fast-MRI has also been found to identify longitudinal liver fat changes in adults during pioglitazone treatment for biopsy-proven NAFLD and in obese children and adolescents after a 1-year nutrition-behavior intervention[25,30].

Fibrosis and inflammation may be present in patients with hepatic steatosis. In adult patients with heterogeneity of underlying pathologies including NAFLD, Fishbein *et al*[8] showed that hepatic MRI, based upon chemical shift imaging, is not influenced by the presence of fibrosis and was able to accurately quantify the hepatic fat content in the patients who also had significant hepatic fibrosis. Our present results confirm and expand on the findings of the above report. In fact, we found that in our obese children with NAFLD, neither inflammation nor fibrosis had an influence on the estimates of steatosis.

Our study has some limitations. Firstly, our results were obtained in a selected population of children with and without NAFLD. Therefore, one could argue that by doing so we would maximize the differences between cases and controls. If we had included patients from the general pediatric population, the results would have been more conclusive. However, obtaining hepatic biopsy for research purposes in such patients would not be feasible or ethical. Secondly, another restriction is that we did not perform MRI measurements at exactly the same locations in the liver that were used for histopathologic assessment. This could affect the results we reported in diagnostic performance. However, we obtained large-wedge liver biopsy specimens which provided accurate data. Lastly, we used a T1-weighted dual-echo chemical shift MRI method to study hepatic steatosis. No corrections for T1, T2*, or fat spectral complexity were made, and consequently only MR signal intensities were evaluated. Recent studies have indicated that T1-weighting (flip angle) and T2-weighting (iron deposition) may interfere with accurate fat quantification[16,31]. However, in the study by van Werven *et al*[21], a strong correlation between T1-weighted dual gradient-echo MR imaging and histopathologic results was demonstrated in the absence of any correction for T1, T2*, or fat spectral complexity. In that study as well as in ours, the mean signal intensity decay of 12 and 9 ROIs throughout the liver was measured, respectively. The T2 correction is important in cases where iron overload problems might lead to T2 changes. As liver iron deposition is a common secondary feature of many chronic liver diseases, signal intensity loss on in-phase gradient-echo MR images caused by the presence of liver iron is a potential pitfall in the determination of liver fat percentage at opposed-phase MR imaging in chronic liver diseases[16]. Thus the T2* correction is very important in cases where iron overload might lead to T2 changes, but none of our young patients with NAFLD had histological evidence of iron accumulation, consistent with a previous report of adult patients with NAFLD who were seen at a referral center without a special interest in disorders of iron storage[32]. In that report, significant iron histological accumulation was not observed in the majority of patients with NAFLD or its various subtypes.

In conclusion, this study with histopathologic validation shows that the modified Dixon method provides high diagnostic and fat-grading accuracy in obese children with NAFLD. Even if the small number of patients included in our study must be taken into account, the results ob-

tained are highly encouraging and may provide a basis for stimulating further studies which would include a larger number of children.

COMMENTS

Background

Nonalcoholic fatty liver disease (NAFLD) has been increasing over the past three decades, both in children and adolescents, presenting a worldwide problem. NAFLD is characterized by lipid accumulation in the liver, and it represents a disease spectrum that ranges from simple hepatic steatosis to steatohepatitis, and eventually to cirrhosis and liver failure.

Research frontiers

Currently, liver biopsy is considered the gold standard to accurately determine, in a semiquantitative manner, the amount of fatty liver infiltration. However, the authors cannot perform liver biopsy as a screening method to detect NAFLD in the general pediatric population. This is an invasive procedure with the potential of complications, being also prone to sampling error and interobserver variability. Consequently, there is a need in children for non-invasive, safe diagnostic tools to detect and quantify hepatic steatosis as well as to identify hepatic fat regression or accumulation over time.

Innovations and breakthroughs

To date, several imaging techniques are used to detect hepatic steatosis. Ultrasonography is a low-cost, widely used technique for the qualitative assessment of steatosis in children. However, it cannot provide reliable quantitative data, and its sensitivity is reduced in subjects with small amounts of fatty liver infiltration. Standard magnetic resonance imaging (MRI), proton magnetic resonance spectroscopy, and computed tomography may not be feasible for children because of their long scan time, reliance on compliance of the patient, and ionizing radiation. In the pediatric population, among the MRI methods, the 2-point Dixon method as modified by Fishbein is an accepted technique for measuring hepatic fat content. It can also be helpful in identifying fat regression or progression in children, and it has been found useful in differentiating increased liver echogenicity due to simple steatosis from that related to glycogen storage disease. However, no previous studies in children have used the degree of hepatic steatosis at histologic analysis as the reference standard.

Applications

The authors' present results obtained in a homogeneous population with NAFLD indicate that the dual-echo MRI may be a good alternative to biopsy for quantifying fat liver content in obese youngsters and for assessing the relation between hepatic fat fraction and metabolic outcomes in these patients. Furthermore, the authors' results highlight the potential application of this method for tracking longitudinal changes in liver fat content in patients under targeted lifestyle intervention or medical therapy.

Terminology

This study with histopathologic validation shows that the dual-echo MRI provides high diagnostic and fat-grading accuracy in obese children with NAFLD. MRI is easy to perform and interpret, and, therefore, may be suitable for widespread use.

Peer review

Dr. Lucia Pacifico and colleagues quantified the amount of liver fat in pediatric NAFLD by using T1-weighted dual-echo MRI, and assessed the validation of MRI quantification by comparing liver biopsy specimens. The noninvasive quantification method with high sensitivity and specificity is very important to assess the degree of fat accumulation in the liver, specifically in uncooperative or high-risk patients for invasive procedures. This study arouses interest for readers and provides an important clue to evaluate the degree of NAFLD or the improvement of the disease in the treatment or follow-up observation.

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BRIEF ARTICLE

Association of upper gastrointestinal symptoms with functional and clinical characteristics in the elderly

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Abstract

AIM: To evaluate the prevalence of upper gastrointestinal symptoms and their association with clinical and functional characteristics in elderly outpatients.

METHODS: The study involved 3238 outpatients ⩾ 60 years consecutively enrolled by 107 general practitioners. Information on social, behavioral and demographic characteristics, function in the activities of daily living (ADL), co-morbidities and drug use were collected by a structured interview. Upper gastrointestinal symptom data were collected by the 15-items upper gastro-intestinal symptom questionnaire for the elderly, a validated diagnostic tool which includes the following five symptom clusters: (1) abdominal pain syndrome; (2) reflux syndrome; (3) indigestion syndrome; (4) bleeding; and (5) non-specific symptoms. Presence and severity of gastrointestinal symptoms were analyzed through a logistic regression model.

RESULTS: 3100 subjects were included in the final analysis. The overall prevalence of upper gastrointestinal symptoms was 43.0%, i.e. cluster (1) 13.9%, (2) 21.9%, (3) 30.2%, (4) 1.2%, and (5) 4.5%. Upper gastrointestinal symptoms were more frequently reported by females ($P < 0.0001$), with high number of co-morbidities ($P < 0.0001$), who were taking higher number of drugs ($P < 0.0001$) and needed assistance in the ADL. Logistic regression analysis demonstrated that female sex (OR = 1.39, 95% CI: 1.17-1.64), disability in the ADL (OR = 1.47, 95% CI: 1.12-1.93), smoking habit (OR = 1.29, 95% CI: 1.00-1.65), and body mass index (OR = 1.06, 95% CI: 1.04-1.08), as well as the presence of upper (OR = 3.01, 95% CI: 2.52-3.60) and lower gastroenterological diseases (OR = 2.25, 95%CI: 1.70-2.97), psychiatric (OR = 1.60, 95% CI: 1.28-2.01) and respiratory diseases (OR = 1.25, 95% CI: 1.01-1.54) were significantly associated with the presence of upper gastrointestinal symptoms.

CONCLUSION: Functional and clinical characteristics are associated with upper gastrointestinal symptoms. A multidimensional comprehensive evaluation may be useful when approaching upper gastrointestinal symptoms in older subjects.

Key words: Upper gastrointestinal symptoms; Elderly; Upper gastro-intestinal symptom questionnaire for the elderly; Gastroesophageal reflux disease; Disability

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INTRODUCTION

Epidemiological and clinical studies suggest that the prevalence of upper gastrointestinal diseases is particularly high in older subjects[1]. Nevertheless, in older patients the clinical identification of upper gastrointestinal diseases on the basis of the presence of symptoms is very difficult and sometimes misleading. It has been reported that older patients with upper gastrointestinal diseases, such as reflux esophagitis[2] or peptic ulcer disease[3], may report a low prevalence of typical or specific symptoms, several patients recounting only nonspecific or no symptoms at all; thus the presence of nonspecific symptoms has been reported as one of the most important reasons for late diagnoses or even severe complications in elderly patients[4,5]. Conversely, many older subjects report upper gastrointestinal symptoms without a clear relationship with well defined disorders of the upper gastrointestinal tract[6]. Indeed, several clinical and functional conditions may influence the symptom perception and referral to doctor, especially in older people[7]. However, very few studies have been performed on the potential association of upper gastrointestinal symptoms and clinical and functional conditions in old age.

Recently, a diagnostic questionnaire, i.e. upper gastro-intestinal symptom questionnaire for the elderly (UGISQUE), was developed and validated in two independent populations of elderly patients who underwent an upper gastrointestinal endoscopy[8]. The UGISQUE included 15 items grouped into five symptom clusters that comprehensively explore both specific and nonspecific symptoms of the upper gastrointestinal tract in older subjects. The findings of this study suggested the concept that the use of a comprehensive diagnostic tool specifically developed for elderly patients may be useful in reducing misleading and under-recognized diagnoses of upper gastrointestinal diseases.

The aim of this study was to evaluate the prevalence of upper gastrointestinal symptoms and their association with clinical and functional characteristics in a large population of elderly outpatients referred to their general practitioner (GP) by using the UGISQUE.

MATERIALS AND METHODS

Study population

The study was conducted according to the Declaration of Helsinki and the guidelines for Good Clinical Practice. Written informed consent was obtained from the patients or from relatives prior to participation in the study.

The study was carried out by 107 GPs and involved elderly outpatients, in the frame of the IPOD project (Identification of symPtOms to Detect GERD and NERD). In the period between April and October 2007, 3238 patients were screened for enrollment, based on the following inclusion criteria: (1) age $\geqslant$ 60 years; (2) ability to provide an informed consent; and (3) willingness to participate to the study. Exclusion criteria were: (1) a cognitive impairment of grade moderate to severe as evaluated by a short portable mental status questionnaire (SPMSQ)[9] score $\leqslant$ 7; and (2) presence of neoplasm at late stages.

Data collection

Data were obtained by a structured interview of patients and were confirmed by the GP's medical records. General practitioners included all patients seen during a 1-wk period (5 working days) who agreed to participate in the study. All subjects aged 65 years and over who consulted their GP for a medical problem during this 2-wk period were included in the study. Elderly patients who were visited in their home or in nursing homes were not included.

The interview was carried out by skilled GPs. A Computer Assisted Personal Interview (CAPI) instrument followed the interview step by step in collecting and recording the demographic, functional and clinical data as well as the information on drug use and gastrointestinal symptoms. Computerized records were e-mailed to the statistics reference center for evaluation.

Measurements

Information on socio-demographic characteristics (age, gender, marital status, education), body mass index (BMI; body weight/height^2), smoking status, alcohol consumption, coffee use, functional status, comorbidity and drug consumption were collected by a structured interview.

Functional status was evaluated by the Barthel Index[10], which defines the level of dependence/independence of eight daily personal care activities (Activities of Daily Living, ADL), including bathing, eating, personal hygiene, dressing, toilet use, transfer, bladder and bowel control.

The cumulative illness rating scale (CIRS)[11] was used to ascertain presence and severity (5-point ordinal scale, score 1-5) of pathology in each of 13 systems, including cardiac, vascular, respiratory, eye-ear-nose-throat, upper and lower gastroenteric disease, hepatic, renal, genito-urinal, musculo-skeletal, skin disorder, nervous system, endocrine-metabolic and psychiatric behavioral problems. In this study we have considered only the comorbidity assessed as the number of concomitant diseases from moderate to severe levels (grade from 3 to 5). Medication use was defined according to the anatomical therapeutics chemical classification (ATC) code system[12] and the number of drugs used by patients was recorded. Patients were defined as drug users if they took a medication of any drug included in the ATC classification code system.

The UGISQUE questionnaire

The UGISQUE (Table 1) includes 15 items for the description of upper gastrointestinal symptoms divided into five symptom clusters: (1) abdominal pain syndrome [1. stomach ache/pain, 2. hunger pains in stomach or belly];

Table 1 Upper gastroIntestinal symptom questionnaire for the elderly

UGISQUE		Symptoms in the last week	Questions	Response scale[1]			
				0	1	2	3
Abdominal pain syndrome	1	Stomach ache or pain	Has he had pain or discomfort in the upper abdomen or the stomach?				
	2	Hunger pains in stomach or belly	Has he had hunger pains? (an empty, hollow feeling in the stomach and the need to eat between meals)				
Reflux syndrome	3	Heartburn	Has he suffered from heartburn? (a nagging, burning sensation in the upper chest or retrosternal region)				
	4	Acid reflux	Has he had acid regurgitation? (a sudden regurgitation of stomach acid content to the esophagus)				
Indigestion syndrome	5	Nausea	Has he suffered from nausea? (a feeling of discomfort in the stomach that can lead to vomiting)				
	6	Rumbling in the stomach	Has he had rumbling stomach? (i.e. growling, bubbling or gurgling sounds)				
	7	Bloated stomach	Has he suffered from bloating? (i.e. a fullness feeling correlated to gas build-up)				
	8	Burping	Has he suffered from burping? (i.e. bringing up excessive air followed by a sense of relief)				
Bleeding	9	Hematemesis	Has he had hematemesis? (vomiting blood) or melena (black stools)				
	10	Melena					
	11	Anemia	Loss of at least 3 g/dL of hemoglobin in the last 3 mo				
Non-specific symptoms	12	Anorexia	Has he suffered from anorexia? (a loss of appetite or interest in food)				
	13	Weight loss	Has he had a weight loss? (involuntary weight loss in the last 3 mo)				
	14	Vomiting	Has he suffered from vomiting? (involuntary, forceful expulsion of gastric content through the mouth)				
	15	Dysphagia	Has he had dysphagia? (sensation of difficulty in passing the food bolus through the esophagus)				

[1]Response scale: (0) absent = no symptoms are reported by patient; (1) mild = awareness of symptoms, but they are easily tolerated; (2) moderate = symptoms interfering with the normal activities; (3) severe = symptoms that induced inability to perform normal activities or symptoms requiring medical attention. UGISQUE: Upper gastroIntestinal symptom questionnaire for the elderly.

(2) reflux syndrome [3. heartburn, 4. acid reflux]; (3) indigestion syndrome [5. nausea, 6. rumbling in the stomach (i.e. vibrations or noise in the stomach), 7. bloated stomach (i.e. swelling in the stomach), 8. burping (i.e. bringing up air or gas through the mouth)]; (4) bleeding [9. hematemesis, 10. melena, 11. anemia]; (5) non-specific symptoms [12. anorexia, 13. weight loss, 14. vomiting, 15. dysphagia].

The UGISQUE includes a response scale with four grades: (0) absent = no symptoms are reported by patient; (1) mild = awareness of symptoms, but they are easily tolerated; (2) moderate = symptoms interfering with the normal activities; and (3) severe = symptoms that induce inability to perform normal activities or symptoms requiring health intervention. Symptomatic patients were defined as those patients who reported moderate or severe discomfort in at least one item. The recall period for symptom assessment was the last week before the interview.

Further details of the UGISQUE methods have been reported elsewhere[8].

Statistical analysis

Subjects were classified according to the absence/presence of UGISQUE symptoms into two groups. Associations between the two groups of subjects and demographic and clinical characteristics were investigated using the χ^2 test or the Fisher exact test for categorical variables. Group mean values were compared through the generalized linear model procedure, after testing for homoschedasticity with the Levene's test; Welch's Anova was considered in case of heteroschedasticity.

A logistic regression model was then developed. The variable on presence of UGISQUE symptoms was considered as the dependent variable, dichotomized into "no symptoms" *vs* "moderate/severe symptoms". As possible predictors, demographic (sex; age; marital status; education), clinical (comorbidities; drug use; BMI; smoking status; alcohol and coffee consumption) and functional (need of assistance in the ADL) characteristics were selected through a stepwise procedure. Relative risks and 95% confidence intervals were calculated to estimate the association of covariates with the dependent variable.

All statistical analyses were performed using SAS, version 9.1.3 package (Cary, SAS Institute).

RESULTS

During the study period 3238 patients were screened for enrollment; 138 patients were excluded because they did not have valid data on UGISQUE. Thus, complete data on 3100 patients were included in the present analysis: 1547 men, 1553 women; with a mean age of 72.2 ± 7.0 years, and an age range of 60-96 years.

Figure 1 shows the presence of gastrointestinal symptoms, according to the five UGISQUE clusters. The overall prevalence of upper gastrointestinal symptoms was 43.0% (1332 subjects out of 3100). In detail, 13.9% of subjects reported symptoms of abdominal pain, 21.9% reported symptoms of reflux syndrome, 30.2% of subjects reported symptoms of indigestion syndrome, 1.2% of subjects reported bleeding symptoms and 4.5% of subjects reported non-specific symptoms.

Table 2 reports the demographic and clinical characteristics of subjects, stratified by the two study groups. No significant differences were found between the two groups in mean age, education and in the prevalence of smoking habit, alcohol and coffee consumption. In symptomatic subjects, a significantly higher prevalence of women ($P <$

Table 2 Socio-demographic and clinical characteristics of patients divided by the presence of upper gastrointestinal symptoms according to the upper gastro-intestinal symptom questionnaire for the elderly clusters in elderly outpatients

	No symptoms (n = 1768)	Yes symptoms (n = 1332)	P-value
Sex (females, %)	45.8	55.8	< 0.0001
Age (yr, mean ± SD)	72.2 ± 7.0	72.2 ± 6.9	0.8635
Marital status (married, %)	68.3	64.2	0.0185
Education (none/primary school, %)	75.8	78.0	0.1503
Body mass index (kg/m^2, mean ± SD)	26.0 ± 3.6	26.6 ± 3.9	< 0.0001
Smoking status (current smoker, %)	11.2	13.0	0.1288
Alcohol consumption (%)	45.7	42.6	0.0957
Coffee consumption (%)	85.1	87.3	0.0743
Heart diseases (%)	29.3	33.2	0.0198
Hypertension (%)	63.2	65.3	0.2274
Vascular diseases (%)	13.6	16.6	0.0223
Respiratory diseases (%)	15.3	22.2	< 0.0001
Eye-ear-nose-throat diseases (%)	13.6	14.9	0.3103
Upper gastroenterological diseases (%)	20.0	44.8	< 0.0001
Lower gastroenterological diseases (%)	6.6	14.4	< 0.0001
Hepatic diseases (%)	5.6	8.1	0.0046
Kidney diseases (%)	4.5	5.0	0.4692
Genital-urinary diseases (%)	24.7	26.6	0.2434
Skeletal, muscle, skin diseases (%)	43.4	50.6	< 0.0001
Nervous system diseases (%)	5.3	6.5	0.1351
Endocrine-metabolic diseases (%)	22.5	22.0	0.7617
Psychiatric diseases (%)	12.6	21.6	< 0.0001
Number of comorbidities (mean ± SD)	2.8 ± 1.7	3.5 ± 1.9	< 0.0001
4 or more comorbidities (%)	28.7	45.3	< 0.0001
Drug consumption (%)	91.7	93.8	0.0285
Number of drugs (mean ± SD)	2.9 ± 1.6	3.3 ± 1.7	< 0.0001
3 or more drugs (%)	52.5	64.0	< 0.0001
Need of assistance in activities of daily living (%)	14.2	21.5	< 0.0001

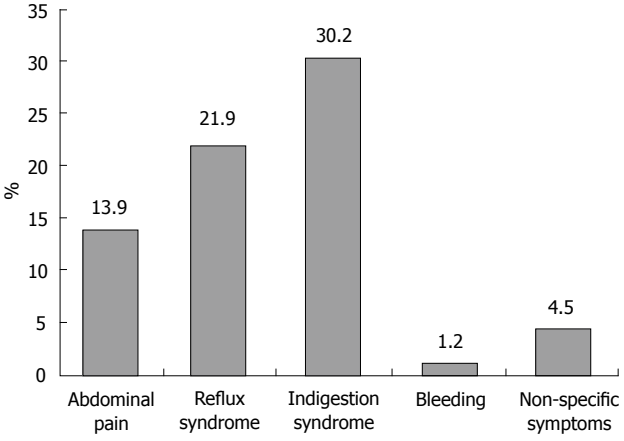

Figure 1 Prevalence of upper gastrointestinal symptoms according to the upper gastro-intestinal symptom questionnaire for the elderly score clusters.

0.0001) and unmarried subjects (P = 0.0185) was found compared to asymptomatic subjects. In the symptomatic group, a significantly higher prevalence of subjects who needed assistance in the ADL than in the asymptomatic subjects was observed (21.5% *vs* 14.2%, P < 0.0001). Moreover, symptomatic subjects had higher prevalence (P < 0.0001) and mean number (P < 0.0001) of concomitant diseases, higher prevalence of drug consumption (P = 0.0285) and higher mean number of drugs taken (P < 0.0001) than asymptomatic subjects.

As regards the concomitant diseases, 45.3% of symptomatic subjects reported 4 or more comorbidities, with respect to 28.7% among asymptomatic subjects (P < 0.0001). Moreover, higher prevalence rates of heart diseases, vascular, respiratory, upper and lower gastroenterological, hepatic, skeletal-muscle-skin and psychiatric diseases were observed in subjects who reported upper gastrointestinal symptoms than asymptomatic subjects.

Table 3 shows the results of a stepwise selection on a logistic regression model, with outcome as to the presence of upper gastrointestinal symptoms according to the UGISQUE clusters. Significant risk factors for upper gastrointestinal symptoms were female gender (OR = 1.39, 95% CI: 1.17-1.64), need of assistance in the ADL (OR = 1.47, 95% CI: 1.12-1.93), actual smoking (OR = 1.29, 95% CI: 1.00-1.65) and BMI (OR = 1.06, 95% CI: 1.04-1.08).

As expected, subjects with upper (OR = 3.01, 95% CI: 2.52-3.60) and lower gastrointestinal diseases (OR = 2.25, 95% CI: 1.70-2.97) were three and two times, respectively, more likely to report upper gastrointestinal symptoms. Moreover, the presence of psychiatric diseases (OR = 1.60, 95% CI: 1.28-2.01) and respiratory diseases (OR = 1.25, 95% CI: 1.01-1.54) were also significant predictors for upper gastrointestinal symptoms according to the UGISQUE clusters.

DISCUSSION

This study reports the results of a wide survey of the prevalence of upper gastrointestinal symptoms and their association with clinical and functional characteristics

Table 3 Risk factors for upper gastrointestinal symptoms according to upper gastro-intestinal symptom questionnaire for the elderly clusters in elderly outpatients

	Odds ratio	95% CI	*P*-value
Sex (female)	1.39	1.17-1.64	< 0.0001
Respiratory disease	1.25	1.01-1.54	0.0430
Upper gastroenterological diseases	3.01	2.52-3.60	< 0.0001
Lower gastroenterological diseases	2.25	1.70-2.97	< 0.0001
Psychiatric diseases	1.60	1.28-2.01	< 0.0001
Need of assistance in activities of daily living	1.47	1.12-1.93	0.0057
Smoking status (actual smoker)	1.29	1.00-1.65	0.0476
Body mass index (kg/m^2)	1.06	1.04-1.08	< 0.0001

in a large population of elderly outpatients. The results showed that demographic, behavioral, functional and clinical characteristics of subjects were significantly associated with the presence of upper gastrointestinal symptoms in old age. These findings suggest that a comprehensive clinical and functional evaluation may be useful in approaching upper gastrointestinal symptoms in older subjects.

The mean age of the IPOD sample was not significantly different from the mean age of the Italian population who were 60-96 years old, as reported by ISTAT for 2006 (72.2 ± 7.0 years *vs* 72.1 ± 10.8 years, respectively; $t = 0.7204$, $P = 0.4713$) [13].

Co-morbidity data, assessed by the CIRS, shows a population that is affected by pathologies in 97.2% of cases, of whom 35.4% reported 4 or more comorbidities. In agreement with previous studies in geriatric populations[14,15], the most frequent diseases were hypertension (63.9%), bone and joint diseases (45.8%), heart diseases (30.7%) and diseases of the upper gastrointestinal tract (30.8%). The high prevalence of comorbidities also reflects the wide use of drugs found in this population. In fact, 92.1% of subjects took at least one drug, with 57.2% of the subjects taking 3 or more. This high prevalence of drug consumption is in agreement with other Italian[16,17] and American studies[18], that have reported drug use prevalence ranging from 90% to 96% in older outpatient populations.

In this study, the cognitive state of subjects was assessed to exclude people who were unable to respond appropriately to the UGISQUE questionnaire. Thus, by excluding subjects with moderate and/or severe cognitive impairment, only 17% of subjects included in the study reported needing assistance in one or more items of ADL. These findings are in agreement with previous data from the Italian national multicenter study of the SOFIA project[16].

In this study we used the UGISQUE, a recently developed questionnaire for the collection of upper gastrointestinal symptoms in elderly patients who underwent an upper gastrointestinal endoscopy. Findings from this study suggest that UGISQUE may also be a clinically useful diagnostic tool for evaluating upper gastrointestinal symptoms in elderly outpatients. Indeed, the survey demonstrates that more than 43% of subjects reported at least one symptom of the upper gastrointestinal tract, i.e. 13.9% of subjects reporting abdominal pain, 21.9% reflux symptoms, 30.2% indigestion symptoms, 1.2% bleeding symptoms and 4.5% non-specific symptoms of anemia (1%), dysphagia (2.7%) and vomiting (0.4%). The presence of this last cluster of symptoms seems to reflect a peculiarity of clinical presentation of the upper gastrointestinal disorders in elderly subjects, as previously reported in endoscopic studies carried out in older populations[2-5] and in agreement with previous data from Italy[19] and Europe[20,21].

Logistic regression demonstrated that female gender was a significant risk factor for reporting upper gastrointestinal symptoms; this finding is in agreement with previous studies performed in general populations[22]. Moreover, disability in the ADL was a significant predictor of upper gastrointestinal symptoms. All these findings confirm a previous study[16], performed in 5500 elderly outpatients, that reported a significantly higher prevalence of symptoms in females, patients who were taking a higher number of drugs, and those who had higher disability.

In agreement with previous studies that reported a significant association between high BMI value and gastrointestinal disorders in young populations[23-25], in this present study, for the first time, we also observed such an association between BMI and upper gastrointestinal symptoms in elderly people. Indeed, changes in gastroesophageal anatomy and physiology caused by obesity, including a diminished lower esophageal sphincter (LES) pressure, the development of a hiatal hernia, and increased intragastric pressure[26], may explain this association.

As expected, the presence of gastroenterological diseases was significantly associated with the risk of presenting upper gastrointestinal symptoms according to the UGISQUE clusters. Very interestingly, however, the presence of psychiatric disorders as well as respiratory diseases was also significantly associated with the presence of upper gastrointestinal symptoms in this population. While it has been reported that psychological distress, depression and anxiety may provoke symptoms of many organ systems, including upper gastrointestinal symptoms that prompt patients to consult a physician[27], at present, this seems to be the first study that has reported such an association in older subjects. This finding is in agreement with previous data reporting a significant association of upper gastrointestinal symptoms with the use of psycholeptic drugs (88% of which were benzodiazepines) in elderly outpatients[16] and supports the concept that subjects with anxiety syndromes and sleep disturbances

may have a greater frequency of functional gastrointestinal disorders, including abdominal pain and/or indigestion syndrome[28]. As regards the significant association between upper gastrointestinal symptoms and respiratory diseases, data do exist that suggest a pathophysiological[29] and clinical[30] link between upper gastrointestinal symptoms and respiratory diseases, especially asthma[31] and chronic obstructive pulmonary disease[32]. The data are in agreement also with a previous finding of a higher use of selective β_2 adrenoreceptor/adrenergic agonist drugs in older subjects with upper gastrointestinal symptoms than in asymptomatic subjects[16].

All these findings suggest that investigation of psychological and/or respiratory problems may be helpful for elderly patients with upper gastrointestinal symptoms.

In conclusion, demographic, functional and clinical characteristics of patients are significantly associated with the presence of upper gastrointestinal symptoms in old age. These findings suggest that a comprehensive clinical and functional evaluation may be useful in approaching upper gastrointestinal symptoms in older subjects.

COMMENTS

Background

Epidemiological and clinical studies suggest that the prevalence of upper gastrointestinal symptoms is particularly high in older age. However, very few studies have been performed on the potential association of upper gastrointestinal symptoms and clinical and functional conditions in old age. Recently, the upper gastroIntestinal symptom questionnaire for the elderly (UGISQUE) was developed and validated in different populations of elderly patients who underwent an upper gastrointestinal endoscopy.

Research frontiers

Gastrointestinal symptoms are widely diffused and frequently misdiagnosed in the elderly population. Their impact on clinical and functional conditions may influence the performance in the activities of daily living, therapeutic compliance, nutrition status, and finally, the quality of life. A multidimensional approach may improve clinical and functional evaluation of the older patient with gastrointestinal symptoms to better identify therapeutic and health care programs.

Innovations and breakthroughs

Specific functional and clinical characteristics, such as disability in the activities of daily living (ADL), body mass index and the presence of gastroenterological, psychiatric and respiratory diseases, are significantly associated with the presence of upper gastrointestinal symptoms in older patients. A comprehensive clinical and functional evaluation by means of diagnostic tools specific for older people (ADL, UGISQUE) may be useful in approaching upper gastrointestinal symptoms in older subjects

Applications

The functional and clinical definition of older patients with gastrointestinal symptoms could lead to better care in clinical practice. The UGISQUE is easy to administer and effective in predicting gastrointestinal disorders in older patients. Further prospective studies on the application of the UGISQUE for predicting gastrointestinal adverse drug reactions and other adverse outcomes, such as disability in the activities of daily living, are needed to evaluate the role of a multidimensional approach in improving the care of older patients.

Peer review

This cross-sectional study analyzed relationships of upper gastrointestinal symptoms and functional or clinical characteristics in elderly outpatients. This manuscript is well-written.

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BRIEF ARTICLE

Prevalence, genotypes and factors associated with HCV infection among prisoners in Northeastern Brazil

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Abstract

AIM: To determine hepatitis C virus (HCV) seroprevalence and its genotypes, and to identify the factors associated with HCV infection.

METHODS: This cross-sectional study, conducted in two prisons (one male and one female) in the State of Sergipe, Brazil, comprised 422 subjects. All of the prisoners underwent a rapid test for the detection of HCV antibodies. Patients with a positive result were tested for anti-HCV by enzyme linked immunosorbent assay and for HCV RNA by qualitative polymerase chain reaction (PCR). The virus genotype was defined in every serum sample that presented positive for PCR-HCV. In order to determine the factors independently associated with positive serology for HCV, multivariate logistic regression was used.

RESULTS: HCV seroprevalence was 3.1%. Of the 13 subjects with positive anti-HCV, 11 had viremia confirmed by PCR. Of these, 90.9% had genotype 1. A total of 43 (10.2%) were injecting drug users, and HCV seroprevalence in this subgroup was 20.6%. The variable most strongly associated with positive serology for HCV was use of injecting drugs [odds ratio (OR), 23.3; 95% confidence interval (CI), 6.0-90.8]. Age over 30 years (OR, 5.5; 95%CI, 1.1-29.2), history of syphilis (OR, 9.8; 95%CI, 1.7-55.2) and history of household contact with HCV positive individual (OR, 14.1; 95%CI, 2.3-85.4) were also independently associated with HCV infection.

CONCLUSION: Most of the HCV transmissions result from parenteral exposure. However, there is evidence to suggest a role for sex and household contact with an infected subject in virus transmission.

Key words: Hepatitis C; Prisoners; Drug abusers; Cross sectional analysis; Brazil

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INTRODUCTION

Hepatitis C virus (HCV) is one of the main causes not only of chronic viral hepatitis, but also of cirrhosis and end-stage liver disease in the world[1]. Hepatitis C, given its treatment costs, high morbidity and mortality, generates a significant burden in healthcare systems. According to the World Health Organization, there are over 170 million people with chronic hepatitis C and approximately 3 to 4 million new cases each year[2].

HCV seroprevalence in the general population has wide geographical variation. Studies performed in Brazil have shown an anti-HCV prevalence of 5.9% in the

Amazon region, 0.9% in the State of Rio de Janeiro and 0.34% in the State of Santa Catarina[3].

There are six HCV genotypes, with several subtypes. For each genotype, there is a different pattern of treatment response and, consequently, a distinct therapeutic approach[4,5]. There is a wide geographical variation when it comes to genotype distribution, so that genotypes 1, 2 and 3 are more frequent in Europe, the USA and Japan; genotype 4 in central Africa, Egypt and the Middle East; genotype 5 in South Africa; and genotype 6 in Asia. Brazil in general has a higher prevalence of genotype 1, followed by genotype 3[6].

Most HCV transmissions are due to parenteral exposure. It has been estimated that HCV is 10 times more infectious than HIV, per unit of blood, requiring less exposure to reach high prevalence[1]. Other routes have been described, such as sexual and vertical transmission, but these are less common than the parenteral one. Risk factors already proposed include use of injecting drugs (ID), tattoos, occupational blood exposure and hemodialysis[4,5]. It is important to highlight that HCV prevalence is higher in certain groups, such as prisoners[4]. Although these subjects represent only 0.8% of the American population, approximately 39% of the cases of chronic HCV infection have a history of imprisonment[7]. There are several international studies which determined hepatitis C prevalence in prisons, but studies in Latin America are scarce.

The following factors are related to higher prevalence of HCV in prisoners: duration of incarceration, use of ID, adverse socioeconomic situation and poor health care. Therefore, there is a potential public health issue, since the prison system works as a concentrator of hepatitis C subjects and a dissemination center of this infection. Risk behavior may precede imprisonment and continue afterwards[8,9].

A large number of HCV carriers are asymptomatic and remain undiagnosed for a long time, resulting in further complications, such as liver cirrhosis, liver failure and hepatocellular carcinoma. These asymptomatic patients also represent a natural reservoir of the disease, and a source of dissemination[2].

Prisons in the State of Sergipe, Brazil, do not currently screen for HCV and there are no statistics concerning HCV status of the prisoners incidentally diagnosed. Given the regional variation of HCV prevalence among prisoners, the lack of data in Sergipe and its importance in order to implement effective strategies to prevent HCV transmission, we conducted a study of the prevalence of HCV infection among prisoners, as well as HCV genotypes in viremic subjects, and factors associated with positive serology for HCV.

MATERIALS AND METHODS

Study population

This was a cross-sectional study performed in two prisons (one male and one female) in the State of Sergipe, Brazil. The study was conducted in the male prison in September 2009 and in the female one in February 2010. Subjects eligible for this study included all prisoners who agreed and signed the consent form.

Data collection

Structured and individual interviews were privately conducted. Before the interview, it was explained that any collected information would be kept confidential. Subjects' names were not collected. Each questionnaire received a code number, in order to allow further connection to its respective blood sample, and was formed by closed questions, including sociodemographic characteristics and risk behaviors, such as the ones involving drug use and sexual practices, before and during the imprisonment.

Blood sample collection

After the interviews, the subjects underwent a rapid test for the detection of HCV antibodies (kit HCV Rapid Test Bioeasy). Peripheral blood from those with a positive result in the rapid test was collected by a finger prick with a single use lancet. Then, six blood spots (two to confirm serology and four for molecular biology) were blotted onto high-quality filter paper (Schleicher & Scheull 903). For each circle, approximately three drops of blood were used. Afterwards, filter paper was left to dry at room temperature for 30 min or until the blood spot was completely dry. The material was kept in aluminum envelopes, along with a bag containing silica gel, and posted to Genoma Center. Only patients with positive anti-HCV had their results confirmed by qualitative polymerase chain reaction (PCR). Those with positive qualitative PCR had the HCV genotype determined.

Statistical analysis

Continuous variables are reported as mean ± SD, and analyzed using the Mann-Whitney *U* test. Categorical variables are presented as percentages and analyzed using Chi-square (χ^2) or Fisher's exact tests. $P < 0.05$ were considered to be statistically significant. In order to identify parameters independently associated with positive serology for anti-HCV, a logistic regression model was determined. Variables with $P < 0.1$ in univariate analysis were included in the multivariate analysis. Before indicating which variables would be inserted in the initial model, multicollinearity issues were solved. Backward selection of variables was performed, with entry and retention set at a significance level of 0.05. The discrimination capability of the final model was evaluated through the area under the ROC curve (AUC), and the goodness-of-fit of the logistic model was verified by the Hosmer-Lemeshow test ($P > 0.05$). Variables that continued in the model were tested for possible interaction among them. Each interaction (between two variables) was individually tested and then added to the final model if they showed statistical significance. Statistical analyses were performed using the Statistical Package for the Social Sciences, version 17 (Chicago, IL, USA).

This study was approved by the Research Ethics Committee of the Federal University of Sergipe in April 2009

Table 1 Characteristics of prisoners with positive serology for hepatitis C virus in Sergipe, Brazil

	n (total = 13[1])	%
Viremia (RNA)	11	84.6
Genotype		
1a	6	54.5
1b	1	9.1
1	3	27.3
3	1	9.1

[1]One patient refused to provide a blood sample for polymerase chain reaction.

Table 2 Characteristics of 422 prisoners in Sergipe, Brazil

	n (total = 422)	%
Gender		
Male	303	71.8
Female	119	28.2
Age (yrs)		
Mean (SD)	32.7 (8.8)	21.1
⩽ 25	89	28.4
25-30	120	19.4
30-35	82	14.5
35-40	61	16.6
> 40	70	
Education level		
Uneducated	45	10.7
Less than high school	312	73.9
High school or more	65	15.4
Religion		
Catholic	238	56.4
Protestant	86	20.4
Other	98	23.2
Race		
White	141	33.5
Black	106	25.2
Multiracial	160	38.0
Other	14	3.3
Marital status		
Single	167	39.6
Married	117	27.7
Widowed	15	3.6
Stable union	111	26.3
Divorced	12	2.8
HCV seropositivity	13	3.1

HCV: Hepatitis C virus.

(N° CAAE 0038.0.107.000-09). Prison authorities did not have access to any questionnaires or blood samples.

RESULTS

Prevalence and genotypes

Of 382 men, 303 (79%) agreed to participate in the research, and of 137 women, 119 (87%) participated. All included subjects underwent the rapid test for the detection of antibodies to HCV, but one of the subjects with a positive result for this test did not provide a blood sample for qualitative PCR. HCV seroprevalence was 3.1%. From the 13 subjects with positive anti-HCV, eleven had confirmed viremia by PCR. Of these, 10 (90.9%) had genotype 1 (Table 1).

Table 3 Characteristics of injecting drug users in prisoners in Sergipe, Brazil

	n (total = 43)	%
Drug		
Cocaine	21	48.8
Heroine	2	4.7
Benzydamine	12	27.9
Other	11	25.6
Duration of use (mean in years)	5.05 (5.7)	
Use in the last 2 mo	5	11.6
Started to use during imprisonment	3	7.0
Uses inside the prison	5	11.6
Needle sharing	14	32.6
Anti-HCV (+)	9	20.9

HCV: Hepatitis C virus.

Subject characteristics

The mean age of the subjects was 32.7 (± 8.8) years, and the most frequent age group was 25-30 years (28.4%). A total of 303 (72%) were men (recruited in the State Penitentiary of Areia Branca); and 119 (28%) were women (recruited in the Female Penitentiary of Sergipe). Seventy-two (60.5%) women and 39 (12.8%) men had drug dealing or drug use as the reason for imprisonment. Many of the subjects were multiracial (38%) and single (39.6%). More than half of the population declared themselves as Catholics (56.4%) (Table 2).

Sexual practices and drug use

A total of 150 (35.5%) subjects reported previous sexually transmitted disease (STD), of which gonorrhea was the most frequently declared. Two hundred and forty-seven (58.5%) participants affirmed that they had paid or been paid for sex, and 109 (25.8%) rarely or never used condoms. Regarding drug use, 311 (73.7%) subjects had used illegal drugs, while 10.2% stated that they had used ID. Among ID users (IDU) (n = 43), 32.6% shared needles and syringes or other injecting equipment, 7% started injecting in prison and 11.6% continued injecting at the time of the interview. HCV seroprevalence among IDU was 20.6% (Table 3).

Univariate analysis

As shown in Tables 4 and 5, we studied the association between serologic HCV status and sexual practices, drug use, sociodemographic and behavioral characteristics. Positive serology for HCV was significantly associated with the following characteristics: previous imprisonment; household contact with a HCV carrier; history of tattooing, though there was no significance considering only tattooing inside prison; previous syphilis; and use of illegal drugs, including inhaled cocaine, marijuana and ID. However, use of crack was not associated with HCV infection. Moreover, those with positive anti-HCV presented significantly higher mean age, higher mean CAGE score and higher mean duration of use of inhaled cocaine, marijuana and ID. There was no significant association between HCV and marital status (data not shown), gender, ethnicity, religion and sexual orientation. Rarely or never having

Table 4 Socio-demographic and behavioral characteristics of 422 prisoners by serologic hepatitis C virus status, Sergipe, Brazil

Variable	HCV (-)	HCV (+)	*P*-value
Gender (male)	295 (72.1%)	8 (61.5%)	0.531
Mean age (yrs)	32.56 (8.8)	36.77 (6.4)	0.019
Ethnicity (white)	137 (33.6%)	4 (30.8%)	0.833
Christian religion	314 (76.8%)	10 (76.9%)	0.990
Family income (R$)	777 (1205)	684 (472)	0.923
Years of schooling (mean)	6.5 (5.4)	6.4 (2.9)	0.565
Mean incarcerated time (mo)	43.5 (40)	30.6 (30)	0.253
Previous imprisonment	139 (34.0%)	11 (84.6%)	< 0.001
History of alcohol use	298 (73.0%)	10 (73.9.0%)	0.756
CAGE (mean)	1.00 (1.2)	1.69 (1.2)	0.033
Household contact with HCV carrier	17 (4.2%)	4 (30.8%)	0.002
History of tattooing	244 (59.7%)	13 (100%)	0.003
Tattooing inside prison	110 (27.0%)	6 (46.2%)	0.202
History of piercing	39 (9.5%)	-	0.620
Previous blood transfusion	39 (9.5%)	-	0.620
Previously shared razors, toothbrushes, nail trimmers or scissors	241 (58.9%)	8 (61.5%)	0.850
Getting wounded by a sharp weapon in a struggle	129 (31.5%)	3 (23.1%)	0.762
Total	409 (96.9%)	13 (3.1%)	

HCV: Hepatitis C virus; CAGE: Cut down, Annoyed by criticism, Guilty e Eye-opener.

Table 5 Sexual practices and drug use of 422 prisoners by serologic hepatitis C virus status, Sergipe, Brazil

Variable	HCV (-)	HCV (+)	*P*-value
Never or rarely used condom	103 (25.3%)	6 (46.2%)	0.109
Sexual orientation (Heterosexual)	315 (77.2%)	13 (100%)	0.081
Number of partners in the last year (mean)	2.44 (8.1)	2.54 (5.3)	0.594
Age at first sexual intercourse (mean in years)	14.54 (2.3)	15.00 (1.7)	0.456
Sexually transmitted diseases	144 (35.2%)	6 (46.2%)	0.557
History of genital herpes	6 (1.5%)	1 (7.7%)	0.198
History of syphilis	17 (4.2%)	3 (23.1%)	0.019
History of gonorrhea	109 (26.7%)	2 (15.4%)	0.528
Partner			
HCV (+)	4 (1.0%)	2 (15.4%)	0.012
Illegal drug user	223 (54.5%)	10 (76.9%)	0.110
Injecting drug user	24 (5.9%)	1 (7.7%)	0.553
Previous imprisonment	124 (30.3%)	5 (38.5%)	0.548
Ever paid or been paid for sex	242 (59.2%)	5 (38.5%)	0.136
History of illegal drug use	298 (72.9%)	13 (100%)	0.025
Inhaled cocaine	194 (47.4%)	11 (84.6%)	0.008
Duration of inhaled cocaine (mean in mo)	107.6 (273.1)	86.1 (80.3)	0.006
Marijuana	277 (67.7%)	13 (100%)	0.012
Duration of marijuana use (mean in mo)	129.5 (220.5)	198.5 (101.4)	0.002
Crack	129 (31.5%)	5 (38.5%)	0.561
Duration of crack use (mean in mo)	77.9 (254.1)	162.0 (371.9)	0.369
History of injecting drug use	34 (8.3%)	9 (69.2%)	< 0.001
Duration of injecting drug use (mean in mo)	26.1 (148.0)	261.2 (445.7)	< 0.001
Use of injecting drugs inside prison	5 (1.2%)	-	0.855
Ever shared needles and syringes or other injecting equipment	11 (2.7%)	3 (23.1%)	0.007
Total	409 (96.9%)	13 (3.1%)	

HCV: Hepatitis C virus.

used a condom, and a history of paying or being paid for sex were not associated with a higher HCV seroprevalence. An association was not observed between STD and HCV, except for syphilis. Regarding partner characteristics, the only variable that was significantly associated with HCV was a positive anti-HCV partner. Both groups (positive and negative anti-HCV) had similar mean duration of imprisonment, mean number of sexual partners in the last year, mean age at the time of the first sexual intercourse, mean family income and average years of education.

Multivariate analysis

Multivariate logistic regression was performed using HCV status as the dependent variable. Among variables that presented collinearity issues (data not shown), the ones with greater clinical impact were chosen. Continuous variables were turned into dichotomous ones, using the receiver operating characteristic (ROC) curve to choose the cut-off point that presented the best discrimination capability. Table 6 shows the variables independently associated with positive serology for HCV, and the strongest

Table 6 Multivariate logistic regression of characteristics associated with positive serology for anti-hepatitis C virus

Characteristic	Crude odds ratio	Adjusted odds ratio	95%CI	*P*-value
Injecting drug user	24.8	23.3	6.0-90.8	0.0000
History of household contact with HCV carrier	10.2	14.1	2.3-85.4	0.004
Previous syphilis	6.9	9.8	1.7-55.2	0.009
Age > 30 yr	5.6	5.5	1.1-29.2	0.043

Hosmer-Lemeshow test (P = 0.420). HCV: Hepatitis C virus; CI: Confidence interval.

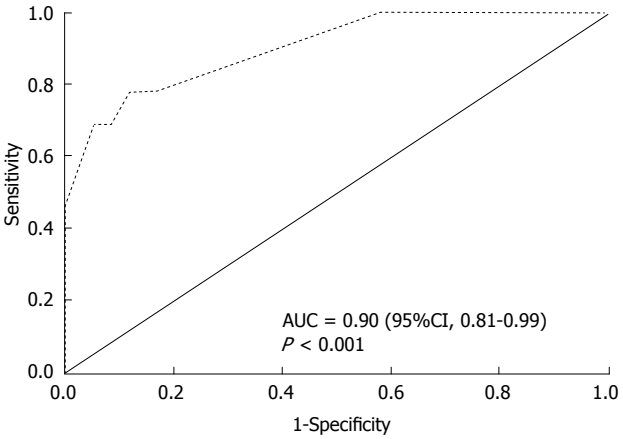

Figure 1 Multivariate model receiver operating characteristics curve. AUC: Area under the curve; CI: Confidence interval.

association verified was between positive serology for HCV and use of ID (OR, 23.3; 95% confidence interval (CI), 6.0-90.8). The chance of presenting with positive anti-HCV was 14 times higher among subjects that had lived with an HCV carrier compared with those without this history, even after adjusting for other variables, such as use of ID. Age over 30 years and previous syphilis were also independently associated with positive serology for HCV. None of the tested interactions was statistically significant (data not shown). The Hosmer-Lemeshow test had P = 0.420, so the final model was considered to be adequate. Moreover, the area under the ROC curve was 0.90 (95%CI, 0.81-0.99; $P < 0.001$), and the discrimination capability of the final model was considered to be good (Figure 1).

DISCUSSION

To our knowledge, this is the first study to determine HCV seroprevalence, and factors associated with this infection in inmates in Northeastern Brazil. HCV seroprevalence observed in this research was 3.1%, which is higher than that found in the general population (1.14%)[4]. Nevertheless, this percentage is below expectation, especially if we consider the following aspects: the prevalence among prisoners described in other regions of Brazil or even in other countries; the absence of damage control programs in the evaluated prisons; and higher prevalence is expected in an already imprisoned population[10]. In Brazil, the studies conducted by Guimarães *et al*[5], Burattini *et al*[8] and Coelho *et al*[11] verified positive serology for HCV in 41%, 34% and 9% of inmates, respectively. Catalan-Soares *et al*[12], in a study involving 63 prisoners, observed lower HCV seroprevalence (6.3%), but it was still twice that found in the present study. Moreover, Strazza *et al*[9], in a study in a Brazilian female prison, found an HCV seroprevalence of 16.2%. In the USA, in an incarcerated population, 16%-41% presented serological evidence of HCV infection, and approximately 12%-35% had chronic hepatitis C[7]. Experiences in Spain, England and France reported a prevalence of 48%, 30% and 30%, respectively[13].

It is important to point out that, depending on the studied region, even inside one country, HCV seroprevalence presents a wide variation. HCV seroprevalence seems to increase along with the proportion of IDU. Vescio *et al*[10], in a meta-analysis, concluded that the most important source of heterogeneity among studies is the different proportion of IDU in each population. In addition, according to the same study, HCV seroprevalence among IDU also has an important influence on this heterogeneity. In our research, 10.2% of the inmates declared that they had already used ID. This proportion varies from 3% to 69% throughout the world[10]. Perhaps the explanation for the proportion of IDU not being as high as expected in our population is linked to the low percentage of imprisonments motivated by drug dealing or drug use, and also to other social and cultural characteristics not assessed in the present investigation.

It has been reported that HCV-RNA may be detected in 40%-90% of subjects with positive anti-HCV[2]. In our population, there was a high proportion of positive HCV-RNA-84.6% of the inmates with positive anti-HCV-that is, subjects capable of infecting others. This information corroborates the hypothesis that prisoners are important carriers of HCV and a potential source of transmission[7,13], especially when many of them will return to society.

Genotyping of hepatitis C provides not only epidemiological data, but also information from the perspective of the therapeutic response. Treatment offers better results for genotypes 2 and 3[14]. Genotyping was performed in all 11 cases in which HCV-RNA was detected. We only identified genotypes 1 and 3, and genotype 1 was the most frequent (90.9%). Other studies also showed genotype 1 as the most frequent[14-18], but always with a higher frequency of genotype 3 when compared with that in the present study.

There was no significant difference in HCV seroprevalence with respect to gender. Previous results in the medical literature are conflicting, in spite of one meta-analysis demonstrating a discreet predominance of positive anti-HCV among women[10]. However, this meta-analysis did not consider confounding variables that could be respon-

sible for such an association. One proposed confounding factor is the higher proportion of women incarcerated for drug dealing or drug use[19]. Regarding ethnicity, previous studies showed that Caucasians had a higher chance of presenting positive for anti-HCV[20,21], but race did not influence the serological status in our population.

Age over 30 years was independently associated with positive serology for HCV, which was also observed in other studies[11,16,20]. This finding may be explained by a higher risk of exposure to HCV over the years. Guimarães *et al*[5] found a different result, in which younger subjects had a higher chance of infection, but this finding might represent a local peculiarity.

Self-reported use of drugs, including ID, has been shown to be both valid and reliable[22]. Despite statistical significance in univariate analysis, inhaled cocaine did not remain in the final model. In the medical literature, HCV transmission through sharing materials used for inhaling cocaine remain controversial[23]. Use of ID remained in the final model and it was the factor most strongly associated with positive serology for HCV. This finding is consistent with those of other studies[9-11,20,24-28] and supports the effectiveness of HCV parenteral transmission. Use of ID during imprisonment has been reported by 3%-28% of inmates[7]. In the present study, only a minority of IDU (11.6%) referred to injecting inside prison. As previously mentioned[10], difficulty in obtaining equipment for use of ID can lead to sharing, making HCV transmission easier. In our population, 32.6% declared that they shared needles, so a needle exchange program might be effective.

It has been demonstrated that HCV seroprevalence was three times higher in prisoners who had tattoos, when compared to those who did not[10]. In spite of observing an association between tattoos and HCV in univariate analysis, we did not identify an independent effect of this variable in HCV seroprevalence. In accordance with Hellard *et al*[29], in this study tattoos were strongly associated with use of drugs, presenting multicollinearity issues in multivariate analysis. However, it is important to point out that tattooing inside prison was not associated with positive anti-HCV, unlike previous findings[29]. Therefore, this might not be an important route of transmission in the studied population.

Other proposed routes of transmission do not seem to be relevant in the studied population. All of the subjects with positive anti-HCV denied a history of blood transfusion. Sharing personal care items and a history of getting wounded by a sharp weapon in a struggle occurred equally in prisoners in both groups-positive and negative anti-HCV.

It has been suggested that previous imprisonment would be associated with HCV infection[5]. Despite its significance in univariate analysis, this variable was not independently associated with a positive serology for HCV. Subjects with previous imprisonment, when compared to those without this background, had a higher proportion of IDU, and for this reason would present higher HCV seroprevalence. For IDU, imprisonment is a fairly common event, due to the illegality of their behavior or to crimes committed because of the high cost of drugs on the black market[30].

Sexual transmission of HCV is controversial[10]. Some authors consider this route of transmission ineffective[31,32], which is corroborated by the fact that use of condoms did not seem to protect the studied prisoners from HCV infection. An association between HCV and syphilis has been described[5,33,34] and we observed that a history of previous syphilis was independently associated with positive serology for HCV, even after adjustment for ID use and other confounding factors. Syphilis may be a marker of sexual promiscuity, but variables that evaluate this aspect, such as number of partners in the last year, other previous STD and having already paid or been paid for sex, were not associated with HCV infection. We suggest that HCV is not associated with STD in general, but with genital ulcers, inherent in syphilitic infection. As previously suggested[35], blood containing HCV would penetrate more effectively through injured genital skin. Other studies corroborate the hypothesis of genital ulcers influencing HCV transmission[23,35]. Therefore, in spite of not being the main route, sexual transmission seems to have a role in this population.

Some studies[25,35] stated that homosexuality would lead to a higher chance of HCV infection. This association was not confirmed in our study. All the subjects with positive anti-HCV denied homosexual practices. This finding demonstrates that perhaps the association found in other studies might be related to risk behaviors, instead of homosexuality itself, which is in accordance with Fox *et al*[26] and Mahfoud *et al*[15].

A previous partner infected by HCV and household contact with an HCV carrier were associated with positive serology for HCV. However, only household contact with an infected subject was retained in the final model. One possible explanation would be that, although both groups referred equally to sharing personal care items, household contact may lead to blood to blood contact by common use of such objects sporadically or in an unobserved manner.

Most HCV infections are acquired before imprisonment[10,36], but transmission inside prisons has been reported[22,37], which justifies implementation of prevention programs, especially in populations with a high proportion of susceptible subjects, such as the one in this study.

Our study has some limitations. This research included populations from two institutions, which makes external validity difficult for other populations in the world or even in other institutions in Northeastern Brazil. Some prisoners may not have answered some questions correctly, especially the ones concerning STD, chronological aspects and with legal implications, such as the use of ID inside prison. Strengths of the study include: a short period of data collection, showing the real HCV prevalence at that moment; interviews were conducted before test results were available, minimal ascertainment bias; and high sensitivity of the rapid test used, which avoids underestimation of HCV seroprevalence. It has been described that this test has both sensitivity and specificity close to 100%[38,39].

The data shown corroborate the hypothesis that, in the studied prisoners, parenteral HCV transmission is the main route. However, there is evidence to suggest a role for sex and household contact in HCV transmission. This study demonstrated low HCV seroprevalence, with a high proportion of subjects having genotype 1. The large number of susceptible individuals in the studied population, the poor response of genotype 1 to antiviral treatment and the progress of chronic infection make prevention programs more important. It has been shown that treatment is cost-effective[40], even in an imprisoned population[41]. Entering the prison system could be an opportunity to treat and break the transmission cycle. In addition, treatment adherence and side effects could be closely monitored.

Data on hepatitis C in Brazil are still scarce, so more epidemiological studies are necessary in order to guide and monitor prevention programs. We defend the offer of anti-HCV tests for those with a higher chance of infection, such as those with a previous history of syphilis, those aged over 30 years, IDU, or those who had lived with an HCV carrier, to improve the positive predictive value of the tests. This active research should be guided, if possible, by local studies. Even the ones not eligible for treatment may reduce transmission and progress to end-stage liver disease after receiving counseling.

COMMENTS

Background

Most studies have shown that hepatitis C virus (HCV) prevalence is higher in certain groups, such as prisoners. Duration of incarceration, use of injecting drugs, adverse socioeconomic situation and poor health care are related to higher prevalence of HCV in this population.

Research frontiers

There is a wide regional variation of HCV prevalence among prisoners and studies that aimed to determine HCV prevalence in prisoners in Latin America are scarce. Most HCV transmission results from parenteral exposure, but other routes have been described. Sexual transmission is still controversial. There is a potential public health issue, since the prison system works as a concentrator of hepatitis C and a dissemination center of this infection. Many HCV carriers are asymptomatic and represent a natural reservoir of the disease, and a source of dissemination

Innovations and breakthroughs

The data shown corroborate the hypothesis that parenteral transmission is the main route. There is evidence to suggest the role of sexual and household contact in HCV transmission. Household contact may lead to blood to blood contact, by common use of personal objects sporadically or in an unobserved manner. This study also demonstrated a low HCV seroprevalence, probably due to the low proportion of injecting drug users.

Applications

Since this study describes HCV prevalence in a regional prison, it may allow the development of strategies to guide and monitor prevention programs. Household contact with an infected subject must not be neglected, and, in the future, may be a risk factor to be considered in routine evaluation.

Peer review

The obtained results show that most of the HCV transmissions are due to parenteral exposure and that transmission through sex and household contact with an infected subject play an important role. The paper is well written and the results appear to be well described and critically discussed (also in consideration of other studies).

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BRIEF ARTICLE

Risk of fracture in celiac disease: Gender, dietary compliance, or both?

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Abstract

AIM: To determine the incidence of peripheral fractures in patients with celiac disease (CD) and the effect of treatment on fracture risk.

METHODS: We compared the incidence and risk of peripheral fractures before and after diagnosis between a cohort of 265 patients who had been diagnosed with CD at least 5 years before study entry and a cohort of 530 age- and sex-matched controls who had been diagnosed with functional gastrointestinal disorders. Data were collected through in-person interviews with an investigator. The overall assessment window for patients was 9843 patient-years (2815 patient-years after diagnosis).

RESULTS: Compared with the control group, the CD cohort showed significantly higher incidence rate and risk of first peripheral fracture before diagnosis [adjusted hazard ratio (HR): 1.78, 95% CI: 1.23-2.56, $P < 0.002$] and in men (HR: 2.67, 95% CI: 1.37-5.22, $P < 0.004$). Fracture risk was significantly associated with the classic CD presentation with gastrointestinal symptoms ($P < 0.003$). In the time period after diagnosis, the risk of fractures was comparable between the CD cohort and controls in both sexes (HR: 1.08, 95% CI: 0.55-2.10 for women; HR: 1.57, 95% CI: 0.57-4.26 for men).

CONCLUSION: CD patients have higher prevalence of fractures in the peripheral skeleton before diagnosis. This is associated with male sex and classic clinical presentation. The fracture risk was reduced after the treatment.

Key words: Celiac disease; Fracture risk; Peripheral fractures; Gluten-free diet; Sex difference

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INTRODUCTION

In the past two decades, the effect of celiac disease (CD) on bone health has been extensively studied[1]. Osteopenia or osteoporosis detected by bone mineral density measurements has been seen in > 50% of patients at the time of their diagnosis of CD[2-5]. Data have accumulated to show that low bone mineral density is more common in adults and is present even if patients have atypical or asymptomatic CD at the time of diagnosis[1,6-8]. The impact of CD treatment on bone density has received some attention but remains under-explored[6,9-13]. Few studies have evaluated the risk of fractures, a more relevant clinical endpoint than bone mineral density, in CD patients[1,14-21]. A recent systematic review with a meta-analysis that pooled 20 995 CD patients and 97 777 controls, from eight studies published between 2000 and 2007, concluded that CD patients have a 43% higher risk of fractures compared with people without CD [pooled odds ratio: 1.43, 95% CI: 1.15-1.78][22]. A more recent study, published after the systematic review, confirmed the significant association between CD and increased fracture risk[23]. It should be noted that available studies are limited by heterogeneity in study methodology, patient population, and potential biases; thus, results have varied widely[22]. Nevertheless, evidence suggests that physicians should carefully examine the bone health of patients with CD.

Current evidence is mixed on fracture risk in patients who are maintaining a gluten-free diet (GFD) to treat CD[24]. In a seminal study that we have conducted previously[14], we found a lower rate of any type of fractures among treated CD patients compared with untreated CD patients. However, three subsequent studies with different study designs have reported different findings[18,20,21,23]. All of these studies showed that the risk of fractures in diagnosed and treated CD patients was significantly higher than in healthy controls. A fifth study did not show any significant difference between patients, before and after diagnosis[16]. Moreover, a Swedish population-based study[21] recently has reported that the elevated risk of fractures among CD patients remains unchanged 20 years after diagnosis. These studies employed different methodologies, which makes it difficult to extrapolate their findings to the general CD patient population.

Theoretically, dietary treatment can improve patients' bone health and reduce the risk of falls, which in turn, may reduce the risk of fractures[1]. Given the equivocal evidence, a better understanding of the effect of GFD on patients' fracture risk is of clinical importance to physicians and patients. The present study aimed to assess the risk of fractures in a large cohort of CD patients and the effect of GFD on this risk.

MATERIALS AND METHODS

Patients and controls

A cohort of 265 adult patients (> 18 years old) with a diagnosis of CD and a cohort of 530 age- and sex-matched controls with functional gastrointestinal disorders were recruited at the gastroenterology units in four medical centers in Buenos Aires, Argentina from March 2007 to November 2009. The CD diagnosis was based on a combination of positive clinical findings (presence of symptoms or risk factors such as family history), characteristic CD enteropathy in duodenal biopsy at the time of diagnosis, positive CD-specific serology, and a positive clinical and/or histological response to a GFD. The presence of positive CD-related serological tests at diagnosis (anti-gliadin antibodies, anti-tissue transglutaminase antibodies and/or antiendomysium antibodies) was considered sufficient for a diagnosis of CD without follow-up assessments. Patients were enrolled in the study if their diagnosis of CD had been established at least 5 years prior to their entry to the study. Confirmation of the CD diagnosis was required at the time of enrollment irrespective of the patient's compliance with the GFD. We excluded 163 patients who were diagnosed with other disorders that could independently reduce bone health (e.g. uncontrolled thyroid dysfunction, rheumatoid arthritis, inflammatory bowel disease, diabetes), who took medications that may affect bone metabolism (e.g. steroids, calcium, vitamin D, alendronate, anticonvulsants, thyroid hormones, estrogen or androgen replacement), and who had complicated CD. Two controls subjects attending the same gastroenterology unit were enrolled for each CD patient in the study. These control subjects were selected if a definitive diagnosis of functional gastrointestinal disorder based on Rome Ⅲ criteria was confirmed by their medical records, and if they had the same age and sex as the enrolled CD patient.

Study design and data collection

Medical history related to CD and fractures was taken from the CD patient and control cohorts using a standard questionnaire through in-person interviews conducted by the investigators, who were experienced with CD. The interview included demographic information; age at which the patient began to experience CD-like symptoms such as diarrhea, weight loss and anemia; age at diagnosis of CD; gynecological and obstetric history; and fracture history, including the type and severity of trauma that produced the fracture and the site of the fracture. All study participants were further questioned about their smoking habits, long-term medications, and hormone replacement therapy. Participants were asked whether they had ever broken a bone and which bone they had fractured. All data reported at the time of the interview were checked with those reported in patient

records. If any discrepancy was detected, patients were contacted by telephone to confirm observations. If the discrepancy still persisted and no documentation of the event was available, the patient was excluded from the study. Trauma was considered as: (1) severe, if it involved a traffic accident, was sports-related, or caused by falling from a height; (2) moderate, if the fracture resulted from slipping or stumbling, or from a fall on level ground; and (3) mild, if minimal trauma was involved. Body weight was determined for all enrolled patients, and body mass index (BMI) was calculated.

CD patient adherence to GFD was estimated based on multiple assessments: (1) opinion of the patient's primary treating physician; (2) patient's self-report; and (3) a validated questionnaire[25]. The degree of adherence was characterized by one investigator as one of the following categories: (1) strict (adherence for > 90% of the time); (2) partial (50%-90% of the time); or (3) poor (< 50% of the time).

Each study unit tabulated data in a centralized Excel spread sheet. The data were periodically verified *via* comparison with patients' medical records and, if necessary, corrected by three investigators who were not involved in data collection. If discrepancies were noted for a study subject, the subject was contacted by the data reviewer and the most accurate information available was accepted as valid. Data on each year of diagnosis and clinical presentation of CD were confirmed by the patient's medical records. Based on the clinical presentation at the time of CD diagnosis, a patient was categorized as presenting with classic (predominantly gastrointestinal symptoms), atypical (extra-intestinal symptoms), or silent (asymptomatic cases detected through screening) CD. The periods before and after diagnosis for control subjects were categorized according to the index CD case.

Statistical analysis

Results are reported as median and range, mean and 95% CI, or mean and standard error of the mean ± SE as appropriate for the data distribution. In the statistical analysis, the time period "before diagnosis" for both populations was defined as the period from a patient's date of birth to 1 year after the date of diagnosis of CD in the index case. Conversely, the time period "after diagnosis" was defined as the period between 1 year after the diagnosis and the time of study enrollment. We included the first year after diagnosis as part of the "before diagnosis" period to minimize the potential residual effect produced by a long-term disease and slow recovery on GFD. It has previously been observed that the risk of complications may be elevated in the immediate period before and after CD diagnosis[26]. Time at risk of fractures for patients and controls was defined as the period between birth (before diagnosis) or diagnosis of CD (after diagnosis) and the time (age) of the first fracture or the enrollment in the study, whichever came first. The rate of factures was compared between the CD and control cohorts.

Comparisons between cohorts were performed using Student's *t* test or Mann-Whitney test. Multivariate linear regression analyses were performed. Data were also reported as incidence rate (IR), which represents the number of events/1000 subject-years at risk, and as the excess number of events (IR of CD patients minus IR of controls). Cox regression analysis was conducted to estimate and compare the risk of fractures between cohorts. Results were reported as hazard ratio (HR) and 95% CI. Separate analysis of fractures was performed by the before/after CD diagnosis period and by sex. The risk of fractures before diagnosis was also analyzed by clinical presentation (classic CD *vs* atypical/silent forms). The HR was adjusted for potential confounders, including age, age at diagnosis, BMI, smoking, and gynecological and obstetric history. The effect of GFD treatment on fracture risk was analyzed by the degree of compliance with the GFD. Statistical significance was defined as 95% CI not including 1.0.

RESULTS

Study sample characteristics

Table 1 summarizes the demographic and clinical characteristics of the CD and control cohorts. The sex and age distributions were well matched between the cohorts. Most subjects were female (84%). Among the CD patients, the median age at CD diagnosis was 30 years, and 65% of the cases were diagnosed at ⩽ 16 years of age.

CD patients had significantly lower BMI at study enrollment compared with controls ($P < 0.001$). Female CD patients and controls were comparable in age at menarche or menopause. The overall assessment period was 9843 patient-years for the CD cohort and 20160 person-years for the control cohort.

Table 2 presents CD patients' clinical characteristics and fracture history according to gender. Female patients were on average older at study entry and at CD diagnosis than male patients ($P < 0.04$ and $P < 0.003$, respectively). Male CD patients had significantly higher BMI at the time of enrolment ($P < 0.05$) and a greater proportion of time at risk after diagnosis (61% *vs* 37%). According to our assessment of patient adherence to GFD, 85 (38%), 48 (22%) and 90 (40%) female patients and 19 (45%), 7 (17%) and 16 (38%) of male patients were deemed as poor, partial, and strict adherents, respectively.

Rates of fractures in CD patients and controls

Overall, CD patients reported a significantly higher rate of having experienced at least one fracture (23%) compared with controls (15%) (Table 1). Twenty-eight percent of the CD patients with a history of fractures had more than one fracture, compared with only 12% of controls ($P < 0.04$). The mean number of fractures was 1.46 per CD patient and 1.13 per control subject ($P < 0.0001$). Multiple fractures appeared to be limited to a subset of cases. Ten of the 11 control subjects with multiple fractures had two; however, 17 CD patients with multiple fractures reported up to four different fractures in the pe-

Table 1 Demography, clinical information and data on fractures in the peripheral skeleton of celiac disease patients and disease controls (Functional gastrointestinal disorders) at the time of the study

	CD patients	Control population	*P* value
No. of patients (F/M)	265 (223/42)	530 (446/84)	
Median age (yr) (range)	42 (18-85)	43 (16-87)	
Age at diagnosis (yr) median (range)	30 (1-80)	-	
BMI (kg/m^2, mean ± SE)	22.5 ± 0.2	24.3 ± 0.2	0.001
Age at menarche (yr) median (range)	13 (9-17)	12 (9-20)	
Age at menopause (yr) median (range)	48 (30-54)	49 (36-59)	
Person-years before diagnosis	7028	14532	
Person-years after diagnosis	2815	5628	
Total No. of fractures	89	93	0.0001
Total No. of cases with at least one fracture	61	82	0.02
No. of patients with at least one fracture before diagnosis	40	45	0.006
No. of patients with at least one fracture after diagnosis	21	37	
Age at first fracture before diagnosis (yr), median (range)	10 (2-61)	15 (1-74)	
Age at first fracture after diagnosis (yr), median (range)	21 (5-75)	37 (6-71)	
Type of trauma producing fracture (No. of cases)			
Mild	27	24	
Moderate	24	34	
Severe/sports	10	24	

CD: Celiac disease; BMI: Body mass index.

ripheral skeleton. Compared with controls, CD patients had a lower median age at the time of the first fracture ($P < 0.05$). Cole's fracture was the most common site in the peripheral skeleton for CD patients, as well as controls (54% *vs* 42%, respectively); possibly because most cases and controls were < 50 years old. One CD patient and no controls reported hip fracture. Finally, compared with controls, more CD patients with fractures reported that the event was caused by mild trauma (29% *vs* 44%, respectively, $P < 0.05$). No differences were observed between cohorts in terms of moderate and severe/sport-related traumas.

Among CD patients, the rate of fractures was higher in male (59%) than female (26%) population ($P < 0.0001$) (Table 2). Male patients had the first fracture at an earlier age than females ($P < 0.04$). Mild trauma was the most common cause of first fracture in women (48% of cases with at least one fracture *vs* 37% in men) and a severe/sports injury was more common in men (32% *vs* 9.5% in women).

Table 2 Clinical characteristics and fracture history of celiac disease patients according to gender

	Female	Male	*P* value
No. of patients	223	42	
Median age (yr), range	42 (18-62)	35 (18-66)	0.04
Age at diagnosis (yr), median (range)	31 (1-80)	19 (1-52)	0.003
BMI (kg/m^2, mean ± SD)	22.5 ± 0.5	23.7 ± 0.6	0.01
Person-years before diagnosis	6380	647	
Person-years after diagnosis	2371	444	
Total no. of fractures	57	32	0.0001
Total no. of cases with at least one fracture	42	19	0.0005
No. of patients with at least one fracture before diagnosis	29	11	0.05
No. of patients with at least one fracture after diagnosis	13	8	0.01
Age at first fracture before diagnosis (yr), median (range)	14 (2-61)	10 (6-32)	0.04
Age at first fracture after diagnosis (yr), median (range)	54 (5-75)	13 (5-60)	
Type of trauma producing first fracture (No. of cases)			
Mild	20	7	
Moderate	18	6	
Severe/sportive	4	6	

BMI: Body mass index.

Table 3 Crude risk of fracture, adjusted risk of fractures and incidence rates (events/1000 subjects per year) in the peripheral skeleton in celiac disease patients compared to control population according to gender

	CD patients	Controls	HR (95% CI)	*P*
Before diagnosis				
Overall population				
IR	8.67	5.64	1.53 (1.05-2.14)	0.01
Adjusted HR			1.78 (1.23-2.56)	0.002
Females				
IR	6.58	5.09	1.28 (0.87-1.88)	NS
Adjusted HR			1.52 (0.99-2.32)	0.052
Males				
IR	29.35	10.20	2.67 (1.37-5.22)	0.004
Adjusted HR			2.63 (1.24-5.59)	0.01
After diagnosis				
Overall population				
IR	7.45	6.04	1.28 (0.74-2.21)	NS
Adjusted HR			No significant change	
Females				
IR	5.48	5.30	1.08 (0.55-2.10)	NS
Adjusted HR			No significant change	
Males				
IR	18.02	9.83	1.57 (0.57-4.26)	NS
Adjusted HR			No significant change	

Hazard ratios (HRs) were adjusted by age at enrollment, age at diagnosis, body mass index (BMI), smoking habits and menopause. CD: Celiac disease; IR: Incidence rate.

Risk of fractures before diagnosis

As shown in Table 3, the risk of fractures in the peripheral skeleton before the diagnosis of CD was higher in the CD than in the control cohort. Compared with controls, the excess number of fractures estimated in the CD cohort was 3.03 per 1000 patients/year. Although the excess of fractures (1.49 events) in female CD patients was marginally higher than in the matched female controls, the excess number of fractures was significantly higher

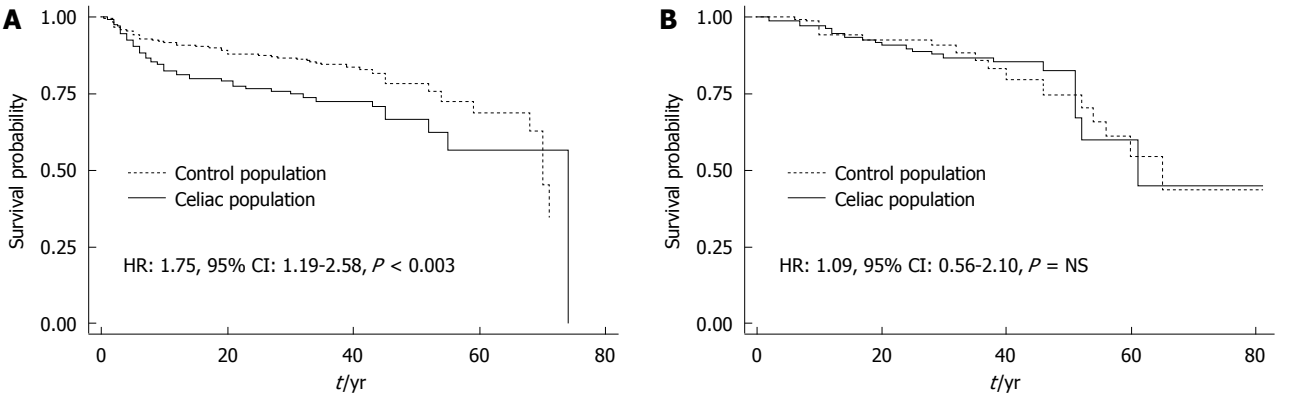

Figure 1 Kaplan-Meier curves of time to first fracture for patients according to clinical presentation in celiac disease patients and matched control population before the time of celiac disease diagnosis. A : Classic presentation celiac disease (CD) patients; B: Atypical/silent presentation CD patients. HR: Hazard ratio; NS: Not significant.

in male CD patients (19.15 events) than in male controls. The risk of fractures before diagnosis was linked to confounders such as age at study entry, age at CD diagnosis, smoking, menopause and BMI only in female patients (Table 3). However, none of these confounders individually modified the estimated risk above 10%. Among CD patients, fractures before CD diagnosis occurred at younger age in male than in female patients ($P < 0.04$) (Table 2).

Figure 1 depicts the survival curves of time to first fracture in CD patients by their clinical presentation at the time of CD diagnosis as compared with controls. The IR of fractures in the classic CD patients was almost twice that of their matched controls (10.14 *vs* 5.73 per 1000, respectively; HR 1.75, 95% CI: 1.19-2.58, $P < 0.003$). In contrast, the incidence of fractures in atypical/silent CD patients did not differ significantly from that of their matched controls (5.44 *vs* 5.84, respectively; HR: 1.09, 95% CI: 0.57-2.10, P = NS).

Risk of fractures after diagnosis and effect of GFD

Compared with the time period before diagnosis, the IR for the first peripheral fracture after CD diagnosis was comparable between the control and CD cohorts. After CD diagnosis, the IR of fractures for the CD cohort decreased from the pre-diagnosis period (-1.22 events per 1000 patients/year). Furthermore, compared with matched controls, all CD cases had an excess of fractures of 1.41 events (HR: 1.28, 95% CI: 0.74-2.21, P = NS) in the after-diagnosis period. Compared with the before-diagnosis period, female CD patients had a lower IR (-1.10 events) after diagnosis, and their risk of fractures was comparable to that of controls (excess of fractures in cases: 0.18 events) (P = NS) (Table 3). Male patients had a significant decrease in fractures from before to after CD diagnosis (-11.33 events/1000 patients per year). However, in the post-diagnosis period, male CD patients continued to have an excess number of fractures (8.19 events) compared with controls, which was not statistically significant (HR: 1.57, 95% CI: 0.57-4.26, P = NS). Female patients categorized as poorly adherent with the GFD (n = 90) had an IR very similar to the before-diagnosis IR (6.41 events/1000 patients per year). Although only one of 16 (7.2%) strictly adherent male patients had at least one fracture after diagnosis, seven of 19 (36.8%) poorly adherent male patients had a fracture. The small number of male patients prevented us from estimating their IR and fracture risk. Figure 2 shows the survival curve of time for first fracture for patients and matched control population before and after the diagnosis of CD according to gender.

DISCUSSION

Previous studies have demonstrated that CD patients have an increased risk of fractures in the peripheral skeleton. Whether this risk can be modified by a GFD is still unclear. Our seminal study has suggested that the prevalence of fractures decreases after initiation of a GFD[14]. However, this conclusion has been challenged by other studies[18,20,21,23]. Some authors have suggested that an early diagnosis and therapeutic intervention for CD before bone damage occurs is the only way to significantly lower the risk of fractures in CD patients.

The present study confirmed the increased risk of fractures overall among CD patients compared to controls with functional gastrointestinal disorders, and this increased risk was most prominent before their CD diagnosis. Additional supportive findings included the increased incidence of fractures produced by mild trauma events (for female cases) and a history of multiple fractures (up to four different events) seen in a subset of CD patients. The increased risk for female CD patients was more pronounced and statistically borderline when data were adjusted for potential confounders such as age at study entry, age at CD diagnosis, smoking, menopause, and BMI. Thus, older age, later diagnosis, cigarette smoking, and lower BMI were factors that contributed to the higher incidence of fractures in the peripheral skeleton. The effect of these confounders was not significant in male patients before CD diagnosis and in the overall patient cohort after CD diagnosis. Our study also con-

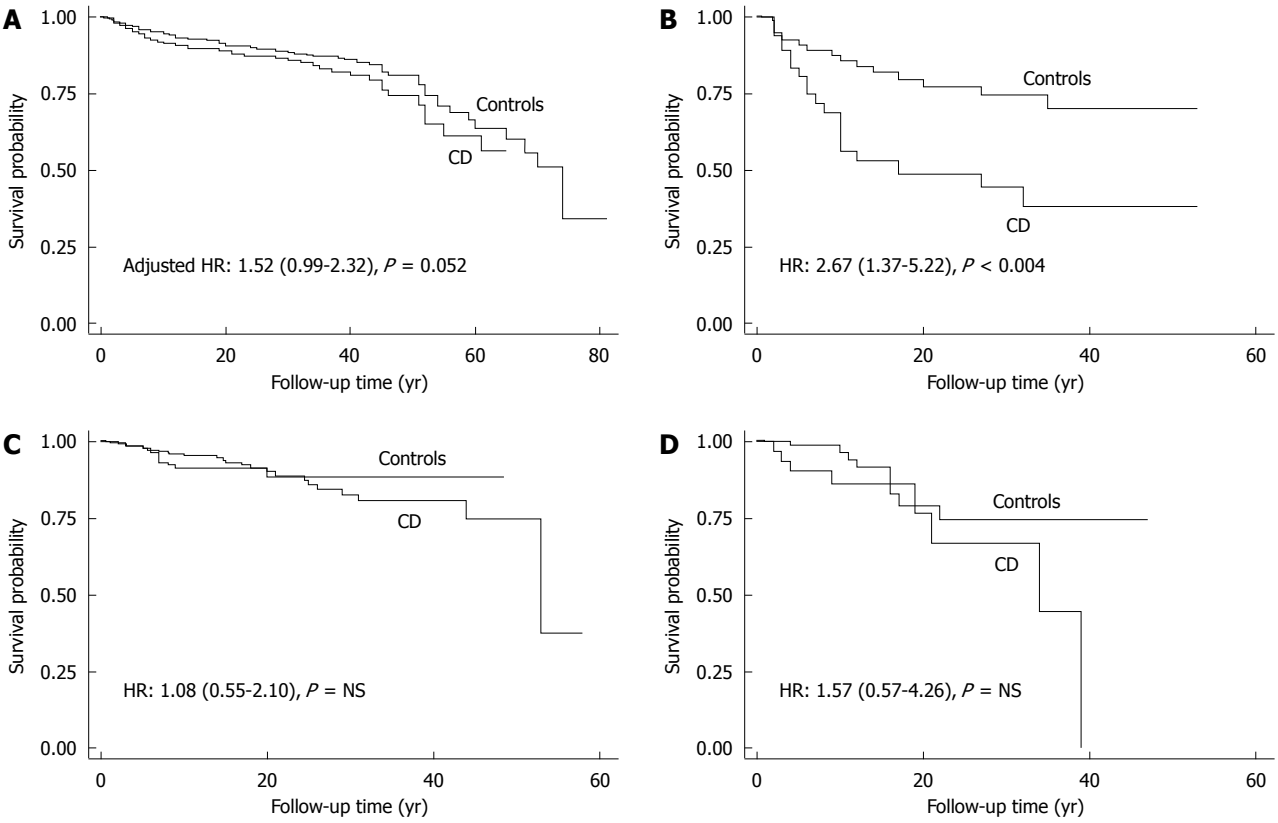

Figure 2 Kaplan-Meier curves showing time of first fracture according to gender in celiac disease population *versus* control group. A: Females before diagnosis; B: Males before diagnosis; C: Females after diagnosis; D: Males after diagnosis. CD: Celiac disease; HR: Hazard ratio; NS: Not significant.

firmed our previous observation that the increased risk of peripheral fractures before CD diagnosis was associated with the classic clinical presentation but not with atypical/silent forms.

Of note, the present study is believed to be the first to identify sex as a relevant risk factor for fracture risk in CD patients; especially before they are diagnosed. The fact that the IR in male controls was more than twice that in female controls indicates that males have a higher exposure to trauma, regardless of whether they have CD. Furthermore, the IR in male CD patients was more than fourfold higher than that in female CD patients, and almost threefold higher than that in matched male controls. In addition, male CD patients had their first fractures before diagnosis at a younger age than female CD patients. Our findings do not support the hypothesis that the increased IR in male CD patients is due to osteoporotic fractures (i.e. events caused by mild trauma).

Our present study also provides original evidence for the profound impact of treatment with GFD on the risk of fractures in the peripheral skeleton. The improvement in bone health was seen in both sexes. These findings are in line with previous evidence that has shown that gluten restriction can reverse the systemic and local physiological mechanisms in bone deterioration of CD patients[3,11-13,27]. Although normalization of bone mass is unlikely in adult CD patients, significant re-mineralization of axial and peripheral skeleton has been shown in several studies[3,6,10-13]. It should be noted that reducing the risk of fracture does not solely depend on increasing bone mass and mineral density[27-32]. Other risk factors, such as structural alteration of bones with impairment of the mechanical quality (stiffness of cortical bones), deterioration of protective factors from trauma (body mass, fat and muscle compartments), and neuromuscular dysfunction, also contribute to bone weakness in CD patients[28,30]. In this context, improving body mass and fat/muscle composition, nutritional status, and bone architecture through long-term GFD treatment may reduce the overall risk of fractures in CD patients. Our study provides further support to the clinical benefits of GFD. Although the conclusion is limited by sample size, our data suggest that greater adherence to a GFD may be beneficial in male as well as female patients.

The sex differences observed in the risk of fractures in CD patients have not been reported before and deserve further comment. A previous study on bone structure and strength in CD patients detected some sex differences in mineral and bone metabolism, localization of bone damage (predominantly cortical/subcortical bone mass of the radius), mechanical quality of bones, and changes induced by 1-year treatment with a GFD[27], which may be related to differences in the development of the male and female mammalian skeleton. At 1-year follow-up, gluten-free treatment appeared to correct only the metabolically induced disturbances, which were predominant in women. However, the current results suggest that long-term adherence to a GFD may significantly reduce fracture risk in male patients as well.

Although intriguing, the current study results were limited by a relatively small number of male patients; larger studies are needed to confirm these findings. Another limitation was that fractures were based on self-report and may have been subject to recall errors; however, the risk of failed recall is expected to be similar between patients and controls. Misclassification of the type of trauma may have biased the results toward a positive association between bone disorders and osteoporotic fractures in CD patients. However, this association is well-established in female patients and not corroborated in male patients; therefore, the conclusions are not likely to have been altered. The assessment of GFD adherence is difficult, particularly in retrospective analyses. Our assessment relied on patients' self-reports and detailed interviews conducted by expert physicians, and was characterized by an independent researcher unaware of other clinical information.

In conclusion, this cohort study confirms the increased risk of fractures in the peripheral skeleton in undiagnosed CD patients and an association of bone damage with the classic, but not the atypical/silent clinical presentation of CD. In addition, this study is believed to be the first to demonstrate a higher excess risk of fracture in male patients compared with female patients before CD diagnosis. Sex differences in the pathogenesis of bone weakness should be further explored. Finally, the study is also believed to be the first to recognize a beneficial effect of a GFD in reversing the elevated risk of fractures, and patients who adhere to long-term GFD can achieve a similar risk of fracture to those without CD, which provides a further argument for strict adherence to the diet to prevent complications of CD.

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COMMENTS

Background

Reduced bone health is seen in > 50% of celiac disease (CD) patients at the time of diagnosis. Very few studies have evaluated the risk of fractures in CD patients. A recent systematic review and meta-analysis of eight studies, published between 2000 and 2007, concluded that CD patients had a 43% higher risk of fractures compared with people without the disorder.

Research frontiers

Although several studies have shown a positive effect of a gluten-free diet (GFD) on bone density and other bone-protective factors, the impact of treatment on the risk of fractures remains controversial. Some studies have suggested that the risk of fractures detected before diagnosis of CD remains elevated several years after diagnosis. In this study, the authors explored the incidence of fractures in the peripheral skeleton of CD patients before diagnosis and the effect of CD treatment on fracture risk.

Innovations and breakthroughs

This study confirms that, before diagnosis, CD patients have a significantly higher rate of fractures in the peripheral skeleton compared with controls with functional gastrointestinal disorders. In addition, the risk is associated with the classic presentation of CD (predominantly gastrointestinal symptoms). This study is believed to be the first to demonstrate that the increased incidence of fractures in CD patients is associated with male sex and that, with treatment GFD, the fracture risk becomes comparable to controls.

Applications

The study further supports the importance of adherence to a GFD to reduce the risk of bone complications in CD patients.

Peer review

The paper provides relevant and novel information, but some issues deserve discussion. I would strongly suggest to engage in a much more in depth discussion and speculation on their opposite findings in CD patients.

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BRIEF ARTICLE

P16 gene hypermethylation and hepatocellular carcinoma: A systematic review and meta-analysis

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Abstract

AIM: To quantitatively investigate the effect of p16 hypermethylation on hepatocellular carcinoma (HCC) and hepatocirrhosis using a meta-analysis of available case-control studies.

METHODS: Previous studies have primarily evaluated the incidence of p16 hypermethylation in HCC and corresponding control groups, and compared the incidence of p16 hypermethylation in tumor tissues, pericancer liver tissues, normal liver tissues and non-tumor liver tissues with that in other diseases. Data regarding publication information, study characteristics, and incidence of p16 hypermethylation in both groups were collected from these studies and summarized.

RESULTS: Fifteen studies, including 744 cases of HCC and 645 non-tumor cases, were identified for meta-analysis. Statistically significant odds ratios (ORs) of p16 hypermethylation were obtained from tumor tissues and non-tumorous liver tissues of HCC patients (OR 7.04, 95% CI: 3.87%-12.78%, $P < 0.0001$), tumor tissues of HCC patients and healthy liver tissues of patients with other diseases (OR 12.17, 95% CI: 6.64%-22.31%, $P < 0.0001$), tumor tissues of HCC patients and liver tissues of patients with non-tumorous liver diseases (OR 6.82, 95% CI: 4.31%-10.79%, $P < 0.0001$), and cirrhotic liver tissues and non-cirrhotic liver tissues (OR 4.96, 95% CI: 1.45%-16.96%, $P = 0.01$). The pooled analysis showed significantly increased ORs of p16 hypermethylation (OR 6.98, 95% CI: 4.64%-10.49%, $P < 0.001$) from HCC tissues and cirrhotic tissues.

CONCLUSION: P16 hypermethylation induces the inactivation of p16 gene, plays an important role in hepatocarcinogenesis, and is associated with an increased risk of HCC and liver cirrhosis.

Key words: P16 hypermethylation; Hepatocellular carcinoma; Liver cirrhosis; Meta-analysis; Odds ratio

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INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the major

causes of cancer death worldwide[1]. The HCC incidence is still increasing in developed countries although considerable progress has been made in diagnostic and therapeutic modalities[2]. The molecular genetics of HCC have recently been extensively characterized[3]. Among these molecular genetics, aberrant DNA cytosine methylation is one of the most consistent epigenetic changes in human cancers. Generally, the overall DNA methylation level is lower in cancer cells than in normal cells. However, some loci tend to show increased DNA methylation in cancer cells[4].

The *p16INK4A* gene is located on chromosome 9p21 and is one of the most frequently altered genes observed in various human neoplasms[4,5]. It is a cell cycle-related gene encoding a p16 protein that binds competitively to cyclin-dependent kinase 4 protein (Cdk4), thereby inhibiting the interaction of Cdk4 and cyclin D1 to stimulate passage through the G1 phase of the cell cycle[6]. The disruption of p16-mediated cell cycle control seems to play a role in hepatocarcinogenesis because inactivation of the *p16INK4A* gene resulting from methylation of the p16INK4A gene, has been reported in HCC[7].

Although previous reports indicated that inactivation of the *p16INK4A* gene is mainly induced by the methylation of the p16 gene, and it is one of the important genetic alterations in HCCs, the reported rates of p16 methylation in HCCs were remarkably diverse. Moreover, whether it is associated with the incidence of hepatocirrhosis is still unclear. The various results of these studies underpin the need for assessing the evidence of the relationship between p16 inactivation and HCC. Hence, we conducted a systematic review and meta-analysis to quantitatively evaluate the effects of p16 hypermethylation on the incidence of HCC.

MATERIALS AND METHODS

The meta-analysis was performed according to the Preferred Reporting Items for Systematic Reviews and Meta-Analyses[8] and the recommendations of the Cochrane Collaboration[9].

Data source and search

To avoid publication bias, both published and unpublished studies, with an English or Chinese language restriction, were included, and several methods were used to identify all relevant studies. The databases screened were PubMed (1976 onward), EMBASE (1966 onward), Cochrane Library (no date restriction), Biological Abstracts (no date restriction), Science Citation Index (no date restriction), China National Knowledge Infrastructure (no date restriction), and the Chinese BioMedical Literature Database (no date restriction). Medical Subject Headings were used in the searching in both Chinese and English languages. The keywords used were p16 methylation, HCC and hepatocirrhosis. Relevant reviews and meta-analysis of the role of p16 methylation in the incidence of HCC and hepatocirrhosis were examined for potential inclusive studies. We also searched http://www.jamas.gr.jp and http://www.cdc.gov websites for studies completed but not yet published.

Study selection

The following studies were included in this meta-analysis: studies primarily evaluating the incidence of p16 hypermethylation in HCC and corresponding control groups, and comparing the incidence of p16 hypermethylation in tumor tissues, pericancer liver tissues, normal liver tissues, and non-tumor liver tissues with other identified diseases. The bibliographies of the search results were manually scanned and independently reviewed by two authors (Xie F and Zang JJ) to identify relevant studies that met the inclusion criteria (full text or abstract). If there was any disagreement between the two authors, it was settled by discussion with a third author (He J) until a consensus was reached. One author (Xu JF) contacted the authors of the article for missing data if necessary.

Data extraction

Data extraction was independently conducted by two reviewers (Xu JF and Qin YY) using a standardized approach. Data for publication information (year of publication and name of first author), study characteristics (sample size and distributions of age and sex), and rates of p16 hypermethylation were collected using standard data extraction forms. Point estimates for selected variables were extracted and checked by the other two reviewers (Xie F and Qin YY). Disagreement was adjudicated by a third reviewer (He J) after referring back to original articles.

Statistical analysis

Odds ratios (ORs) were used as a measure of the relationship between p16 hypermethylation and the risk of HCC for case-control studies and the corresponding 95% CIs. The pooled ORs were combined by the Mantel-Haenszel methods. When there were trials with no events in one or both arms, the Peto method was used[6,10].

An OR > 1 indicated a higher incidence of p16 methylation in HCC tissues than in corresponding controls. The percentage of variability across studies attributable to heterogeneity beyond chance was assessed by χ^2 test ($P < 0.1$) and I^2 statistics[11]. When there was no statistically significant heterogeneity, a pooled effect was calculated with a fixed-effects model; otherwise, a random-effects model was employed. We also assessed the probability of publication bias with funnel plots[12] and Egger's test[13]. A P value of less than 0.1 indicated statistically significant publication bias. In addition, we conducted a sensitivity analysis to evaluate whether the results were statistically affected. Statistical significance was defined as a two-tailed P value of 0.05. All statistical analyses were conducted with RevMan version 5 from the Cochrane Collaboration.

RESULTS

Search results

Fifteen[14-28] articles met the inclusion criteria according

Table 1 Demographic data of studies included in meta-analysis

Study	HCC tissue/control	No. of patients	Country or area	Median age (Yr)	Sex (M/F)	Year of publication
Formeister *et al*[14]	Tumor/non-tumor tissues	43/45	America	66.28 ± 8.1	37/12	2010
Zhu *et al*[15]	Tumor/non-tumor tissues	88/88	China	52.7 ± 10.62	78/10	2010
Zhang *et al*[16]	Tumor/liver cirrhosis/normal liver tissue	120/120/10	China	52.8 ± 10.2	106/14	2008
Xu *et al*[28]	Tumor/non-tumor tissues from other patients	30/5	China	NR	NR	2006
Liu *et al*[17]	Tumor/pericancer tissues	50/50	China	48.5	46/4	2006
Qin *et al*[18]	Tumor/pericancer/non-tumor tissues	20/20/20	China	NR	NR	2004
Lee *et al*[19]	Tumor/dysplastic nodule/liver cirrhosis/chronic hepatitis tissues	60/22/30/34	Korea	53.8	47/13	2003
Schagdarsurengin *et al*[20]	Tumor/non-tumor/liver cirrhosis/normal liver tissues	14/14/6/8	Germany	NR	NR	2003
Zhang *et al*[21]	Tumor/pericancer/normal tissues	83/10/12	China	NR	NR	2002
Yu *et al*[22]	Tumor/pericancer tissues	29/29	China	NR	NR	2003
Saito *et al*[24]	Tumor/non-tumor tissues	59/48	Japan	61 ± 12	42/7	2001
Zhang *et al*[27]	Tumor/pericancer tissues	35/35	China	NR	NR	2002
Kondo *et al*[23]	Tumor/non-tumor tissues	40/40	Japan	20-77	32/8	2000
Wong *et al*[25]	Tumor/non-tumor tissues from other patients	25/35	Hong Kong	NR	NR	2000
Liew *et al*[26]	Tumor/non-tumor tissues	48/30	Hong Kong	NR	NR	1999

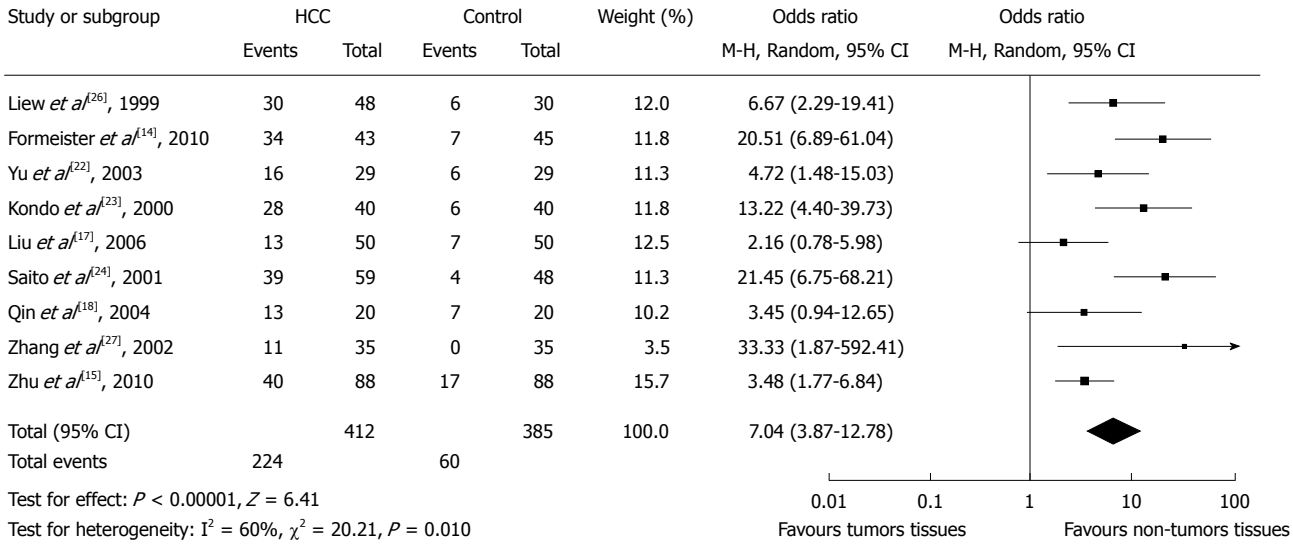

Figure 1 Pooled analysis of p16 hypermethylation in tumorous liver tissues and non-tumorous liver tissues of hepatocellular carcinoma patients. HCC: Hepatocellular carcinoma.

to the aforementioned search strategies and provided data regarding p16 hypermethylation in 744 cases of HCC tumor tissues and 645 cases of non-tumor tissues. Hypermethylation profile of tumorous and paired non-tumorous liver tissue samples from nine studies, HCC tumor tissues and normal tissues (normal liver tissues or blood samples) from five studies, and HCC tumor tissues and abnormal and non-tumorous tissues (dysplastic nodule, liver cirrhosis, and chronic hepatitis) from four studies was compared, respectively. Twelve eligible trials were conducted in Asia from 1999 to 2010, and the other three were conducted in the United States and Germany. Three of them were published in Chinese, and the others were published in English. The median sample size was 79 patients (range, 22-176). The median age of the study participants ranged from 48.5 to 66.2 years. All of the specimens in the 15 studies were surgically obtained from HCC patients or non-HCC patients who underwent liver surgery. The characteristics of the included studies are shown in Table 1.

P16 hypermethylation in tumorous liver tissues and non-tumorous liver tissues of HCC patients

Data for this comparison were available in nine studies which included 412 and specimens of HCC tissues and 385 non-tumorous pericancer tissues. Overall, 224 (54.5%) and 60 (15.6%) cases of p16 hypermethylation were observed in tumorous and non-tumorous tissues of HCC patients, respectively. The pooled analysis showed significantly increased ORs of HCC for p16 hypermethylation compared with controls (OR 7.04, 95% CI: 3.87%-12.78%, $P < 0.0001$). There was, however, evidence of heterogeneity across the studies (P for heterogeneity = 0.01, $I^2 = 60\%$, Figure 1). The heterogeneity

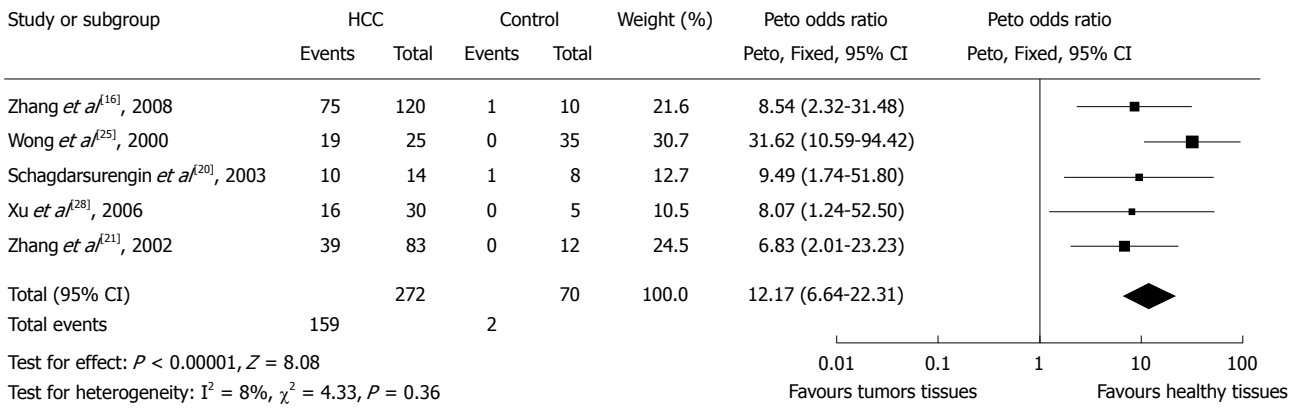

Figure 2 Pooled analysis of p16 hypermethylation in tumorous liver tissues of hepatocellular carcinoma patients and healthy liver tissues of patients with other diseases. HCC: Hepatocellular carcinoma.

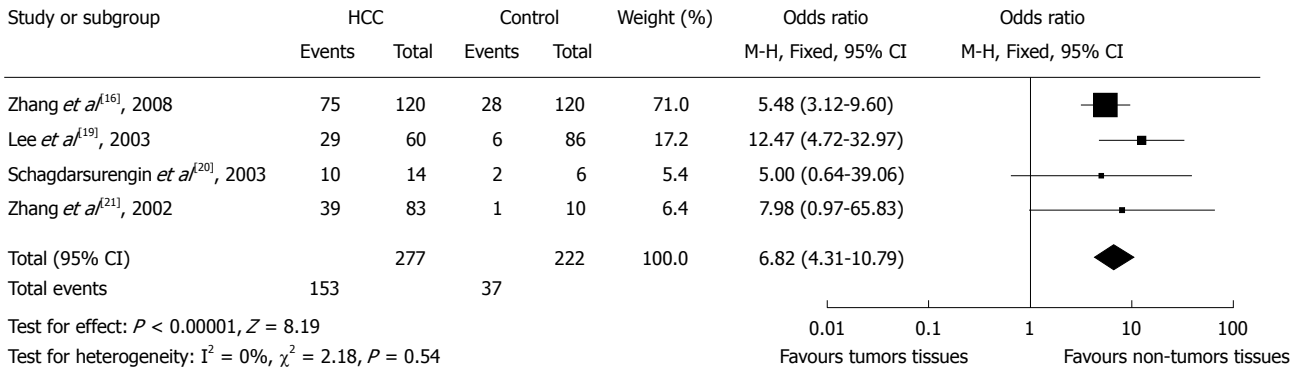

Figure 3 Pooled analysis of p16 hypermethylation in tumorous liver tissues of hepatocellular carcinoma patients and liver tissues of patients with non-tumorous liver diseases. HCC: Hepatocellular carcinoma.

was incorporated into the random-effects model. Funnel plots did not show any evidence of publication bias.

P16 hypermethylation in tumorous liver tissues of HCC patients and healthy liver tissues of patients with other diseases

Five studies calculated the OR of p16 hypermethylation in HCC patients and non-HCC healthy patients (Figure 2). There were 159 cases of methylated p16 genes among 272 (58.5%) HCC patients and 2 in 70 (2.9%) non-HCC patients, indicating an OR for p16 hypermethylation of 12.17 (95% CI: 6.64%-22.31%, $P < 0.0001$). There was no evidence of heterogeneity across the studies (P for heterogeneity = 0.36; $I^2 = 8\%$). Funnel plots did not show any evidence of publication bias.

P16 hypermethylation in tumorous liver tissues of HCC patients and liver tissues of patients with non-tumorous liver diseases

Four studies calculated the OR of p16 hypermethylation in liver tissues of HCC patients and those of patients with liver diseases (Figure 3). There were 153 cases of hypermethylated p16 genes in 277 (55.2%) HCC patients and 37 in 222 patients (16.7%) with liver diseases, indicating an OR for p16 hypermethylation of 6.82 (95% CI: 4.31%-10.79%, $P < 0.0001$). There was no evidence of heterogeneity across the studies (P for heterogeneity = 0.54; $I^2 = 0\%$). There was no evidence of publication bias in the funnel plots.

Among these studies, data on the comparison of p16 hypermethylation in HCC tissues and cirrhotic tissues were also extracted. Overall, 133 (60.7%) and 30 (15.9%) cases of p16 hypermethylation were observed in 219 HCC tissues and 189 cirrhotic tissues, respectively. The pooled analysis showed significantly increased OR (6.98, 95% CI: 4.64%-10.49%, $P < 0.001$, data not shown).

P16 hypermethylation in cirrhotic liver tissue and non-cirrhotic liver tissue

Five studies did this comparison, which included 185 specimens of cirrhotic tissues and 87 specimens of non-cirrhotic tissues. Overall, 42 (22.7%) and 8 (9.2%) cases of p16 hypermethylation were observed in cirrhotic tissues and non-cirrhotic tissues, respectively. The pooled analysis showed significantly increased OR of liver cirrhosis for p16 hypermethylation compared with matched controls (OR 4.96, 95% CI: 1.45%-16.96%, $P = 0.01$, Figure 4). There was no evidence of heterogeneity across the studies (P for heterogeneity = 0.74; $I^2 = 0\%$). There was no evidence of publication bias in the funnel plots.

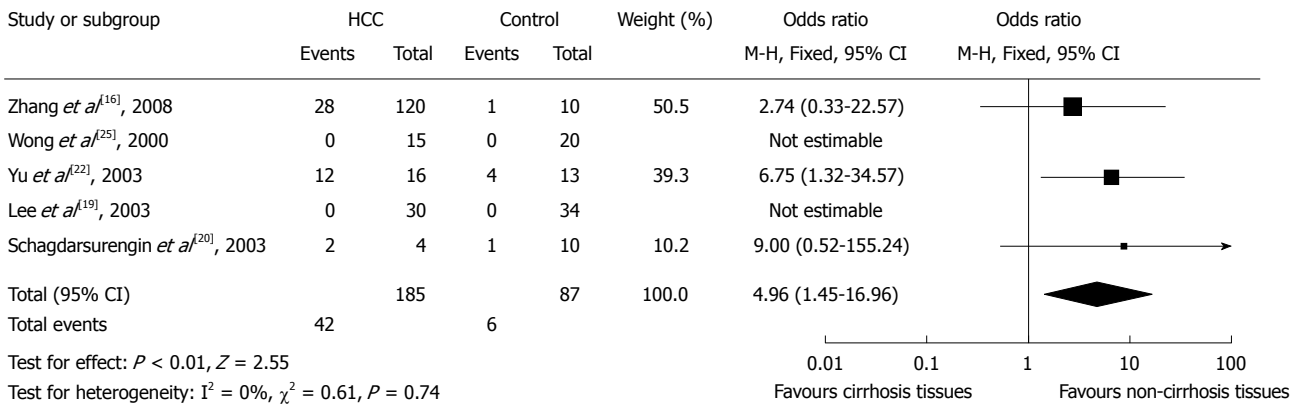

Figure 4 Pooled analysis of p16 hypermethylation in cirrhotic liver tissues and non-cirrhotic liver tissues. HCC: Hepatocellular carcinoma.

DISCUSSION

Gene-specific promoter alterations are common epigenetic aberrations found in human liver tumors; however, the epigenetic changes of p16 gene hypermethylation specific to the underlying disease etiology remains elusive. Based on 15 studies and a total of 744 cases of HCC tumor tissues and 645 cases of non-tumor tissues, this pooled analysis comprehensively assessed the relationship between p16 gene hypermethylation and the incidence of HCC or liver cirrhosis. Using the pooled crude ORs from the included studies, we found that p16 gene hypermethylation was associated with 6.16-, 12.17-, and 6.82-fold increased risks of HCC compared with non-tumorous tissues of HCC patients, healthy liver tissues of patients with other diseases, and liver tissues of patients with non-tumorous liver diseases, respectively. Moreover, a 4.96-fold increased risk of liver cirrhosis was also found when compared with non-cirrhotic tissues.

The relationship between p16 gene hypermethylation and the incidence of HCC has been verified by other studies that assessed p16 mRNA expression and its promoter CpG island methylation. Kaneto *et al*[29], using methylation-specific PCR and immunohistochemistry, detected methylation of the p16 promoter in HCC (72.6%, 16/22) and loss of expression in all methylation-positive HCCs. Roncalli *et al*[30] reported that methylation of the p16 promoter with complete loss of immunoreactivity occurred in 27 of 33 HCCs (82%). Our results, which were consistent with those of other reports, suggested that p16 gene methylation might play an important role in hepatocarcinogenesis and it might be the major mechanism of *p16* gene inactivation.

This review quantitatively assessed the relationship of p16 gene methylation between HCC tissues and non-HCC tissues using well-designed case control studies. To our knowledge, this has not been presented in other meta-analyses or reviews[31-33]. Consistent results were shown in sensitivity analyses, and no evidence of publication bias was found.

This study has several potential limitations. First, the possibility of information and selection biases and unidentified confounders cannot be completely excluded because all of the included studies were observational. Second, the searching strategy was restricted to articles published in English or Chinese. Articles with potentially high-quality data that were published in other languages were not included because of anticipated difficulties in obtaining accurate medical translation. Third, most studies included in this meta-analysis were conducted in Eastern Asia, where HCC more frequently occurs. Fourth, comparisons of p16 hypermethylation in cirrhotic non-tumorous liver tissues and normal tissues, chronic hepatitis tissues, or non-cirrhotic HCC tissues were involved in five of the included studies. However, there was no distinction between cirrhotic liver tissues with or without HCC. Thus, we could not perform comparisons under these circumstances. Hence, cautions should be taken when our findings are interpretated among the general populations.

In conclusion, we found that p16 hypermethylation was associated with an increased risk of HCC and liver cirrhosis. P16 hypermethylation, which induced the inactivation of the p16 gene, plays an important role in hepatocarcinogenesis.

COMMENTS

Background

The inactivation of the *p16INK4A* gene is one of the important genetic alterations in hepatocellular carcinoma (HCC), which is mainly induced by the hypermethylation of *p16* gene. However, the role of p16 hypermethylation in HCC or hepatocirrhosis is unclear. Hence, the authors performed a systematic review and meta-analysis to quantitatively evaluate the effects of p16 hypermethylation in the incidence of HCC and hepatocirrhosis.

Research frontiers

Inconsistent results have been reported on the effect of p16 hypermethylation on HCC or hepatocirrhosis and its corresponding controls, and the incidence of p16 hypermethylation.

Innovations and breakthroughs

This is the first systematic review and meta-analysis to investigate quantitatively the effect of p16 hypermethylation in HCC or hepatocirrhosis.

Applications

P16 hypermethylation induces the inactivation of *p16* gene and plays an important role in hepatocarcinogenesis, and it is associated with an increased risk of HCC and liver cirrhosis. Detection of p16 hypermethylation using a methylation-specific PCR is favorable for the differential diagnosis of HCC from liver cirrhosis.

Peer review

The authors aimed to quantitatively evaluate the effects of p16 hypermethyl-

ation on the incidence of HCC and hepatocirrhosis by systematic review and meta-analysis. The authors found that p16 hypermethylation was associated with an increased risk of HCC and liver cirrhosis. The article is well organized. The methods utilized were appropriate and they presented convincing evidence.

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BRIEF ARTICLE

Coordination and nursing care of pediatric patients undergoing double balloon enteroscopy

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Abstract

AIM: To review safety, efficacy, and proper nursing care of double-balloon enteroscopy (DBE) in pediatric patients with small intestinal disease.

METHODS: Our study included 37 patients with abdominal pain, diarrhea, passage of blood in the stools, and other symptoms, who underwent DBE from December 2006 to July 2010. DBE was retrograde in 36 procedures, antegrade in six, and from both ends in five. The diagnostic significance and salient points in nursing care are discussed in this article.

RESULTS: At least one lesion was discovered in 28 out of 37 patients, which yielded a positive diagnosis in 75.7% of cases. Good bowel preparation and skilled nursing care not only shortened the procedure time, but could also alleviate patient discomfort and enhance the quality of examination. No serious procedure-related complications were observed in any cases.

CONCLUSION: DBE is a new modality of endoscopic procedure that improves the standard of diagnosis and treatment of small bowel diseases in children. Good nursing care is essential to the successful execution of the procedure.

Key words: Children; Double-balloon enteroscopy; Nursing care; Small intestinal disease

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INTRODUCTION

Small intestinal diseases are not rare in children, but limitations in investigative approaches affect our understanding of pediatric small bowel disorders. The small intestine is deep-seated, up to 6 m long in adults, and has many turns and convolutions, which makes direct visualization through the traditional means of upper and lower gastrointestinal endoscopic procedures difficult. Sonde or push enteroscopes can examine the small intestine only up to 80-100 cm beyond the ligament of Treitz, thus, a full endoscopic examination of the small bowel has always been difficult.

Double-balloon enteroscopy (DBE) provides a significant advance in diagnosis and management of small intestinal diseases[1-5]. It also poses a challenge to the nursing profession. Nurses are responsible for assisting the endoscopist with completing the examination, minimizing the suffering of patients undergoing the procedure, and preventing the occurrence of complications during and after the examination. Our hospital acquired DBE equipment in 2006 and has achieved satisfactory results in its

application. Here, we report our experience using DBE in the management of small intestinal diseases, with an emphasis on indications and special aspects of nursing care.

MATERIALS AND METHODS

Ethics

This study was approved by the Institutional Ethics Committee of the Children's Hospital of Fudan University, Shanghai, China and informed consent was obtained from all patients and their parents.

Clinical data

During December 2006 to July 2010, we had 37 patients who underwent DBE, with 26 boys and 11 girls. Patient age ranged from 4 to 16 years, specifically: 4-8 years old, nine patients; 8-12 years old, nine patients; and 12-16 years old, 19 patients. Retrograde DBE was performed in 36 cases, antegrade DBE in six, and DBE from both ends in five. Demographic characteristics of the 37 children who underwent DBE examination are in Table 1. Procedures indicated included (Table 2): occult gastrointestinal bleeding, recurrent abdominal pain, chronic diarrhea, and hypoproteinemia. All patients underwent traditional investigations that included gastroscopy, colonoscopy, abdominal computer tomography (CT) or magnetic resonance imaging (MRI), and radioisotope scan for Meckel's diverticulum if symptom cause could not be identified.

Table 1 Demographic characteristics of 37 children who underwent double-balloon enteroscopy

Age group (yr)	*n*	Mean age (yr)	Male/female	Antegrade/retrograde/both
4.0-8.0	9	5.6	3/6	0/8/1
8.1-12.0	9	10.4	3/6	1/6/2
12.1-16.0	19	13.9	5/14	0/17/2

Table 2 Preliminary indication for double-balloon enteroscopy in 37 children

Preliminary indication	*n*
Occult gastrointestinal bleeding	12
Recurrent abdominal pain	10
Chronic diarrhea	13
Other	2

Methods of examination

DBE can be administered through the mouth (antegrade), or through the anus (retrograde). The route of insertion is determined by the clinical features and results of other ancillary examinations including CT/MRI scans, angiography, barium examination of the small bowel, and radioisotope scanning. The procedure is usually conducted in a fully equipped operating room with full anesthetic capabilities, with the anesthesiologist administering general anesthesia *via* an endotracheal tube. The lower ileum can usually be reached in the transoral, antegrade approach, whereas the upper jejunum can be reached *via* the transanal, retrograde approach. Sequential application of the antegrade and retrograde examinations can achieve full examination of the small intestine.

Pre-procedure nursing care

Psychological care: Psychological preparation of an adult patient undergoing DBE is very important. If patients are poorly prepared, an unsuccessful examination may result. For the pediatric patient, psychological preparation is equally or more important. Most of our patients undergoing DBE suffered from illnesses of long duration and had received gastroscopy, colonoscopy, and many other investigations without a definitive diagnosis. Moreover, because DBE requires a long procedural time, and most patients and parents demonstrated anxiety, a preprocedural routine that carefully detailed the aspects of the examination to the parents as well as patients was imperative. The aim, method, significance, and other details of the procedure were carefully explained, to gain confidence and cooperation before the examination.

Dietary and bowel preparation: We recommended a restrictive diet for the patients prior to the procedure. Two days before the examination, they were instructed to consume a low-residue, semi-liquid diet. A light laxative such as senna or lactulose was administered with adequate fluid. On the day of examination we usually gave, in addition to the laxative, an enema of 500-1000 mL of warm saline until clear fluid passed.

Others: Six hours before the procedure we carefully enquired if the patient had any contraindications. Any serious cardiological or pulmonary disorder and significant gastrointestinal blood loss was noted and evaluated for suitability to undergo the procedure. We routinely checked the liver and renal function, electrocardiogram, complete blood count and clotting factors preoperatively. Venous access, cardiac monitoring, oxygen saturation monitoring, and other routine monitoring procedures were set up for the anesthesiologist. Other facilities such as suction tubes, suction pump, oxygen supply, Ambu bag, and instruments and medications for resuscitation were also routinely checked to ensure patient safety.

Nursing care during the procedure

The double balloon endoscope is different from regular gastroscopy or colonoscopy, and is much longer and softer. The small intestine is long and convoluted, and situated deeper in the abdomen; hence, manipulation of the endoscope is difficult. The assistance of nursing personnel during the procedure is very important. Before the procedure, a small amount of water was added into the space between the overtube and the endoscope, as a lubricant to facilitate the pushing and pulling of the scope. K-Y Jelly (Johnson and Johnson Co.) was routinely used as a lubricant to reduce the friction between the mucosa and the endoscope. For antegrade DBE, the initial part of

the insertion of the endoscope was similar to that for routine gastroscopy. The endoscope was first introduced into the duodenum as far as the third part of the duodenum. Then, the balloon tip was inflated to anchor the tip of the endoscope at this part of the intestine. The overtube was slid to the most anterior position and the balloon inflated, anchoring it firmly to this part of the duodenum. The balloon at the tip of the endoscope was deflated and gradually inserted further into the small intestine beyond the ligament of Treitz. The balloon tip was again inflated and the overtube balloon was deflated. The overtube was slid to the anterior position and the entire endoscope, together with the overtube, was pulled out to shorten the inserted length, and pleated the small intestine onto the shaft of the endoscope. The entire procedure was repeated several times to increase the depth of insertion. For retrograde insertion, the endoscope was inserted into the anus, rectum, and sigmoid colon as in a regular colonoscopy examination. The balloons were inflated and deflated as previously described to facilitate the advance of the endoscope to the cecum. The overtube balloon was inflated to anchor it securely at the cecum, and the tip of the endoscope with its deflated balloon was inserted into the ileocecal valve. The endoscope was manipulated to have a safe length inside the ileum, and the balloon tip inflated. The overtube balloon was deflated and the overtube was slid carefully to the anterior position through the ileocecal valve. The overtube balloon was inflated again to allow secure anchoring at the terminal ileum. The endoscope balloon was deflated and the tip of the endoscope gradually advanced deeper into the small intestine. The process of inflation and deflation of the balloons and advancing of the endoscope were repeated to achieve deeper insertion of the endoscope into the small intestine, until it could go no further or the suspected lesion was reached.

During the procedure, nursing assistance was needed for maintaining the endoscope and overtube at the proper position during various phases of the procedure, and for inserting the overtube to the 1.55-m mark on the surface of the endoscope. When a pathological lesion was detected during the procedure, the endoscopic nurse assisted in obtaining biopsies, injection of dye, removal of polyps *via* diathermy snare, and other tasks. The procedure usually took more time than regular gastroscopy or colonoscopy, hence, the period of anesthesia was also longer. Patient vital signs were carefully monitored, and the condition of the abdomen closely observed. Excessive inflation of air can cause gross distension of the abdomen; in this case, the operator must be alerted and air removed from the intestinal lumen. In this series, we did not encounter any perforation or major bleeding after the procedure.

Post-procedural nursing care

After the examination, vital signs were closely monitored in the recovery room until the patient was fully conscious. For patients undergoing antegrade examination, the head was turned towards one side, and any secretion or vomit was cleared from the oral cavity and pharynx to prevent aspiration. When fully conscious, patients may complain of a slight headache or sore throat. This was thoroughly explained to patients and parents. The long procedure time and repeated insertion and withdrawal of the overtube can result in frictional injury to the pharynx that usually does not require special treatment. Management was usually supportive, including rinsing the mouth with chilled saline, which can be effective in soothing the oral and pharyngeal mucosa and reducing discomfort. For patients who underwent retrograde DBE, rectal bleeding can be a complication and was watched for; nursing care to the anus was also performed. The patients were usually kept nil by mouth for 6 h after the procedures until they were fully conscious. Feeding was initiated with a fluid diet, and after eating, patients were monitored for nausea, vomiting, and abdominal pain. Changes in level of consciousness, stool characteristics, and other symptoms were closely observed. Any deterioration was reported to the doctors responsible for the patient. The small intestine is very long, so after the procedure, gas tends to be retained in the intestine, which results in distension. Patients were encouraged to pass gas through the anus or by burping, and early ambulation also enhanced the passage of gas from the system.

Table 3 Endoscopic findings and diagnoses from double-balloon enteroscopy in 37 patients

Endoscopic findings or diagnoses	Cases (%)
Inflammatory bowel disease	13 (35.1)
Meckel's diverticulum	5 (13.5)
Ulcerations or erosions	4 (10.8)
Non-specific ileitis	2 (5.4)
Jejunal polyp	1 (2.7)
Amebiasis	1 (2.7)
Anaphylactoid purpura	1 (2.7)
Congenital small intestinal lymphangiectasia	1 (2.7)
Overall positive rate	28/37 (75.5)

Equipment cleansing and sterilizing after use

After the procedure, the enteroscope was immediately cleaned as a preliminary procedure, and then fully treated in the endoscope treatment room with water, enzyme, antiseptic and finally rinsing with 75% ethyl alcohol and water. The enteroscope was dried with air current and hung in the endoscope cabinet for future use.

RESULTS

Among the 37 cases, lesions were detected in 28 (75.5%) (Table 3). Lesions were mainly inflammatory bowel disease, Meckel's diverticulum, jejunal polyp, anaphylactoid purpura, and congenital small intestinal lymphangiectasia. Of 10 cases that were investigated for abdominal pain, no mucosal abnormality in the small intestine was detected in seven (positive rate of 30%), and these were probably cases of functional disorders that resulted in abdominal pain. In 10 of 12 patients with occult gastro-

intestinal bleeding, the bleeding source was found (positive rate of 83.3%). The positive rate for patients with suspected intestinal bleeding was higher than for patients with abdominal pain.

In all procedures, patients who underwent examinations had no complications during or after DBE, and the average procedure time was 101 ± 53.0 min (antegrade: 91 min; retrograde: 104 min).

DISCUSSION

Yamamoto *et al*[1], and May have been pioneers in applying DBE for clinical use. They generally regard DBE as a safe procedure and the appearance of bleeding or perforation are rare complications. Recent reports have confirmed the safety of DBE in pediatric patients[6,7]. A majority of patients may develop abdominal distension, mild abdominal pain or sore throat, but these symptoms are mostly self-limiting and resolve spontaneously without any specific treatment. In this study, no major complication resulted after the procedure, which confirmed that DBE is a relatively safe procedure in the pediatric age group.

In our series of 37 patients who underwent a total of 42 DBE procedures, the positive rate was 75.5%, and the preliminary indication for DBE examination was occult gastrointestinal bleeding. In 12 patients with intestinal hemorrhage, five were diagnosed with Meckel's diverticulum, and in these, conventional diagnostic methods including ^{99m}Tc scaning, did not yield a definitive diagnosis. Meckel's diverticulum is usually located 50-100 cm from the ileocecal valve, therefore, it is out of the range of conventional endoscopic procedures. If Meckel's diverticulum is highly suspected, but ^{99m}Tc scanning is negative, DBE examination may be considered. In the present study, the positive rate for patients with suspected intestinal bleeding was higher than for patients with abdominal pain.

In our study, 15 patients underwent DBE examination for suspected Crohn's disease, and diagnosis was confirmed in 13. Characteristic changes were found in all 13 patients, such as aphthous ulcers, intestine stenosis and discontinuity inflammatory lesions. Lesions of Crohn's disease are beyond the reach of traditional colonoscopy, therefore, DBE examination may be a good choice for patients with suspected Crohn's disease. Our research demonstrated that DBE has high diagnostic value for Crohn's disease.

DBE is a reliable procedure for the investigation of small intestinal pathology, and its safety and reliability have been reported in various clinical studies[8-11]. In the present study, a significant 75.5% of patients had a positive diagnosis after examination, which was comparable to other centers, both in China and internationally[12-14]. Videoendoscopy is superior to other modalities of investigation for the diagnosis and management of gastrointestinal disorders. DBE, as a successor to traditional gastroscopy and colonoscopy, is a major advance in gastrointestinal endoscopy[15-18]. Through cycles of insertion, anchoring and pulling, the small intestine can be shortened by telescoping it onto the shaft of the endoscope, which enables examination of regions beyond the reach of the endoscope length. Compared to the maximum depth of insertion of a traditional enteroscope, which is 80-100 cm from the ligament of Treitz, DBE can be inserted much farther. Normally, the mid-ileum can be reached, and the terminal ileum can be reached in some patients. The double balloon endoscope provides a wide visual field and images of high clarity and definition. Moreover, similar to a regular gastroscope or colonoscope, with DBE, it is possible to insufflate air, aspirate, and perform biopsies and therapeutic procedures when necessary. It is now considered a gold standard for the diagnosis and management of small intestinal diseases that cannot be replaced with other means.

After 42 DBE procedures, we made the following observations from a nursing perspective. To assist endoscopists with performing DBE and to minimize the suffering of sick children, the endoscopic nurse should: (1) meticulously examine the instrument before the procedure, paying special attention to the installation of the endoscope balloon to ensure that it is functioning properly and free from leakage; (2) provide psychological support and intestinal preparation; (3) closely monitor the vital signs, and fully cooperate with the endoscopist during the procedure, to control the insertion and withdrawal of the endoscope and overtube, and occasionally introduce water or lubricant to the space between the endoscope shaft and overtube, to reduce friction; (4) be aware of markings on the endoscopic shaft to prevent damage to the endoscopic balloon; and (5) ensure that after the procedure, the child fasts for 6 h before a fluid diet is introduced. The child can usually be fed normally on the second day.

In conclusion, in adults, DBE is a well-established procedure that is used in many countries. Its application in the pediatric age group is relatively recent; hence, few reports are available on this topic. This study investigated the nursing perspective in cases conducted in our hospital under intravenous or general anesthesia. We conclude that DBE is a safe and reliable procedure in the pediatric age group, with few complications and little suffering. High quality nursing care and good coordination with the endoscopists are essential to the successful conduction and completion of the procedure. We look forward to conducting a prospective study on patients undergoing DBE, preferably with a large sample size. We hope that, as nurses, we can better collaborate with physicians, so that procedure time can be shortened and patient suffering can be minimized.

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COMMENTS

Background

Technical challenges have obstructed the diagnosis and treatment of small intestinal disease. The small intestine is long, tortuous, far from both ends of

the digestive tract, and unfixed in position. An innovative form of enteroscopy, double-balloon enteroscopy (DBE), allows full-length visualization, biopsy, and endoscopic treatment of previously inaccessible lesions. The diagnostic and therapeutic benefits of DBE have been well documented in the adult population. To date, little has been published to evaluate the safety and efficacy of DBE in pediatric patients and the impact of nursing on this procedure, which has its own unique set of indications, limitations, and potential complications.

Research frontiers

DBE constitutes a new procedure for digestive endoscopy that makes direct visualization of the entire small bowel possible, with the simultaneous ability to take biopsy specimens and carry out endoscopic interventions. However, more studies are needed to evaluate the diagnostic value of DBE in children with suspected small intestinal disease, and determine the role of appropriate nursing care in reducing the incidence of complications, shortening the examination, and improving the lesion-detection rate.

Innovations and breakthroughs

Publications on pediatric DBE operation and nursing care are limited. In this report, a descriptive, qualitative study was conducted on 37 pediatric patients who underwent 42 DBE examinations for suspected small intestinal diseases. The clinical significance and salient points for nursing are summarized.

Applications

In this study, the pre-procedural, intraprocedural, and post-procedural nursing care were described in detail. In addition, the points of nursing care for pediatric patients undergoing DBE are summarized, which may offer a reference strategy for future DBE operations.

Terminology

Antegrade DBE is administered through the mouth, whereas retrograde DBE is inserted through the anus. The route of insertion is determined by the clinical features and results of other ancillary examinations. If the suspected lesion is low in the intestinal, retrograde DBE should be chosen.

Peer review

In this paper, the authors review the safety, clinical efficacy and nursing care of DBE in children. This topic is interesting in the pediatric age group but before publication, the authors should discuss the indications for pediatric DBE in more detail.

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BRIEF ARTICLE

Epidemiological and clinical features of hepatitis B virus related liver failure in China

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Abstract

AIM: To examine the epidemiologic and clinical characteristics of hepatitis B virus (HBV) related liver failure in patients in China.

METHODS: This study was conducted with a retrospective design to examine 1066 patients with HBV-related liver failure in the southwest of China.

RESULTS: There were more male than female patients. Young and middle-aged people comprised most of the patients. Farmers and laborers comprised the largest proportion (63.09%). Han Chinese accounted for 98.12%, while minority ethnic groups only accounted for 0.88% of patients. A total of 43.47% patients had a family history of HBV-related liver failure and 56.66% patients had a history of drinking alcohol. A total of 42.59% patients with HBV-related liver failure had definite causes. With regard to the clinical manifestation of HBV-related liver failure, the symptoms were: hypodynamia, anorexia and abdominal distension. Total bilirubin (TBIL) and alanine aminotransferase (ALT) levels were altered in 46.23% of patients with evident damage of the liver. Univariate logistic regression analysis showed that the patients' prognoses were correlated with ALT, aspartate aminotransferase, albumin, TBIL, prothrombin activity (PTA), and alpha-fetoprotein levels, and drinking alcohol, ascites, hepatorenal syndrome, infection and $\geqslant$ 2 complications. Multifactor logistic regression analysis showed that the activity of thrombinogen and the number of complications were related to the prognosis.

CONCLUSION: Alcohol influences the patients' prognosis and condition. PTA and complications are independent factors that can be used for estimating the prognosis of HBV-related liver failure.

Key words: Hepatitis B virus related liver failure; Chronic hepatitis B; Epidemiology; Prognosis

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INTRODUCTION

Hepatitis B virus (HBV) infection is a severe threat of public health worldwide. Two billion people have been infected with HBV out of a total population of 6 billion, including chronic HBV infection in 350 million people[1,2]. One million people have died of liver disease related to HBV, 75% of which are distributed in the Asian-Pacific area[3]. China has a high occurrence of HBV infection. A survey of national epidemiology announced in April 2008 by the Ministry of Health showed that 93 million people in China have been infected with HBV.

The features of HBV-related liver failure include a lot of complications in patients, difficulty of treatment and a high fatality rate. Therefore, a large sample investigation about the natural history and the clinical process of HBV-related liver failure are required. This study analyzed the epidemiologic and clinical characteristics of HBV-related liver failure, based on a sample of 1066 cases in the southwest region of China.

MATERIALS AND METHODS

Case selection

All the 1066 cases were chosen from inpatients of the General Infectious Disease Institute of Southwest Hospital, the Third Military Medical University of China PLA, from February 2003 to December 2009. The patients were mostly from Chongqing and Sichuan, including the southwest regions in Guizhou and Yunnan.

The selection criteria included: (1) patients with chronic hepatitis B; (2) serum total bilirubin (TBIL) ≥ 171 μmol/L, prothrombin activity (PTA) ≤ 40% and complete data. The exclusion criteria were: (1) liver transplantated patients; (2) a short time of hospitalization (< 72 h); (3) patients with missing clinical and laboratory data; and (4) patients with associated tumors and other major diseases.

Methods

Epidemiologic survey: A questionnaire was given to the patients, which required information such as age, sex, ethnic group, career, family history, history of drinking alcohol, inducement of HBV-related failure, symptoms, physical signs, laboratory examinations, and complications. Questions that were not properly answered were not included in the statistical analysis. The daily alcohol intake (g) is equal to: alcohol intake × 0.8 × spirit (%), which was classified into low, medium, and high degrees (Table 1).

Laboratory examinations: Serum biochemical tests of alanine aminotransferase (ALT), AST, total bilirubin and albumin levels were measured by a Hitachi 7060 full-automatic chemical analyzer. α-fetoprotein (AFP), hepatitis B surface antigen (HbsAg), hepatitis B core antibody (HbcAb), hepatitis B e antigen (HbeAg) and hepatitis B e antibody (HbeAb) were measured using a German Roche Elecsys 2010 full-automatic electrochemiluminescence analyzer.

Serum HBV DNA was measured by a PE5700 instrument (ABI) and the reagent kits were from Cloning Biological High-tech Co., Ltd. (Shanghai, China). HBV DNA ≥ 1000 copy/mL (3.0 logl0) was positive.

Statistical analysis

SAS V8.0 statistical software was used for analysis. Data were shown as means ± SD. Potential factors that may have influenced the prognosis were examined by logistic analysis. $P < 0.05$ indicates statistical significance.

Table 1 Classification of alcohol intake

Classification	Male (g/d)	Female (g/d)
Low	< 50	< 25
Medium	50-100	25-50
High	> 100	> 50

Table 2 Characteristics of 1066 cases with hepatitis B virus related liver failure

Item	Group	*n* (%)
Gender	Male	901 (84.52)
	Female	165 (15.48)
Age (yr)	< 20	13 (1.22)
	20-29	192 (18.01)
	30-39	338 (31.71)
	40-49	305 (28.61)
	50-59	152 (14.26)
	> 60	66 (6.19)
Occupation	Farmer	345 (32.39)
	labor	327 (30.70)
	Soldier	225 (21.13)
	Office clerk	58 (5.45)
	Teacher	42 (3.94)
	Student	23 (2.16)
	Merchant	20 (1.88)
	Driver	11 (1.03)
	Doctor	9 (0.85)
	Nurse	3 (0.28)
	Painter	1 (0.09)
	Policeman	1 (0.09)
Ethnic groups	Han	1046 (98.12)
	Tujia	16 (1.50)
	Miao	3 (0.28)
	Gelao	1 (0.09)

RESULTS

Epidemiology

Among the 1066 patients with HBV-related liver failure, there were 901 males (84.52%) and 165 females (15.48%), with a male: female ratio of 5.46:1.

The mean age was 39.76 ± 11.69 years (range, 12-75 years). The highest morbidity was in the age group of 30-39 years (31.71%, Table 2). The age group with the second highest morbidity was between 40 and 49 years (28.61%), followed by 20-29 years (18.01%), 50-59 years (14.26%), > 60 years (6.19%) and < 20 years (1.22%) (Table 2).

With regard to the occupation structure of the patients with HBV-related liver failure, farmers comprised the highest proportion , followed by laborers, cadres, teachers, students, businessmen, drivers, doctors, nurses and a painter and a policeman (Table 2).

A total of 1046 (98.12%) patients belonged to the Han ethnic group, followed by the Tujia minority ethnic group (1.50%), the Miao minority ethnic group (0.28%), and the Gelao minority ethnic group (0.09%) (Table 2).

Family history and history of alcohol drinking

A total of 463 patients (43.47%) had a family history of HBV-related liver failure and 56.66% of patients had a history of drinking alcohol. Two hundred patients seldom drank alcohol (18.76%), 171 patients drank alcohol lightly

Table 3 Inducement of chronic hepatitis B into severe hepatitis/liver failure

Inducement	*n* (cases)	Percentage (%)
Overlapping contagious virus infection	192	18.01
Overlapping hepatitis D virus infection	109	10.23
Overlapping hepatitis A virus infection	29	2.72
Overlapping hepatitis E virus infection	27	2.53
Overlapping hepatitis G virus infection	18	1.69
Overlapping hepatitis C virus infection	10	0.94
Drinking alcohol	87	8.16
Fatigue	56	5.25
Secondary infection	54	5.12
Gallbladder disease	38	3.56
History of dirty diet	10	0.94
Pregnancy	9	0.84
Medication damaging Liver	6	0.56
Hyperthyreosis	2	0.19
Uncertain inducement	612	57.41
One type of inducement	371	34.80
Two types of inducement	72	6.75
⩾ Three types of inducement	11	1.03

Table 4 Clinical manifestations in patients with hepatitis B virus related liver failure

Clinical manifestations	*n* (cases)	Percentage (%)
Symptoms		
Hypodynamia	417	41.62
Anorexia	407	40.62
Abdominal distension	224	22.18
Nausea and vomiting	215	21.04
Diarrhea	50	4.73
Physical signs		
Liver palms	387	37.46
Jaundice	271	26.54
Spider nevus	228	21.86
Hypersplenotrophy	161	15.25
Hepatomegaly	158	14.85
Hepatic pain	150	14.18
Edema	126	12.27

(16.04%), 108 patients drank alcohol moderately (10.13%) and 125 patients drank alcohol heavily (11.73%).

Inducement of chronic hepatitis B into severe hepatitis/liver failure

The incidence rate of HBV-related liver failure was highest in the presence of other contagious viruses that infect the liver. Among 192 cases (18.01%), 109 cases were also infected by HDV (Table 3). The second highest cause of inducement of disease was drinking alcohol, followed by fatigue and other infections. Over half of the patients had no ascertainable cause of disease. In those patients in whom the cause of disease was known, most only had 1 factor that induced the disease. None of the patients had more than 3 types of inducement of disease.

Clinical manifestations

On admission, the patients' main clinical manifestations were hypodynamia, loss of appetite and abdominal distension (Table 4).

Table 5 Laboratory data in patients with hepatitis B virus related liver failure

Laboratory indexes	Mean
ALT (IU/L)	272.51 ± 541.51
AST (IU/L)	262.13 ± 440.55
Glutamyltranspeptidase (IU/L)	91.24 ± 55.74
Alkaline phosphatase (IU/L)	187.41 ± 96.01
ALB (g/L)	32.08 ± 7.95
TBIL (μmol/L)	396.56 ± 190.52
Direct bilirubin (μmol/L)	234.48 ± 100.75
PTA (%)	15.62 ± 12.98
Glucose (mmol/L)	5.35 ± 3.86
Blood urea nitrogen (mmol/L)	59.45 ± 970.09
Creatinine (μmol/L)	204.40 ± 613.78
AFP (ng/mL)	191.26 ± 221.36
HBV DNA (copies/mL)	$4.3 \pm 8.8 \times 10^7$

ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; ALB: Albumin; TBIL: Total bilirubin; PTA: Prothrombin activity; AFP: α-fetoprotein; HBV: Hepatitis B virus.

Table 6 Complications in patients with hepatitis B virus related liver failure

Complications	*n* (cases)	Percentage (%)
Ascites	509	47.83
Hepatic encephalopathy	506	47.48
Ⅰ level	34	3.86
Ⅱ level	178	20.23
Ⅲ level	122	13.86
Ⅳ level	172	19.55
Hernia of brain	29	2.72
Hydrocephalus	78	7.32
Hemorrhage of digestive tract	234	21.95
Hepatorenal syndrome	210	19.70
Spontaneous bacterial peritonitis	103	9.66
Infection	417	39.12
Electrolyte imbalance	141	13.23

Laboratory examinations

The laboratory data of the patients are shown in Table 5. A total of 11.54% (123 cases) of the cases were HBeAg positive, 22.52% (240 cases) were HBeAb positive, 78.30% (834 cases) were HBV DNA positive, and the mean value was $4.3 \pm 8.8 \times 10^7$copies/mL.

Complications

The most common complications were ascites, hepatic encephalopathy, and infection. Hemorrhage of the digestive tract and electrolyte imbalance were the next most common complications (Table 6).

Prognosis

The patients were divided into 2 groups: the improved group and the deteriorated group (including exacerbation and death). The description "improved" was used

to define those patients who were able to be discharged from hospital because clinical symptoms improved and liver function recovered. The description of "deteriorated" was defined as patients who died or deteriorated when they were voluntarily discharged from the hospital, except for those who died or further deteriorated because of other diseases or accidents. Three hundred and forty-five cases improved (34.36%) and 721 cases were deteriorated (67.64%) (369 cases died and 352 cases were exacerbated). The rate of death due to the disease was 34.62%.

Logistic single factor regression analysis

Logistic regression analysis was performed for 42 factors that might have influenced the prognosis, using prognosis (improvement and unsuccessfully treated) as the dependent variable. The result showed that the patients' prognoses were related to ALT, AST, ALB, TBIL, PTA, and AFP levels, and drinking alcohol, ascites, hepatorenal syndrome, infection and $\geqslant 2$ complications.

Logistic multifactor regression analysis

Logistic multifactor regression analysis was performed for prognostic factors that were screened out by single factor regression analysis. We found that PTA ($P = 0.03$) and the number of complications ($P = 0.01$) were independently related to the prognosis of HBV-related liver failure.

DISCUSSION

In China, the main transmission route of HBV is vertical transmission and the secondary way is by blood products. Among the patients in our study, there were more males than females, while the number of males with HBV-related liver failure is increasing. With regard to profession, farmers and laborers comprised the largest proportion at 63.09%, with farmers occupying even larger proportion than laborers. The cause of the illness might be related to people's life style and working environment, inaccurate comprehension of the disease due to poor medical conditions and minimal schooling, and missing the best time for treatment because of not visiting a doctor in time. Among the ethnic groups, the Han accounted for 98.12% of patients, while minority ethnic groups only accounted for 0.88%. This finding could be because economic conditions are better and the population of the Han is higher compared with the ethnic minorities in the southwest of China. The result of single factor analysis showed that the patients' prognosis was not related to sex, age, occupation and ethnic groups.

The recurrence and aggravation of chronic HBV are due to various inducements during the long repetitive chronic process. Based on our data analysis, illness conditions deteriorated in 42.59% of patients in whom the cause of disease was known. The main factors responsible for inducing the illness were as follows: superinfection with other contagious viruses that infected the liver, drinking alcohol, fatigue and being complicated with other infections. With regard to superinfection with other contagious viruses that infect the liver, internationally, it is regarded that HGV virus infection does not cause liver failure, but it is rather found in patients co-infected with HBV. In China, drinking alcohol is common because of the rich "alcohol culture" and gradually enriched material conditions. Young and middle-aged people are busy with work and are under great social pressure. These factors, which have resulted in a trend for a lower average age for HBV-related liver failure, are the reasons for inducing and exacerbating the illness. Infection was found to be another cause of HBV-related liver failure, with 10 cases having liver failure due to an unclean diet history. Eight of these 10 cases had diarrhea and the patients may have been complicated with gastrointestinal infection. If the inducement of HBV-related liver failure is fatigue, it is related to damage of the patient's immune system. In 57.41% of patients, their illness deteriorated and there did not appear to be any definite cause of HBV-related liver failure. This may be related to several factors such as social environment, job competition, mental stress and emotional factors. In summary, infection (including being complicated with other hepatitis virus infections and other infections) is the biggest inducement of the disease, which is similar to the findings in other reports[4,5]. In addition, the factor of alcohol further increased the possibility of HBV-related liver failure.

Our data showed that the characteristics of severe hepatitis in the southwest of China are similar to acute liver failure and acute-on-chronic liver failure abroad, and these included acute onset, inducement for initiating or worsening of the disease, superinfection by hepatitis B and D viruses, and being complicated by infection and fatigue. Clinical manifestations of HBV-related liver failure involve two main types: alimentary tract symptoms, such as yellowing of the skin and sclera, hypodynamia, anorexia, abdominal distension, and physical signs of hepatitis such as liver palms, hepatic face, and spider nevus. Some of the patients did not have encephalopathy at the early stage of the disease, and this occurred after hospitalization. Some of the patients had ascites as the main clinical manifestation at admission, and most of them had secondary onset of hepatic encephalopathy. The prognosis of patients with hepatic encephalopathy greater than stage II was worse.

According to the laboratory data, liver function indicated damage to the liver and PTA was decreased. In the early stage of the disease, ALT and AST levels were increased. TBIL was also increased. The results of single factor analysis showed that patients' prognoses were related to ALT, AST, ALB, TBIL, PTA and AFP levels, which is consistent with other studies in China and in other countries[6-8]. Multifactor logistic regression analysis showed that PTA was independently related to the prognosis. PTA is the most important biochemical index used to determine the aggravation of chronic hepatitis B[9]. The lower the level of PTA, the higher the rates of hemorrhage and fatality[9]. The prognosis is bad if PTA is < 30%, and if this

is the case, the majority of patients die[9]. The quantity of serum bilirubin reflects the degree of damage to liver cells. TBIL appeared to be related to HBV-related liver failure, but multifactor analysis showed that TBIL was not a factor that affected the prognosis. It is generally acknowledged that the higher the level of AFP, the better the prognosis of patients with liver failure. The US Acute Liver Failure Study Group has shown that a 1-fold higher level of AFP is not related to a good prognosis; however, patients' prognoses are relatively good when AFP levels are increased 3 days after hospitalization[10].

HBV-related liver failure/severe hepatitis B is a serious disease. The incidence rate of complications is high. It is critical to prevent complications to improve the survival rate[11]. Our study results showed that 70.73% of patients had up to several complications. A total of 48.78% of patients had 2 or more complications. The type, quantity and the degree of severity of complications are important factors that can influence the outcome of HBV-related liver failure/severe hepatitis B. In our study, single factor analysis showed that the patients' prognoses were related to ascites, hepatorenal syndrome, infection and $\geqslant$ 2 complications. Multifactor analysis showed that the number of complications was an independent risk factor of HBV-related liver failure. In the USA and European countries, the first manifestation of hepatic failure is often hepatic encephalopathy. However, according to our data, ascites is the main manifestation in China. Infection is usually the earliest complication occurring in the midterm stage of the disease. Our data showed that infection was a complication that occurred in the early stage of HBV-related liver failure. Infection was related to the prognosis and it also aggravated the disease. Previous studies have shown that 60% to 80% of liver failure patients have secondary bacterial or fungal infection[12-17]. Riordan and Williams demonstrated that approximately 80% of patients with severe HBV are complicated with infection, which is difficultly controlled[18]. Because of the complexity of the pathogenesis of liver failure, the present system for estimating the prognosis cannot predict the results, although there is a great deal of patients' data available.

Liver failure is severe liver damage caused by various factors, which cause obstruction or decompensation of function, such as composition, detoxification, drainage and biotransformation[19]. Various clinical syndromes can appear, including the obstruction of coagulation mechanisms, icterus, hepatic encephalopathy and ascites[19]. According to the speed of pathological development, histology of liver failure and the patient's condition, liver failure can be classified into 3 types: acute liver failure (ALF), acute-on-chronic liver failure (ACLF) and chronic liver failure (CLF)[19,20]. According to morbid physiology, liver failure is mainly divided into two types that separately result in necrosis caused by the inflammation of liver cells and the decompensation of liver cells. ALF belongs to the type of liver failure that results in necrosis caused by inflammation of liver cells[19]. ACLF and CLF belong to the type of liver failure with decompensation of liver cells[19]. Patients with ALF have symptoms such as abnormal cruor (usually an international normalized ratio $\geqslant$ 1.5), a change in consciousness to varying degrees (encephalopathy), and the duration of disease is less than 26 wk[21,22]. Patients with ACLF have acute decompensation on the basis of chronic liver disease (TBIL $\geqslant$ 171 μmol/L)[23]. Patients with CLF have chronic decompensation of liver function (TBIL < 171 μmol/L) caused by a decrease in liver function on the basis of the final phase of hepatism[19]. According to the diagnostic standard discussed above[19-23], in our study, 654 cases had ACLF, 296 cases had CLF, and 116 cases had ALF.

The term "liver failure" is used in European countries and the USA because it is associated with function, whereas it is called severe chronic hepatitis in China and Japan because it is associated with inflammation. Hepatitis virus that appears to be acute liver failure is called severe hepatitis[24]. The main difference between the terms "liver failure" used in the USA and European countries and "severe hepatitis" used in China is whether to include hepatic encephalopathy in the diagnosis. Some patients with liver failure do not have hepatic encephalopathy[25]. Severe damage of the liver may develop into liver failure before hepatic encephalopathy occurs.

Although there are differences, liver failure has been divided into ALF, including the acute and sub-acute types, and CLF, including the chronic acute and chronic decompensated types, and this point of view gradually becomes consistent among international academic communities. Because of the large amount of etiologies of liver failure, physicians use a combination of clinical diagnoses (e.g. chronic severe hepatitis) and morbid physiology diagnoses (e.g. CLF). Liver decompensation is the main manifestation of chronic liver failure. Patients with this disease may not have hepatic encephalopathy, but patients with acute liver failure must have hepatic encephalopathy[26-30].

In conclusion, the morbidity of chronic HBV is steadily increasing. Once chronic HBV develops into HBV-related liver failure/chronic severe hepatitis, the liver is seriously damaged with complex symptoms, it develops rapidly, it has many complications, it is difficult to treat and it has a high death rate. We advise patients with hepatitis to enhance self-protection and prevent bad life-style habits, so that they can be diagnosed in the early stage and be cured in a timely manner with positive results and treatment of complications, so as to ultimately reduce the death rate.

COMMENTS

Background

Hepatitis B virus (HBV) infection becomes a severe threat for public health worldwide. The features of HBV-related liver failure include: a serious condition of the patients, a high incidence of complications, difficulty of treatment and a high fatality rate. Large-sample studies are needed to determine the natural history and clinical process of HBV-related liver failure.

Research frontiers

This study investigated the inducement of liver failure/severe hepatitis B and the independent risk factors associated with its prognosis.

Innovations and breakthroughs

This study explored the inducement and prognosis of hepatitis B virus related liver failure as well as the diagnostic classification of liver failure.

Applications

This study is useful in clinical management and prognosis prediction of hepatitis B virus related liver failure.

Terminology

HBV-related liver failure/chronic severe hepatitis: HBV-related liver failure refers to patients with liver failure caused by chronic hepatitis B virus (HBV) infection. Liver failure is severe liver damage caused by various factors, which cause obstruction or decompensation of function, such as composition, detoxification, drainage and biotransformation. Chronic severe hepatitis refers to patients with evidence of chronic liver disease that leads to acute decompensation of liver function.

Peer review

The manuscript is accepted after minor revisions.

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BRIEF ARTICLE

Effects of refluxate pH values on duodenogastroesophageal reflux-induced esophageal adenocarcinoma

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Abstract

AIM: To determine the effects of duodenogastric juice pH on the development of esophageal adenocarcinoma (EAC).

METHODS: An animal model of duodenogastroesophageal reflux was established using Sprague-Dawley (SD) rats undergoing esophagoduodenostomy (ED). The development of EAC was investigated in rats exposed to duodenogastric juice of different pH. The rats were divided into three groups: low-pH group (group A), high-pH group (group B) and a sham-operated group as a control (group C) (n = 30 rats in each group). The incidence of esophagitis, Barrett's esophagus (BE), intestinal metaplasia with dysplasia and EAC was observed 40 wk after the treatment.

RESULTS: The incidence rate of esophagitis, BE, intestinal metaplasia with dysplasia and EAC was higher in groups A and B compared with the control group after 40 wk ($P < 0.01$), being 96% and 100% ($P > 0.05$), 88% and 82.4% ($P > 0.05$), 20% and 52.1% ($P < 0.05$), and 8% and 39% ($P < 0.05$), respectively.

CONCLUSION: Non-acidic refluxate increases the occurrence of intestinal metaplasia with dysplasia and EAC while the low-pH gastric juice exerts a protective effect in the presence of duodenal juice. The non-acid reflux is particularly important in the progression from BE to cancer. Therefore, control of duodenal reflux may be an important prophylaxis for EAC.

Key words: Esophageal reflux; Esophageal adenocarcinoma; pH-metry; Pathogenesis

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Cheng P, Li JS, Gong J, Zhan LF, Chen RZ. Role of pH refluxate pH in duodenogastroesophageal reflux-induced esophageal adenocarcinoma. *World J Gastroenterol* 2011; 17(25): 3060-3065 Available from: URL: http://www.wjgnet.com/1007-9327/full/v17/i25/3060.htm DOI: http://dx.doi.org/10.3748/wjg.v17.i25.3060

INTRODUCTION

The incidence rate of esophageal adenocarcinoma (EAC) has been increasing more rapidly than that of other malignancies[1]. This rapid increase may be related to the increasing occurrence of gastroesophageal reflux disease (GERD) and Barrett's esophagus (BE)[2,3]. BE is the main risk factor and an acquired condition for these tumors[4]. Gastric acid has been regarded as the major risk factor for GERD, and acid suppression is the first-line treatment[5]. However, the role of the gastric acid in the development

of GERD remains controversial.

Gastric juice refluxing into the esophagus contains gastric, biliary and pancreatic secretions that have refluxed into the stomach from the duodenum. Early studies showed that reflux of combined duodenal and gastric juices into the esophagus caused severe esophagitis[6], and reflux of duodenal juice alone resulted in a similar degree of esophageal injury[7]. It has been shown[8-12] that esophageal exposure to duodenal juice is a key factor in the genesis of BE and EAC. Some researchers have suggested that the obvious increase in the incidence of EAC might be related to the acid suppression[13]. However, to dynamically monitor the duodenal juices and clarify the role of duodenal juice reflux in the pathologic process has attracted much attention. Some studies have confirmed that duodenal juice reflux could induce BE and EAC in rats[10].

Improved animal models are therefore needed to examine the role of non-acidic reflux in EAC induced by duodenal juice reflux in the absence of exogenous carcinogens. The aim of this study was to investigate the roles of gastric and duodenal juices in the genesis of EAC in a rat model.

MATERIALS AND METHODS

Experimental animal

Ninety 8-wk-old Sprague-Dawley (SD) rats weighing 200-250 g were purchased from the Experimental Animal Center of Xi'an Jiao Tong University. The male pairing female rats were randomly divided into three groups, each with 30 rats.

Animal model

A rat model of duodenogastroesophageal reflux, with different pH values of reflux contents, was established in accordance with the method of Zhang *et al*[14]. Surgical diversion of duodenal secretions into the esophagus in the experimental group was induced by end-to-side esophagoduodenostomy (ED). The rats were divided into a low-pH group (group A) and a high-pH group (group B), with 30 rats in each group. A sham-operated group (group C, n = 30) was used as a control group (Figure 1).The esophagus was separated from the posterior vagal trunk and left gastric vessels, tied with silk at the gastroesophageal junction, and dissected 2 mm proximal to the tie. The anterior vagus nerve was protected from damage when the esophagus was cut with 16 interrupted stitches of 7-0 polypropylene. The purpose of the anastomosis was to induce the reflux of both gastric and duodenal juices into the esophagus. In group A, the anterolateral wall of the duodenum at the distal end 1 cm from the pylorus was opened longitudinally, and anastomosed with the cut end of the esophagus. In group B, the anterolateral wall of the duodenum at the distal end 2 cm from the pylorus was opened longitudinally and anastomosed with the cut end of the esophagus. In group C, the lower esophagus and the first portion of the duodenum were dissociated. Surgery was performed after an acclimatization period of 4 d. Rats were kept in hanging cages under a 12 h light-dark cycle at a temperature of 21℃ and a humidity of 60%. Water and standard chow were provided ad libitum. Food was discontinued the evening before surgery, and water was discontinued on the morning of surgery. Rats were anesthetized with an intramuscular injection of xylazine hydrochloride (18 mg/kg) and ketamine (72 mg/kg), with further doses administered intraperitoneally during surgery, as required. Before closure, 0.5-1.5 mL of 0.9% sodium chloride was instilled into the peritoneal cavity. Water was permitted when the rats awoke, and chow was provided the next day. The rats were housed in cages at 22-25℃ with free access to standard rat pellet food and water for 40 wk. Rats were treated following the guidelines for the care and use of laboratory animals of the National Animal Welfare Committee.

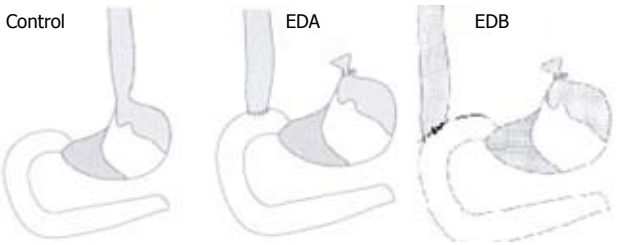

Figure 1 Animal model establishment. Esphagoduodenostomy group A (EDA) for duodeno-gastro-esophageal reflux of low pH value and esphagoduodenostomy group B (EDB) for high pH value respectively, and control for the sham operation group.

Intraluminal pH was measured using a pH glass electrode of Digitrapper MK Portable pH Monitor(Sweden Medtronic Synectics Company, Stockholm, Sweden). It was positioned in the distal end of the esophagus, the forestomach, the opisthogaster and the duodenum 1 and 2 cm from the pylorus in the process of esophagoduodenostomy. It was also measured after rats were killed 40 wk after operation.

Tissues and specimens

Rats were killed 40 wk after operation. The esophagus was opened longitudinally and gross pathologic changes were observed. Esophagitis, BE and EAC were differentiated, and samples of the three abnormal tissues (0.2 × 0.2 cm^2) were removed, and fixed in formalin. Paraffin sections were stained with hematoxylin-eosin and observed under a light microscope.

Statistical analysis

The incidence rates of esophagitis, BE and EAC between the groups were analyzed and compared using χ^2 tests with SPSS software, and differences in numerical data were compared between groups using t test. The level of significance was set at $P < 0.05$.

RESULTS

The number of the surviving rats in the three groups was 25, 23 and 29, respectively. The overall mortality was 14.4%.

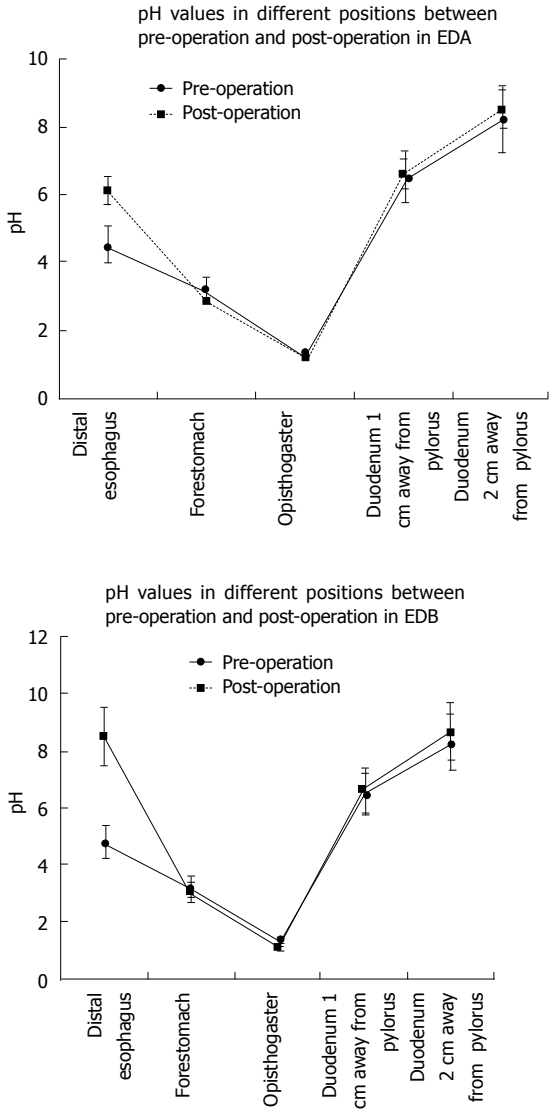

Figure 2 pH in different positions of esophagus, stomach and duodenum.

pH values in different parts of esophagus, stomach and duodenum

The pH values increased from the proventriculus down to the duodenum 2 cm away from the pylorus ($P < 0.05$), (Figure 2). The preoperative pHs at the distal end of the esophagus in groups A and B were significantly lower than the postoperative values ($P < 0.05$).

Postoperative pH values in the distal esophagus in groups A and B

The pH value in the distal esophagus in group A, in which the duodenum was cut 1 cm from the pylorus, was 6.14 ± 0.36, which was significantly lower than that in group B (8.27 ± 0.46, $P < 0.01$). There was no significant difference in preoperative pH values in the distal esophagus between the two groups ($P = 0.12$).

Gross observations

In the sham-operated group, the esophageal wall was thin, with a smooth mucosa, and the esophageal lumen was

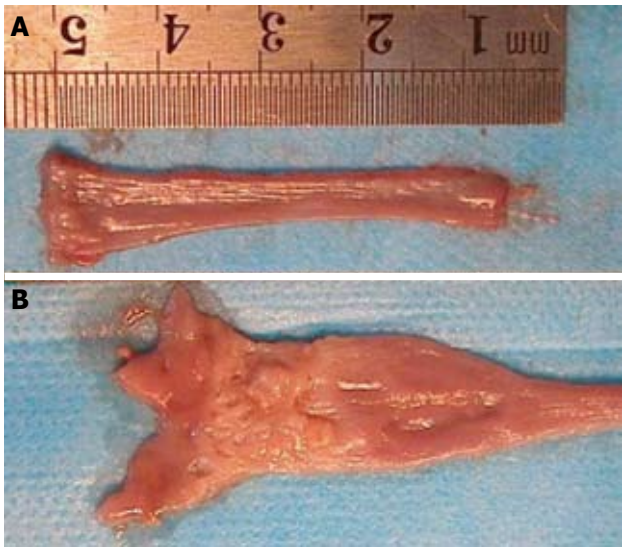

Figure 3 Gross specimens of the sham operated-group(shown in picture A) and animal model group(shown in picture B).

uniform in size along its length. Blood vessels were visible below the mucous membrane, and congestive inflammation was occasionally visible in the distal esophagus. In most animals in both groups, the lumen of the middle and the lower parts of the esophagus was dilated. Esophagitis appeared as mucosal hyperplasia, with a thickened, rough surface with small and large kernels in longitudinal rows, becoming less pronounced from the distal to the proximal end. Hyperemia, edema, mild erosion and indistinct blood vessels below the mucous membrane were visible at the proximal end. BE occurred mostly in the distal esophagus at the stoma between the esophagus and duodenum, and appeared as an unclear boundary between the esophagus and the duodenal mucosa. The esophagus was inflamed at the proximal end, smooth and velvet-like, with a clear boundary from the duodenum. The area of BE generally extended for about 0.5-2 cm, with chronic proliferation and inflammation at the proximal end of the esophageal mucosa. Small sheets of BE pathology were seen in some cases of esophagitis. The upper esophagus was normal in BE, and all esophageal adenocarcinomas (EACs) developed near the proximal end of the stoma in BE, with nodular hyperplasia, ulcer and fish-like appearance. The esophagus at the upper end of the tumor was obstructed, with obvious dilation and changes in the features of BE. The hyperplasia was reduced at the proximal end of the obstruction, and appeared as congestion of 1-2 mm and presented edema changes (Figure 3).

Histologic characteristics

Normal esophageal epithelium appeared as stratified squamous epithelium, with neat rows, some showing keratinization. Esophagitis appeared as hyperplasia of the scaly epithelial basal cells, excessive keratinization and papillomatosis, visible neutrophilic granulocytes, infiltrated lympho-epithelioid cells, mucosal erosion and edema of the submucosa in the mucosa and the lower layers of the mucosa. BE

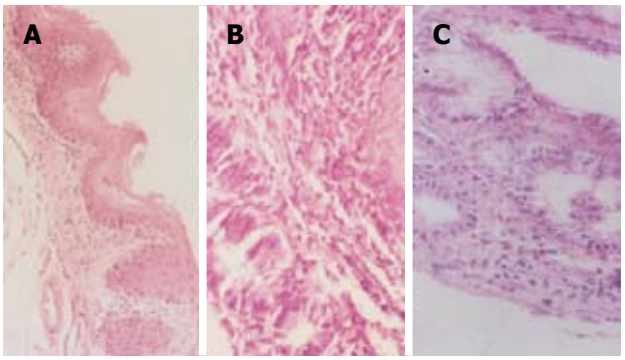

Figure 4 The changes of the esophagus mucosa in the sham operated group and the animal model groups under the light microscope (200 ×). A: Normal esophagus in the sham operated group; B: Esophagitis and barrett esophagus in the model group; C: Intestinal metaplasia with dysplasia and esophageal adenocarcinoma in the model group.

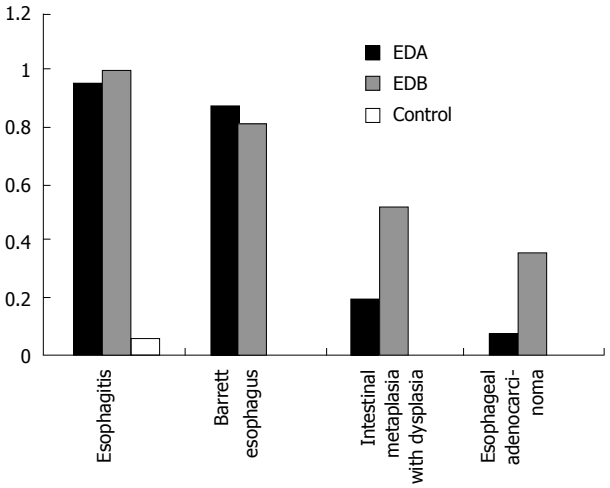

Figure 5 The incidence rates of diseases of esophagitis, Barrett esophagus, intestinal metaplasia with dysplasia and esophageal adenocarcinoma 40 wk after operation. The incidence of esophagitis, barrett esophagus, intestinal metaplasia with dysplasia and esophageal adenocarcinoma 40 wk after operation in the sham, operated group (Control), esophagoduodenostomy group A (EDA) and esophagoduodenostomy group B (EDB) group.

showed replacement of the squamous mucosa with simple columnar epithelium. Intestinal metaplasia with dysplasia showed replacement of the mucosa with simple columnar epithelium, changes in the size and shape of hyperplastic cells, with large, hyperchromatic nuclei and increased nucleoplasm, irregular arrangement of the cells, disappearance of cell polarity, and irregular shape and arrangement of the glandular cells. However, these changes were not characteristic of cancer and no obviously abnormal cells could be seen and no pathologic invasion to the basilar membrane. EAC showed severe intestinal metaplasia with dysplasia, pathologic invasion to the basilar membrane, and some to the blood or lymphatic vessels (Figure 4).

Incidence rates of esophagitis, BE, intestinal metaplasia with dysplasia, and EAC

The incidence rate of esophagitis in groups A and B 40 wk after the treatment was 96% and 100%, respectively (χ^2 = 0.930, P = 0.330). The equivalent incidence rate of BE was 88% and 82.4%, respectively χ^2 =0.280, P = 0.60), and of intestinal metaplasia with dysplasia was 20% and 52.1%, respectively (χ^2 = 5.420, P = 0.02). The incidence rate of EAC was 8% and 39% in the two groups, respectively (χ^2 = 6.570, P = 0.01). All these rates were significantly higher than in the sham-operated control group (P < 0.001) (Figure 5).

DISCUSSION

GERD occurs when the contents of the stomach and duodenum are regurgitated into the esophagus, causing pathologic lesions of the mucosa, and pathologic changes in the esophagus[15]. Gastroesophageal reflux can result in the development of EAC. The incidence rate of EAC has increased significantly in recent years, more rapidly than that of other tumors[16], and the annual increase in the incidence rate of GERD reflects the increasing incidence rate of EAC. Clinical epidemiological studies have shown that gastroesophageal reflux correlates closely with EAC[17].

The mechanism that gastroesophageal reflux induces EAC has been the subject of numerous studies[18], and a recent research has shown that reflux of both gastric and duodenal juices can damage the esophageal mucosa[19]. However, the contributions of the specific components of gastroesophageal reflux to the development of EAC remains unclear[20]. The current study used an animal model, in which the pH values of the duodenogastric reflux could be varied, to investigate the effects of gastric acid and duodenal juice on the EAC induced by gastroesophageal reflux, with the aim of identifying the specific responsible factors.

Gastric acid is believed to be an important contributory factor in reflux esophagitis[10,21]. However, recent studies suggest that the role of other refluxes in the morbidity of gastroesophageal reflux cannot be ignored[22]. With the development of biliary monitoring technology, the effects of duodenogastric reflux can be better understood. The duodenal contents include bile, pancreatic juice, and intestinal juice, of which cholic acid, trypsin and hemolytic lecithin could damage the esophageal mucosa[23,24]. Exposure to acid and duodenal contents for a prolonged period would lead to the damage of the esophageal mucosa, the development of BE, and even EAC. Animal experiments have shown that bile can damage the esophageal mucosa[9]. Cholic acid synergizes with gastric acid, and could damage the esophageal mucosa by increasing the acidic environment, thus reinforcing the damaging effects of acid and pepsase, instead of being destroyed by acid after combining cholic acid and trypsin[25].

An animal model was established in this study to investigate the duodenogastric reflux of contents with different pH values, their damage to the esophageal mucosa and their effects on the development of BE and EAC. The pH value of the duodenogastric reflux 2 cm away from the pylorus was significantly higher than that 1 cm away (P < 0.05). This difference was related to the relative proportions of sodium bicarbonate secreted by the pancreas and gastric acid secreted by the stomach. The pH values dif-

fered depending on the position in the esophagus relative to the anastomosis of the esophagus and duodenum; the pH value 2 cm away from the pylorus was higher than that 1 cm away from the pylorus ($P < 0.01$).

These results confirm that the esophagus was stimulated by the contents of the stomach and duodenum with different pH values; a higher pH indicated a higher proportion of duodenal juice, while a lower pH indicated a higher proportion of gastric juice.

Esophagitis, BE, intestinal metaplasia with dysplasia and EAC developed in both the treated groups after 40 wk. The incidence rates of intestinal metaplasia with dysplasia and EAC were higher in the high-pH group, compared with the low-pH group ($P < 0.01$). There were no significant differences in the incidence rate of esophagitis or BE.

The results of this study showed that the reflux of gastric juice and duodenal contents could induce EAC in rats. More acidic duodenogastric reflux was associated with lower incidence rate of intestinal metaplasia with dysplasia and EAC, compared with more basic duodenogastric reflux. These results suggest that duodenal juice reflux increases the incidence rate of intestinal metaplasia with dysplasia and EAC, thus playing an important role in the pathogenesis of EAC, while gastric juice regurgitation had an opposite effect. The results imply that non-acid reflux is particularly important in the progression from BE to cancer. Therefore, control of duodenal reflux may be an important prophylaxis of the EAC.

COMMENTS

Background

The incidence rate of esophageal adenocarcinoma (EAC) is rising faster than that of any other cancers. Clinical epidemiological studies have shown that gastroesophageal reflux correlates closely with EAC. However, the relationship between the specific reflux components and the induction of EAC remains unclear.

Research frontiers

Gastroesophageal reflux can cause EAC, and the mechanisms have been the subject of extensive research. The specific gastroesophageal reflux components responsible for EAC remain largely unknown. In this study, the authors demonstrated that non-acidic reflux increases the incidence rates of intestinal metaplasia with dysplasia and EAC, while acidic reflux had an opposite effect.

Innovations and breakthroughs

Recent reports have highlighted the importance of duodenal juice in the pathogenesis of EAC. The duodenum contains bile, pancreatic juice, and intestinal juice, of which cholic acid, trypsin and hemolytic lecithin could damage the esophageal mucosa. This is the first study to report a relationship between the pH of the duodenogastric refluxate and the incidence of EAC. The results of this study therefore suggest that duodenal juice plays an important role in the pathogenesis of EAC and gastric juice had an opposite effect.

Applications

Better understanding of the roles of the specific esophageal reflux components in the pathogenesis of EAC may represent a future strategy for the prevention of EAC.

Peer review

The article is original and well-thought. The topic of the research is important, as it would add to the body of evidence regarding the role of alkaline reflux in esophageal carcinoma. The manuscript is clearly laid out and well written. The methodology/design is suitable to answer the questions posed.

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CASE REPORT

Gastric carcinoid in a patient infected with *Helicobacter pylori*: A new entity?

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Abstract

There are four types of gastric carcinoid tumors, classified according to their histology and malignant potential. Only a few cases of carcinoid tumors in patients infected with *Helicobacter pylori* (*H. pylori*) have been reported so far. We report a patient infected with *H. pylori* presenting with a small solitary gastric carcinoid tumor with very low proliferative rate and normal gastrin levels. The tumor was endoscopically removed and the patient received an eradication therapy against *H. pylori*. No signs of metastatic disease have been found so far during more than 3 year of follow-up. Infection with *H. pylori* may cause chronic gastritis with normal or elevated gastrin levels, leading to the development of gastric carcinoids by mechanisms unrelated to gastrin. Enterochromaffin-like cell tumors related to a chronic *H. pylori* infection may be considered as a distinct type of gastric carcinoid tumors.

Key words: Gastric carcinoids; Gastrin; Gastritis; *Helicobacter pylori*

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INTRODUCTION

Gastric carcinoids are rare neuroendocrine tumors of the stomach that arise from the enterochromaffin-like (ECL) cells[1]. Initially, three types of gastric carcinoids were reported[2,3]. The first two types, which are multiple, are related to high gastrin levels; type Ⅰ arise in patients with autoimmune chronic atrophic gastritis type A and type Ⅱ occur in patients with the Zollinger-Ellison Syndrome. Type Ⅲ is a solitary tumor with no known correlation to gastrin production. More recently a highly aggressive variant has been described, named type Ⅳ gastric carcinoid tumor[1].

Helicobacter pylori (*H. pylori*) has been reported to cause chronic atrophic gastritis[4] and alteration of the gastric secretion[5]. Chronic gastritis caused by *H. pylori* can be a risk factor for gastric cancer[4], but the occurrence of ECL cell tumors in the stomach of patients infected with *H. pylori* is rare[6]. We here present a patient infected with *H. pylori* presenting with a solitary gastric carcinoid tumor.

CASE REPORT

A 60-yr-old woman from Sweden had been suffering from abdominal pain for several years and flushing since 2003. She had no family history for MEN I, Zollinger-Ellison syndrome or autoimmune gastritis. Gastroscopy in May 2006 due to oral lichen showed a polyp-like lesion in the

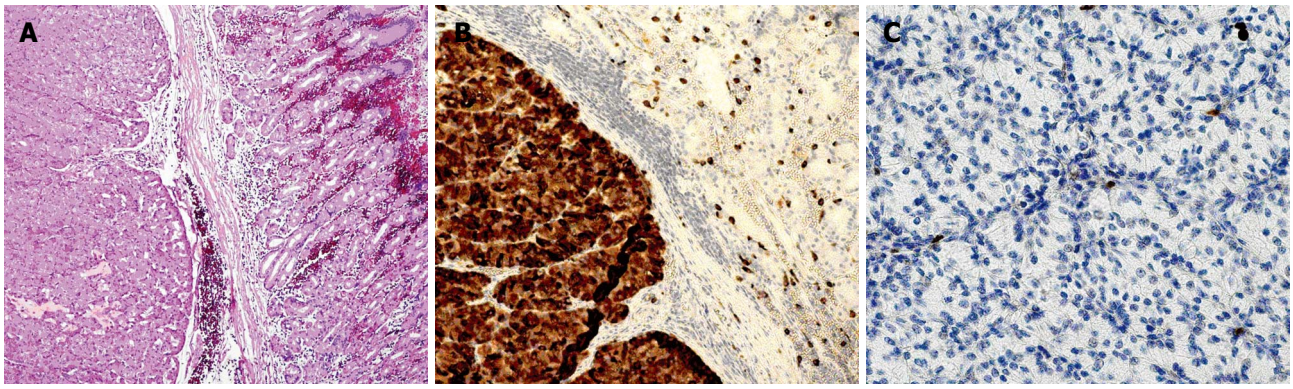

Figure 1 Gastric carcinoid. A: Infiltration of the muscularis mucosae; Hematoxylin-eosin stain. Magnification, × 50; B: Tumor and normal mucosa adjacent to tumor immunostained for VMAT-2. Virtually all tumor cells positive. Magnification, × 100; C: Tumor immunostained for Ki-67. < 1% tumor cells positive. Magnification, × 200.

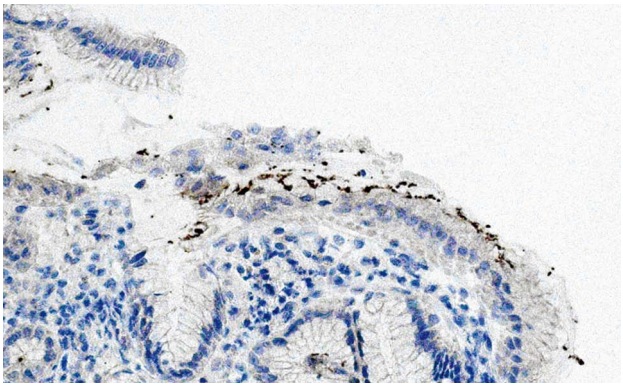

Figure 2 Signs of *Helicobacter pylori* infection in biopsy from antral mucosa. Magnification, × 200.

gastric body near the cardia. Microscopic examination showed a neuroendocrine tumor positive for chromogranin A, VMAT-2 and synaptophysin, and with serotonin positivity in the majority of the cells. Ki67 was positive in < 1% of the tumor cells (Figure 1). The tumor was considered to be a type Ⅲ ECL-oma. Inflammation and *H. pylori* were present in the gastric mucosa (Figure 2). The patient was referred to our Department and a new gastroscopy in September 2006 showed inflammation in the antrum, corpus and fundus, atrophy in the antrum and corpus, and ECL-cell hyperplasia in the corpus, where a polyp considered as ECL-oma of type Ⅰ was found. Gastroscopy in November 2006 showed gastritis and positivity for *H. pylori* but no ECL hyperplasia. Gastric pH was 3.5. The patient had normal urinary histamine metabolites, normal U-5'HIAA, normal fasting serum gastrin and normal plasma chromogranin A and B. She received eradication treatment against *H. pylori*. A gastroscopy in February 2007 showed chronic inflammation without atrophy in the mucosa. There was a 0.5 cm polyp in the upper corpus surrounded by ECL hyperplasia. The tumor cells were positive for chromogranin A and VMAT 2, but negative for serotonin. Ki67 was < 1%. The tumor was considered to be a type Ⅲ ECL-oma due to lack of mucosal atrophy. The patient underwent an endoscopic mucosal resection of the polyp in April 2007. The pathology report showed a 7 mm ECL cell carcinoid with 5 mm depth that did not invade the muscularis propria. The tumor cells were positive for chromogranin A, synaptophysin and VMAT-2 but negative for gastrin and serotonin; Ki67 was < 1%. The tumor was considered as a type Ⅲ ECL-oma. A gastroscopy in September 2007 showed inflammation in the antrum with focal metaplasia but no signs of *H. pylori*, and another gastroscopy in December 2008 showed no inflammation or atrophy. The patient has not had any signs of metastatic disease in the liver or elsewhere. Repeated CT scans and ultrasounds, as well as an octreoscan in 2006 and a 5-HTP PET scan in January 2008, have been negative. Urinary 5-HIAA, plasma chromogranin A and B and serum gastrin and pancreatic polypeptide have been normal at all control visits. She has no evidence of pernicious anemia and thyroid hormone levels have been normal. At the latest control visit in January 2010, gastroscopy was macro- and microscopically normal. Staining for *H. pylori* was negative.

DISCUSSION

We report a patient with normal gastrin levels presenting with a small solitary gastric carcinoid with very low proliferative rate and without evidence of metastatic disease during more than 3 years of follow-up. The normal gastrin levels suggest that the carcinoid tumor was not type Ⅰ or Ⅱ. The absence of metastatic disease and the small dimension of the polyp, together with the low proliferative rate, indicate that it was not a type Ⅲ carcinoid. The patient was infected with *H. pylori* and had signs of chronic gastritis, gastric atrophy and ECL cell hyperplasia, which resolved after eradication of the *Helicobacter* infection. There have been no recurrences after the eradication treatment and endoscopic polypectomy. Although careful interpretation is needed, a causal relationship seems plausible. It is well known that chronic acid suppression may induce ECL cell proliferation[7]. However, our patient did not receive any proton pump inhibitors or other acid suppressive therapy, neither before the development of the carcinoid tumor nor during the follow-up period. It has previously been shown that longstanding *H. pylori* infection causes chronic inflammation of the gastric mucosa in animals[8]. A long-term *H. pylori* infection is also associated with atrophy of the gastric mucosa, and atrophy is a

risk factor for malignancy[4]. *H. pylori*-induced gastritis may play an important role in the development of gastric adenocarcinoma in humans[4] and animal models[9]. Development of gastric carcinoid tumors in subjects infected with *H. pylori* is believed to be rare[6], but has been described in animals[9-11] and, more rarely, in humans. Five humans infected with *H. pylori* without atrophic gastritis or Zollinger-Ellison syndrome who developed gastric carcinoids have been reported in Japan[12]. In Europe, Solcia reported four cases[13] of gastric carcinoids in *H. pylori*-infected humans, of whom all had chronic atrophic gastritis type A. Infection with *H. pylori* was, however, found to be much more common in patients with early gastric carcinomas than in carcinoid patients[13]. *H. pylori* thus seems more likely to cause neoplasms with higher malignant potential than the indolent carcinoids. Since the chronic gastritis in our patient resolved and no tumor recurrences have occurred after eradication treatment, it is nevertheless possible that her gastric carcinoid was actually caused by *H. pylori*-induced chronic gastritis.

H. pylori may affect the acid secretion of the parietal cells by causing mucosal inflammation[14]. Gastric acid secretion depends on the localization and the degree of the inflammation[14]. Acute infection with *H. pylori* results in hypochlorhydria, whereas chronic infection can cause either hypo- or hyperchlorhydria, depending on the distribution of the infection and the degree of corpus gastritis[5]. Recent studies suggest that inflammatory cytokines, produced in response to the bacteria, can play a role in the perturbations in acid and gastrin secretion induced by *H. pylori* [5]. Gastrin is associated with enterochromaffin-like (ECL) cell proliferation and is a factor implicated in the pathogenesis of ECL-cell tumors type Ⅰ and Ⅱ[3]. The patients in Japan with *H. pylori*-associated gastric carcinoids mentioned above all had high gastrin levels. Our patient, however, developed ECL-cell hyperplasia and a gastric carcinoid tumor despite normal gastrin levels. This observation suggests that *H. pylori* may facilitate gastric ECL cell proliferation by other mechanisms, independent of gastrin hypersecretion. The mucosal inflammation induced by *H. pylori* has been shown to cause excessive apoptosis, which in turn leads to proliferation[15,16]. Lipopolysaccharides also appear to influence tumor ECL cell proliferation[16,17]. Another factor involved in ECL cell proliferation is REG protein, which may be produced by *H. pylori* infection[18].

In conclusion, we postulate that *H. pylori* may lead to chronic gastritis, with normal or elevated gastrin levels, and cause the development of gastric carcinoids by mechanisms unrelated to gastrin. ECL cell tumors related to a chronic *H. pylori* infection may be considered as a distinct type of gastric carcinoid tumors, as they seem to have distinct histopathological, pathogenetic and clinical characteristics compared to the other types of gastric carcinoids.

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CASE REPORT

Liver transplantation for acute hepatic failure due to chemotherapy-induced HBV reactivation in lymphoma patients

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Abstract

Hepatitis B (HBV) reactivation induced by chemotherapy is problem encountered recently in the management of malignant diseases. Chemotherapy-induced HBV reactivation may ultimately lead to terminal acute liver failure. Liver transplantation (LT) currently remains the only definitive treatment option for such cases, but is generally denied to patients suffering from malignancy. Here, the authors describe 2 cases of cancer-free and HBV graft re-infection-free survival after LT performed for terminal liver failure arising from HBV reactivation induced by chemotherapy for advanced stage lymphoma. These 2 cases, and some other reports in the literature, may suggest that patients suffering from hematologic malignancies and terminal liver disease can be considered for LT if the prognosis of their hematologic malignancy is good.

Key words: Liver transplantation; Contraindication; Cancer; Liver failure; Chemotherapy; Hepatitis B virus

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Noterdaeme T, Longrée L, Bataille C, Deroover A, Lamproye A, Delwaide J, Beguin Y, Honoré P, Detry O. Liver transplantation for acute hepatic failure due to chemotherapy-induced HBV reactivation in lymphoma patients. *World J Gastroenterol* 2011; 17(25): 3069-3072 Available from: URL: http://www.wjgnet.com/1007-9327/full/v17/i25/3069.htm DOI: http://dx.doi.org/10.3748/wjg.v17.i25.3069

INTRODUCTION

Reactivation of a previous hepatitis B virus (HBV) infection is a known complication in patients undergoing chemotherapy or immunosuppressive treatment. Such reactivations have been observed in HB surface antigen (HBsAg) positive and negative subjects, with an incidence of 26% to 47%[1,2]. Although lamivudine prophylaxis is considered as the treatment of choice in such situations, in some cases it may not prevent reactivation of the underlying infection[3]. Chemotherapy-induced

HBV reactivation may then lead to terminal liver failure, with very limited treatment options, as life-saving liver transplantation (LT) is generally not performed in patients suffering from preexisting extrahepatic malignancies[4]. We report 2 cases of long-term cancer-free and HBV graft re-infection-free survival after LT for HBV reactivation induced by chemotherapy administered for advanced staged lymphoma.

CASE REPORTS

Case 1

A 49-year-old Caucasian male was diagnosed with advanced nodular sclerotic Hodgkin's lymphoma stage IIIb-IV in January 2006. In the following month, chemotherapy was initiated using 4 cycles of escalated BEACOPP regimen (bleomycin, etoposide, adriamycin, cyclophosphamide, oncovin, procarbazine, prednisone). After 3 cycles, blood analyses indicated an acute HBV infection (HBsAg+, anti-HBc+). Indeed, prior to chemotherapy, the patient had been an HBsAg carrier, a status he had failed to mention initially. Further blood tests confirmed the revised diagnosis of chronic HBV infection (anti-HBs-, anti-HBe+). His hepatic function was closely monitored and the last cycle of escalated BEACOPP was administered in April 2006. A follow-up positron emission tomography (PET) revealed significant lymphoma regression, after which the patient was switched to a baseline BEACOPP pattern, to minimize adverse effects.

One month later, after the first cycle of baseline BEACOPP chemotherapy, the patient was admitted to hospital with fatigue, anorexia, generalized edema and jaundice. Blood analysis showed significant alteration of liver function (aspartate aminotransferase: 505 IU/L, alanine aminotransferase: 300 IU/L, lactate dehydrogenase: 579 IU/L, T-bilirubin 45.9 mg/L), and further chemotherapy had to be postponed. Polymerase chain reaction for HBV-DNA was performed with positive results (HBV-DNA > 10 000 000 copies). Lamivudine therapy was then initiated. In June 2006 the patient presented with liver failure (MELD (Model for End-stage Liver Disease) score 35, Quick < 20%, Factor V < 20%, international normalized ratio > 5) and hepatic encephalopathy. Additional serology performed for other pathogens remained negative (human immunodeficiency virus, HCV, cytomegalovirus, Epstein-Barr virus, herpes simplex virus, toxoplasmosis), so that hepatic failure could only be attributed to the reactivation of the underlying HBV infection. He was then referred to our university hospital for LT evaluation, despite the fact that the last cycles of chemotherapy had not yet been administered. As the last PET showed no residual lymphoma activity, an emergency LT was performed. Immunosuppression was initialized using tacrolimus, mycophenolate mofetil and prednisone. Graft reinfection was prevented using anti-Hbs immunoglobulin injections and lamivudine. No further chemotherapy was administered. In the first 4 years of follow-up, regular computed tomography (CT) and PET scans did not show any evidence of lymphoma activity. Immunosuppressive treatment was gradually tapered as in other LT recipients. At 4-year follow-up, the patient was alive and well, cancer-free and HBV-free, on long-acting tacrolimus monotherapy and HBV prevention bi-therapy (lamivudine + anti-HBs immunoglobulin injections).

Case 2

A 53-year-old female originating from central Africa was diagnosed with big cell lymphoma type B of the stomach, with thoracic involvement in December 2006. Staged as IIIa, the patient underwent poly-chemotherapy using a R-ACVBP regimen (rituximab, doxorubicin, cyclophosphamide, vindesine, bleomycin, prednisone). The last chemotherapy was administered in March 2007, and she was considered in full remission (negative PET, CT and gastroscopy). Despite her hepatitis B status (HBAg+, anti-HBc+, anti-HBs-, anti-HBe+) being known before chemotherapy initiation no preventive therapy was taken, and HBV reactivation was diagnosed in November 2007 (positive PCR). The patient immediately received lamivudine, which proved ineffective, resulting in terminal hepatic failure (MELD-score 29, Quick < 23%, Factor V < 30%) in March 2008. The patient was then referred for LT evaluation. An urgent LT has then been performed since stage 2 to 3 encephalopathy became apparent. Because of severe coagulopathy, hemostatis was difficult during the LT procedure, and splenectomy had to be performed at the same time. Pathology of the spleen did not reveal lymphoma. Graft HBV reinfection was prevented by anti-Hbs immunoglobulin injections and lamivudine, and initial immunosuppression consisted of tacrolimus, mycophenolate mofetil and prednisone, rapidly tapered. At 2-year follow-up, the patient was alive and well, and her immunosuppressive medication has been adjusted to long-acting tacrolimus monotherapy. She did not develop any sign of HBV or lymphoma recurrence.

DISCUSSION

This article reports 2 patients with lymphoma who underwent successful LT for chemotherapy-induced HBV reactivation. These cases demonstrate that life-saving LT should not be denied as an absolute contraindication in patients with lymphoma and chemotherapy-induced HBV reactivation. This concept confirms other reports suggesting that patients suffering from hematological diseases show low recurrence rates after LT[5,6]. These cases offer the opportunity to reconsider current LT limitations, particularly in those instances where transplantation would usually be denied. The authors consider that patients suffering from preexisting malignant diseases should not be excluded by default for this life-saving procedure, but that potential benefits and risks must be evaluated individually particularly in malignant lesions affecting a younger and fitter population. This

view was also recommended by the King's College Hospital group[7].

Reactivation of a previous HBV infection is an entity regularly encountered with chemotherapy. Cases of fatal fulminant or subacute HBV liver failure following chemotherapy for lymphoma have been reported[8-10]. This is particularly true in cases in which rituximab and corticosteroids are included in the protocol[1,2,8]. The pathophysiology remains to be determined, but reports suggest that immunosuppressants favor viral reproduction, and that a massive immunological reaction occurs as soon as normal immune system function is reestablished at the end of chemotherapy. This overwhelming immune response is the origin of hepatic acute cytolysis[1]. Reports suggest that every patient undergoing chemotherapy should be checked for previous HBV infection, and that HBV preventive treatment throughout the patient's chemotherapy should be performed in case of previous HBV infection. However, at the time of treatment of the 2 patients mentioned in this case report, lamivudine prophylaxis was not reimbursed by the Belgian health system so that it was administered only after reactivation had already occurred. Nonetheless in some cases, lamivudine prophylaxis may not prevent reactivation, and terminal or fulminant liver failure may occur[3]. Urgent LT is the only effective treatment[11,12] but is usually denied because of the underlying malignancy. To the best of the authors' knowledge, only a few cases of LT in this particular setting have been reported so far[5,8,13]. This suggests that patients suffering from hematologic diseases seem to constitute a subgroup in which the reoccurrence rate after LT seems to be low. These 2 cases do support these observations. To some extent these observations can be explained by the new treatment possibilities and the recent outcome improvements that have been made over the last few decades in the management of hematologic diseases. New chemotherapy regimens, as well as new methods (e.g. PET) to assess the efficiency of ongoing treatments, are being continuously developed, allowing tailored therapies for each patient, rendering this condition highly curable. Current studies suggest that an escalated BEACOPP regimen is the treatment of choice for advanced Hodgkin lymphoma, and has an overall chance of 96% to achieve full remission with a 5 year survival rate peaking at 92%[14-16]. Other complications such as veno-occlusive disease or graft-*versus*-host disease following bone marrow transplantation may also cause terminal liver failure in patients treated for hematologic malignancies. Though experience is limited, reports indicate that LT may also be a feasible and effective approach in such cases[17-19].

Recurrence of the underlying preexisting malignancy may occur after LT, promoted by the necessary immunosuppression and by a direct effect of calcineurin inhibitors[4,20]. However, in cancer patients with good prognosis, as in the 2 lymphoma patients described here, LT may be life-saving. In the absence of large studies, each patient should be assessed individually to evaluate if organ transplantation can be beneficial in terms of survival and quality of life. Further studies with longer follow-up are needed to establish prognostic factors to identify those patients in whom LT can be considered as an effective approach.

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Intrahepatic biliary cystadenoma: Is there really an almost exclusively female predominance?

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Abstract

Biliary cystic tumors, such as cystadenomas and cystadenocarcinomas, are rare cystic tumors of the liver, accounting for less than 5% of all intrahepatic cysts of biliary origin. Biliary cystadenomas have been known to occur predominantly in women (> 85%), and 38%-44% of biliary cystadenocarcinomas have occurred in males. We wrote this letter to comment on a brief article (*World J Gasteroenterol* 2011 January 21; 17(3): 361-365) regarding a case of intrahepatic biliary cystic neoplasm treated with surgery. The adenoma-carcinoma sequence is the possible mechanism of carcinogenesis. If the carcinogenesis of biliary cystadenocarcinoma occurs in the adenoma-carcinoma sequence, we believe that the male-to-female ratio of cystadenoma should be higher than the incidence rate that has been reported to date.

Key words: Biliary cystadenoma; Cystadenocarcinoma; Carcinogenesis; Incidence

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TO THE EDITOR

Biliary cystic tumors, such as cystadenomas and cystadenocarcinomas, are rare cystic tumors of the liver, accounting for less than 5% of all intrahepatic cysts of biliary origin. Biliary cystadenomas have been known to occur predominantly in women (> 85%), and this almost exclusively female predominance suggests a strong hormonal influence. Emre *et al*[1]. reported nine patients with intrahepatic biliary cystic liver neoplasms, all of them were female. And 38%-44% of biliary cystadenocarcinomas occur in males with a higher mean age compared with cystadenomas[2].

In spite of the improvement in imaging techniques, the differential diagnosis of simple hepatic cysts and intrahepatic biliary cystadenoma is still problematic.[1] If the malignancy is suspected, surgery is recommended; if benign disease is suspected, many clinicians might misdiagnose cystadenomas as simple cysts and recommend observation and/or follow-up examinations rather than surgical treatment. Cases of biliary cystic neoplasm reported in the literature are diagnosed mostly based on pathologic findings after operation. Thus, we examined whether there may be a bias toward the gender-related incidence of biliary cystadenoma.

Between May 2004 and December 2009, 10 patients underwent surgery for intrahepatic biliary cystic neoplasm at Chonnam National University Hospital, Gwangju, Korea. Eight patients had biliary cystadenomas, and two had cystadenocarcinomas. The patients with cystadenomas consisted of five females (62.5%) and three males (37.5%). Both patients with cystadenocarcinomas were males. In our report, the female predilection of biliary cystadenoma is much weaker than in other reports[1,2].

The role of biliary cystadenoma in the pathogenesis of biliary cystadenocarcinoma is controversial. Transformation into a cystadenocarcinoma has been reported, although it is rare[3]. Thus, the adenoma-carcinoma sequence is a possible mechanism of carcinogenesis. Biliary cystadenomas have been known to occur predominantly in women. If the carcinogenesis of biliary cystadenocarcinoma occurs in the adenoma-carcinoma sequence, we believe that the male-to-female ratio of cystadenoma should be higher than the incidence rate that has been reported until now. The current knowledge and predictions about intrahepatic biliary cystic neoplasms are based on a limited number of case reports. The precise mechanisms of carcinogenesis remain unknown. Thus, accumulation of larger groups of patients and further examinations will be necessary to analyze the pathogenesis and incidence of intrahepatic biliary cystic neoplasms (cystadenomas and cystadenocarcinomas).

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January 20-22, 2011
Gastrointestinal Cancers Symposium 2011, San Francisco, CA 94143, United States

January 27-28, 2011
Falk Workshop, Liver and Immunology, Medical University, Franz-Josef-Strauss-Allee 11, 93053 Regensburg, Germany

January 28-29, 2011
9. Gastro Forum München, Munich, Germany

February 4-5, 2011
13th Duesseldorf International Endoscopy Symposium, Duesseldorf, Germany

February 13-27, 2011
Gastroenterology: New Zealand CME Cruise Conference, Sydney, NSW, Australia

February 17-20, 2011
APASL 2011-The 21st Conference of the Asian Pacific Association for the Study of the Liver
Bangkok, Thailand

February 22, 2011-March 04, 2011
Canadian Digestive Diseases Week 2011, Vancouver, BC, Canada

February 24-26, 2011
Inflammatory Bowel Diseases 2011-6th Congress of the European Crohn's and Colitis Organisation, Dublin, Ireland

February 24-26, 2011
2nd International Congress on Abdominal Obesity, Buenos Aires, Brazil

February 24-26, 2011
International Colorectal Disease Symposium 2011, Hong Kong, China

February 26-March 1, 2011
Canadian Digestive Diseases Week, Westin Bayshore, Vancouver, British Columbia, Canada

February 28-March 1, 2011
Childhood & Adolescent Obesity: A whole-system strategic approach, Abu Dhabi, United Arab Emirates

March 3-5, 2011
42nd Annual Topics in Internal Medicine, Gainesville, FL 32614, United States

March 7-11, 2011
Infectious Diseases: Adult Issues in the Outpatient and Inpatient Settings, Sarasota, FL 34234, United States

March 14-17, 2011
British Society of Gastroenterology Annual Meeting 2011, Birmingham, England, United Kingdom

March 17-19, 2011
41. Kongress der Deutschen Gesellschaft für Endoskopie und Bildgebende Verfahren e.V., Munich, Germany

March 17-20, 2011
Mayo Clinic Gastroenterology & Hepatology 2011, Jacksonville, FL 34234, United States

March 18, 2011
UC Davis Health Informatics: Change Management and Health Informatics, The Keys to Health Reform, Sacramento, CA 94143, United States

March 25-27, 2011
MedicReS IC 2011 Good Medical Research, Istanbul, Turkey

March 26-27, 2011
26th Annual New Treatments in Chronic Liver Disease, San Diego, CA 94143, United States

April 6-7, 2011
IBS-A Global Perspective, Pfister Hotel, 424 East Wisconsin Avenue, Milwaukee, WI 53202, United States

April 7-9, 2011
International and Interdisciplinary Conference Excellence in Female Surgery, Florence, Italy

April 15-16, 2011
Falk Symposium 177, Endoscopy Live Berlin 2011 Intestinal Disease Meeting, Stauffenbergstr. 26, 10785 Berlin, Germany

April 18-22, 2011
Pediatric Emergency Medicine: Detection, Diagnosis and Developing Treatment Plans, Sarasota, FL 34234, United States

April 20-23, 2011
9th International Gastric Cancer Congress, COEX, World Trade Center, Samseong-dong, Gangnam-gu, Seoul 135-731, South Korea

April 25-27, 2011
The Second International Conference of the Saudi Society of Pediatric Gastroenterology, Hepatology & Nutrition, Riyadh, Saudi Arabia

April 25-29, 2011
Neurology Updates for Primary Care, Sarasota, FL 34230-6947, United States

April 28-30, 2011
4th Central European Congress of Surgery, Budapest, Hungary

May 7-10, 2011
Digestive Disease Week, Chicago, IL 60446, United States

May 12-13, 2011
2nd National Conference Clinical Advances in Cystic Fibrosis, London, England, United Kingdom

May 19-22, 2011
1st World Congress on Controversies in the Management of Viral Hepatitis (C-Hep), Palau de Congressos de Catalunya, Av. Diagonal, 661-671 Barcelona 08028, Spain

May 21-24, 2011
22nd European Society of Gastrointestinal and Abdominal Radiology Annual Meeting and Postgraduate Course, Venise, Italy

May 25-28, 2011
4th Congress of the Gastroenterology Association of Bosnia and Herzegovina with international participation, Hotel Holiday Inn, Sarajevo, Bosnia and Herzegovina

June 11-12, 2011
The International Digestive Disease Forum 2011, Hong Kong, China

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Surgery and Disillusion XXIV SPIGC, II ESYS, Napoli, Italy

July 7-16, 2011
International Scientific Conference on Probiotics and Prebiotics-IPC2011, Kosice, Slovakia

June 22-25, 2011
ESMO Conference: 13th World Congress on Gastrointestinal Cancer, Barcelona, Spain

June 29-2, 2011
XI Congreso Interamericano de Pediatria "Monterrey 2011", Monterrey, Mexico

September 2-3, 2011 Falk Symposium 178, Diverticular Disease, A Fresh Approach to a Neglected Disease, Gürzenich Cologne, Martinstr. 29-37, 50667 Cologne, Germany

September 10-11, 2011
New Advances in Inflammatory Bowel Disease, La Jolla, CA 92093, United States

September 10-14, 2011
ICE 2011-International Congress of Endoscopy, Los Angeles Convention Center, 1201 South Figueroa Street Los Angeles, CA 90015, United States

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Falk Symposium 179, Revisiting IBD Management: Dogmas to be Challenged, Sheraton Brussels Hotel, Place Rogier 3, 1210 Brussels, Belgium

October 19-29, 2011
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19th United European Gastroenterology Week, Stockholm, Sweden

October 28-November 2, 2011
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November 11-12, 2011
Falk Symposium 180, IBD 2011: Progress and Future for Lifelong Management, ANA Interconti Hotel, 1-12-33 Akasaka, Minato-ku, Tokyo 107-0052, Japan

December 1-4, 2011
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Organization as author

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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 DOI:10.1097/01.ju.0000067940.76090.73]

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6 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303 DOI:10.1136/bmj.325.7357.184]

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15 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/index.htm

Patent (list all authors)

16 **Pagedas AC**, inventor; Ancel Surgical R&D Inc., assignee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 20020103498. 2002 Aug 1

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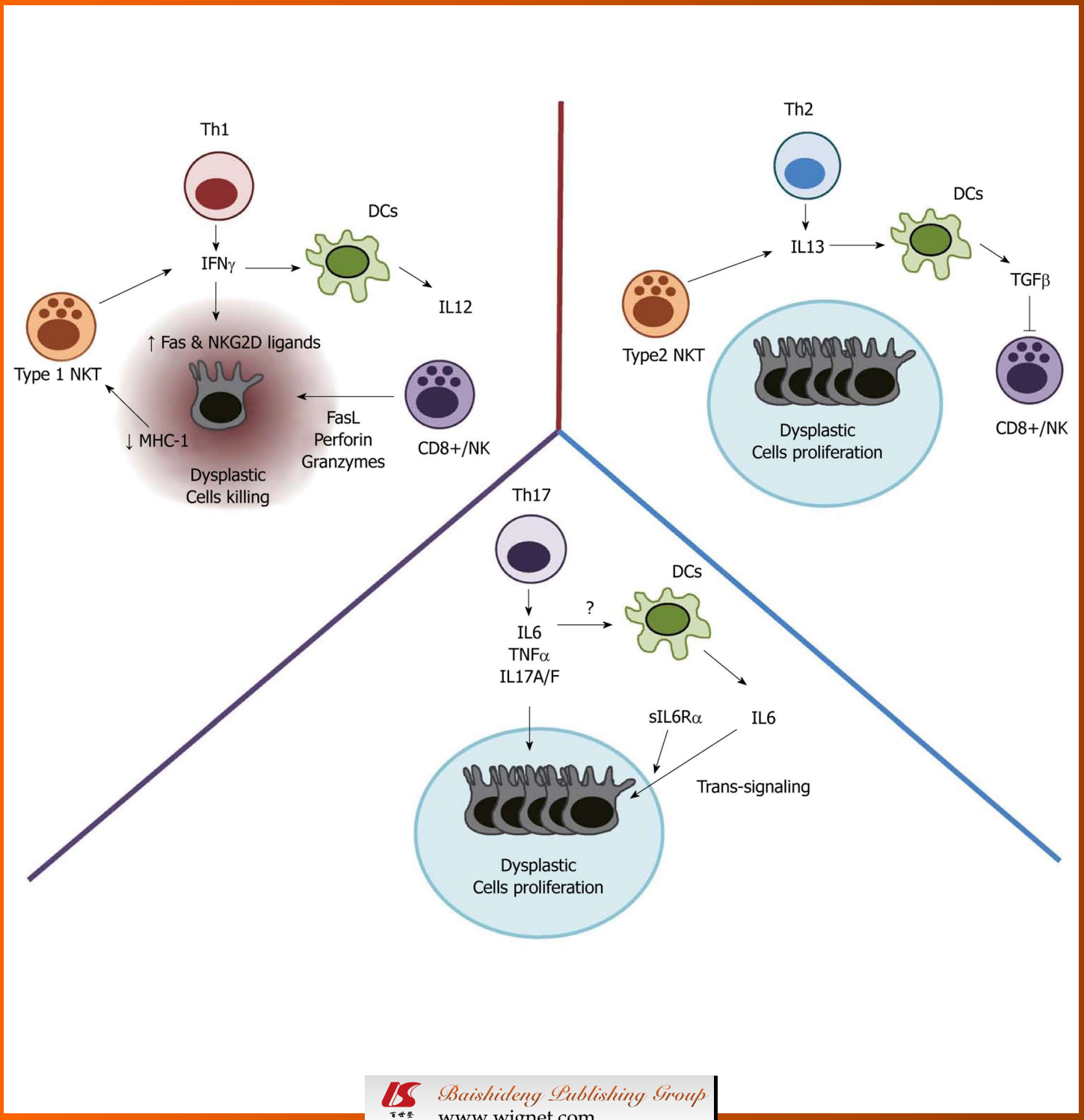

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EDITORIAL

Anti-angiogenesis in hepatocellular carcinoma treatment: Current evidence and future perspectives

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Abstract

Hepatocellular carcinoma (HCC) is among the most common cancer diseases worldwide. Arterial hypervascularisation is an essential step for HCC tumorigenesis and can be targeted by transarterial chemoembolization (TACE). This interventional method is the standard treatment for patients with intermediate stage HCC, but is also applied as "bridging" therapy for patients awaiting liver transplantation in many centers worldwide. Usually the devascularization effect induced by TACE is transient, consequently resulting in repeated cycles of TACE every 4-8 wk. Despite documented survival benefits, TACE can also induce the up-regulation of proangiogenic and growth factors, which might contribute to accelerated progression in patients with incomplete response. In 2007, sorafenib, a multi-tyrosine kinase and angiogenesis inhibitor, was approved as the first systemic treatment for advanced stage HCC. Other active targeted compounds, either inhibitors of angiogenesis and/or growth factors, are currently being investigated in numerous clinical trials. To overcome revascularisation or tumor progression under TACE treatment it seems therefore attractive to combine TACE with systemic targeted agents, which might theoretically block the effects of proangiogenic and growth factors. Over the last 12 mo, several retrospective or prospective cohort studies combining TACE and sorafenib have been published. Nevertheless, robust results of the efficacy and tolerability of such combination strategies as proven by randomized, controlled trials are awaited in the next two years.

Key words: Hepatocellular carcinoma; Sorafenib; Anti-angiogenesis; Transarterial chemoembolization

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INTRODUCTION

The incidence of hepatocellular carcinoma (HCC) is rising with a world-wide annual incidence above 600 000[1]. Treatment of HCC is challenging because HCC mainly occurs within liver cirrhosis[1], and therapy options and prognosis are determined by tumor biology as well as impaired liver function. Several clinical staging systems have been proposed[2]. However, the most commonly used in Western countries is the Barcelona Clinic Liver Cancer (BCLC) system[3,4]. According to this algorithm, treatment is stratified according to tumor stage, liver function, and performance status. Intermediate stage HCC (BCLC stage B) without options for surgical treatment or ablation is treated by transarterial chemoembolization (TACE). TACE has been shown to expand median survival from 16 to 19-20 mo[5,6]. In patients with advanced (BCLC stage C) and especially end-stage HCC (BCLC stage D), survival depends not only on progression of

tumor disease but depends incremental on accompanying liver dysfunction, also. Without intervention, survival of patients with advanced HCC rarely exceeds 6 mo, and median survival in patients with end-stage HCC (BCLC stage D, Okuda stage Ⅲ, performance status 3-4) is commonly below 3-4 mo[4,7-9]. According to the modified BCLC system, the dual kinase inhibitor sorafenib is considered the standard of care for patients with advanced HCC[10]. However, the survival benefit is limited to approximately 3 mo, whereas disease stabilization can be achieved in 27%-78% as shown in prospective trials[11-14].

Typically, HCC is a hypervascularized tumor with characteristic early arterial enhancement during dynamic imaging, which is the rational for TACE. By TACE, however, mainly central vessels of a tumor nodule are occluded, while progression may occur via neovascularization in the tumor periphery. In theory, this might be prevented or at least attenuated by concomitant systemic treatment with anti-angiogenic agents (Figure 1).

ANGIOGENESIS IN PATHOGENESIS OF HEPATOCELLULAR CARCINOMA

Chronic hepatitis and hepatic fibrogenesis are closely connected to angiogenesis[15]. Different cytokines, growth factors, and metalloproteinases are involved in these processes. Vascular endothelial growth factor (VEGF) was shown to be crucially involved in angiogenesis as well as fibrogenesis[15]. Despite other factors, hepatic tissue hypoxia seems to be a relevant trigger for angiogenesis in necroinflammatory liver disease, especially by induction of VEGF, resulting in increasing arterial contribution to hepatic perfusion[16,17]. At this stage, the majority of neo-vessels originate from the portal vein, supporting short-circuits between the portal vein system and the hepatic veins[16,18]. Despite the predominant occurrence of HCC in liver cirrhosis rather than in non-cirrhotic liver disease[1], it is still unknown whether HCC arises from hepatic stem cells or from hepatocytes *via* malignant transformation. The latter concept is supported by the observation that development of HCC from dysplastic nodules has been described[19,20]. Arterial hypervascularization seems to be pathognomonic for established HCC, and HCC nodules larger than 2 cm regularly show arterial enhancement[21,22]. Therefore, neovascularization seems to be crucial for HCC tumorigenesis.

Consistently, increased expression of angiopoietin-1/-2 mRNA in tumor tissue was reported, suggesting a critical role of neo-vascularisation for HCC pathogenesis[23]. Moreover, augmented expression of VEGF was found in HCC, and higher serum VEGF levels were associated with poor prognosis of patients with HCC[24-29]. In contrast, a recent study showed that neither VEGF-A nor VEGFR were up-regulated in HCC tissue, and angiotensin-1/-2 expression were only modestly changed[30]. Of note, sinusoidal capillarization suggesting vascular remodeling was observed within the same study[30]. These inconsistent data further highlight that tumor angiogenesis is a complex process and most likely heterogeneous. The angiopoietin/VEGF system seems to play an important role in angiogenesis of HCC, but other, yet incompletely understood pathways may also be involved.

THERAPEUTIC INHIBITION OF ANGIOGENESIS IN HEPATOCELLULAR CARCINOMA

Inhibition of angiogenesis is an established and successful treatment strategy in a variety of malignant diseases. The liver is predominantly supplied by the portal venous system, whereas HCC nodules are characterized by typical arterial hypervascularization. This accounts for the rationale for use of hypervascularization as a diagnostic criterion as well as development of angiogenesis inhibition treatment strategies. In the absence of targeted agents, embolization of arterial tumor vessels was established in the 1980s. Currently, TACE is commonly used in patients with HCC BCLC stage 0/A as bridging therapy until liver transplantation and as non-curative therapy in patients with HCC BCLC stage B and C[6].

Indeed, TACE may lead to reduction of tumor vascularization and viable tumor volume[6]. Recently, this has also been confirmed for a modified TACE technique using doxorubicin eluting beads (DEB)[31]. Furthermore, VEGF levels as a surrogate marker for angiogenesis were shown to correlate with therapeutic outcome after TACE. Pretreatment VEGF levels were significantly higher in patients not responding to TACE compared to patients with disease stabilization. Moreover, pretreatment VEGF serum levels > 240 pg/mL were an independent prognostic factor for survival[32].

It has been suggested that tumor progression after TACE may be caused by activation of angiogenesis due to TACE-induced hypoxemia[33]. Plasma VEGF levels were shown to increase shortly after TACE, reaching a peak value one day after TACE[34-37]. Additionally, increase of plasma VEGF levels after TACE was correlated with the development of metastasis and a reduced progression free survival[35,37]. Unfortunately, reliable biomarkers predicting response to TACE are missing. Nevertheless, a median survival of 35 mo has been reported in patients with complete tumor response[38]. In this study low VEGF levels were associated with a longer survival, while higher VEGF levels were detectable in patients without tumor response. Of note, prior TACE was reported to induce angiogenesis in surgical specimens, whereas patients who underwent surgery without prior TACE had no induction of angiogenesis[39]. Whether the use of DEB-TACE, which can induce higher rates of tumor response, also leads to upregulation of proangiogenic factors is under debate[40,41].

Sorafenib, the first systemically agent approved for HCC, is a multikinase inhibitor with activity against VEGFR2, PDGFR, c-Kit receptors, b-RAF, and p38[42], signal transduction pathways which seem to be involved in pathogenesis of HCC[43]. However, there are limita-

Table 1 Efficacy of systemic targeted monotherapy in hepatocellular carcinoma according to current phase Ⅰ-Ⅲ studies

Author	Year	Phase	Investigational drug	*n*	RR	DS	PFS/TTP	PFS-6m	OS
O'Neil *et al*[59][1]	2009	Ⅱ	AZD 6244	16	0	37.5	NR	NR	NR
Malka *et al*[60]	2007	Ⅱ	Bevacizumab	30	12.5	54	3.5/NR	17	NR
Schwartz *et al*[61]	2006	Ⅱ	Bevacizumab	30	6.7	57	NR/6.4	NR	NR
Siegel *et al*[58]	2008	Ⅱ	Bevacizumab	46	13	NR	6.9/NR	NR	12.4
Raoul *et al*[62]	2009	Ⅱ	Brivanib	55	11	10	NR/2.8	NR	10
Gruenwald *et al*[63]	2007	Ⅱ	Cetuximab	27	0	44	2.0/1.9	22.2	NR
Zhu *et al*[64]	2007	Ⅱ	Cetuximab	30	0	17	1.4/NR	NR	9.6
Philip *et al*[65]	2005	Ⅱ	Erlotinib	38	9	50	3.2/NR	32	13
Thomas *et al*[66]	2007	Ⅱ	Erlotinib	40	0	43	3.1/NR	NR	6.25 (10.75)[2]
Blaszkowsky *et al*[67]	2010	Ⅲ	Everolimus	25	4	44	3.8/3.9	8%	8.4
O'Dwyer *et al*[68]	2006	Ⅱ	Gefitinib	31	3	22.5	2.8/NR	NR	6.5
Lin *et al*[69]	2008	Ⅱ	Imatinib	15	0	13.3	NR/NR	NR	NR
Ramanathan *et al*[70]	2006	Ⅱ	Lapatinib	37	5	35	2.3/NR	2.3	6.2
Rizell *et al*[71]	2008	Ⅱ	Sirolimus	21	4.8	23.8	NR/NR	NR	6.5
Abou-Alfa *et al*[12]	2006	Ⅱ	Sorafenib	137	2.2	33.6	NR/4.2	NR	9.2
Cheng *et al*[72]	2009	Ⅲ	Sorafenib	226 (150 treated)	3.3	54	NR/2.8	NR	6.5
Furuse *et al*[13]	2008	Ⅰ	Sorafenib	27	4	83	NR/4.9	46.2	15.6
Llovet *et al*[11]	2008	Ⅲ	Sorafenib	602 (299 treated)	2	71	NR/5.5	NR	10.7
Yau *et al*[14]	2009	Ⅱ	Sorafenib	51	8	18	3.0/NR	NR	5
Zhu *et al*[73]	2009	Ⅱ	Sunitinib	34	2.9	47	3.9/4.1	NR	9.8
Faivre *et al*[74]	2009	Ⅱ	Sunitinib	37	2.7	35	3.7/5.3	NR	8
Hoda *et al*[75]	2008	Ⅱ	Sunitinib	23	6	35	NR/NR	NR	NR
Koeberle *et al*[54]	2010	Ⅱ	Sunitinib	45	2	40	2.8/2.8	NR	9.3
Kanai *et al*[57]	2010	Ⅰ/Ⅱ	TSU-68	35	8.6	42.8	NR/2.1	NR	13.1

[1]Trial stopped; [2]Recorded from therapy start (recorded from diagnosis). DS: Disease stabilization (%); NR: Not reported; OS: Overall survival (mo); PFS/TTP: Progression free survival/time to progression (mo); PFS-6m: Progression free survival rate at 6 mo (%); RR: Response rate [complete + partial response (%)].

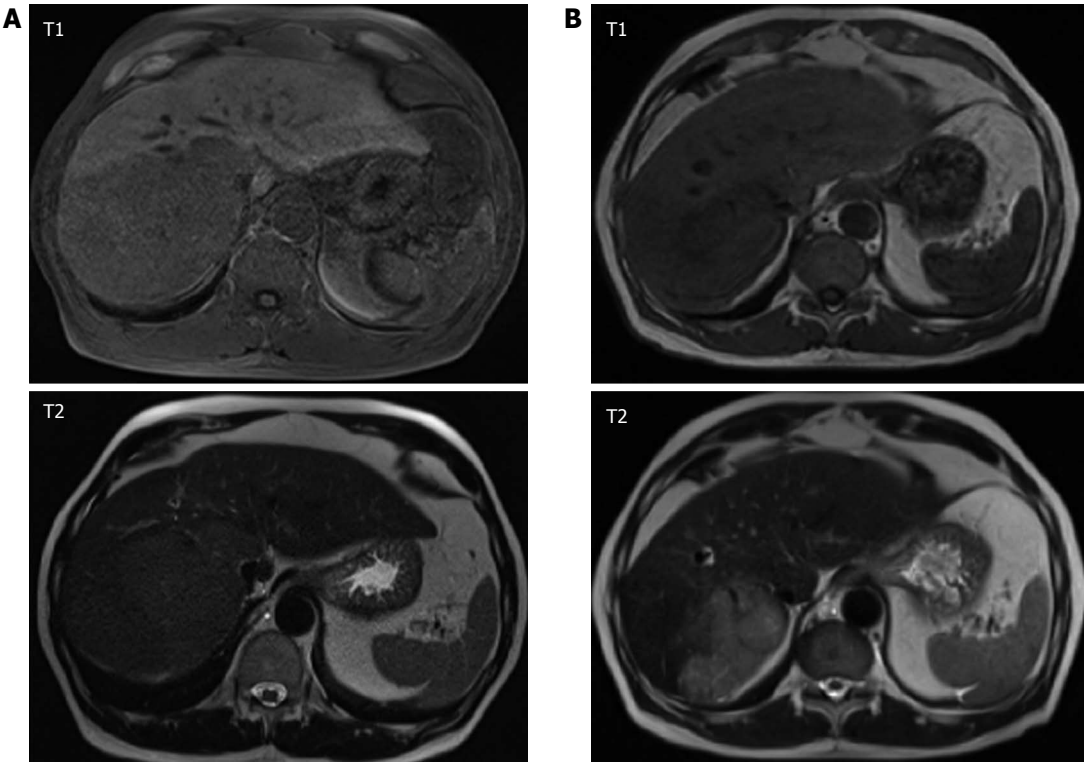

Figure 1 Dynamic gadolinium-enhanced magnetic resonance imaging (MRI; T1, T2 weighting), in a 67 year old patient with hepatocellular carcinoma evolved from liver cirrhosis due to hemochromatosis (A) before initiation of anti-angiogenic therapy and (B) after 70 d or three cycles of transarterial chemoembolization and continuous administration of sorafenib, respectively. Patient showed partial response according to RECIST criteria. Serum alfa-fetoprotein level decreased from 276 to 115 ng/mL.

tions on the therapy with sorafenib, founded on restricted efficacy and potential side effects, mainly fatigue, diarrhea and hand-food syndrome. In comparison to TACE valid predictive biomarkers are missing, also[11].

Table 2 Efficacy of combination therapy with systemic acting agents and targeted therapy in hepatocellular carcinoma according to current phase Ⅰ-Ⅱ studies

Author	Year	Phase	Investigational drug	*n*	RR	DS	PFS/TTP	PFS-6m (%)	OS
Sun *et al*[76]	2007	Ⅱ	Bevacizumab/CapOx	30	11	78	4.5/NR	40	NR
Thomas *et al*[55]	2009	Ⅱ	Bevacizumab/erlotinib	40	25	42.5	9.0/NR	NR	15.7
Hsu *et al*[77]	2008	Ⅱ	Bevacizumab/capecitabine	45	9	42	4.1/NR	NR	10.7
Zhu *et al*[78]	2006	Ⅱ	Bevacizumab/GemOX	33	20	27	5.3/NR	NR	9.6
Berlin *et al*[79]	2008	Ⅱ	Bortezomib/doxorubicin	39	2.3	25.6	2.4/NR	NR	5.7
Asnacios *et al*[80][1]	2008	Ⅱ	Cetuximab/GemOx	45	20	40	4.7/NR	NR	9.5
Louafi *et al*[81][1]	2007	Ⅱ	Cetuximab/GemOx	35	24	4.5	NR/NR	40	9.2
Knox *et al*[82][2]	2008	Ⅱ	G3139/doxorubicin	17	0	35	NR/1.8	17.2	5.4
Abou-Alfa *et al*[83][3]	2010	Ⅱ	Sorafenib/doxorubicin	96	4	77	6.9/8.6	2.7	13.7
Richly *et al*[84]	2009	Ⅰ	Sorafenib/doxorubicin	18	6.3	69	4.0[4]/NR	NR	NR

[1]Overlap of patient cohorts cannot be excluded from information provided in the abstracts; [2]Trial stopped due to lack of efficacy; [3]Trial stopped due to superiority of sorafenib; [4]Calculated from a median duration of disease control rate (combined endpoint for complete and partial response as well as stable disease) of 17.4 wk. CapOx: Capecitabine and oxaliplatin; DS: Disease stabilization (%); GemOx: Gemcitabine and oxaliplatin; NR: Not reported; OS: Overall survival (mo); PFS/TTP: Progression free survival/time to progression (mo); PFS-6m: Progression free survival rate at 6 mo (%); RR: Response rate [complete + partial response (%)].

Table 3 Efficacy of sorafenib and transarterial chemoembolization in hepatocellular carcinoma (sequential therapy not included) according to current phase Ⅰ-Ⅱ studies

Author	Year	Phase	Investigational drug	*n*	RR	DS	PFS/TTP	OS
Chow *et al*[45]	2010	Ⅱ	Sorafenib + SIRT	35	31.4	77.1	NR/NR	10.8
Chung *et al*[47][1]	2010	Ⅱ	Sorafenib + TACE	50	NR[2]	96	NR/NR	NR
Dufour *et al*[48]	2010	Ⅰ	Sorafenib + TACE	14	NR[3]	NR[3]	NR[3]	NR[3]
Erhardt *et al*[46][1]	2009	Ⅱ	Sorafenib + TACE	44	NR[4]	63.6	8.0/16.1	11.7
Reyes *et al*[49][1]	2009	Ⅱ	Sorafenib + DEB-TACE	50	NR[5]	NR	NR/NR	NR

[1]Interim analysis; [2]20/50 patients received 2 cycles of transarterial chemoembolization (TACE), only, and 18 of these 20 patients achieved complete response compared to 2 patients with progressive disease. 30/50 patients received more than 2 cycles of TACE and achieved partial response or stable disease; [3]Primary objective of this prospective trial was evaluation of safety and tolerability of a continuous regimen of sorafenib combined with TACE; [4]According to 31 patients who received at least 1 cycle of TACE, 2/31 (6.5%) showed complete response, 15/31 (48.4%) showed partial response, and 11/31 (35.5%) showed stable disease. PFS, TTP, and OS are given for all 44 patients enrolled at time point of interim analysis; [5]Patients who completed DEB-TACE showed 100% objective tumor response and 100% partial response or stable disease according to EASL or RECIST criteria, respectively. DEB-TACE: (Drug eluting beads)-transarterial chemoembolization; NR: Not reported; DS: Disease stabilization (%); OS: Overall survival (mo); PFS/TTP: Progression free survival/time to progression (mo); PFS-6m: Progression free survival rate at 6 mo (%); RR: Response rate [complete + partial response (%)]; SIRT: Selective internal radio therapy.

STRATEGIES FOR COMBINATION OF TACE AND TARGETED AGENTS IN HCC

Combination of local and systemic inhibition of angiogenesis seems to be a consequential step to improve outcome in intermediate and advanced stage HCC[44]. Tolerability of combination therapy with sorafenib and conventional TACE as well as DEB-TACE was shown within different trials[45-49]. Currently, the combination of conventional TACE and sorafenib as well as combination of sorafenib and DEB-TACE (SPACE trial) is being evaluated in phase Ⅱ and Ⅲ trials[50]. Moreover, sorafenib was combined with selective internal radiation therapy within a multicenter phase Ⅱ study showing good efficacy in patients with advanced HCC but without extra-hepatic metastasis[45]. So far, no increased toxicity has been reported. The combination of brivanib, a dual VEGFR and fibroblast growth factor inhibitor[51], and TACE is currently evaluated within the multicenter phase Ⅲ BRISK TA Study.

Another interesting approach could be the inhibition of VEGF driven angiogenesis by targeting VEGF with siRNA as shown in a proof-of-concept study recently[52]. Furthermore, promising results were reported for other agents alone or in combination with TACE, e.g. tegafur/uracil, the multi-tyrosine kinase inhibitor TSU-68, sunitinib, erlotinib, and the VEGF antibody bevacizumab[53-57]. However, none of these agents is approved for HCC. Of these, bevacizumab is the currently most commonly clinical used VEGF inhibitor in a variety of malignant entities. However, despite encouraging results in earlier trials, even as single agent treatment, bleeding complications were reported in up to 11% of patients treated with bevacizumab[58]. For the combination of bevacizumab with TACE, severe bleeding and septic complications have been reported in 25% of patients, and the AVATACE-1 trial investigating TACE in combination with bevacizumab has been terminated due to safety concerns in the treatment arm, which does not justify a further clinical development of bevacizumab in this indication. This highlights that large phase Ⅲ trials are required for new agents in HCC, which seems challenging given the increasing number of phase Ⅰ and Ⅱ studies addressing HCC in the last years (Tables 1-3).

In summary, inhibition of angiogenesis in HCC seems a very promising approach for future treatment

of HCC. Multimodal approaches with combination of local and systemic therapy may further improve survival in intermediate and advanced stage HCC.

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REVIEW

Pediatric nonalcoholic fatty liver disease, metabolic syndrome and cardiovascular risk

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Abstract

Nonalcoholic fatty liver disease (NAFLD) encompasses a range of liver histology severity and outcomes in the absence of chronic alcohol use. The mildest form is simple steatosis in which triglycerides accumulate within hepatocytes. A more advanced form of NAFLD, nonalcoholic steatohepatitis, includes inflammation and liver cell injury, progressive to cryptogenic cirrhosis. NAFLD has become the most common cause of chronic liver disease in children and adolescents. The recent rise in the prevalence rates of overweight and obesity likely explains the NAFLD epidemic worldwide. NAFLD is strongly associated with abdominal obesity, type 2 diabetes, and dyslipidemia, and most patients have evidence of insulin resistance. Thus, NAFLD shares many features of the metabolic syndrome (MetS), a highly atherogenic condition, and this has stimulated interest in the possible role of NAFLD in the development of atherosclerosis. Accumulating evidence suggests that NAFLD is associated with a significantly greater overall mortality than in the general population, as well as with increased prevalence of cardiovascular disease (CVD), independently of classical atherosclerotic risk factors. Yet, several studies including the pediatric population have reported independent associations between NAFLD and impaired flow-mediated vasodilatation and increased carotid artery intimal medial thickness-two reliable markers of subclinical atherosclerosis-after adjusting for cardiovascular risk factors and MetS. Therefore, the rising prevalence of obesity-related MetS and NAFLD in childhood may lead to a parallel increase in adverse cardiovascular outcomes. In children, the cardiovascular system remains plastic and damage-reversible if early and appropriate interventions are established effectively. Therapeutic goals for NAFLD should address nutrition, physical activity, and avoidance of smoking to prevent not only end-stage liver disease but also CVD.

Key words: Nonalcoholic fatty liver disease; Metabolic syndrome; Cardiovascular risk; Children

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INTRODUCTION

Over the last two decades, the rise in the prevalence rates of overweight and obesity may explain the emergence of nonalcoholic fatty liver disease (NAFLD) as the leading

cause of liver disease in pediatric populations worldwide[1]. NAFLD comprises a disease spectrum ranging from simple steatosis to steatohepatitis (NASH), with varying degrees of inflammation and fibrosis, progressing to end-stage liver disease with cirrhosis and hepatocellular carcinoma[2,3]. NAFLD affects from 2.6% to 9.8% of children and adolescents, and this figure increases up to 74% among obese individuals[4-8]. NAFLD is strongly associated with obesity, insulin resistance, hypertension, and dyslipidemia, and is now regarded as the liver manifestation of the metabolic syndrome (MetS)[9], a highly atherogenic condition. When compared to control subjects who do not have steatosis, patients with NAFLD have a higher prevalence of atherosclerosis, as shown by increased carotid wall intimal thickness, increased numbers of atherosclerotic plaques, and increased plasma markers of endothelial dysfunction, that are independent of obesity and other established risk factors[10-13]. Consistent with these observations natural history studies have reported that the increased age-related mortality observed in patients with NAFLD is attributable to cardiovascular as well as liver-related deaths[14-17].

Pathologic studies have shown that atherosclerosis is an early process beginning in childhood, with fatty streaks observed in the aorta and the coronary and carotid arteries in children and adolescents[18,19]. There is a positive correlation between the extent of early atherosclerotic lesions in the aorta and the coronary and carotid arteries and cardiovascular risk factors, including obesity, dyslipidemia, hypertension, and diabetes[20-22]. Yet the exposure to cardiovascular risk factors of children and adolescents is independently associated with an increased carotid atherosclerosis in early to middle adulthood[23,24]. Thus, the possible impact of NAFLD on cardiovascular disease (CVD) deserves particular attention in view of the implications for screening/surveillance strategies in the growing number of children and adolescents with NAFLD. In the present review, we examine the current evidence on the association between NAFLD and atherosclerosis in the pediatric population, discuss briefly the possible biological mechanisms linking NAFLD and early vascular changes, and address the approach to treatment of NAFLD to prevent not only end-stage liver disease but also CVD.

NAFLD AND THE METABOLIC SYNDROME

NAFLD is closely associated with abdominal obesity, atherogenic dyslipidemia, hypertension, insulin resistance and impaired glucose tolerance, which are all features of the MetS. Approximately 90% of patients with NAFLD have at least one of the features of MetS, and about 33% meet the complete diagnosis, placing NAFLD as the hepatic representation of MetS[25]. The relationship of NAFLD with MetS features has been confirmed in adults in several studies[9,26-29]. Evidence for a relationship between MetS and NAFLD in children is also emerging[30-32]. The Korean National and Nutrition Examination Survey found that participants aged 10-19 years who presented with three or more risk factors for MetS, had an odds ratio (OR) of 6.2 (95% CI, 2.3-16.8) for an elevated serum alanine aminotransferase (ALT), which they used as an indicator of fatty liver[30]. A single center study from Italy reported MetS to be present in 65.8% of children (3-18 years) with biopsy-proven NAFLD and found grade of fibrosis to be the only histological feature significantly associated with MetS on univariate analysis[31]. A case-control study comparing 150 overweight children with biopsy-proven NAFLD to 150 age-, sex-, and obesity-matched children without evidence of NAFLD, found that, after adjustment for age, sex, race, ethnicity, and hyperinsulinemia, children with MetS had an OR of 5.0 (95% CI, 2.6-9.7) for NAFLD compared with children without MetS[32]. This is the most compelling data to support a significant relationship between NAFLD and MetS, not explicable merely by the coexistence of overweight or obesity in these two conditions, and lend support to the hypothesis that fat accumulation in the liver has an important role in the pathogenesis of other obesity-related comorbidities[33].

NAFLD AND CARDIOVASCULAR DISEASE

Increases in morbidity and mortality from CVD are probably among the most important clinical features associated with NAFLD[13]. Published studies have shown that mortality among patients with NAFLD is higher than that in the general population, mainly due to concomitant CVD and liver dysfunction[14-17]. Using the resources of the Rochester Epidemiology Project, Adams *et al*[14] conducted a population-based cohort study to examine the natural history of patients diagnosed with NAFLD on the basis of imaging studies (83%) or liver biopsy (17%). Mean (SD) follow-up was 7.6 (4.0) years culminating in 3192 persons/years follow-up. Death occurred in 12.6% of patients and was most commonly due to malignancy and ischemic heart disease, which were also the two most common causes of death in the Minnesota general population of the same age and sex. Liver disease was also an important contributor of death among patients with NAFLD, being the third most common cause and accounting for 13% of all deaths. In contrast, "chronic liver disease and cirrhosis" was the 13th leading cause of death among the Minnesota general population, accounting for less than 1% of all deaths[14]. This implies that the increased overall mortality rate among NAFLD patients compared with the general population was at least in part due to complications of NAFLD. In a cohort study involving 129 consecutively enrolled patients diagnosed with biopsy-proven NAFLD, Ekstedt *et al*[15] compared survival and causes of death with a matched reference population. Mean follow-up (SD) was 13.7 (1.3) years. Mortality was not increased in patients with steatosis. In contrast, survival of patients with NASH was significantly reduced. A comparison of the causes of death of patients with NASH with those of the corresponding reference population showed it was significantly more common for patients with NASH to die from liver-related causes (2.8% *vs* 0.2%) and from cardiovascular disease

(15.5% *vs* 7.5%). No significant differences in causes of death were found between non-NASH patients and the corresponding reference population[15]. In a cohort study involving 173 patients retrospectively identified as having a diagnosis of biopsy-proven NAFLD, Rafiq *et al*[16] showed that after a median follow-up of 18.5 years, patients with histologic NASH had significantly higher liver-related mortality than the non-NASH NAFLD cohort (17.5% *vs* 2.7%). The most common causes of death were coronary artery disease, malignancy, and liver-related death. In a very recent study involving a cohort of 118 subjects with NAFLD who underwent liver biopsy because of elevated liver enzymes, Söderberg *et al*[17] confirmed that, after a 28-year follow-up, overall survival was reduced in subjects with NASH, whereas bland steatosis with or without severe fibrosis was not associated with any increase in mortality risk in comparison with the general population. The main causes of death among patients with NAFLD were CVD, followed by extrahepatic cancers and hepatic diseases. All these data provide evidence of an increased risk for cardiovascular mortality in patients with NASH. However, most studies which examined the natural history of NAFLD were retrospective cohort studies with relatively small numbers of patients with histologically proven NAFLD who were seen at tertiary referral centers - features that limit the generalizability of the findings to a community-based practice where patients may have a milder disease. Indeed, among people with NAFLD, those who are referred to hepatologists may have a more advanced liver disease than those detected in the community or population based screening but are not referred. Therefore, the magnitude of mortality risk in NAFLD depends on the setting and method of ascertainment. Future longitudinal studies with larger and less selected cohorts of patients are needed to identify through reliable, noninvasive means the true impact of the wide spectrum of NAFLD in the general population on the long-term overall and cardiovascular mortality.

Data on the prognosis and clinical complications of NAFLD in children remain scant[3]. Although coronary artery disease and stroke usually occur in middle and late age, autopsy studies have shown that the atherosclerotic process in the vascular wall begins in childhood and is accelerated in the presence of risk factors[18-24]. Given the large number of children affected, it is imperative that we establish a better understanding of the natural history of pediatric NAFLD in terms of the progression of liver disease as well as its complications (including long-term cardiovascular risk profile). Feldstein *et al*[34] recently reported the first longitudinal study describing the long-term survival of children with NAFLD who underwent a follow-up of up to 20 years. That study demonstrated that NAFLD in children is a disease of progressive potential. Some children presented with cirrhosis, others progressed to advanced fibrosis or cirrhosis during follow-up, and some developed end-stage liver disease with the consequent need for liver transplantation. Feldstein *et al*[34] also showed that NAFLD in children is associated with significantly shorter long-term survival than the expected survival in the general population of the same age and sex. Children with NAFLD had a 13.8-fold higher risk of dying or requiring liver transplantation than the general population of the same age and sex. The recorded deaths were not liver-related.

Recent epidemiological studies in adult subjects have also demonstrated that NAFLD is associated with an increased risk of incident CVD that is independent of the risk conferred by traditional risk factors and components of the MetS[35-42]. Yet, several studies (including the pediatric population) have reported independent associations between NAFLD and impaired flow-mediated vasodilatation (FMD) and increased carotid-artery intimal medial thickness (cIMT) - two reliable markers of subclinical atherosclerosis - after adjusting for cardiovascular risk factors and MetS[10,12,43-47].

NAFLD AND MARKERS OF SUBCLINICAL ATHEROSCLEROSIS IN CHILDREN

The relation between obesity and atherosclerosis development has been evaluated in many pediatric studies[48], but few studies focused on the relation between NAFLD and atherosclerosis (Table 1)[32,47,49-56]. In an autopsy study involving 817 children (aged 2 to 19 years) who died of external causes (accident, homicide, suicide) from 1993 to 2003, Schwimmer *et al*[49] showed that the prevalence of atherosclerosis was increased by a factor of 2 among those with NAFLD. Atherosclerosis was assessed as absent, mild (aorta only), moderate (coronary artery streaks/plaques), or severe (coronary artery narrowing). Fatty liver was present in 15% of the children. For the entire cohort, mild atherosclerosis was present in 21% and moderate to severe atherosclerosis in 2%. Atherosclerosis was significantly more common in children with fatty liver than those without the disease (30% *vs* 19%, $P < 0.001$). Body mass index (BMI) was not independently correlated to the presence of atherosclerosis, but fatty liver status and BMI did interact significantly ($P < 0.01$). Consequently, for obese subjects the odds of having atherosclerosis was more than 6 times higher in children with fatty liver than those without[49].

Despite this, there are currently few data regarding the possible association between liver histopathologic changes and atherogenic risk in children[32,52,56]. In the Bogalusa heart study in children, investigators found that the extent to which the intimal surface was covered with atherosclerotic lesions was significantly associated with elevation of concentrations of total cholesterol (TC), low-density lipoprotein cholesterol (LDL-c), triglycerides (TG), and lower concentration of high-density lipoprotein cholesterol (HDL-c). Ratios of cholesterol ester-rich lipoprotein level (TC/HDL-c and LDL-c/HDL-c) are well-established predictors of C VD[57]. More recently, the TG/HDL-c ratio has been shown to be a strong predictor of MetS and CVD[58,59]. In a case-control study, Schwimmer *et al*[32] showed that children with a biopsy-proven NAFLD had a significantly higher fasting glucose, insulin, TC, LDL-c,

Table 1 Published studies on the association between nonalcoholic fatty liver disease and markers of atherosclerosis in the pediatric population

Authors	Study population and sample size (No.)	Diagnosis	Outcomes	Main results
Schwimmer *et al*[49]	Children (817) who died of external causes (accident, homicide, suicide) from 1993 to 2003. Fatty liver was present in 15% of the children	Autoptic liver biopsy	Atherosclerosis was assessed as absent, mild (aorta only), moderate (coronary artery streaks/plaques), or severe (coronary artery narrowing)	For the entire cohort, mild atherosclerosis was present in 21% and moderate to severe atherosclerosis in 2%. Atherosclerosis was significantly more common in children with fatty liver than those without the disease (30% *vs* 19%; $P < 0.001$)
Schwimmer *et al*[32]	Overweight children with (150) and without (150) NAFLD matched for gender, age, and severity of obesity	Liver biopsy	Prevalence of cardiovascular risk factors (abdominal obesity, dyslipidemia, hypertension, insulin resistance, and glucose abnormalities)	NAFLD was strongly associated with multiple cardiovascular risk factors independently of both BMI and hyperinsulinemia
Pacifico *et al*[50]	Obese children with (29) and without (33) NAFLD; Healthy lean children (30)	Liver ultrasound	cIMT	cIMT was significantly higher in obese children with NAFLD compared with obese children without NAFLD and control group. Yet, the severity of fatty liver was associated with cIMT independently of anthropometric and metabolic features
Demircioglu *et al*[51]	Obese children with mild (32), moderate-severe (22) NAFLD, and without NAFLD (26); Healthy lean subjects (30) matched for age and gender	Liver ultrasound	cIMT	cIMT measured at left sites of common carotid artery, carotid bulb, and internal carotid artery was significantly higher in obese children compared with controls. Moreover, there was an increase in the mean cIMT of each segment with the increase in steatosis grade
Kelishadi *et al*[54]	Obese adolescents with (25) and without (25) components of MetS; Normal weight adolescents with (25) and without (25) components of MetS	Liver ultrasound and elevated ALT	cIMT	cIMT was significantly associated with insulin resistance and NAFLD
Manco *et al*[52]	Overweight and obese children with (31) and without (49) NAFLD matched for gender, age, and BMI	Liver biopsy	cIMT	cIMT was similar in cases and controls on the right side but significantly higher on the left site. There was no association between cIMT and severity of steatosis as well as fibrosis, and NAFLD activity score
Caserta *et al*[53]	Randomly selected adolescents (642) of whom 30.5% and 13.5% were, respectively, overweigth and obese. Overall prevalence of NAFLD, 12.5%	Liver ultrasound	cIMT	NAFLD, BMI (or waist circumference) and systolic blood pressure were independently associated with increased cIMT
Nobili *et al*[56]	Children with NAFLD (118)	Liver biopsy	Atherogenic lipid profile (TG/HDL-c, TC/HDL-c, and LDL-c/HDL-c ratios)	The severity of liver injury was strongly associated with a more atherogenic lipid profile, independently of BMI, insulin resistance, and presence of MetS
Pacifico *et al*[47]	Obese children with (100) and without (150) NAFLD; Healthy lean children (150)	Liver ultrasound and elevated ALT	cIMT and FMD	Obese children had more functional and morphologic vascular changes than healthy lean controls, regardless of liver involvement. However, obese children with NAFLD had significantly decreased FMD response and increased cIMT compared to obese children without NAFLD independently of other cardiovascular risk factors and MetS
Weghuber *et al*[55]	Obese children with (14) and without (14) NAFLD	Proton MR spectroscopy	FMD	FMD was comparable between the two groups

NAFLD: Nonalcoholic fatty liver disease; BMI: Body mass index; cIMT: Carotid intima-media thickness; TG: Triglycerides; HDL-c: High-density lipoprotein cholesterol; TC: Total cholesterol; LDL-c: Low--density lipoprotein cholesterol; ALT: Alanine aminotransferase; FMD: Flow mediated dilation; MetS: Metabolic syndrome; MR: Magnetic resonance.

TG, systolic and diastolic blood pressure than age-, sex-, and BMI-matched peers without NAFLD. These data confirm that fat accumulation in the liver may play a more important role than obesity itself in determining the risk for "weight-related" metabolic comorbidities[33]. Thus, the authors concluded that NAFLD may serve as a marker to stratify the cardiovascular risk of overweight and obese children and adolescents[32]. Furthermore, in a study involving 118 consecutive children with biopsy-proven NAFLD undergoing extensive metabolic profiling, Nobili and colleagues found that the NAFLD activity and fibrosis scores had a significant positive correlation with TG/HDL-c, TC/HDL-c, and LDL-c/HDL-c ratios[56]. After adjusting for potential confounders including BMI, homeostatic model assessment index, impaired glucose tolerance, and presence of MetS, both NAFLD activity

score and stage of fibrosis remained independent predictors of an atherogenic lipid profile. The lipid ratios were found to be markedly higher in children with established NASH compared with those patients with simple steatosis or borderline disease, indicating that severity of liver injury in children with NAFLD is strongly associated with increased atherogenic risk[56].

Recent improvements in imaging technology have identified early vascular changes that can be assessed by the use of ultrasonography. These early changes include impairment of FMD, arterial stiffness, and increased cIMT. The measurement of FMD and cIMT by high-resolution ultrasound is increasingly used for cardiovascular risk evaluation in young individuals with obesity, MetS or its components, and pre-diabetes[23,60,61]. Pacifico *et al*[50] first showed that the severity of ultrasonographically diagnosed NAFLD in obese children was significantly associated with carotid atherosclerosis, independently of anthropometric and metabolic features. Demircioglu *et al*[51], in a subsequent study, also found an association between ultrasonographically detected NAFLD and cIMT measured at sites of the common carotid artery, carotid bulb and internal carotid artery. In addition, there was an increase in the mean of cIMT of each segment with the increase in hepatosteatosis grade[51]. Kelishadi *et al*[54] also demonstrated that cIMT was significantly associated with insulin resistance and NAFLD, suggesting that the liver and the vessels share common mediators. This is in contrast to the case-control study by Manco *et al*[52] including a mixed population of overweight and mildly obese children of whom 31 had biopsy-proven NAFLD, whereas 49 had no ultrasound evidence of NAFLD and no abnormal levels of aminotransferases. Although cIMT was statistically significantly higher on the left side in NAFLD cases, the authors concluded that this difference was unlikely to be clinically relevant because of the substantial overlap of cIMT values between cases and controls. Also, there were no differences in the frequency of MetS components between the groups. Finally, there was no association between histologic severity of NAFLD and cIMT[52]. However, a recent study by Patton *et al*[30] showed the potential power of MetS as a prognostic indicator of disease severity in NAFLD. Of the MetS features, central obesity and insulin resistance were most consistently associated with NAFLD histology[30].

The association between NAFLD and carotid atherosclerosis has also been determined in a large, randomly selected adolescent population from Reggio Calabria, a town in southern Italy[53]. The authors found that NAFLD, BMI, waist circumference, and systolic blood pressure were independent markers of increased cIMT. Likewise, in a very recent study with a large sample size it has been shown that obese children with NAFLD have a significantly lower FMD response and increased cIMT compared to obese children without NAFLD independently of other cardiovascular risk factors and MetS, and that obese children exhibit more functional and morphologic vascular changes than healthy lean controls, regardless of liver involvement[47]. The larger number of subjects in that study may in part account for the associations the authors were able to identify between NAFLD and functional vascular changes, in contrast to the study by Weghuber *et al*[55]. in which a very small sample of obese children with and without NAFLD had a similar FMD response.

Although longitudinal studies are needed to clarify the extent to which pediatric NAFLD and its severity influence long-term cardiovascular outcomes in the general population, overall the above cross-sectional findings suggest that childhood NAFLD is associated with early atherosclerosis.

POSSIBLE BIOLOGICAL MECHANISMS LINKING NAFLD AND ACCELERATED ATHEROSCLEROSIS

The biologic mechanisms by which NAFLD contributes to accelerated atherosclerosis, independently of other risk factors, are still poorly understood. Increased visceral adipose tissue and insulin resistance are the undisputed major contributors to NAFLD, MetS, and atherosclerosis[9,62,63]. The adipose tissue inflammation with consequent release of multiple proinflammatory molecules is one of the earliest steps in the chain of events involved in the development of insulin resistance and atherosclerosis, in particular in obese and overweight persons[64-66]. While insulin resistance promotes fatty acid accumulation in the liver, the latter causes hepatic insulin resistance characterized by a lack of suppression of endogenous liver glucose production. Therefore, NAFLD might act as a stimulus for further increased whole-body insulin resistance and dyslipidemia (with a characteristic overproduction of triglyceride- and cholesterol-rich remnant particles), leading to accelerated atherosclerosis[33]. It is also conceivable that other atherogenic mechanisms could be involved in patients with NAFLD including enhanced oxidative stress and chronic, subclinical inflammation, which are thought to be causal factors in the progression from simple steatosis to more advanced forms of NAFLD[27-29,62]. Indeed, patients with NAFLD frequently have higher plasma markers of oxidative stress and inflammation, at least partially derived from the diseased liver, as well as decreased adiponectin concentrations, an adipose-secreted cytokine with antiatherogenic properties[11,67-69].

Recent research has suggested a role for increased fructose consumption as a risk factor for NAFLD[70,71]. Strong evidence exists that fructose consumption may promote hepatic de novo lipogenesis and intrahepatic lipids, inhibition of mitochondrial β-oxidation of long-chain fatty acids, triglyceride formation and steatosis[72]. In addition, compared to glucose consumption, sustained dietary fructose has been reported to significantly increase plasma concentrations of fasting small dense LDL-c, oxidized LDL-c, and postprandial remnant-like-particle-triglyceride and -cholesterol in overweight and obese subjects[73]. These changes may be associated with increased risk of CVD[74,75].

Finally, NAFLD could be linked to accelerated athero-

genesis through the presence of abnormal lipoprotein metabolism, especially during the post-prandial phase[76,77]. Apolipoprotein (APO) B is a large protein involved in the transport of triglycerides and cholesterol from the liver to peripheral tissues. Diminished synthesis of APO B, a rate-determining step in the very low density lipoproteins (VLDL) assembly, would impair the ability of the hepatocyte to export triglycerides and cholesterol esters. Impaired VLDL secretion would also result in increased levels of atherogenic triglyceride- and cholesterol-rich remnant particles. Recent studies have suggested a genetic basis for abnormal lipoprotein metabolism in patients with NAFLD. Two single-nucleotide polymorphisms in the gene encoding APOC3 may be associated with hypertriglyceridemia[78-80]. APOC3 variants C-482T and T-455C lead to increased plasma concentrations of APOC3, which in turn inhibit lipoprotein lipase and triglyceride clearance, thus conferring a predisposition to both fasting and postprandial hypertriglyceridemia due to an increase in chylomicron-remnant particles[81].

THERAPEUTIC APPROACH

The only accepted therapy for pediatric NAFLD is lifestyle modification with diet and physical exercise. The close association of NAFLD with MetS and obesity in children provides the rationale for the therapeutic role of weight reduction in the treatment of fatty liver disease. Fortunately, this approach may also be beneficial in improving cardiovascular risk profile.

Weight-loss oriented lifestyle interventions in the overweight pediatric population have been shown to increase glucose tolerance and improve the MetS risk factors[82]. In children with presumed NAFLD, several studies demonstrate a normalization of serum ALT associated with weight loss[5,83,84]. However, the relative efficacy of weight loss and degree of weight loss needed to induce histologic improvement in pediatric NAFLD is unknown. Studies in adults with NAFLD suggest that weight loss also leads to significant improvement in liver histology. In particular, a weight loss greater than 5% has been associated with significant improvement in liver histology[85]. There is only one clinical trial using liver histology as the primary end point in children and adolescents with NAFLD[83]. The study demonstrated that 2 years of lifestyle intervention with a diet tailored on individual caloric requirement and increased physical activity was associated with a mean weight loss of approximately 5 kg, resulting in a significant improvement in liver histology as well as in insulin resistance, serum levels of aminotransferases, and lipid levels. No information exists on recommending any type of diet. A low-carbohydrate diet has been shown to lead to a reduction in serum ALT and fatty liver content in adult patients[86]. A randomized controlled study in obese adolescents has demonstrated that a diet based on a reduced glycemic load is more effective than a low fat diet in achieving weight loss[87], but similar data are not available in children with NAFLD. Current data on the role of a low fructose diet in children with NAFLD are inconclusive[71]. A 6-month pilot study in children with NAFLD showed that a low fructose diet was associated with a significant decrease in oxidized LDL-c[88]. However, no effect on serum ALT concentrations was found. N-3 long-chain polyunsaturated fatty acids (LCPUFA) including eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) have been reported to control some of the metabolic stigmata of obesity[89]. Dietary N-3 LCPUFA lower blood triglycerides and have anti-inflammatory as well as insulin-sensitizing effects[89]. A recent randomised clinical trial has shown that the supplementation of DHA improves liver steatosis and insulin sensitivity in children with NAFLD[90]. Their role in prevention of CVD is also emerging[91]. At this time, however, the available information is insufficient to derive dietary intake recommendations for EPA and DHA[92]. Finally, diet duration and amount of weight loss have not been definitively assessed in children[83,93,94].

A general consensus exists about the key role of physical activity and its synergic effect when combined to diet modifications. Increasing energy expenditure is an additional way to reducing daily calories. Liver biopsy has shown improvement of histologic features in children with NAFLD who were engaged in a moderate daily exercise program (45 min/d aerobic physical exercise) associated to dietary changes[83].

Owing to the likely role of insulin resistance and oxidative stress in the development and progression of NAFLD, most studies on pharmacological treatment have focused on the use of metformin or antioxidants. These drugs have been found to be effective in pilot studies[5]. Recently, in a multicenter, randomized, placebo-controlled clinical trial of treatment with metformin, vitamin E, or placebo for 96 wk in 173 nondiabetic children with histologically confirmed NAFLD, the Nonalcoholic Steatohepatitis Clinical Research Network found that compared with placebo, neither vitamin E nor metformin was associated with a sustained reduction in serum ALT[95,96]. Compared to placebo, vitamin E significantly improved hepatocellular ballooning and NAFLD activity score and, in the subset of children with NASH at baseline, significantly increased resolution of NASH. Metformin had no significant effect on any secondary histologic outcome[96]. Neither vitamin E nor metformin had significant effects on fibrosis, lobular inflammation, or portal inflammation scores. No significant differences in safety were reported between groups[96]. However, the likelihood that vitamin E would need to be taken indefinitely[97] underlines the importance of long-term prospective studies involving patients with NASH to assess the effect of vitamin E on liver-related and cardiovascular mortality[98].

Given the role of obesity in the pathogenesis of NAFLD, bariatric surgery has been proposed as a potential treatment strategy. In obese adult patients, bariatric surgery has been shown to induce weight loss, ameliorate cardiovascular risk factors, resolve hepatic steatosis and, in most studies, inflammation[99-101]. NASH was improved in the majority of affected patients and was intimately associ-

ated with insulin resistance over both the short and long term[101]. The issue of NAFLD and bariatric surgery in children is complex[71]. Though adolescents are increasingly undergoing surgical treatment of obesity, the guidelines for eligibility are not standardized[102]. In addition, children with a clinical diagnosis of NAFLD are different from those typically undergoing bariatric surgery[103]. Children with a clinical diagnosis of NAFLD tend to be younger and less obese than adolescents undergoing surgical treatment of obesity[103]. Although several studies report resolution or improvement of comorbidities after bariatric surgery in adolescents[104,105], liver outcome data are needed. In a series of 41 adolescents, Nadler *et al*[105] reported improvement in liver function enzymes 1 to 2 years after surgery.

CONCLUSION

The current body of evidence suggests that NAFLD is associated with a significantly greater overall mortality than in the general population, as well as with increased CVD prevalence, independently of classical atherosclerotic risk factors. These observations raise the possibility that NAFLD may be not only a marker but also an early mediator of atherosclerosis.

Children with NAFLD may also be at a higher risk for atherosclerosis. Therefore, the rising prevalence of obesity-related MetS and NAFLD in childhood may lead to a parallel increase in adverse cardiovascular outcomes. In children, the cardiovascular system remains plastic and damage-reversible if early and appropriate interventions are established effectively. Therapeutic goals for NAFLD should address nutrition, physical activity and avoidance of smoking to prevent not only end-stage liver disease but also CVD.

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REVIEW

Intestinal inflammation and colorectal cancer: A double-edged sword?

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Abstract

Chronic inflammation is thought to be the leading cause of many human cancers including colorectal cancer (CRC). Accordingly, epidemiologic and clinical studies indicate that patients affected by ulcerative colitis and Crohn's disease, the two major forms of inflammatory bowel disease, have an increased risk of developing CRC. In recent years, the role of immune cells and their products have been shown to be pivotal in initiation and progression of colitis-associated CRC. On the other hand, activation of the immune system has been shown to cause dysplastic cell elimination and cancer suppression in other settings. Clinical and experimental data herein reviewed, while confirming chronic inflammation as a risk factor for colon carcinogenesis, do not completely rule out the possibility that under certain conditions the chronic activation of the mucosal immune system might protect from colonic dysplasia.

Key words: Colorectal cancer; Inflammation; T cells; Cytokines; Immunosurveillance

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INTRODUCTION

Chronic inflammation is thought to be the leading cause of many human cancers including colorectal cancer (CRC). Ulcerative colitis (UC) and Crohn's disease (CD), the two major forms of inflammatory bowel disease (IBD), are associated with an increased risk of developing colitis-associated colorectal cancer (CAC). The risk of CRC in UC patients is 2% after 10 years, 8% after 20 years and 18% after 30 years of active disease[1]. Although more recently other studies have estimated a lower risk in this class of patients, the overall incidence rate ratio for developing CRC calculated in UC patients was found by Bernstein *et al*[2] to be 2.75 [95% confidence interval (95% CI), 1.91-3.97] compared to the general population.

While the relationship between UC and CRC is well established, the risk associated with CD has been unclear until recently. Indeed, the heterogeneous nature of CD which can involve any part of the gut in a non-continuous way, with many patients having no colonic involvement, makes it difficult to estimate the actual risk of developing CRC in these patients. A milestone Swedish study demonstrated a relative risk of CRC of 5.6 for CD patients with exclusive localization in the colon and 3.2 for patients with ileo-colitis[3]. In contrast, patients with exclusive ileal localization of the disease had no increased risk. A meta-analysis of CRC risk in CD revealed an overall relative risk of 2.5 (95% CI, 1.3-4.7)[4]. In the subset of patients with exclusive colonic

localization, the risk was 4.5 (95% CI, 1.3-14.9) while the risk in patients with ileal disease was not significantly increased. Interestingly, when comparing Crohn's colitis with UC of similar extent, the relative risk of developing CRC is similar between the two groups.

Several risk factors concur to determine the probability of developing CRC in single patients. The observation that the cumulative risk increases over the years indicates that disease duration does play a role[1,3]. In addition, the extension of the disease has been shown to increase the risk of CAC; this being 1.7 in patients with ulcerative proctitis, 2.8 in those with left-sided colitis and 14.8 in patients with extensive colitis[3]. Also, the severity of inflammation independently correlates with the risk of developing CAC[5]. The same independent risk factors have been linked to the risk of developing CRC in CD. In addition, in CD patients, perianal disease, bypasses and strictures might be sites of increased risk of neoplastic transformation[6-8].

Overall, these data indicate that chronic inflammation of the colon such as that observed during either UC or CD increases the risk of developing CRC. However, the mechanisms involved in this process are still poorly understood. The current opinion regarding the pathogenesis of IBD is that, in genetically susceptible individuals, there is an overreaction of the immune system toward antigens of the gut microbiota leading to chronic inflammation[9]. UC and CD are characterized by different immune responses. While UC is caused by an atypical T helper (Th)2-mediated immune response characterized by high levels of IL-5 (but not IL-4) and IL-13, in CD there is a prevalent activation of Th1 cells with high expression of TNF-α and IFN-γ[10-13]. More recently, a new subset of IL-17-producing T helper cells, the Th17 cells, has been shown to play a role in the pathogenesis of CD[14,15]. Finally, in addition to CD4+ T cells, CD8+ T cells, natural killer, natural killer T cells and regulatory T cells have also been implicated in the pathogenesis of IBD[16-18].

Given the role played by these cell subsets and by the cytokines they express in the induction and maintenance of gut inflammation, their role has also been investigated in the pathogenesis of CAC. Here we review some of the recent data that implicate immune cells and inflammatory cytokines in the pathogenesis of CAC.

INFLAMMATION AND TUMOR INITIATION: A DOUBLE-EDGED SWORD

Chronic inflammation is thought to induce dysplasia by inducing DNA modifications in intestinal epithelial cells. Indeed, chronic accumulation of activated immune cells such as neutrophils, macrophages and dendritic cells is accompanied by the release of oxygen and nitrogen reactive species, which are known to induce genomic mutations[19,20]. Moreover, chronic inflammation is associated with DNA methylation and histone modification[21-23]. All these processes have been associated with the altered expression of genes involved in carcinogenesis such as *p53, APC, K-ras* and *Bcl-2*[24]. Once initiated, dysplastic cells are subjected to the effect of cell-derived growth factors and cytokines which contribute to tumor growth. However, lines of evidence have also indicated that, under certain conditions, immune cell subsets and cytokines fight to maintain dysplastic cells in check thus preventing tumor progression. A change in the immune response and/or an adaptation to the selective pressure of the immune system, referred to as immune-editing, at a certain point will select dysplastic cell clones able to grow sustained by the presence of growth factors and proinflammatory cytokines released in the surrounding microenvironment, thus changing the role of the immune system from a negative regulator of tumor growth to cancer promoter[25]. Although most of the data sustaining this mechanism derive from models of sporadic cancer, it is possible that a similar alteration of the balance between immune system and dysplastic cells might also occur during long-standing intestinal inflammation.

CD4+ T cells and colitis-associated carcinogenesis

Whether T cells are required for the development of colitis-associated CRC is an open question. In the azoxymethane/dextran sulphate sodium (AOM/DSS) experimental model of CAC, RAG1-deficient mice that do not have B and T cells did not develop tumors even in the presence of colitis[26]. These results indicate that lymphocytes are required to promote tumor growth in the context of colitis. However, it is worth considering that an enhanced activity of natural killer (NK) cells, which are still present in RAG1-/- mice, might be responsible for tumor protection in these mice. Indeed, depletion of suppressive subsets of T cells (i.e. regulatory T cells) has been shown to increase NK cell activity and tumor rejection[27-29]. Experiments with RAG1-/-//γ-chain-/- double knockout mice which lack B, T and NK cells would help to address this issue.

With regard to T helper cell subsets, the role of Th1 and Th2 cells in CAC has been shown by Osawa *et al*[30]. The authors compared CAC development in IL4-/- and IFN-γ-/- deficient mice which have a biased Th2 and Th1 immune response, respectively. Interestingly, Th1-biased IFN-γ-/- mice developed more tumors than wild type. Since in these mice there was high expression of IL-4 and IL-5, the authors concluded that Th2-derived cytokines promote tumor growth. Indeed, a Th2 response has been correlated with progression of experimental and human sporadic CRC[31,32] while a Th1 response has been associated with a better prognosis[33]. Moreover, the Th2-related cytokines IL-4 and IL-13 have been shown to induce the upregulation of activation-induced cytidine deaminase (AID), an enzyme involved in DNA mutation in epithelial cells *in vitro*[34]. Accordingly, AID levels are highly expressed in tumor samples from UC patients. The higher susceptibility to develop CAC shown by IFN-γ-/- mice might be related to a decreased immunosurveillance[34]. Indeed, IFN-γ has been shown to be involved in the activation of cytotoxic T cells and NK cells which play a central role in the antitumor immune response[35,36].

The initial observation that UC patients have a higher risk of CAC in comparison to CD fits well with the con-

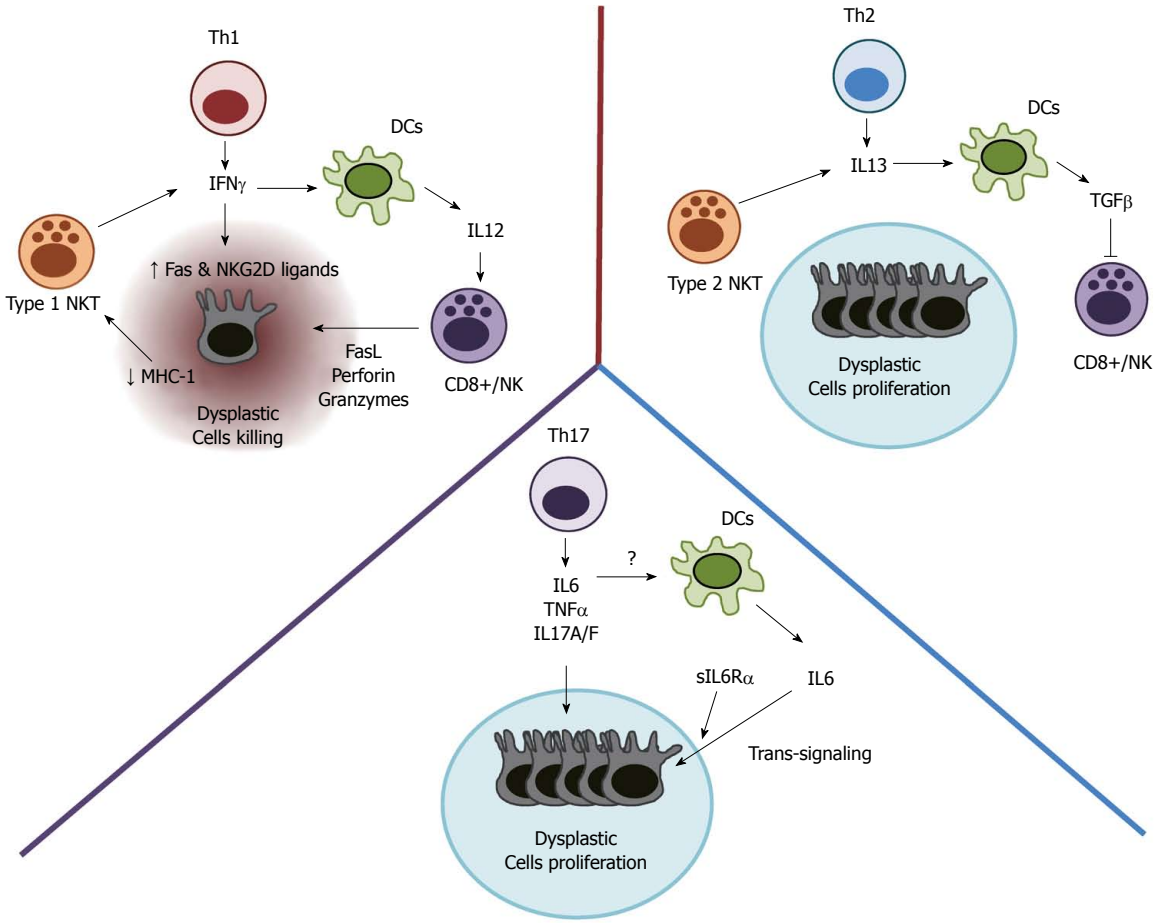

Figure 1 Different T helper-mediated immune responses might be associated with distinct effects on dysplastic cell survival. While Th2 and Th17 immune responses might promote dysplastic cell proliferation and tumor growth, Th1 cells could induce cell death thus preventing tumor progression. Th: T helper; NK: Natural killer; NKT: Natural killer T cells; IL: Interleukin; TGF-β: Transforming growth factor-β; DC: Dendritic cells; MHC: Major histocompatibility complex; Fas: Tumor necrosis factor receptor superfamily, member 6; NKG2D: Killer cell lectin-like receptor subfamily K, member 1; TNF-α: Tumor necrosis factor-α; sIL6R-α: Soluble IL-6 receptor-α.

cept that the Th2 immune response, observed in UC, promotes cancer development while the CD-associated Th1 response is protective. However, as mentioned above, the initially observed lower incidence of CAC in CD in comparison to UC is considered to be due to the methodological approach used in these studies. An alternative explanation for this apparent contradiction could derive from recent advances in the pathogenesis of CD. CD has been long thought to be a Th1-mediated immune disease. This concept derives from initial studies on the role of IL-12, a cytokine involved in the differentiation of Th1 cells[13,37]. In these studies, neutralization of p40, a subunit of IL-12, was effective in preventing gut inflammation both in experimental models and in humans[38-40]. However, we now know that p40 is shared by another cytokine, IL-23, which is a heterodimer composed of p40 and p19. IL-23 has been shown to be involved in the maintenance of Th17 cells, a novel class of T helper cells[41]. Accumulating evidence suggests that Th17 cells might play a role in the pathogenesis of CD. Gain of function polymorphisms of the IL-23 receptor gene are associated with CD[42]. IL-23 p19-/- mice are less susceptible to colitis in comparison with IL-12 p35-/- mice which do not express IL-12[43]. Mice deficient in receptor-related organ receptor (ROR)γ t, the lineage commitment transcription factor of Th17 cells, are resistant to inflammation in different models of colitis[44]. Most of the proinflammatory effect of Th17 has been attributed to the expression of IL-17. IL-17 is known to induce the expression of proinflammatory factors such as TNF-α, IL-6, IL1, iNOS, metalloproteinases and chemokines, which also play a role in CAC[45]. Finally, IL-23 expression is increased in several types of human cancer including CRC[46], and IL-23 p19-/- mice are demonstrated to be more resistant to tumor development[47]. Overall, these data suggest that tumor-promoting Th17 cells rather than Th1 cells might sustain inflammation in CD, thus explaining the increased risk of CAC in CD patients (Figure 1).

Cytotoxic T cells

CD8+ T cells, NK and natural killer T (NKT) cells have been shown to play a role in cancer immunity. Their role, initially limited to the capacity to kill dysplastic target cells, has been recently extended demonstrating a more complex contribution to the antitumor immune response.

A central role in cancer immunosurveillance is attrib-

uted to CD8+ cytotoxic T cells. After presentation of tumor-related antigens by antigen-presenting cells, CD8+ T cells become activated and release different cytotoxic molecules responsible for target cell killing. Activated CD8+ T cells express high levels of IFN-γ and FasL[48]. While FasL, a membrane bound molecule, induces apoptosis by interacting with Fas expressed on the surface of dysplastic cells[49], IFN-γ has been shown to enhance the expression of Fas in colorectal cancer cell lines thus enhancing the killing process[50]. Perforin, granzyme A and granzyme B are also expressed by CD8+ T cells and their effect on target cells is to induce a permeabilization of the cell membrane and cell death[51,52].

NK cells are large granular lymphocytes with both cytotoxicity against tumor and cytokine-producing effector function. NK cells are involved in the rejection of *in vivo* implanted tumors in a manner dependent on the presence or absence of signals on the target cells. The lack of MHC class I expression on the surface of target cells or the upregulation of NKG2D ligands can determine NK cells activation[53-55]. NKG2D ligands are expressed at various levels in CRC cell lines and the expression of one of them, MICA, was associated with a better prognosis in CRC patients[56]. Once activated, NK cells express high levels of IFN-γ, perforin and granzymes which induce apoptosis in target cells. Interestingly, IL-21, a cytokine highly expressed in both UC and CD, has been shown to activate NK cells[57]. However, whether IL-21-induced activation of NK cells plays a role in anti-tumor immunity is still unclear[58-60].

In contrast to NK cells, which lack the T cell receptor (TCR), NKT cells express a limited variety of TCRs. Although NKT cells have NK-like cytolytic activity, they are considered regulators of the immune response, being able to express both Th1- and Th2-related cytokines. In tumor immunity, NKT cells have been considered as both enhancers and suppressors of the anti-tumor activity. A subset of NKT cells (type I NKT), characterized by the expression of V-α-14-Jα-18 TCR-α chain, has been shown to enhance tumor immunity by IFN-γ expression and NK cell activation[61,62]. Moreover, IFN-γ indirectly promotes the activation of CD8+ T cells by inducing the expression of IL-12 in antigen-presenting cells[63]. Tumor infiltration by type I NKT cells in CRC patients has been positively correlated with the disease-free survival[64]. In contrast to type I NKT cells, type Ⅱ NKT cells, which do not express the V-α-14-Jα-18 TCR-α chain, have been associated with suppression of antitumor immunity. Type Ⅱ NKT cells express IL-13, which has been shown to induce the expression of the immunosuppressive cytokine TGF-β in myeloid cells[56,65]. Interestingly, the selective activation of type Ⅱ NKT cells enhanced CT26 cell growth in a mouse model of CRC metastasis[66]. In UC, activation of type Ⅱ NK cells and expression of IL-13 characterize the "atypical" Th2 immune response observed in these patients[11]. It is tempting to speculate that activation of type Ⅱ NKT cells in UC might contribute to CAC development by selectively dampening the antitumor immune response while sustaining mucosal inflammation and cancer development.

Innate immune cells

The role of innate immune cells in sporadic CRC progression has started to be unveiled (For review, see Mantovani *et al*[67]). However, whether these cells are also important in the development of CAC is still unclear.

The role of innate immunity in the development of CAC is suggested by recent findings that Toll-like receptors (TLRs) are important in inflammation and CAC. TLRs form a family of membrane-bound receptors expressed by cells of different lineages such as epithelial cells, macrophages and dendritic cells. TLRs "sense" the presence of bacterial compound present in the extracellular space. The interaction between the microbiota and the intestinal mucosa through TLRs is required to maintain intestinal homeostasis. Recent genetic studies suggest that polymorphisms in the genes encoding TLRs are associated with increased risk of IBD and disease extension[68,69]. Moreover, TLR4 is demonstrated as being upregulated in intestinal epithelial cells of patients with active IBD[70]. With regard to CAC, it was shown that intestinal bacteria are required for tumor development in models of CAC. Furthermore, deficiency of MyD88, a molecule involved in TLR intracellular signaling, significantly exacerbated chemically-induced colitis[71] and reduced tumor number and size in sporadic and colitis-associated CRC models[72,73].

CAC: the role of cytokines and chemokines

As mentioned above, immune cells actively contribute to CAC by expressing soluble factors (e.g. cytokines and chemokines) and the role of some of these has been extensively investigated.

IL-6

IL-6 is a multifunctional cytokine important for immune responses, cell survival, apoptosis, and proliferation. IL-6 has been linked to IBD pathogenesis. Atreya *et al*[74] have demonstrated that IL-6 expression in the mucosa of IBD-affected patients induces T cell resistance to apoptosis, thus contributing to chronic inflammation. Accordingly, a correlation between IL-6 levels and the clinical activity of IBD has been demonstrated[75,76]. With regard to CAC, IL-6 expressed during colitis was shown to promote tumor growth in mice[26]. In this model, selective inhibition of the TGF-β signaling in T cells was associated with an enhanced expression of IL-6. In turn, IL-6 trans-signaling, mediated by the interaction of IL-6 and the soluble form of the IL-6 receptor α (sIL-6α) with the gp130 receptor expressed on the surface of dysplastic cells, enhanced tumor cell proliferation. Moreover, in a similar model, Grivennikov *et al*[77] demonstrated that IL-6 expressed by lamina propria myeloid cells protects normal and transformed epithelial cells from apoptosis in a STAT-3-dependent manner, demonstrating the critical oncogenic function of this cytokine-activated transcription factor. Recently, higher expression of IL-6 and STAT3 was observed in both patients with active UC and those who had progressed to CAC, compared with patients with inactive disease or control patients[78]. In the same study, patients with either inactive or active UC, compared with

control individuals, showed increased expression of suppressor of cytokine signaling 3 (SOCS3), which limits the ability of IL-6 to activate STAT3. On the other hand, the expression of SOCS3 was decreased in patients with UC who had progressed to CRC. In the AOM/DSS mouse model of CAC, IEC-specific SOCS3 gene disruption led to increased size, number and load of colonic tumors and this was associated with increased STAT3 and nuclear factor-κB (NF-κB) activation in colon[79].

TNF-α

Tumor necrosis factor (TNF)-α is a pivotal cytokine in the pathogenesis of IBD and anti-TNF-α monoclonal antibody (MAb) therapy is routinely used in UC and CD patients[80]. Although TNF-α has been classically considered as an anticancer agent, it is currently recognized that chronically elevated TNF-α in tissues may promote tumor growth, invasion and metastasis[81]. Indeed, mice deficient for the p55 TNF-α receptor subunit were protected from tumor development in the AOM/DSS model of CAC[82]. Moreover, in the same study, repetitive anti-TNF-α treatment not only suppressed colitis in mice but also prevented CAC.

The effect of TNF-α signaling in CAC is mostly due to the intracellular activation of NF-κB. NF-κB is a pleiotropic transcription factor with a key role in innate and adaptive immunity and is required for the expression of various proinflammatory factors[83]. In addition to its critical function in inflammation, NF-κB activation can support carcinogenesis by increasing cell proliferation and angiogenesis, inhibiting cell death, and promoting cell invasion and metastasis[84]. Greten *et al*[85] have shown that blocking NF-κB activation in the intestinal epithelium dramatically reduced the incidence of CAC, and this was associated with enhanced epithelial cell apoptosis during early tumor development. Interestingly, no reduction of intestinal inflammation was observed in these mice, thus indicating that prosurvival signals provided by NF-κB in epithelial cells play a role in CAC initiation independent of the inflammation severity.

IL-10

IL-10 is an immunomodulatory cytokine and its main biological function is to limit and terminate inflammatory responses. Experimental data indicate that IL-10 might play a role in the pathogenesis of IBD and CAC. Indeed, patients carrying mutations of IL-10 receptor that abrogate IL-10 signaling develop more aggressive disease. Moreover, IL-10-deficient mice spontaneously develop colitis[86] and CAC[87] when infected with certain enteric bacteria such as *H. hepaticus*. In this model, colitis and CAC could be prevented by administering exogenous IL-10, thus indicating that IL-10 is pivotal in the control of inflammation and inflammation-related cancer in the gut. Analogous to IL-6, IL-10 activates STAT3 in target cells. However, the final effect is inhibition of NF-κB activation[88,89] and reduction of the expression of proinflammatory cytokines such as TNF-α, IL-6 and IL-12[90]. IL-10 has also been shown to act as an antiangiogenic factor[91].

A source of IL-10 is represented by regulatory T cells (Tregs), a class of immunosuppressive T cells. Interestingly, in a model of sporadic CRC, Erdman *et al*[92] showed that the transfer of wild type Tregs, but not IL-10-/- Tregs, in CRC-susceptible $Apc^{min-/+}$ mice prevented the development of adenomas and induced rapid tumor regression. These data suggest that, besides its role as a negative controller of the immune system, IL-10 might directly suppress the growth of transformed epithelial cells. Accordingly, Tregs transfer was associated with induction of epithelial cell apoptosis and downregulation of Cox-2, a molecule involved in dysplastic cell survival and proliferation.

TGF-β

Another important immunosuppressive cytokine is transforming growth factor (TGF)-β. TGF-β tightly controls the activation of the immune system and the inhibition of TGF-β signaling causes autoimmune diseases involving several organs including the gut. Moreover, the inhibition of TGF-β signaling operated by the intracellular inhibitory molecule Smad7 in gut lamina propria cells has been shown to contribute to chronic gut inflammation observed in IBD[90].

TGF-β plays an important role in epithelial cell differentiation and growth arrest. Accordingly, TGF-β signaling is found to be altered in sporadic CRC[93]. In contrast, the role of TGF-β in CAC is still unclear. Using a T cell-specific dominant negative TGF-β receptor II transgenic mouse, Becker *et al*[26] demonstrated that TGF-β signaling-mediated negative control of IL-6 expression in T cells is required to inhibit dysplastic epithelial cell proliferation. Conversely, IL-6 has been shown to inhibit TGF-β signaling by inducing Smad7 expression[94]. Smad3 is a key intracellular mediator of the anti-inflammatory and immunosuppressive activity of TGF-β in the colon. Accordingly, Smad3-deficient mice develop CAC that is dependent on the presence of enteric bacteria[95]. Despite the role of TGF-β as immunosuppressant and inhibitor of dysplastic cell growth, TGF-β signaling acts, under certain conditions, as a tumor promoter. Indeed, TGF-β-induced suppression of tumor-specific CD8+ T cells might favor tumor growth and progression[96].

Chemokines

Chemokines and their receptors play an integral role in IBD by regulating the accumulation of immune cells at the site of intestinal inflammation[97].

Monocyte chemoattractant protein 1 (MCP-1, CCL2), a member of the CCβ family of chemokines, is a known chemotactic factor regulating the recruitment of monocytes/macrophages and other inflammatory cells to sites of inflammation *via* activation of the CCR2 receptor[98]. The expression of MCP-1 is increased in the mucosa of patients with IBD[99]. Popivanova *et al*[100], using a model of CAC, showed that CCL2 blockade reduces the infiltration of COX-2-expressing F4/80-positive cells and suppresses COX-2 expression by infiltrating macrophages, resulting in retardation of cancer progression.

The role of chemokines in CAC is further supported by recent studies on chemokine decoy receptor D6. D6, like all decoy receptors, does not induce a conventional intracellular signal but mediates high-affinity ligand binding and efficient ligand degradation. D6 expression is increased in patients with IBD and with CAC compared with healthy subjects. In the AOM/DSS model of CAC, D6-deficient mice showed increased expression of chemokines and higher accumulation of inflammatory cells in comparison to the wild type, resulting in the development of more severe colitis and higher incidence of CAC[101].

CONCLUSION

Clinical and experimental data indicate that chronic inflammation increases the risk of developing CAC, acting at different stages of the carcinogenesis process. The constant release of free radicals is known to be genotoxic leading to the dysregulation of important oncogenes and onco-suppressors. Moreover, it is also known that the release of cytokines such as IL-6 and TNF-α during chronic colitis can promote tumor growth and that low expression of immunosuppressive cytokines such as TGF-β and IL-10 can exacerbate this process. However, it is also clear that what we call macroscopically chronic inflammation may be the result of very different kinds of immune responses and their impact on CAC development is still unclear.

Many lines of evidence indicate that IFN-γ expressed by Th1 cells protects from tumorigenesis in different experimental models. Indeed, IFN-γ is critical in the activation of cytotoxic cells and antitumor activity. Moreover, IFN-γ renders dysplastic cells more susceptible to cell-mediated cytotoxicity. In contrast, Th2- and Th17-mediated immune responses in the gut seem to promote CAC development. Therefore, it is tempting to speculate that different Th-driven "chronic inflammations" of the gut could be associated with different risk of CAC (Figure 2). If this concept will turn out to be true, not only generic immunosuppressive therapy but also modulation of the ongoing intestinal immune response could be considered as an approach in the prevention of CAC in IBD patients.

In clinical practice, many anti-inflammatory drugs and immune-modulators are routinely used in the therapy of IBD. Their efficacy is based on the capacity to reduce clinical manifestations related to disease and to reduce the *in situ* macroscopic/microscopic inflammation. However, in most of the cases little is known about their impact on the immune response at a molecular level and the consequent effect on colon carcinogenesis. An exception is represented by 5-ASA. Clinical data indicate that the long term use of 5-ASA might prevent CAC in UC patients, acting as an anti-inflammatory agent and interfering with cancer cell growth[102]. However, whether 5-ASA might act in part by modulating the activity of the immune system, sustaining immunosurveillance, has not yet been investigated. The impact of other anti-inflammatory drugs and immune-modulators on UC-related colon carcinogenesis, and their capacity to improve or dampen the immunosurveillance against dysplastic cells, are still unknown. The long term evaluation of patients undergoing different therapeutic regimens will help address this issue.

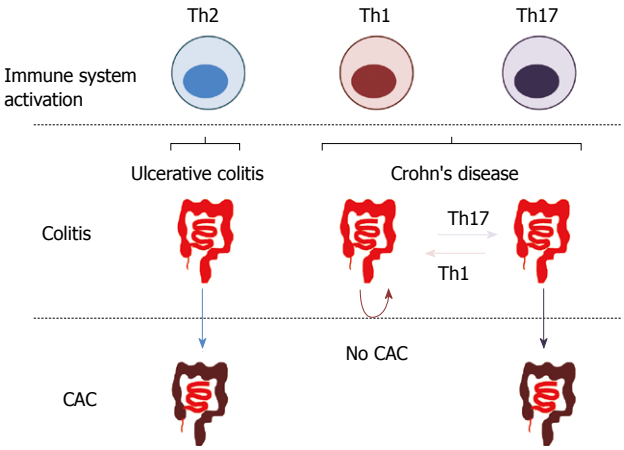

Figure 2 Hypothetical relationship between inflammatory bowel disease and colitis associated colorectal cancer. The T helper (Th)2 immune response characterizing ulcerative colitis determines an elevated risk of developing colitis associated colorectal cancer (CAC). In Crohn's disease, while a Th17-mediated immune response could cause inflammation and enhance CAC risk, the shift towards a Th1-mediated colitis could lower the incidence of CAC.

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REVIEW

Dual protective role of HO-1 in transplanted liver grafts: A review of experimental and clinical studies

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Abstract

Liver transplantation is considered as the most effective treatment for end-stage liver disease. However, serious complications still exist, particularly in two aspects: ischemia and subsequent reperfusion of the liver, causing postoperative hepatic dysfunction and even failure; and acute and chronic graft rejections, affecting the allograft survival. Heme oxygenase (HO), a stress-response protein, is believed to exert a protective function on both the development of ischemia-reperfusion injury (IRI) and graft rejection. In this review of current researches on allograft protection, we focused on the HO-1. We conjecture that HO-1 may link these two main factors affecting the prognosis of liver transplantations. In this review, the following aspects were emphasized: the basic biological functions of HO-1, its roles in IRI and allograft rejection, as well as methods to induce HO-1 and the prospects of a therapeutic application of HO-1 in liver transplantation.

Key words: Liver transplantation; Heme oxygenase-1; Allograft rejection; Ischemia/reperfusion injury

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INTRODUCTION

Transplantation remains the main therapeutic option for patients with end-stage liver disease. Thanks to the clinical use of immunosuppressants, acute rejections have been brought under substantial control. However, the adverse effects of these drugs, such as the development of blood hypertension, hyperlipidemia, diabetes, renal failure, and *de novo* tumors in transplanted patients, are significant, increasing the postoperative mortality. The severe side effects of the immunosuppressants limit their success in attenuating acute rejection. In addition, surgery and preservation of the allografts result in a cascade of ischemia-reperfusion injury (IRI) in the transplantation, for which there are still no effective therapeutic interventions. Consequently, the strategies to simultaneously attenuate IRI and induce donor-specific tolerance would considerably improve the quality of life and survival of the transplant recipients.

The liver, an immunologically privileged organ, bears inherent tolerogenic properties in the event of orthotopic

liver transplantation (OLT). Liver allografts could be established and maintained even without immunosuppressants[1]. In humans, liver transplants can also confer protection on other organ grafts stemming from the same donor[2]. Based on the aforementioned characteristics of the liver, it seems more feasible to induce a donor-specific tolerance in liver transplantations than in the case of transplantations of other solid organs.

More attentions have been paid to heme oxygenase (HO)-1 because of its cytoprotective, antioxidant, maintaining microcirculation, modulating the cell cycle and anti-inflammatory functions[3]. In the process of a liver transplantation, many cell types, including Kupffer cells, endothelial cells, and dendritic cells (DCs), can induce an HO-1 overexpression to prevent IRI and rejections[4-6]. Since HO-1 seems to be involved in both processes, it may act as a linkage between IRI and rejection in liver transplantation in order to induce donor-specific tolerance.

BASIC BIOLOGICAL FUNCTIONS OF HO-1 AND ITS BYPRODUCTS

HOs are rate-limiting enzymes in the heme catabolism. The heme catabolism by HO-1 produces carbon monoxide (CO), free iron, and biliverdin that is subsequently converted to bilirubin by biliverdin reductase[7]. Three HO isozymes have been identified: HO-1, HO-2 and HO-3. HO-1 is an inducible enzyme, while the other two are expressed constitutively[8].

HO-1 is a bona fide 32-kDa stress protein (Hsp32), variously manifested in endothelial, epithelial, smooth muscle and other cell types. HO-1 plays a protective role in many disease models *via* its anti-inflammatory, anti-apoptotic, and anti-proliferative actions[3]. Three products of the heme metabolism are considered to be beneficial due to their immunomodulatory, anti-apoptotic, and vasoactive properties.

CO, despite its potential toxicity, has recently caused a great interest because of its function as a signaling molecule with vasodilatory effects mediated by cGMP, and its antiapoptotic and anti-inflammatory effects[9,10]. CO can travel freely throughout intracellular and extracellular compartments and exert a wide spectrum of modulating physiological effects on multi-systems[11].

Bilirubin, a byproduct, is found to exert a beneficial influence on many diseases, including atherosclerosis, inflammatory, autoimmune, degenerative diseases, and cancer, in which it serves as a highly lipophilic antioxidant[12]. It can slightly reduce ethanol-induced lipid peroxidative injury by decreasing MDA content[9]. In addition, Takamiya *et al*[13] have demonstrated that HO-1 stabilizes mast cells (MCs) in order to exercise an anti-inflammatory action through bilirubin.

Furthermore, Fe, the third product, despite its cytotoxic pro-oxidant effects, induces an over-expression of ferritin, which in turn has strong antioxidant effects through the depletion of free iron and also by other less characterized effects that result in the induction of tolerogenic dentritic cells[14].

HO-1 ATTENUATES LIVER IRI IN LIVER TRANSPLANTATION

IRI is a continual process that culminates in hepatocellular injury. Clinically, it remains a major obstacle to liver transplantation and can lead to hepatic dysfunction and even post-transplantation failure. As a result, the mechanisms and prevention of cellular injury during hepatic ischemia and subsequent reperfusion needs to be elucidated[15].

Kupffer cells, the resident macrophage population within the liver, play key roles in IRI. They are activated after reperfusion by various stimuli in an autocrine fashion by Toll-like receptor 4 signaling[16], or by complement activation[17,18]. After being activated, they release inflammatory cytokines and free radicals, such as reactive oxygen species (ROS), tumor necrosis factor α (TNFα), interleukin 1 (IL-1), nitric oxide (NO), thromboxanes, and leukotrienes[19], and recruit neutrophils to the liver. TNF and IL-1 can also recruit and activate CD4+T-lymphocytes which maintain Kupffer cell activation by secretion of the granulocyte stimulating factors or interferon (IFN)-γ[20,21]. Of all the hepatic cells, the nonparenchymal sinusoidal endothelial cells (SECs) are most susceptible to IRI[22]. SECs are activated by tissue anoxia that disturbs the intracellular energy metabolism and enzyme function, leading to their apoptosis. These cause marked microcirculatory disturbances, leukocyte and platelet adhesion, diminished blood flow and continuation of the ischemic process, resulting in massive hepatic necrosis[23]. Thus, the inhibition of SEC apoptosis may be a useful therapeutic strategy to reduce the risk of ischemia injury in liver preservation. Yue *et al*[5] have found that the apoptosis of SECs was attenuated after the TAT-HO-1 was transducted into the liver, which may be associated with an increased expression of Bcl-2 and a reduced expression of Bax.

It is well known that it is of critical importance to attenuate IRI in liver transplantation. Both HO-1 and its products of degradation play a role in attenuating IRI (Figure 1). The findings that $Hmox^{-}/^{-}$ animals are more susceptible to IRI injury than the $Hmox^{-}/^{+}$ and $Hmox^{+}/^{+}$ animals indicate that HO-1 may play a potent protective role in IRI[24]. A further study has shown that donor livers with an enhanced HO-1 expression lowered the serum ALT/AST levels of the recipient, alleviated allograft injury, and suppressed cytokine release[4]. Luke Devey described a mechanism that HO-1 could drive macrophage differentiation down an "anti-inflammatory" pathway[24]. Therefore, preconditioning the donor liver, especially its Kupffer cells with a strong induction of HO-1, plays a potential protective role. Kupffer cells are not only the main factor associated with liver IRI, but also a major site of expression of the hepatic HO-1. Based on these findings, we can assume that HO-1 in Kupffer cells is

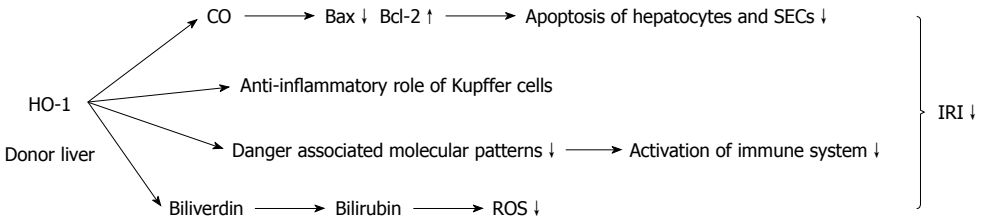

Figure 1 Function of heme oxygenase 1 and its degradation product in ischemia and reperfusion injury during liver transplantation. HO-1: Heme oxygenase 1; CO: Carbon monoxide; SEC: Sinusoidal endothelial cell; ROS: Reactive oxygen species; IRI: Ischemia-reperfusion injury.

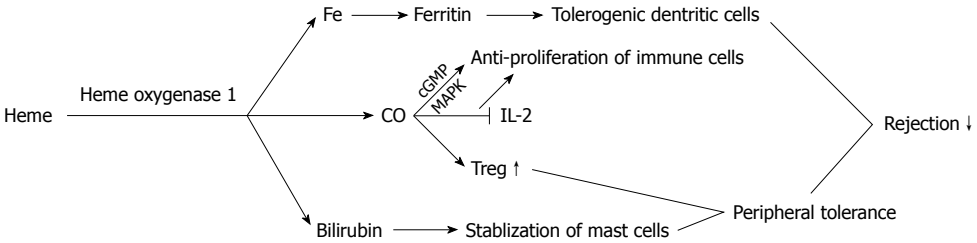

Figure 2 Function of heme oxygenase 1 degradation product to reduce rejection. CO: Carbon monoxide; Treg: Regulatory T cells; IL: Interleukin.

induced exclusively to exert a protective function in the event of IRI. Additionally, HO-1 can modulate each stage of the immune activation pathway such as limiting the production of damage-associated molecular patterns, modulating T cell activation, and enhancing immunological tolerance[25].

As previously described, CO mediates a cytoprotective and anti-inflammatory effect in I/R related oxidative injury. It significantly reduces the messenger RNA (mRNA) levels of the proapoptotic Bax, while it upregulates the anti-apoptotic Bcl-2. Bax and Bcl-2 are both found to be expressed in hepatocytes and SECs at the sinusoidal space. Therefore, CO reduces the IRI-mediated apoptosis through an overexpression of Bcl-2 and diminished Bax expression. This protective role of CO is mediated by an activation of the soluble guanylyl cyclase, as demonstrated by the fact that 1H-(1,2,4)oxadiazole (4,3-α) quinoxaline-1-one (ODQ; a soluble guanylyl cyclase inhibitor), completely reversed its beneficial effect[26].

The oxidation of bilirubin by ROS results in the conversion of bilirubin to biliverdin, the latter being a precursor of bilirubin in the heme degradation that is recycled to bilirubin in mammals by biliverdin reductase. This recycling process between bilirubin and biliverdin is believed to be behind one of the explanations for bilirubin's powerful antioxidant effects in the redox cycle[27]. However, the exact mechanism of the protective role of HO-1 remains to be fully explained.

Contrary to the aforementioned protective role of HO-1 against oxidant-induced injury and the induction of HO-1 as an adaptive response against oxidative damage, Froh *et al*[28] reported that a cobalt protoporphyrin (CoPP)-induced HO-1 over-expression increases liver injury, as demonstrated by an over-expression of hepatic ALT, aggravation of cell necrosis, and fibrosis. They suggested that high levels of HO-1 may sensitize cells to oxidative stress due to an accumulation of free divalent iron, thereby increasing oxidative injury. Since Kupffer cells are the main source of HO-1 in the liver, an increased expression of HO-1 may also aggravate the activation of Kupffer cells, thus increasing the formation of inflammatory and fibrogenic mediators. The controversial role of the HO-1 expression in human liver allografts of either cytoprotection or increased cytotoxicity ought to be investigated in more detail in the future.

HO-1 REDUCES REJECTIONS IN LIVER TRANSPLANTATION

The spontaneous graft tolerance of the liver is an active process which depends upon the transfer of donor leukocytes in the liver. Migration within the recipient's lymphoid system results in an early immune activation of recipient lymphocytes, with their subsequent deletion from exhaustion. Regulatory T cells and antigen presenting cells (APCs) are both involved in inducing donor-specific tolerance.

One highly significant tolerance inducing mechanism is the suppression of allogeneic responsiveness by regulatory T cells. Regulatory T cells (Tregs) are a subset of T lymphocytes that play a core role in immunological suppression and the termination of immune responses. Deficiency or dysfunction of these cells may lead to autoimmunity or an aggravated pathogen-induced inflammation[29]. The end-products of HO-1 degradation do not have much organ specificity to reduce rejection (Figure 2). CO could provide one mechanism by which the regulatory capacity of Tregs is generated. CO has been shown to have broad anti-proliferative effects in human CD4+T cells[30]. Generally, CO is thought to perform those anti-proliferative actions by modulating the activity of guanylate cyclase to increase the cellular cGMP

levels, and through the MAPK (mitogen activated protein kinase) pathway. More specifically, CO was also shown to block the production of IL-2, a principal cytokine responsible for T cell proliferation[31]. CO, functioning as a nonspecific suppressor, also fits with the observation that Treg cells are capable of suppressing the proliferation of various immune cell types without major histocompatibility complex restriction and in a non-Ag-specific manner.

Recently, the existence of interactions between Tregs and MCs has been demonstrated[32]. The secretion of immunosuppressive mediators, such as transforming growth factor β, forms the basis of the pivotal role of MCs in inducing allograft tolerance. IL-9 is the functional link by which activated Tregs recruit and activate MCs to mediate regional immune suppression. This is demonstrated by the fact that by neutralizing IL-9, allograft rejection in tolerant mice is greatly accelerated[33]. However, the degranulation of intragraft or systemic MCs causes the loss of Tregs and MCs from the graft, and impairs Treg function. As a result, rejection occurs in the established tolerant allograft[34]. Therefore, it seems important to stabilize the MCs in order to sustain allograft tolerance. As described above, the liver more easily induces tolerance than other organs. Although MCs are not abundant in the liver, an inhibition of MC degranulation seems important, because of the hepatic immunological privilege. Takamiya *et al*[13] illustrated that an overexpression of HO-1 suppresses compound 48/80-, IgE-induced MC degranulation through bilirubin. Yasui *et al*[35] demonstrated that the upregulation of HO-1 within the MCs also inhibits their cytokine production associated with a selective suppression of the DNA-binding activity of the AP-1 transcription factors. As a result, to induce HO-1 within MCs may be another target to affect Tregs. On the other hand, because MCs are involved in the fibrosis of chronic rejection, inhibiting MC degranulation and cytokine production may control the progression of chronic rejection. Whether the HO-1 overexpression of the donor's or the recipient's MCs is more important, needs to be ascertained.

It is well known that the Treg functions depend on the activity of APCs, and the HO-1 of APCs may also influence the Tregs. George *et al*[36] have demonstrated that lack of HO-1 in the APCs significantly impairs the suppressive function of Treg cells on effector T cells, which indicated the importance of HO-1 within APCs. APCs are involved in initiating rejection and can also be mediated to induce tolerance. Perfusion of donor liver with HO-1 inducers will up-regulate HO-1 expression of many cells in the liver, including hepatic dentritic cells. This pathway contains some mechanism for HO-1 to reduce both acute and chronic rejections (Figure 3). There are two recognition patterns associated with organ transplantation rejection: direct and indirect recognition induced by donor and recipient APCs, DCs are the main APCs in the liver, the only cell type that can activate naïve CD4+T cells. Hepatic DCs differ from those in other organs, because they are less immunostimulatory in response to diverse antigens. Some data have also shown that compared with splenic and blood DCs, freshly isolated mouse liver DCs express lower levels of Toll-like receptor 4 mRNA and are less able to activate allogeneic T cells, or polarize naïve T cells toward Th1 responses to LPS[37]. Immature DCs (imDCs) lack the capability of presenting alloantigen to alloreactive T cells because of a low expression of costimulatory molecules[38]. As it is shown, imDCs express HO-1, but apparently reduce or lose the ability of expression as they become mature[39]. There is expanding evidence that donor hepatic imDCs can downregulate immune responses, thus inducing and maintaining peripheral T-cell tolerance[39]. Furthermore, it has been hypothesized that the presence of large numbers of imDCs within the donor liver that circulate and repopulate the recipient contribute to microchimerism, another mechanism associated with donor-specific tolerance[40]. However, in the event of an acute liver transplant rejection, when the imDCs undergo maturation upon alloantigen stimulation, they induce an acute rejection through direct recognition. Consequently, keeping DCs in their immature state is crucial in order to induce an antigen-specific tolerance in liver transplantation.

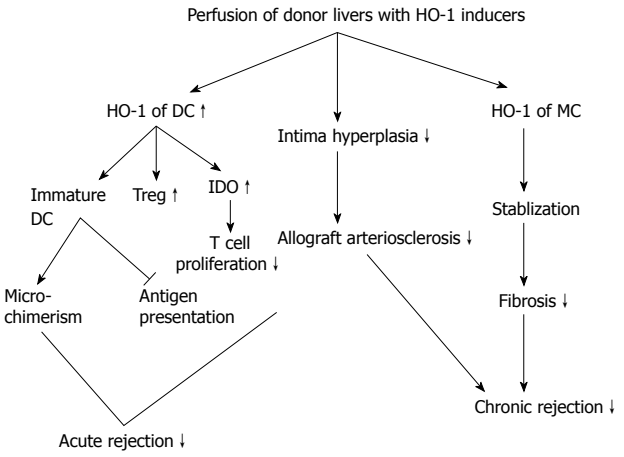

Figure 3 Perfusion of donor liver with heme oxygenase 1 inducers could reduce rejections through many pathways. HO-1: Heme oxygenase 1; DC: Dendritic cell; Treg: Regulatory T cells; IDO: Indoleamine 2,3-dioxygenase; MC: Mast cell.

Many studies have supported the idea that an induction of HO-1 or its products can inhibit DC maturation. Chauveau *et al*[39] have proven that the induction of HO-1 directs DC refractory to an LPS-induced maturation. Recent studies have also demonstrated that an HO-1 overexpression inhibits the secretion of cytokines critical for DC maturation, such as IL-12[41]. Indoleamine 2,3-dioxygenase (IDO) is a further mechanism associated with the immunosuppressive activity of imDCs, whereby IDO can inhibit T cell proliferation through tryptophan degradation, and induce Tregs as well[42,43]. An upregulation of HO-1 resulted in an IDO overexpression through CO[6]. The above-mentioned information all demonstrates that by inducing HO-1, the development of DCs could be directed selectively toward a tolerogenic DC type. In chron-

Table 1 Upregulation of heme oxygenase-1 in donor liver to alleviate ischemia-reperfusion injury and rejection

Effective product	Targets	Results	Ref.
HO-1	KC	Preventing IRI	[4]
HO-1	Attenuating apoptosis of SEC	Alleviating IRI	[5]
HO-1	Inhibiting DC maturation	Reducing rejection	[6]
HO-1	Anti-inflammatory differentiation of KC	Preventing IRI	[24]
HO-1	Modulating oxidative stress and proinflammatory mediators	Alleviating IRI	[30]
CO	Suppressing T cell proliferation	Reducing rejection	[31]
HO-1	Microchimerism	Inducing allograft tolerance	[41]
CO	Inhibiting TLR-induced DC maturation	Reducing rejection	[42]
HO-1	Suppressing intragraft infiltration of KC and neutrophils, preventing proinflammatory cytokine and chemokine expression	Alleviating IRI	[50]
HO-1	Inducing Treg	Inducing allograft tolerance	[56]
Biliverdin	Decreasing P-selectin, ICAM-1, iNOS and IL-6	Alleviating IRI	[60]

HO-1: Heme oxygenase 1; CO: Carbon monoxide; KC: Kupffer cell; SEC: Sinusoidal endothelial cell; DC: Dendritic cell; TLR: Toll-like receptor; Treg: Regulatory T cells; ICAM-1: Intercellular adhesion molecule-1; iNOS: Inducible nitric oxide synthase; IL: Interleukin; IRI: Ischemia-reperfusion injury.

ic rejection, graft arterial vasculature remodels after the transplantation. Chronic graft dysfunction is characterized by the development of intimal hyperplasia and narrowing of the vessel lumen[44]. Cheng *et al*[45] have found that a loss of HO-1 in DCs or *HO-1* gene silencing by small interfering RNA upregulated the MHCII expression through CIITA-driven transcriptional regulation and transcription 1 (STAT1) phosphorylation. They have also illustrated that an inhibition of HO-1 in DCs aggravated the development of transplant arteriosclerosis by increasing intimal hyperplasia, and by activating a CD4(+) T cell allograft response mediated by an MHCII upregulation. Therefore, we conclude that the activity of HO-1 is an important regulatory mechanism affecting multiple levels of the immune response to induce tolerance. Elucidating its effects on specific immune cells will aid the development of therapeutic strategies for a variety of inflammatory disorders, including autoimmune diseases and transplant rejection.

UP-REGULATION OF HO-1 AS POTENTIAL THERAPY ON GRAFT PROTECTION IN LIVER TRANSPLANTATION

Based on the aforementioned information, we can conclude that HO-1 plays a potential protective role in both IRI and graft rejection, whereby HO-1 may act as a link between these two events (Table 1). Thus, by upregulating HO-1 within the donor liver allograft, a preconditioned pre-transplantation hepatic status can be provided that may uphold allograft survival and normal function for a long time.

HO-1 is highly inducible by a variety of stimuli including heme, NO, cadmium, growth factors, and hyperoxia. These diverse stimuli act *via* a similarly broad range of signaling pathways. The nuclear factor (NF)-κB and activator proteins-1 and -2 lie in the promoter region of HO-1.The transcription factor NF-E2-related factor-2 is recognized as the key mediator of HO-1 induction and the protective functions of HO-1, as seen in experimental models both *in vivo* and *in vitro*[46]. Due to the potential toxicity of the conventional HO-1 inducers, such as hemin and CoPP, a number of new strategies, including protein transduction, traditional Chinese medicine, adenoviral transduction and others, have attracted substantial interest as potential HO-1 inducers[5,47,48].

Protoporphyrines are prototypic HO-1 inducers *in vitro*. Depending on the metal atom of the porphyrines, the enzymatic HO-1 function is activated (e.g. iron or cobalt atom). However, since porphyrines are heavy metals, their clinical usage has many limitations.

Simvastatin, clinically used as a lipid-lowering drug, is another inducer of HO-1. Lee *et al*[49] have shown that the protective effect of statins on vessels is produced by HO-1. Uchiyama *et al*[50] further demonstrated that simvastatin increases the HO-1 expression by inducing a nuclear translocation of the heat shock factor 1 in vascular endothelial cells. However, there are still several unresolved problems. Simvastatin is administered orally in clinical applications. Uchiyama *et al*[50], however, induced HO-1 by an intraperitoneal injection of a high-dose of simvastatin in their experiments. In view of its potential for a clinical application, a pilot study is necessary to evaluate whether simvastatin administered orally also induces HO-1.

In recent years, traditional Chinese herbal medicine has become popular in inducing HO-1. Sinomenine, a pure alkaloid extracted from the Chinese medical plant *Sinomenium acutum*, has been investigated for its protective effect on hepatic cells affected by an overexpression of HO-1 to attenuate IRI[5]. *Isodon Serra* (*I. Serra*) is another Chinese medicinal herb that has been found to possess the capacity to induce HO-1. It is a perennial herb that has been used widely for the treatment of arthritis, enteritis, jaundice, hepatitis, lepromatous leprosy, ascariasis and acute cholecystitis[51]. Moreover, it has been used in China to treat esophageal cancer. It has an anti-proliferative effect on melanoma cells and many other kinds of malignant cells[52,53]. However, our studies focused on other functions, besides its anti-tumorigenic activities[54,55]. Matsushima *et al*[56] have proven that crassin acetate, a

coral-derived cembrane diterpenoid, can effectively induce HO-1 mRNA/protein expression and HO-1 enzymatic activity in DCs. Nodosin and Oridonin, extracts obtained from *I. Serra*, also belong to a type of diterpenoid. Our previous studies elucidated that Oridonin has an immunosuppressive effect by regulating the cell mitosis cycle and modulating the signal mechanisms of four cytokines (IL-2, IFN-γ, IL-12 and TNFα)[54]. Oridonin, a potent HO-1 inducer, is a promising immunosuppressive drug. Hu *et al*[55] have shown that Oridonin upregulated the HO-1 expression at both the transcriptional and translational levels, and accordingly promoted HO-1 activity *in vitro* in their experiments. However, the exact mechanisms of our findings remain to be further investigated. Nodosin, another *I. serra* extract, also induces HO-1. We used a Nodosin solution *in vitro* to perfuse the isolated liver, while lactic Ringer's solution was used as control. The results showed that the expression of HO-1 in both the mRNA and the protein is higher in the perfused group than in the control group[57]. The potential role of *I. Serra* extract in the upregulation of HO-1 suggested that it is a novel nontoxic drug candidate for liver allograft protection. More models and methods used for the study of *I. Serra* extracts need to be defined in the future investigations. We believe that in the near future, the full pharmacological activity and detailed mechanisms of *I. Serra* will be further described.

FUTURE PROSPECTS

In a clinical setting, however, the inducible HO-1 system still has several limitations. The different effects of HO-1, which are neither exclusively cytoprotective nor exclusively cytotoxic, should be further investigated. The HO-1 induced cytoprotection might be restricted to a narrow threshold of overexpression. Besides, there are no available reagents that can specifically induce HO-1. Therefore, the unintended effects of treatment with non-specific HO-1 inducers would likely present a disadvantage[10]. Although an adenoviral-based HO-1 gene transfer has been attempted *in vivo*, the efficiency of viral transfection is organ dependent. CO may represent a candidate for the treatment of transplanted patients against IRI. However, its therapeutic window must be carefully considered, because the inhalation of high levels can be toxic or even be lethal. Biliverdin and reduced bilirubin may also represent possible candidates for clinical application. We have recently demonstrated that biliverdin had a protective effect in stringent rat liver models of IRI, as evidenced by an improved portal blood flow/bile production and a reduction in hepatocellular damage. It also improved the survival rate in a syngeneic rat OLT model after prolonged cold ischemia[58]. However, because bilirubin in excess can cause neurotoxicity and can act as a lytic agent binding to erythrocyte membranes, the therapeutic window of biliverdin must be examined in detail prior to its clinical use.

From the above, the question arises if HO-1 or its products can be used clinically. Although CO is toxic, beneficial results can be obtained with relatively low doses for appropriate length of time. In rodents, the administration of biliverdin or bilirubin in the first few weeks of life did not reveal much toxicity. Recent evidence indicates that they are not only non-toxic at physiological concentrations in normal cells, they may also have important anti-oxidant, anti-inflammatory, or anti-apoptotic properties[59,60].

Based on this review, which reveals that HO-1 is associated with both processes of IRI and acute rejection, we can conclude that the preconditioning of the donor liver with an upregulation of HO-1 not only attenuates IRI, but also reduces rejection after liver transplantation. HO-1, therefore, seems to stand out as a potential key therapeutic target to maintain graft function and improve the recipients' prognosis in liver transplantation. However, due to its limitations, the therapeutic role of HO-1 must undergo further critical analysis.

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ORIGINAL ARTICLE

Effect of preoperative FOLFOX chemotherapy on CCL20/CCR6 expression in colorectal liver metastases

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Abstract

AIM: To evaluate the influence of preoperative FOLFOX chemotherapy on CCL20/CCR6 expression in liver metastases of stage Ⅳ colorectal cancer (CRC) patients.

METHODS: Using Real Time-PCR, enzyme-linked immunosorbent assay, Western Blots and immunohistochemistry, we have analyzed the expression of CCL20, CCR6 and proliferation marker Ki-67 in colorectal liver metastasis (CRLM) specimens from stage Ⅳ CRC patients who received preoperative FOLFOX chemotherapy (n = 53) and in patients who did not receive FOLFOX chemotherapy prior to liver surgery (n = 29).

RESULTS: Of the 53 patients who received FOLFOX, time to liver surgery was ⩽ 1 mo in 14 patients, ⩽ 1 year in 22 patients and > 1 year in 17 patients, respectively. In addition, we investigated the proliferation rate of CRC cells in liver metastases in the different patient groups. Both CCL20 and CCR6 mRNA and protein expression levels were significantly increased in patients who received preoperative FOLFOX chemotherapy ⩽ 12 mo before liver surgery (P < 0.001) in comparison to patients who did not undergo FOLFOX treatment. Further, proliferation of CRLM cells as measured by Ki-67 was increased in patients who underwent FOLFOX treatment. CCL20 and CCR6 expression levels were significantly increased in CRLM patients who had undergone preoperative FOLFOX chemotherapy.

CONCLUSION: This chemokine/receptor up-regulation could lead to increased proliferation/migration through an autocrine mechanism which might be used by surviving metastatic cells to escape cell death caused by FOLFOX.

Key words: FOLFOX chemotherapy; CCL20/CCR6 expression; Colorectal liver metastases; Proliferation

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INTRODUCTION

Colorectal cancer (CRC) constitutes one of the most common causes of cancer death in the western world. CRC is also a tumor with a high propensity for metastatic spread, mostly to liver and lungs. Liver metastases develop in approximately 50% of CRC patients at some point in the course of their disease and worsen the prognosis for patient survival dramatically[1,2]. To date, long-term survival of patients with colorectal liver metastases (CRLM) can only be achieved by surgical resection. However, in cases where metastases are considered unresectable, chemotherapy is the treatment of choice, although 5-year survival with chemotherapy alone is very poor. Thus, surgical treatment of resectable liver metastases has become the standard treatment[3].

However, surgical resection can only be successful when liver metastases can be thoroughly resected and provided there is no other non-resectable distant metastasis. Moreover, the primary tumor needs to be resectable. Since all these criteria are often not fulfilled, cancer recrudescence occurs in 45% to 75% of CRLM patients within the first five years after primary tumor resection[3,4]. For these reasons, adjuvant chemotherapy has been evaluated in patients with resectable CRLM. To date, the standard approach with regard to adjuvant chemotherapy for CRC patients is a combination of oxaliplatin, fluorouracil and leucovorin[5,6]; termed FOLFOX. Perioperative FOLFOX chemotherapy has been shown to reduce the risk of cancer relapse by a quarter[7]. Thus, the combination of FOLFOX chemotherapy and surgery is a promising tool to improve the prognosis of CRC patients.

It has been shown that both the chemokine CCL20 and its unique receptor CCR6 are expressed in CRC cells[8-11], providing a basis for efficient autocrine and paracrine loops[12]. CCL20 stimulation of CCR6-bearing CRC cells led to increased proliferation and migration *in vitro*[8,9]. Moreover, our recent data suggest that interactions between CCL20 and the corresponding receptor CCR6 are critical components in the regulation of CRC progression and organ selective CRC metastasis to the liver[10,11].

The purpose of this study was to retrospectively analyze the impact of FOLFOX chemotherapy administered to stage Ⅳ CRC patients on CCL20/CCR6 expression in liver metastases. Eighty-two patients underwent radical surgery of the primary CRC and of synchronous and metachronous liver metastases. Twenty-nine patients underwent liver surgery without preoperative FOLFOX and 53 patients received FOLFOX prior to liver surgery. We compared CCL20/CCR6 expression in liver resection specimens from both groups and correlated their expression with proliferation of CRLM cells.

MATERIALS AND METHODS

Materials

Surgical specimens and corresponding normal tissue from the same samples were collected from patients who underwent surgical resection at our department between 2002 and 2008.

Informed written consent for tissue procurement was obtained from all patients and the study was approved by the local ethics commission of the Ärztekammer des Saarlandes.

Eighty-two patients were included in the study, comprising CRC patients who had FOLFOX chemotherapy before liver surgery (n = 53) and CRC patients who did not have FOLFOX before liver surgery (n = 29). Of the 53 patients who received FOLFOX, time to liver surgery was ⩽ 1 mo in 14 patients, ⩽ 1 year in 22 patients and > 1 year in 17 patients, respectively. In every patient sample the corresponding non-affected normal liver tissue was also analyzed, thus a total of 164 samples were analyzed. In the 53 patients who received FOLFOX chemotherapy before liver surgery, two cancers were classified as pT1, six as pT2, forty as pT3 and five as pT4, with positive nodal involvement in 40 cases, according to the UICC TNM classification[13]. In the twenty-nine patients who received no FOLFOX chemotherapy before liver surgery, six cancers were classified as pT1, two as pT2, nineteen as pT3 and two as pT4, with positive nodal involvement in 10 cases. The clinical data and patient characteristics were obtained from a prospective database and are summarized in Table 1. In the group who did not receive FOLFOX, 8 patients underwent CRLM resection less than 6 mo and 21 patients underwent CRLM resection 6 mo and longer after resection of primary tumor. In the patient group who received FOLFOX chemotherapy, 7 underwent CRLM resection less than 6 mo after resection of primary tumor and 45 patients underwent CRLM resection 6 mo and longer after resection of primary tumor. One patient underwent resection of primary tumor and CRLM resection at the same time (Table 2).

Tissue preparation

Tissue specimens were collected immediately after surgical resection, snap frozen in liquid nitrogen and then stored at -80℃ until they were processed under nucleic acid sterile conditions for protein and RNA extraction. For corresponding normal tissue we used adjacent non-affected tissue to the same resected specimen. All tissues obtained were reviewed by an experienced pathologist and examined for the presence of tumor cells. As minimum criteria for usefulness for our study, we only used tumor tissues in which tumor cells constituted at least > 75% of the tumor biopsy.

Single-strand cDNA synthesis

Total RNA was isolated using RNeasy columns from Qiagen (Hilden, Germany) according to the manufacturer's instructions. RNA integrity was confirmed spectrophotometrically and by electrophoresis on 1% agarose gels. For cDNA synthesis, 5 μg of each patient total RNA sample were reverse-transcribed in a final reaction volume of 50 μL containing 1 × TaqMan RT buffer, 2.5 μmol/L random hexamers, 500 μmol/L each dNTP, 5.5 mmol/L $MgCl_2$, 0.4 U/μL RNase inhibitor, and 1.25 U/μL Multiscribe RT. All RT-PCR reagents were purchased from Applied

Table 1 Clinical characteristics of patients with colorectal liver metastasis

Characteristic	CRLM with FOLFOX[1] (*n* = 53)	CRLM without FOLFOX[2] (*n* = 29)
Localization of primary tumor		
Colon	25	13
Rectum	28	16
Gender		
Male	29	17
Female	24	12
Age at surgery (yr)		
Median	61.4	66.7
Range	35-79	43-77
Largest tumor diameter (cm)		
Median	4.7	4.9
Range	1.3-9.7	1.2-10.1
Tumor (T)-category of primary tumor		
pT1	2	6
pT2	6	2
pT3	40	19
pT4	5	2
Lymph node metastasis (N-category)[3]		
Positive	40	10
Negative	13	19
Grade		
G1	0	1
G2	32	17
G3	21	11

[1]Colorectal cancer patients with FOLFOX chemotherapy before colorectal cancer liver metastasis (CRLM) surgery; [2]Colorectal cancer patients without FOLFOX chemotherapy before CRLM surgery; [3]N-category significantly different between FOLFOX and non-FOLFOX patients ($P < 0.05$).

Biosystems (Foster City, CA). The reaction conditions were 10 min at 25℃, 30 min at 48℃, and 5 min at 95℃.

Real-time PCR

All Q-RT PCR assays containing the primer and probe mix were purchased from Applied Biosystems, (Applied Biosystems, Foster City, CA) and utilized according to the manufacturer's instructions. PCR reactions were carried out using 10 μL 2 × Taqman PCR Universal Master Mix No AmpErase® UNG and 1 μL gene assay (Applied Biosystems, Foster City, CA), 8 μL Rnase-free water and 1 μL cDNA template (50 mg/L). The theoretical basis of the qRT assays is described in detail elsewhere[14]. All reactions were run in triplicate along with no template controls and an additional reaction in which reverse transcriptase was omitted to assure absence of genomic DNA contamination in each RNA sample. For the signal detection, ABI Prism 7900 sequence detector was programmed to an initial step of 10 min at 95℃, followed by 40 thermal cycles of 15 s at 95℃ and 10 min at 60℃ and the log-linear phase of amplification was monitored to obtain C_T values for each RNA sample.

Gene expression of all target genes was analyzed in relation to the levels of the slope matched housekeeping genes phosphomannomutase (PMM1) and β2-microglobulin (β2M)[15]. Data analysis was performed according to the relative standard curve method. Data are presented in relation to the respective housekeeping genes.

Table 2 Period between resection of primary tumor and resection of colorectal liver metastasis

Interval	CRLM with FOLFOX[1] (*n* = 53)	CRLM without FOLFOX[2] (*n* = 29)
< 6 mo	7	8
⩾ 6 mo	45	21
0	1	0

[1]Colorectal cancer patients with FOLFOX chemotherapy before colorectal cancer liver metastasis (CRLM) surgery; [2]Colorectal cancer patients without FOLFOX chemotherapy before CRLM surgery.

Isolation of total protein

Protein lysates from frozen tissue were extracted with radioimmunoprecipitation (RIPA) buffer containing Complete, a protease inhibitor cocktail (Roche, Penzberg, Germany). Total protein quantification was performed using the Pierce BCA protein assay reagent kit (Pierce, Rockford, IL., USA).

Sandwich-type enzyme-linked immunosorbent assay

The chemokine protein levels in the different tissue lysates were determined by sandwich-type enzyme-linked immunosorbent assays (ELISA) according to the manufacturer's instructions. Samples were assayed in duplicate with all values calculated as the mean of the two measurements. CCL20 levels were assayed using a validated commercial ELISA (Duo Set R&D Systems, DY360, Minneapolis, MN, USA). The absorbance was read at 450 nm in a 96-well microtiter plate reader. The chemokine concentration from each tissue lysate was normalized to the total protein content of each sample.

Western blotting analysis

Total protein (25 μg/lane) was separated by SDS-PAGE using a 10% gel and blotted onto nitrocellulose membranes (Hybond ECL, Amersham Biosciences, Piscataway, NJ, USA). Membranes were blocked by incubation in Tris-buffered saline (TBS) containing 5% nonfat dry milk and 0.1% Tween 20 for 2 h at room temperature and then incubated overnight at 4℃ with goat anti-human CCR6 antibody (diluted 1:500, C2099-70B, Biomol, Hamburg, Germany). Blots were then washed and incubated at room temperature for 1 h with donkey anti-goat HRP antibody (diluted 1:5000, sc-2056, Santa Cruz Biotechnology, Santa Cruz, CA, USA). Bands were visualized by ECL Western blotting analysis systems (Amersham Biosciences, Piscataway, NJ, USA). The human cell lysate HL-60 (sc-2209, Santa Cruz Biotechnology, Santa Cruz, CA, USA) served as positive control.

Immunohistochemistry

Resected specimens were routinely fixed with formalin in the immediate postoperative period and paraffin-

embedded within the first three hours after procurement. Before staining, 4 μm thick sections were mounted on Superfrost Plus slides, deparaffinized with xylene, and rehydrated in graded ethanol to deionized water. The sections were treated with an antigen retrieval solution (Target Retrieval, Dakocytomation, Carpinteria, CA) and microwaved. CCL20 and CCR6 staining was performed according to the avidin-biotin-peroxidase reaction (Vectastain ABC ELITE Kit, Vector Laboratories Inc., Burlingame, CA) and Ki-67 staining was performed according to the APAAP method (Dako REAL Detection System, Dako, Glostrup, Denmark, K5000). For CCL20 and CCR6 staining, but not for Ki-67 staining, slides were immersed in 3% hydrogen peroxide for 10 min and then treated with avidin and biotin (Avidin/Biotin blocking kit, Vector Laboratories Inc., Burlingame, CA). Sections for CCL20, CCR6 and Ki-67 staining were incubated with serum followed by an overnight incubation with goat anti-human CCR6 polyclonal antibody (1:125, Biomol, Hamburg, Germany, C2099-70B), goat anti-human CCL20 polyclonal antibody (1:150, R&D, Abingdon, UK AF360) or Ki-67 MIB-1 monoclonal antibody (1:75, Dako, Glostrup, Denmark, M7240). Consequently, immunostaining with the avidin-biotin-peroxidase reaction (Vectastain ABC ELITE Kit, Vector Laboratories Inc., Burlingame, CA) was performed on CCL20 and CCR6 slides and a chromogene aminoethyl-carbazide solution (Tissugnost, Darmstadt, Merck) was used. For detection of Ki-67, the alkaline phosphatase-antialkaline phosphatase complex (APAAP) was used with Fast Red Substrate as chromogen (Dako, Glostrup, Denmark, K0597). Consequently, for all sections counterstaining was performed in hematoxylin solution. Negative controls were performed in all cases omitting primary antibody.

Statistical analysis

All data are presented as mean and SE (standard of the mean). Statistical calculations were done with the MedCalc (MedCalc software, Mariakerke, Belgium) software package[16]. The parametric Student's *t*-test was applied, if normal distribution was given; otherwise, the Wilcoxon's rank sum test was used. Statistical significance was considered on a two-sided significance level (α) of 0.05.

RESULTS

Characteristics of patients

Prior to CRLM resection, 82 patients were enrolled in our study over a 6 year period. The median age of patients at surgery was 61.4 years (35-79) in the FOLFOX group and 66.7 years (43-77) in the patient group without FOLFOX. There were 29 males and 24 females in the FOLFOX group and 17 male and 12 female patients in the non-FOLFOX patient group. The demographic and clinical characteristics of patients are shown in Tables 1 and 2. FOLFOX and non-FOLFOX patients showed no statistically relevant differences with respect to T-stage, grading and timing between primary tumor resection and CRLM resection. However, with respect to N-stage our data revealed a significant difference between FOLFOX and non-FOLFOX patients ($P < 0.05$), as shown in Table 1. Thus, the non-FOLFOX group included a higher percentage of patients without lymph node metastasis (65.5%) compared to the FOLFOX group under investigation (24.5%).

Impact of preoperative FOLFOX chemotherapy on CCL20 expression

Significantly greater levels of CCL20 mRNA and CCL20 protein expression were observed in CRLM tissues compared to corresponding normal tissue in all patients ($P < 0.05$ and $P < 0.001$, respectively) (Figures 1 and 2). However, patients who were preoperatively treated with FOLFOX chemotherapy showed significantly higher levels of CCL20 mRNA and CCL20 protein expression as compared to patients without FOLFOX treatment ($P < 0.05$) (Figures 1A and 2A). When the interval between FOLFOX treatment and surgery was considered, only patients who received FOLFOX chemotherapy $\leqslant$ 12 mo before liver surgery expressed significantly higher amounts of CCL20 mRNA and CCL20 protein, as compared to patients without FOLFOX treatment, respectively ($P < 0.05$) (Figures 1B and 2B).

Immunostaining of the CRLM tissue revealed positive staining for CCL20 in 56% (16/29) of patients without and in 87% (46/53) of patients with preoperative FOLFOX chemotherapy (Figure 3A and B), respectively. CCL20 was immunolocalized with lesser intensity in the tumor tissue sections of CRLM patients without FOLFOX, as shown for a representative patient in Figure 3A, compared to patients who underwent preoperative FOLFOX chemotherapy, as shown in Figure 3B for a representative patient.

Impact of preoperative FOLFOX chemotherapy on CCR6 expression

Both CCR6 mRNA and CCR6 protein expression levels were significantly increased in CRLM tissues of all patients compared to the corresponding normal liver tissue ($P < 0.05$) (Figure 4). Further, CCR6 mRNA and CCR6 protein expression levels were significantly higher in CRLM tissues of patients who underwent preoperative FOLFOX chemotherapy compared to patients without FOLFOX ($P < 0.05$) (Figure 4A). CCR6 up-regulation was limited to those patients who received preoperative FOLFOX chemotherapy $\leqslant$ 12 mo before liver surgery ($P < 0.05$) (Figures 4B and 5).

Immunostaining revealed positive staining for CCR6 in 59% (17/29) of patients without and in 87% (46/53) of patients with preoperative FOLFOX chemotherapy (Figure 3C and D), respectively. CCR6 staining intensities were stronger in the FOLFOX group. However, intense laminar CCR6 immunostaining was found mainly in the benign-appearing tissue sections of CRLM patients. Thus, CCR6 staining localized to a streak of hepatocytes along the tumor invasion front. These hepatocytes appeared clearly distinct from normal hepatocytes (Figure 3C and D).

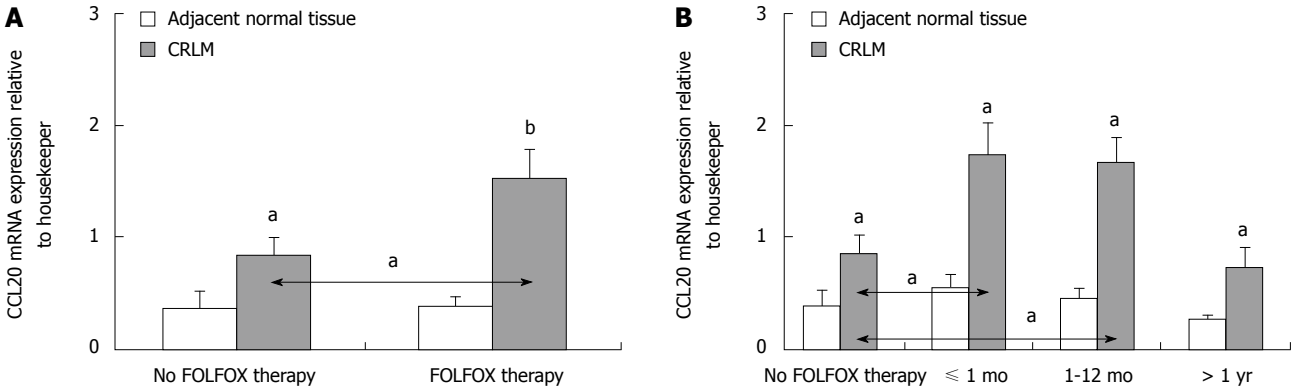

Figure 1 CCL20 mRNA expression relative to PMM1 and β2M in colorectal liver metastasis patients with (*n* = 53) and without (*n* = 29) FOLFOX chemotherapy before colorectal liver metastasis surgery (A) and itemized according to different time periods of FOLFOX treatment before colorectal liver metastasis surgery (no FOLFOX therapy, *n* = 29; FOLFOX treatment ≤ 1 mo, *n* = 14; FOLFOX treatment ≤ 1 year, *n* = 22; FOLFOX treatment > 1 year, *n* = 17) (B). Q-RT-PCR data are expressed as mean ± SE, $^{a}P < 0.05$ and $^{b}P < 0.001$, respectively. CRLM: Colorectal liver metastasis.

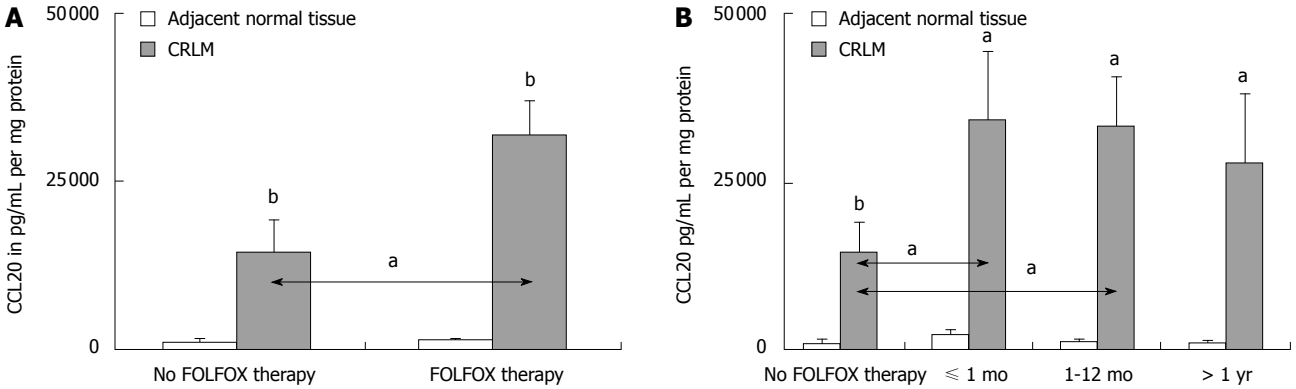

Figure 2 CCL20 protein concentrations (pg/mL pro mg total protein) in colorectal liver metastasis patients with (*n* = 53) and without (*n* = 29) FOLFOX chemotherapy before colorectal liver metastasis surgery (A) and itemized according to different time points of FOLFOX treatment before colorectal liver metastasis surgery (no FOLFOX therapy, *n* = 29; FOLFOX treatment ≤ 1 mo, *n* = 14; FOLFOX treatment ≤ 1 year, *n* = 22; FOLFOX treatment > 1 year, *n* = 17) (B). Protein data are expressed as mean ± SE, $^{a}P < 0.05$ and $^{b}P < 0.001$, respectively. CRLM: Colorectal liver metastasis.

Impact of FOLFOX on proliferation of CRLM cells

Proliferation of CRLM cells as demonstrated by Ki-67 staining revealed a more frequent immunostaining pattern in CRLM tissues of patients who underwent preoperative FOLFOX (Figure 3F) compared to patients without FOLFOX (Figure 3E). In CRLM patients without FOLFOX treatment (*n* = 29) (Figure 3E) we observed weak or no Ki-67 immunostaining in 11 patients (38%), moderate immunostaining intensities in 13 patients (45%) and strong immunostaining intensities in 5 patients (17%). In CRLM patients with preoperative FOLFOX treatment (*n* = 53) (Figure 3F) we observed weak or no Ki-67 immunostaining in 16 patients (30%), moderate immunostaining intensities in 11 patients (21%) and strong immunostaining intensities in 26 patients (49%).

DISCUSSION

At present, standard treatment of CRC patients without distant metastasis consists of surgical resection of the primary tumor followed by adjuvant FOLFOX chemotherapy in patients with lymph node metastasis[17]. For patients with synchronous liver metastasis there are three treatment options: colectomy with synchronous or heterochronous CRLM surgery; perioperative chemotherapy with FOLFOX, FOLFIRI or CapeOX followed by colectomy and CRLM resection; and colectomy followed by chemotherapy and staged CRLM resection[5,6]. As liver resection offers the chance of long-term survival only for patients with resectable CRLM, chemotherapy is often applied to render formerly unresectable CRLM patients resectable. Moreover, superior survival for patients who have undergone resection has been demonstrated by several studies[18,19]. This improved survival may be due to the lower tumor burden.

The impact of chemotherapy on metastatic lymph node lesions was addressed in another study, where patients with 4 or more lymph node metastases around the primary cancer were considered to benefit from perioperative chemotherapy[20]. Chemotherapy options for metastatic CRC have significantly changed in recent years. While the optimal adjuvant systemic chemotherapy has yet to be determined, FOLFOX treatment seems to be one of the most effective CRLM treatment options[21] and perioperative FOLFOX chemotherapy is most commonly used to reduce the risk of cancer relapse in CRC patients[7].

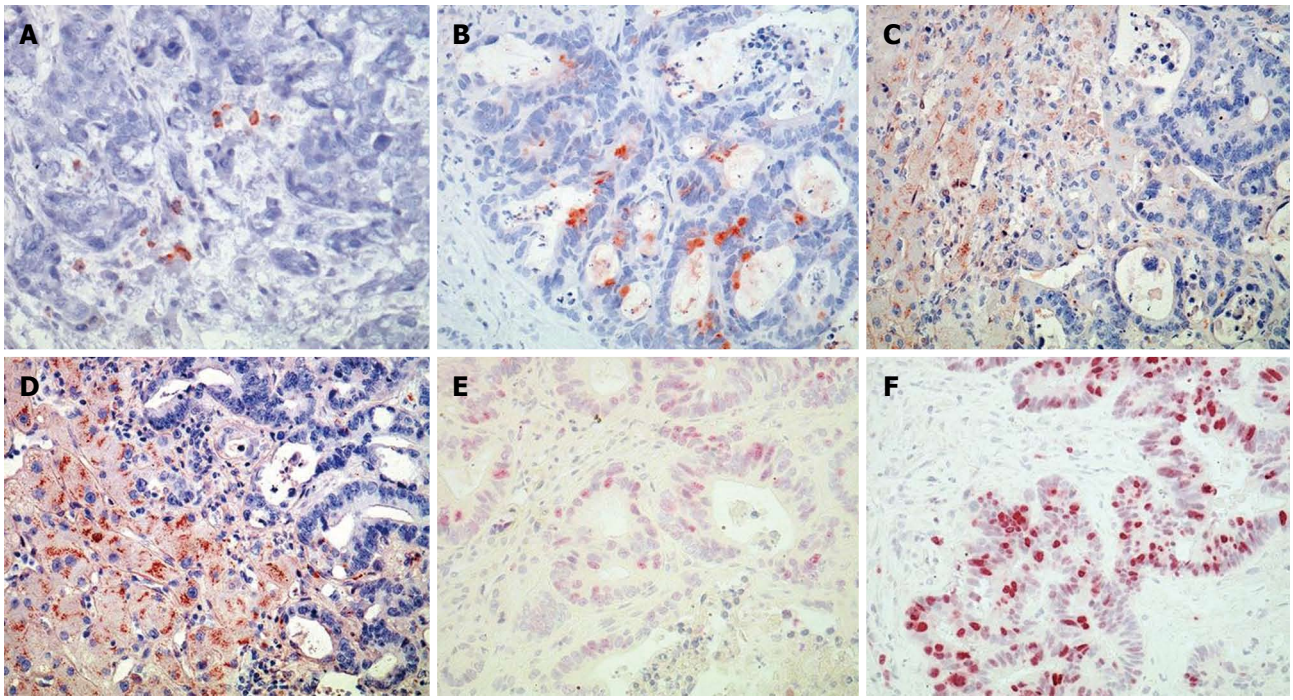

Figure 3 Immunohistochemical staining (original magnification × 200). Representative examples of CCL20 (A, B), CCR6 (C, D) and Ki-67 (E, F) protein expression in colorectal liver metastasis specimens from a colorectal liver metastasis patient without (A, C, E) and with preoperative FOLFOX treatment less than 1 mo before surgery (B, D, F), respectively.

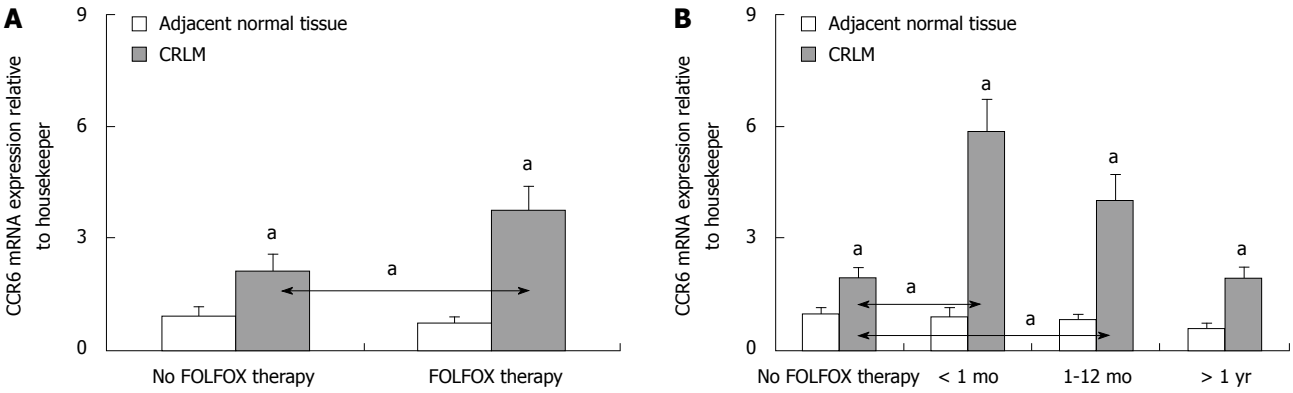

Figure 4 CCR6 mRNA expression relative to PMM1 and β2M in CRLM patients with (*n* = 53) and without (*n* = 29) FOLFOX chemotherapy before CRLM surgery (A) and itemized according to different time points of FOLFOX treatment before CRLM surgery (no FOLFOX therapy, *n* = 29; FOLFOX treatment ≤ 1 mo, *n* = 14; FOLFOX treatment ≤ 1 year, *n* = 22; FOLFOX treatment > 1 year, *n* = 17) (B). Q-RT-PCR data are expressed as mean ± SE, $^{a}P < 0.05$.

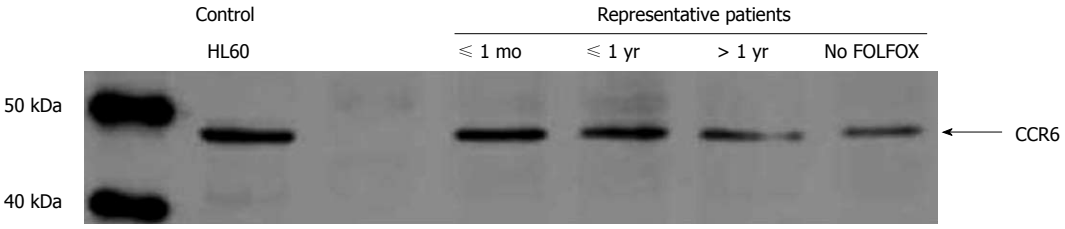

Figure 5 Expression of chemokine receptor CCR6 in colorectal liver metastasis patients itemized according to different time points of FOLFOX treatment before colorectal liver metastasis surgery as determined by Western blotting analysis. Total cell lysates of tumor (P) were immunoblotted with antibodies specifically recognizing chemokine receptor CCR6. Acute leukemia cell line HL60 served as a positive control for the detection of CCR6.

Although pathological effects of chemotherapy for CRLM have been discussed[21-24], the pathological response to chemokine expression in CRLM after chemotherapy has not been reported. Since CCL20 and CCR6 have both been

shown to be expressed on CRC cells[10,11] and CCL20 stimulation of CCR6-bearing CRC cells led to increased proliferation and migration *in vitro*[8,9], we investigated the impact of FOLFOX chemotherapy in stage Ⅳ CRC patients on CCL20/CCR6 expression in liver metastatic tissue.

Our results have shown that both CCL20 and CCR6 expression were significantly increased in patients who had received preoperative FOLFOX chemotherapy ≤ 12 mo before liver surgery as compared to patients who had not received FOLFOX chemotherapy prior to liver surgery. While CCL20 expression was significantly elevated in CRLM tissues, we detected CCR6 signals only sporadically in the tumor cells. Yet, CCR6 expression appeared rather cumulative in deformed hepatic cells along the tumor invasion front, which may represent a stimulative signal for the tumor to further expand into the neighboring hepatic tissue. Further, we demonstrated that CRLM cells of patients who had preoperative FOLFOX chemotherapy are characterized by an increased proliferation rate. This was measured by the expression of the human Ki-67 protein which is strictly associated with cell proliferation. During interphase, the antigen can be exclusively detected within the nucleus, whereas in mitosis most of the protein is relocated to the surface of the chromosomes[25].

Investigating the proliferation of CRLM cells in CRLM resection specimens, we measured proliferation after the event of FOLFOX exposure. FOLFOX treatment promotes apoptosis of CRC cells, thus inhibiting their proliferation. However, after FOLFOX chemotherapy, not only cell proliferation as measured by KI-67 staining, but also CCL20 and CCR6 expression levels, were increased as compared to patients without FOLFOX chemotherapy. *In vitro* data have shown an increased proliferation and migration of CCR6-bearing tumor cells after CCL20 stimulation[8,9]. Thus, the observed up-regulation of CCL20 expression by surviving metastatic CRC cells after FOLFOX treatment might represent a CCL20/CCR6 dependent autocrine mechanism, potentially leading to increased proliferation and migration. Since FOLFOX chemotherapy induces non-specific cell death, this mechanism might be used by surviving metastatic cells within the liver to escape FOLFOX-induced cell death. Interestingly, if the interval between preoperative FOLFOX chemotherapy and liver surgery was > 1 year, the up-regulation of neither CCL20 nor CCR6 remained statistically significant. Generally, this suggests that the up-regulation of CCL20 and CCR6 by FOLFOX is a temporary event.

The disruption of chemokine/chemokine receptor interactions is a promising strategy in the treatment of cancer. A CCR5 inhibitor is already in the clinic for the treatment of human immunodeficiency virus (HIV)-infected patients. Other different chemokine antagonists are currently under investigation in phase Ⅰ-Ⅲ trials for infectious diseases, autoimmune diseases and cancer. Since CCR6 and CCL20 may play a role in CRC, leading to proliferation and migration *via* autocrine or paracrine mechanisms, progression of CRC might be advantaged by CCR6/CCL20 interactions. Thus, the effect of chemotherapy on the expression of cancer-related chemokines and their receptors might explain in part the frequent recurrence of metastasis in patients after this treatment. Therefore, CCL20 and CCR6 interactions may constitute a potential new target for specific treatment interventions in the treatment of CRC. Such a novel approach might be effective in combination with FOLFOX as preoperative treatment prior to resection of CRLM.

COMMENTS

Background

Although long-term survival of patients with colorectal liver metastases (CRLM) can only be achieved by surgical resection, cancer recrudescence can only be avoided if CRLM are thoroughly resected and if no other non-resectable distant metastases are present. As liver resection offers the chance of long-term survival only for patients with resectable CRLM, chemotherapy is often applied to render formerly unresectable CRLM patients resectable. Thus, a combination of perioperative chemotherapy and surgery is frequently applied to improve prognosis in colorectal cancer (CRC) patients.

Research frontiers

As a standard approach of adjuvant chemotherapy, a combination of oxaliplatin, fluorouracil and leucovorin (termed FOLFOX) is most commonly used to reduce the risk of cancer relapse in CRC patients. Although pathological effects of chemotherapy for CRLM have been discussed, the pathological response regarding chemokine expression in CRLM after chemotherapy has not been reported. Thus, application of FOLFOX may enhance the expression of chemokines which are known to be up-regulated with CRC.

Innovations and breakthroughs

Recently, interactions of the chemokine/chemokine receptor pair CCL20/CCR6 became known as critical components in the regulation of CRC progression and organ selective CRC metastasis to the liver. Thus, the authors retrospectively analyzed the impact of FOLFOX chemotherapy in stage Ⅳ CRC patients on CCL20/CCR6 expression in liver metastases. The results have shown that both CCL20 and CCR6 expression levels were significantly increased in patients who had received preoperative FOLFOX chemotherapy ≤ 12 mo before liver surgery as compared to patients who had not received FOLFOX chemotherapy prior to liver surgery.

Applications

As the disruption of chemokine/chemokine receptor interactions is a promising strategy in the treatment of cancer, various chemokine antagonists are currently under investigation in phase I-III trials for infectious diseases, autoimmune diseases and cancer. Since CCL20/CCR6 interactions may play a role in the progression of CRC, the effect of chemotherapy on the expression of cancer-related chemokines and their receptors might explain in part the frequent recurrence of metastasis in patients after this treatment. Thus, CCL20/CCR6 interactions may constitute a potential target for specific CRC treatment interventions, especially in combination with FOLFOX as preoperative treatment prior to CRLM resection.

Peer review

The authors have investigated the expression of CCL20/CCR6 in resected liver metastases of colorectal cancer with regard to preoperative FOLFOX chemotherapy. In their retrospective data analysis, they found a correlation of chemotherapy with an upregulation of CCL20/CCR6 after FOLFOX treatment within 12 mo before surgery. This result is a descriptive observation without further elucidation of the underlying mechanisms. However, it may be a basis for further research in this field to clarify tumor cell resistance against specific chemotherapeutic agents.

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ORIGINAL ARTICLE

Interferon-γ inhibits ghrelin expression and secretion *via* a somatostatin-mediated mechanism

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Abstract

AIM: To investigate if and how the proinflammatory cytokine interferon γ (IFNγ) affects ghrelin expression in mice.

METHODS: The plasma concentration of ghrelin, and gastric ghrelin and somatostatin expression, were examined in wild-type mice and mice infected with *Helicobacter pylori* (*H. pylori*). Furthermore, ghrelin expression was examined in two achlorhydric mouse models with varying degrees of gastritis due to bacterial overgrowth. To study the effect of IFNγ alone, mice were given a subcutaneous infusion of IFNγ for 7 d. Finally, the influence of IFNγ and somatostatin on the ghrelin promoter was characterized.

RESULTS: *H. pylori* infection was associated with a 50% reduction in ghrelin expression and plasma concentration. Suppression of ghrelin expression was inversely correlated with gastric inflammation in achlorhydric mouse models. Subcutaneous infusion of IFNγ suppressed fundic ghrelin mRNA expression and plasma ghrelin concentrations. Finally, we showed that the ghrelin promoter operates under the control of somatostatin but not under that of IFNγ.

CONCLUSION: Gastric infection and inflammation is associated with increased IFNγ expression and reduced ghrelin expression. IFNγ does not directly control ghrelin expression but inhibits it indirectly *via* somatostatin.

Key words: Ghrelin; Interferon-γ; Somatostatin; Inflammatory diseases; *Helicobacter pylori*

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INTRODUCTION

The gastric peptide hormone ghrelin is, in adults, predominantly produced in P/D$_1$ endocrine cells in humans or in A-like endocrine cells in rats and mice, which are located in the oxyntic glands of the gastric corpus[1-5]. Within the oxyntic glands, ghrelin-containing cells are found from the neck to base in both rats and humans[2,6-8]. Ghrelin-producing cells are also found in the antrum of the stomach and proximal small intestine as well as in other organs[2,9-14], but these sites are of lesser importance as the plasma ghrelin concentrations are reduced by 65% after gastrectomy[13]. Plasma ghrelin consists of two forms; the active acylated ghrelin, which is the ligand for the GH secretagogue (GHS) receptor, and the non-acylated ghrelin, which constitutes greater amounts in the blood than the acylated form[11]. Ghrelin is involved in energy homeostasis and ghrelin plasma concentrations are decreased in obesity and increased in states of negative energy balance such as fasting, anorexia or cachexia[11] as well as being inversely correlated to body mass index (BMI) and insulin secretion[11,13]. Ghrelin plasma concentrations increase before meals and decrease after eating[15,16]. However, to what degree ghrelin is important as a meal initiator or cause of increased caloric ingestion in obesity has not yet been determined[17].

Recently, several studies have found that infection with the gram-negative bacteria *Helicobacter pylori* (*H. pylori*) reduces ghrelin concentrations in both humans[7,18,19] and rodents[20]. With regard to various upper gastrointestinal diseases, plasma concentrations of ghrelin were lowest in chronic gastritis and gastric ulcer and highest in acute gastritis[21]. Furthermore, children infected with *H. pylori* have faltering growth[22,23], which suggests that *H. pylori* could alter signals from the stomach related to the control of growth and body weight[24].

The inflammation that occurs in the *H. pylori*-infected host is a Th1-dominated immune reaction which is regulated by, among others, the lymphocyte-derived cytokine interferon-γ (IFNγ)[25]. In the gastrin knockout (KO) mouse, which is another model for chronic gastritis due to bacterial overgrowth, we and others have also found increased gastric production of IFNγ and expression of IFNγ regulated transcripts[26,27]. Furthermore, IFNγ is one of the major cytokines behind the inflammatory response to *H. pylori* as no inflammation occurs during *H. pylori* infection without the presence of IFNγ[25]. Finally, infusion of IFNγ triggers inflammation *in vivo* without *H. pylori*[26]. Since approximately 50% of the world population is infected with this bacteria[28], knowledge of the factors modulating body weight during *H. pylori* infection could have great impact on health in general. Since little is known about the factors that regulate ghrelin expression during *H. pylori* infection and gastric inflammation[29], we examined the effect of IFNγ on ghrelin expression in mice.

MATERIALS AND METHODS

Mice

Groups of wild-type (wt) C57BL/6J mice (aged 12-16 wk), KO mice which are gastrin deficient (aged 12-16 wk or 48-56 wk)[30], histidine decarboxylase (HDC) KO mice (aged 48-56 wk)[31] and matching control mice were used. All mice were male mice that had been backcrossed to the C57BL/6J mouse strain. The mice were kept under specific pathogen-free conditions and monitored according to the Federation of European Laboratory Animal Science Associations recommendations[32] with 12 h light, 12 h dark cycles. The study was approved by the Danish Animal Welfare Committee.

H. pylori infection

C57BL6/J mice (n = 10) were inoculated with a non-mouse-adapted clone of *H. pylori* strain 67:21, originally isolated from an antral biopsy obtained from a Swedish female with gastric ulcer. The strain is VacA$^+$ and contains the entire Cag pathogenicity island (PAI) with genetic stability in the Cag PAI[33]. The mice were inoculated every second day (three times) during a 5-d period. After the mice had been sacrificed, DNA was extracted and analyzed for the presence of *Helicobacter* species using a semi-nested polymerase chain reaction-denaturing gradient gel electrophoresis assay, specific for the genus *Helicobacter*, as described previously[34]. A matched group of uninfected C57BL6/J mice were used as controls. All animal experiments were approved by the Danish Animal Welfare Committee (2005/562-40) and the Danish Forest and Nature Agency (20010077355/6).

IFNγ infusion

Wild-type mice were given a continuous subcutaneous IFNγ infusion (8 μg/kg per hour or 24 μg/kg per hour for 7 d) for each group (n = 6) using osmotic minipumps (Alzet no.2001; Alza Corp., Cupertino, CA). Control mice received a saline infusion instead. The lower dose of IFNγ equals the dose of IFNγ used by Kang *et al*[26].

Tissue and plasma collection

The mice were anesthetized with intraperitoneal 2,2,2 tribromoethanol (Sigma-Aldrich Corp., St. Louis, MO), blood was collected in EDTA-tubes and the stomachs removed. The stomachs of all mice were dissected into fundus and antrum and immediately placed in liquid nitrogen. Plasma and tissue was subsequently stored at -80℃ until further analysis.

Measurement of plasma ghrelin

Plasma ghrelin was measured in EDTA plasma without extraction using RIA no. RK-031-31 (Phoenix Peptides, Belmont, CA). This assay measures the sum of Ser3-octanoyl and Ser3-des-octanoyl ghrelin peptides. The assay has a detection limit of 20 pmol/L, an interassay variation of 13%, and an intra-assay variation of 5%[4].

mRNA extraction and analysis

The stomachs were dissected into fundus and antrum and immediately placed in liquid nitrogen. RNA was extracted using the method described by Chomcynski and

Sacchi[35], and quantitative changes in the specific mRNA were determined by real-time PCR using the Lightcycler (Roche, Mannheim, Germany) as described by Chen *et al*[36]. Quantitations were performed using one of the following primer sets for each analysis: Ghrelin forward primer (FP) 5'-TCTGCAGTTTGCTGCTACTCA-3' and ghrelin reverse primer (RP) 5'-CCTCTTTGACCTCTTCCCAGA-3'; IFNγ FP 5'-CCTTTGGACCCTCTGACTTG-3' and IFNγ RP 5'-CATCCTTTTGCCAGTTCCTC-3'; gastrin FP 5'-CACTTCATAGCAGACCTGTCCA-3' and gastrin RP 5'-CTGGCCTCTGGAAGAGTGTT-3'; somatostatin FP 5'-CCCAGACTCCGTCAGTTTCT-3' and somatostatin RP 5'-TCAGAGGTCTGGCTAGGACAA-3'; iNOS FP 5'-ACCCCTGTGTTCCACCAGGAGATGTTGAA-3' and iNOS RP 5'-TGAAGCCATGACCTTTCGCATTAGCATGG-3' and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) FP 5'-GGTGCTGAGTATGTCGTGGA-3' and GAPDH RP 5'-GTGGTTCACACCCATCACAA-3'. Each run consisted of one negative control, one sample in which the Moloney murine leukemia virus reverse transcriptase had been omitted in the reverse transcription (RT) step, a standard curve generated by 3-fold serial dilution of RT reactions, and seven to nine RT reactions from each of the three strains. Expression of a given transcript was normalized to a GAPDH quantification performed on the same RT reaction as previously described[36].

Immunohistochemical analysis

Stomachs were rinsed in ice-cold PBS, fixed in 4% paraformaldehyde in PBS for 4-6 h and embedded in paraffin. Five-micrometer sections were cut and stained with hematoxylin and eosin. Immunohistochemistry was performed using the rabbit ghrelin antibody H-031-31 diluted 1:1 000 (Phoenix Peptides) detected with Envision-DAB+ (Dako, Glostrup, Denmark) as previously described[37]. The specificity of the immunostaining was tested by absorbing the primary antibodies with antigen before applying them to the slides or omitting the primary antibody when purified antigen was not available. The morphometrical analysis was performed by cell counting in transversely cut sections as described[38].

Cell lines

NCI-H727 cells were grown in RPMI 1640 media (Invitrogen, Carlsbad, CA), 10% FBS (Biowest, Nuaillé, France), *penicillin* (100 U/mL) and *strep*tomycin (100 μg/mL) (Invitrogen) and cultured at 37℃ in 5% CO_2.

Plasmids and transient transfections

A 2.5 Kb fragment containing the mouse ghrelin promoter and exon one was amplified from C57BL6/J genomic DNA using the Expand kit (Roche, Mannheim, Germany) using mGhrMluI primer 5'-ATATACGCGTGTAGAACACTCACCCTAAATCTG-3' and mGhrXhoI primer 5'-ATATCTCGACTGCCTGGGGATGTGGTGCCTG-3'. The fragment was ligated into the pGL3 Bacic reporter vector (Promega, Madison, WI). The promoter sequence was confirmed by sequencing. One day before transfection, 500 000 NCI-H727 cells were seeded in 6-well dishes coated with 0.01% poly-L-lysine. The NCI-H727 cells were transfected using 6 μL TurboFect™ *in vitro* Transfection Reagent (Fermentas, Burlington, Canada); 2 μg ghrelin promoter plasmid and 1 μg pRL-0 (Promega, Madison, WI) were mixed with 200 μL GIBCO™ Opti-MEM I (Invitrogen, Carlsbad, CA) and incubated for 20 min before application to the cells. Twenty-four hours later, the cells were FBS starved in RPMI1640 media containing 0.5% FBS (Biowest, Nuaillé, France) for 24 h before treatment with forskolin (10 mg, Sigma-Aldrich, St. Louis, MO), IBMX (≥ 99.9%, Sigma-Aldrich, St. Louis, MO), octreotide (200 μg/mL, Mayne Pharma, Melbourne, Australia), or IFNγ (0.2 mg/mL, Immukine, Boehringer Ingelheim, Ingelheim, Germany), for 24 h alone or in combination. All treatments were performed in triplicate. IBMX and forskolin were dissolved in 99.9% DMSO (Merck, Darmstadt, Germany). Cells were then harvested and assayed for luciferase activity using the Dual-Luciferase Reporter Assay System according to the instructions by the manufacturer (Promega, Madison, WI) and normalized to Renilla luciferase activity.

Statistical analysis

Student's unpaired *t*-test statistics were used and differences with a $P \leqslant 0.05$ were considered significant. Unless otherwise stated, results are given as mean ± SD.

RESULTS

H. pylori infection is associated with an IFNγ inflammatory response and with suppression of ghrelin expression

The mice were sacrificed 2 mo after inoculation, as earlier studies had shown that a *cag*-dependent inflammation of the corpus mucosa develops at this time and results in a severe active and chronic gastritis[39,40]. At 2 mo, seven out of ten mice tested positive for *H. pylori* using semi-nested PCR with primers for *H. pylori* CagA and urease genes. The non-infected mice were subsequently excluded. All control mice tested negative.

The infection of the mouse stomach by *H. pylori* caused a 2- to 3-fold increase in the fundic expression of IFNγ and of inducible nitric oxide synthase (iNOS) (Table 1). Furthermore, during the 2 mo infection, the *H. pylori*-infected mice did not gain weight in contrast to wild-type mice (Figure 1A). Ghrelin mRNA expression was reduced to 55% in the *H. pylori*-infected mice (Figure 1B). This was associated with a 49% decrease in the plasma ghrelin concentration (Figure 1C). The reduced ghrelin mRNA expression presumably reflects a reduced expression in each cell as opposed to cell atrophy, as the density of fundic ghrelin cells was unaffected (Table 1). In the infected mice the expression of antral somatostatin was suppressed, whereas the fundic somatostatin expression was increased (Table 1).

Ghrelin expression was reduced in old gastrin knockout mice, but not in young gastrin and old histidine decarboxylase knockout mice

To test whether the altered ghrelin expression could also be

Table 1 Fundic somatostatin mRNA increases after 2 mo of *Helicobacter pylori* infection in wild-type mice (mean ± SE)

	Fundus			Antrum		
	Control	*H. pylori*	*P*	Control	*H. pylori*	*P*
IFNγ mRNA	1.0 ± 0.2	1.8 ± 0.3	< 0.05	1.0 ± 0.1	4.1 ± 0.9	< 0.05
iNOS mRNA	1.0 ± 0.1	1.5 ± 0.1	< 0.05	1.0 ± 0.2	2.2 ± 0.5	< 0.05
Somatostatin mRNA	1.0 ± 0.2	1.4 ± 0.2	< 0.05	1.0 ± 0.2	0.4 ± 0.1	< 0.05
Ghrelin cells (#/mm mucosa)	25 ± 3	27 ± 5	NS	8 ± 2	7 ± 3	NS

The expression of interferon γ (IFNγ), iNOS and somatostatin mRNA in arbitrary units in *H. pylori*-infected mice (n = 7) 2 mo after inoculation or in uninfected control mice (n = 7). Ghrelin cell density was unchanged. *H. pylori*: *Helicobacter pylori*; NS: Non-significant.

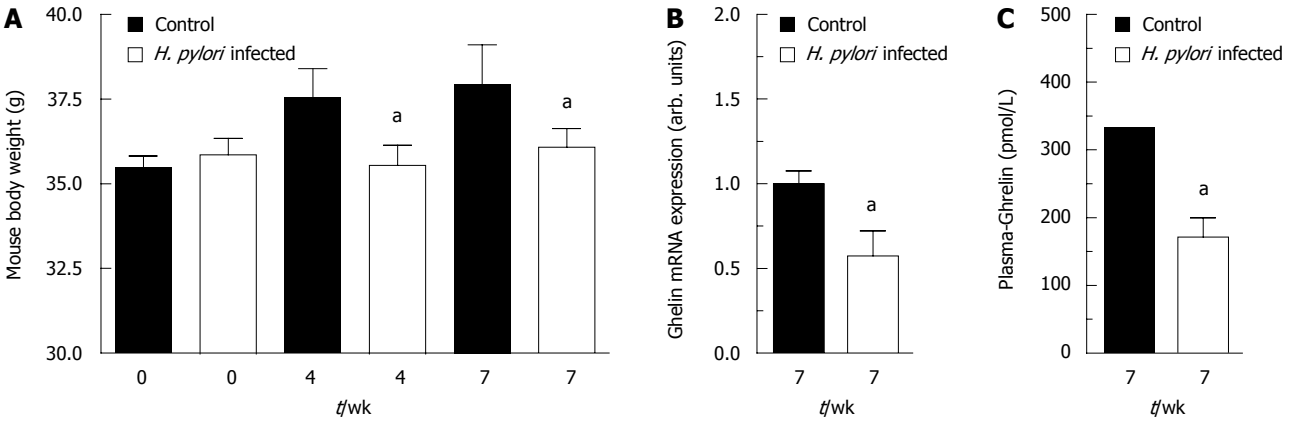

Figure 1 Mice infected with *Helicobacter pylori* have reduced ghrelin expression and do not gain weight. C57BL6/J mice were infected with *Helicobacter pylori* (*H. pylori*) strain 67:21 [this strain is VacA+ and contains a complete genetically stable Cag pathogenicity island (PAI)]. While the control mice gained weight the infected mice did not (A). Mice infected with *H. pylori* had reduced ghrelin expression in the stomach (B) and reduced ghrelin plasma concentrations (C). $^{a}P < 0.05$ *vs* control.

found in other mouse models with gastric inflammation, we examined the expression of IFNγ and ghrelin in two other mouse models; the achlorhydric gastrin KO mice and the hypochlorhydric histidine decarboxylase (HDC) KO mice[41,42].

IFNγ expression was not induced in the old HDC KO mice (Figure 2A), and the ghrelin expression was unchanged in these mice (Figure 2B). Young gastrin KO mice only had moderate inflammation, while the old mice had more inflammation when evaluated by higher IFNγ expression (Figure 2C). Ghrelin expression was unaffected in young gastrin KO mice but reduced in old gastrin KO mice (Figure 2D).

IFNγ suppresses fundic ghrelin mRNA expression and plasma ghrelin concentrations

Since both the *H. pylori* infection and the bacterial overgrowth in the gastrin KO mice were associated with increased expression of IFNγ and reduced ghrelin expression, we examined the effect of IFNγ on ghrelin expression. The fundic ghrelin expression was approximately 30 times higher than the antral (Figure 3A and B). The fundic expression of ghrelin mRNA was halved at both infusion rates of IFNγ (8 μg/kg per hour and 24 μg/kg per hour) examined compared to expression levels in wt mice (Figure 3A and B). In contrast, antral ghrelin expression did not alter significantly under IFNγ infusion at either dose. The reduction in ghrelin expression presumably reflects a reduced expression in each cell as opposed to cell atrophy, as infusion of IFNγ did not change the density of fundic ghrelin cells (Figure 3D). The reduced ghrelin expression was correlated with a 40% reduction in plasma ghrelin concentrations at 7 d of IFNγ infusion (Figure 3C). Furthermore, IFNγ infusion induced the expression of fundic somatostatin, whereas the antral somatostatin expression did not change under the influence of IFNγ (Table 2).

The ghrelin promoter operates under the control of somatostatin but not under that of IFNγ

We next examined the effect of IFNγ and somatostatin on the transcriptional regulation of ghrelin using a 2 kb ghrelin promoter construct. The experiments were carried out in NCI-H727 cells since these are carcinoid cells expressing both somatostatin receptor 2 (SSTR2) and SSTR5. This indicates that they could be a good model for A-like cells in the stomach (Døssing, unpublished data). Treatment with IFNγ did not affect the activity of the ghrelin promoter construct. In contrast, forskolin and IBMX both independently and together activated the 2 kb promoter (Figure 4). Moreover, treatment with octreotide (somatostatin analog) reduced the basal ghrelin promoter activity (Figure 4) as well as forskolin/IBMX-induced ghrelin promoter activation in a dose-dependent manner.

DISCUSSION

Our results show that the gastric expression of ghrelin

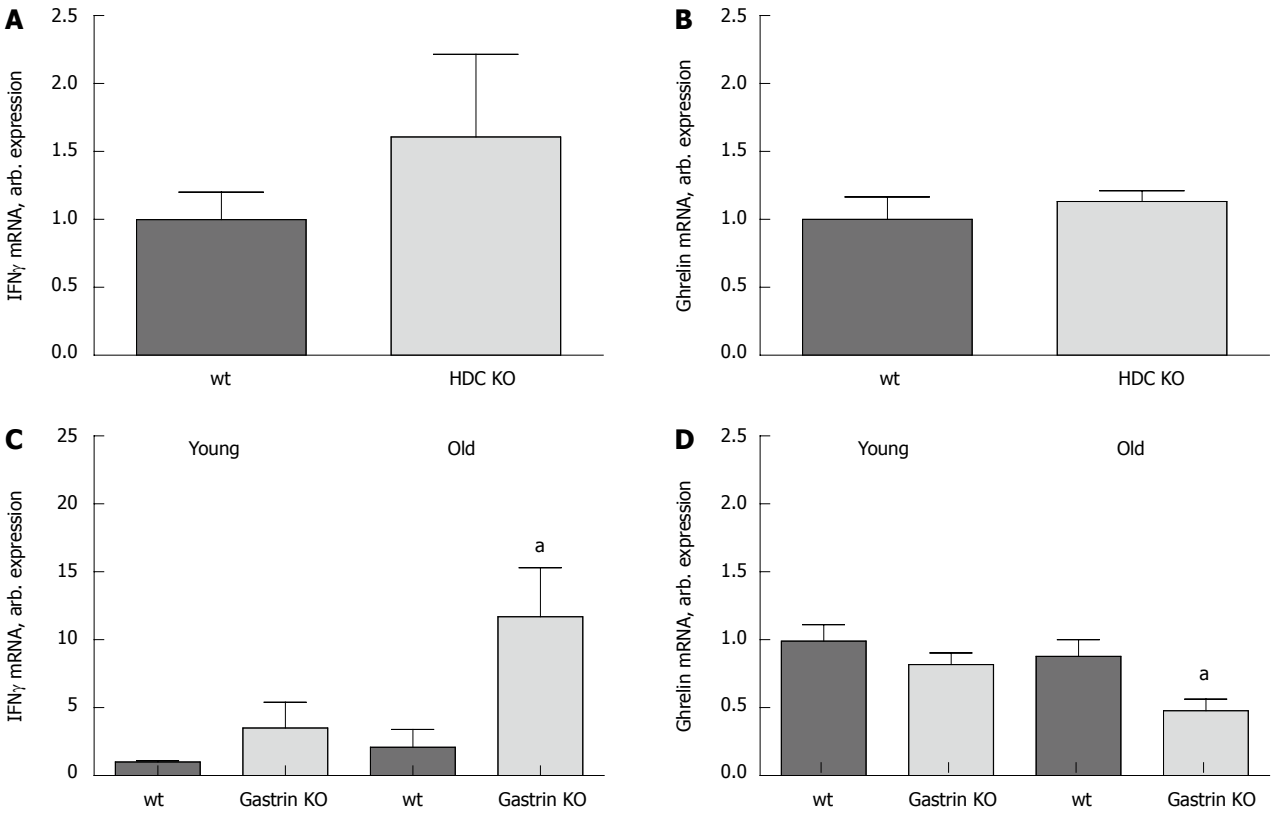

Figure 2 Interferon γ expression is increased and ghrelin expression is reduced in old but not young gastrin knockout mice and histidine decarboxylase knockout mice. The expression of interferon γ (IFNγ) and ghrelin mRNA in young (12-16 wk) and old (48-56 wk) achlorhydric gastrin KO mice and old (48-56 wk) hypochlorhydric histidine decarboxylase (HDC) KO mice (n = 6 in each group) is shown. There is no change in either IFNγ (A) or ghrelin (B) expression in HDC KO mice as compared to wt mice. While the gastric inflammation evaluated by expression of IFNγ increases when the gastrin KO mice get older (C), the expression of ghrelin decreases (D). $^{a}P < 0.05$.

Table 2 Fundic expression of somatostatin mRNA increases during subcutaneous interferon γ infusion (mean ± SE)

	Fundus			Antrum		
	Saline	+ IFNγ	*P*	Saline	+ IFNγ	*P*
Low Dose IFNγ						
Somatostatin mRNA	1.0 ± 0.2	1.9 ± 0.2	< 0.05	1.0 ± 0.2	0.9 ± 0.1	NS
IFNγ mRNA	1.0 ± 0.1	1.1 ± 0.2	NS	1.0 ± 0.1	1.2 ± 0.2	NS
High Dose IFNγ						
Somatostatin mRNA	1.0 ± 0.1	1.6 ± 0.1	< 0.05	1.0 ± 0.1	1.1 ± 0.1	NS
IFNγ mRNA	1.0 ± 0.2	1.3 ± 0.1	NS	1.0 ± 0.1	1.2 ± 0.1	NS

The fundic and antral expression of somatostatin mRNA and endogenous interferon γ (IFNγ) mRNA in arbitrary units in mice infused with either IFNγ or saline (n = 6 in each group). Low dose IFNγ = 8 μg/kg per hour for 7 d and high dose IFNγ = 24 μg/kg per hour for 7 d. NS: Non-significant.

mRNA and the plasma concentration of ghrelin are reduced during gastric infection, either due to bacterial overgrowth in general or to *H. pylori* infection specifically. Both types of infection are associated with an IFNγ inflammatory response. Furthermore, infusion of IFNγ alone could mimic the changes in ghrelin expression and plasma concentration seen during *H. pylori* infection and bacterial overgrowth.

The observation of reduced ghrelin expression in *H. pylori*-infected mice is in agreement with several studies that found reduced ghrelin concentrations in both humans[7,18,19] and rodents[20] infected with *H. pylori*. However, others have reported ghrelin plasma concentration to be unaffected[43,44] or even to increase during *H. pylori* infection[45]. These discrepancies could be due to differences in the severity of the infection. We found gastric ghrelin expression unaffected in young gastrin KO mice and HDC KO mice, both with only mild inflammation as evaluated by the IFNγ response. However, as the gastrin KO mice got older and developed a more severe gastric inflammation, the ghrelin expression decreased. Similar observations have also been observed in humans, where a correlation between increasing degree of chronic inflam-

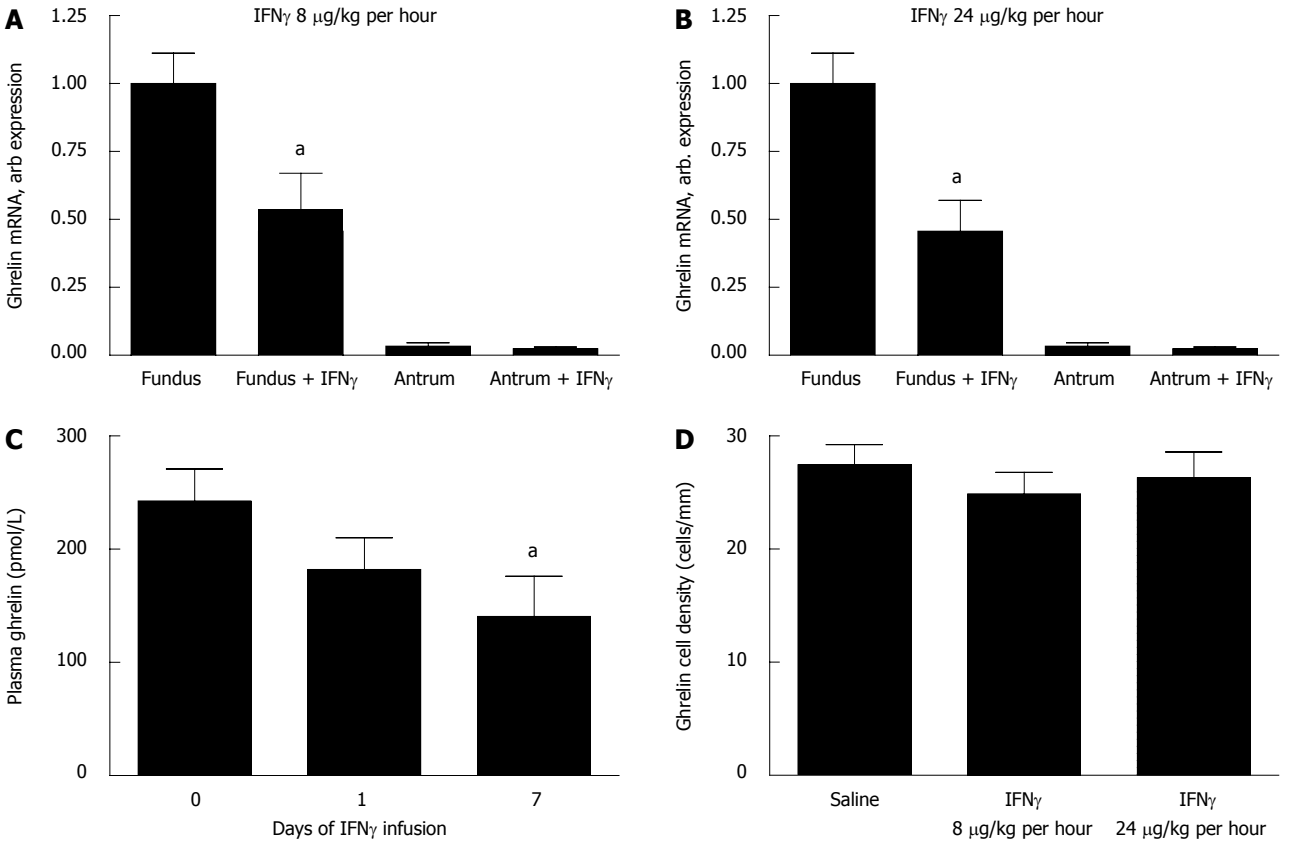

Figure 3 Interferon γ suppresses ghrelin expression in the gastric fundus and ghrelin concentrations in plasma independently of loss of ghrelin-producing cells. Mice infused with interferon γ (IFNγ) as compared to saline infused mice (n = 6 in each group). IFNγ was infused at low dose (LD) = 8 μg/kg per hour for 7 d or high dose (HD) = 24 μg/kg per hour for 7 d. Low (A) and high (B) dose IFNγ infusion decreases the expression of ghrelin mRNA to a similar degree. HD IFNγ represses ghrelin concentrations in plasma gradually over time (C). The density of fundic ghrelin cells is not altered by IFNγ infusion (D). $^aP < 0.05$.

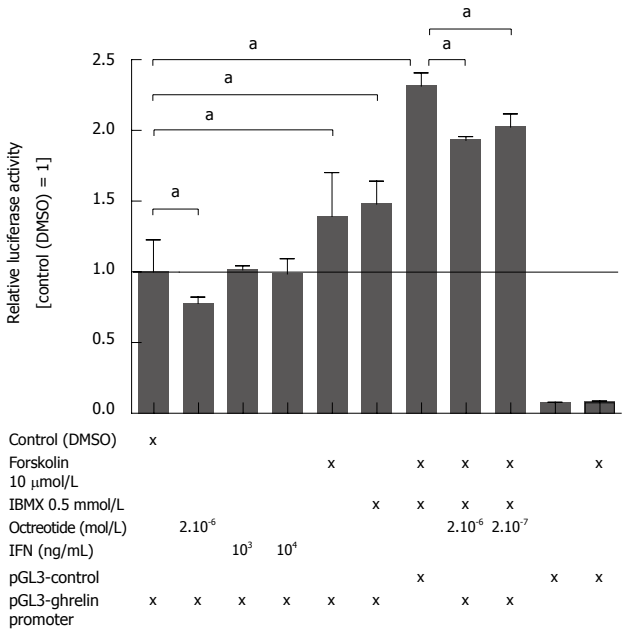

Figure 4 The somatostatin receptor agonist octreotide inhibits activation of the ghrelin promoter. Pulmonary carcinoid NCI-H727 cells were transfected with a 2 kb mouse ghrelin promoter construct and treated for 24 h with forskolin, IBMX, octreotide, and interferon γ (IFNγ) alone or in combination, after which the ghrelin promoter response was measured by the dual-luciferase reporter assay system. Octreotide inhibited both basal and IBMX and/or forskolin-induced ghrelin expression. Forskolin and IBMX were dissolved in dimethyl sulfoxide (DMSO). $^aP < 0.05$.

mation, severity of glandular atrophy and metaplasia and decreasing ghrelin expression was found[8,19].

Reduced gastric ghrelin expression could either be due to a reduced number of ghrelin cells or reduced expression within each cell. Ghrelin-producing cells are primarily found in the oxyntic glands in the stomach[2,3]. The expression of ghrelin mRNA was more than 30 times higher in the fundus than antrum in control mice. Reduced numbers of ghrelin cells in fundic mucosa has indeed been seen in *H. pylori*-infected humans[7] and this correlates with a reduction in plasma ghrelin concentrations.

In our study, the density of ghrelin cells did not change indicating that our observations are due to reduced expression within the cells. The mechanism leading to reduced ghrelin expression during *H. pylori* infection is poorly understood. We found that IFNγ had no direct effect on the ghrelin promoter, suggesting that the IFNγ inhibition of ghrelin expression is mediated *via* other transmitters. In our study, fundic somatostatin was increased both during gastric inflammation and during infusion of IFNγ. Somatostatin is the universal inhibitor of secretion from endocrine cells[46] and also inhibits ghrelin secretion[47]. We show that octreotide (a somatostatin analog) inhibits the activity of the ghrelin promoter. Increased corpus somatostatin has also been found during *H. pylori* infection[48]. However, reduced fundic somatostatin expression has been found in other studies[49,50]. One explanation for

these differences is the degree of fundic atrophy, which affects both ghrelin and somatostatin expression[7].

Immunoregulation of somatostatin has also been demonstrated in *in vitro* studies[51]. These showed that TNFα and IL-1β stimulated somatostatin secretion. IL-4 also stimulated somatostatin secretion and together these changes could explain the hypochlorhydria seen in mice infected with *H. felis*[52]. However, in that study, infusion of IFNγ resulted in a reduction of fundic somatostatin. We have no explanation for the difference in response to IFNγ. The proinflammatory cytokine IL-1β also influences ghrelin levels and seems to suppress excess ghrelin secretion in *H. pylori*-infected mice[53]. Thus, not only IFNγ but other cytokines as well are associated with reduced ghrelin expression.

We have shown that gastric infections either due to *H. pylori* or bacterial overgrowth are associated with reduced fundic ghrelin expression and increased IFNγ production. Infusion of IFNγ in mice alone mimics the changes seen in the mice with gastric infections. Stimulation with IFNγ does not directly inhibit the ghrelin promoter; instead the inhibition is mediated through somatostatin.

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COMMENTS

Background

Ghrelin is involved in energy homeostasis and ghrelin plasma concentrations are decreased in obesity and increased during fasting, anorexia or cachexia. Recently, several studies have found that infection with *Helicobacter pylori* (*H. pylori*) reduces ghrelin concentrations in both humans and rodents. Furthermore, children infected with *H. pylori* have faltering growth, suggesting that *H. pylori* may alter signals from the stomach related to the control of growth and body weight. The mechanism(s) through which inflammation modulates ghrelin expression are, however, poorly understood.

Research frontiers

Chronic gastritis induced by *H. pylori* is a Th1-dominated immune reaction which is regulated by, among others, the lymphocyte-derived cytokine interferon-γ (IFNγ). In the gastrin knockout (KO) mouse which is a model for chronic gastritis due to bacterial overgrowth, increased gastric production of IFNγ has been found. Since little is known about the factors that regulate ghrelin expression during *H. pylori* infections and gastric inflammation, the authors examined if, and through which mechanisms, IFNγ modulates ghrelin expression in mice.

Innovations and breakthroughs

H. pylori-infected mice and old gastrin KO mice with inflammation due to bacterial overgrowth of the stomach display an increased expression of IFNγ and a decreased expression of ghrelin. The changes in ghrelin and somatostatin expression can be duplicated by infusion of IFNγ alone. IFNγ does not directly suppress ghrelin expression but inhibits it indirectly by increasing somatostatin secretion.

Applications

A better understanding of the mechanisms that control ghrelin expression during inflammation by either *H. pylori* alone or by gastric bacterial infections in general aids in the understanding of factors modulating growth and body weight during infection. This could have great impact on general health in the population.

Terminology

IFNγ is a cytokine that is important for innate and adaptive immunity against bacterial infections and for tumor control. The most important functions of IFNγ come from its immunostimulatory and immunomodulatory effects. Ghrelin is a hormone produced in the oxyntic glands of the gastric corpus. It is a growth hormone promoting intestinal cell proliferation, and is involved in energy homeostasis. Ghrelin expression was, in this study, found to be inhibited by octreotide, which is an analog of somatostatin. Somatostatin acts as a general inhibitor of secretion from, and growth of, endocrine cells. Somatostatin is widely distributed throughout the body including several locations in the digestive system such as the stomach, intestine and delta cells of the pancreas.

Peer review

This is the first experimental study on the effect of IFNγ on ghrelin expression. It is a very well designed study, using a careful combination of several animal models and different techniques, and the discussion is well structured. The study is valuable in the context of providing evidence on the indirect regulation of ghrelin expression and secretion by IFNγ mediated through somatostatin.

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BRIEF ARTICLE

Ethanol injection is highly effective for hepatocellular carcinoma smaller than 2 cm

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Abstract

AIM: To analyze the long-term prognosis in a cohort of western cirrhotic patients with single hepatocellular carcinoma treated with ethanol injection.

METHODS: One-hundred forty-eight patients with solitary hepatocellular carcinoma were enrolled. The tumor diameter was lower than 2 cm in 47 patients but larger in the remaining 101 patients. The impact of some pre-treatment clinical and laboratory parameters and of tumor recurrence on patients' survival was assessed.

RESULTS: Among the pre-treatment parameters, only a tumor diameter of less than 2 cm was an independent prognostic factor of survival. The occurrence of new nodules in other liver segments and the neoplastic portal invasion were linked to a poorer prognosis at univariate analysis. Patients with a single hepatocellular carcinoma smaller than 2 cm showed a better 5-year cumulative survival (73.0% *vs* 47.9%) (P = 0.009), 3-year local recurrence rate (29.1% *vs* 51.5%) (P = 0.011), and 5-year distant intrahepatic recurrence rate (52.9% *vs* 62.8%) (P = 0.054) compared to patients with a larger tumor.

CONCLUSION: The 5-year survival rate of patients with single hepatocellular carcinoma < 2 cm undergoing ethanol injection is excellent and comparable to that achieved using radiofrequency ablation.

Key words: Hepatocellular carcinoma; Cirrhosis; Percutaneous ethanol injection; Prognosis

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INTRODUCTION

Hepatocellular carcinoma (HCC) is the sixth more common cancer worldwide, and is the leading cause of death among patients with cirrhosis in Europe and the USA[1-3]. Since the rate of tumors diagnosed early in a subclinical stage is still low, screening and surveillance strategies in cirrhotic patients based on an ultrasound (US) study of the liver and serum assay of α-fetoprotein (AFP) have been developed and applied in both the Eastern and

Western world. Accordingly, a trend towards a progressive decrease of the mean diameter of HCC diagnosed by screening during the last few years has been demonstrated, and this seems to improve patients' prognosis by increasing their access to curative treatments[4].

Surgical resection and liver transplantation provide the best effective cure in HCC. However, resection is usually offered to cirrhotics with a single lesion and preserved liver function without severe portal hypertension, while liver transplantation is an effective option for patients with tumors within the Milan criteria (one nodule < 5 cm or no more than 3 nodules < 3 cm), but can only be considered in a limited number of patients due to the problem of organ shortage[5]. US-guided percutaneous ablation is currently regarded as the first line approach in the treatment of early-stage HCC deemed unsuitable for surgery or liver transplantation. Percutaneous ethanol injection (PEI) was the first percutaneous treatment introduced in clinical practice; it has been widely used during the last 20 years with excellent results[6], and was recommended as the standard ablation therapy of HCC according to the European guidelines for the management of HCC published in 2001[7]. At the end of the nineties, radiofrequency ablation (RFA) became available and progressively replaced PEI[8,9]. Accordingly, a question about the usefulness of PEI in the treatment of HCC has been recently raised. In agreement with Forner *et al*[10], we think that PEI is still useful for the treatment of lesions located at risky sites for RFA, for residual areas of viable tumors after RFA, and as a bridge treatment in HCC patients listed for liver transplantation. Furthermore, according to the present guidelines for the management of HCC[11], the efficacy of PEI is probably similar to that of RFA in patients with compensated cirrhosis and single tumors smaller than 2 cm in which 5-year survival has been shown to be higher than 70% in eastern series[9,12,13].

The aim of this study was to assess the factors affecting long term prognosis in a single-centre cohort of western cirrhotic patients with single HCC treated with PEI, focusing on the subgroup of patients with small tumors smaller than 2 cm.

MATERIALS AND METHODS

Two hundred-eighteen cirrhotic patients with single HCC treated with PEI in our centre during the period 1991-2008 were evaluated. In all patients cirrhosis was confirmed by histological and/or clinical findings (blood chemistry, US, and/or endoscopic signs of liver cirrhosis and/or portal hypertension). Most patients were diagnosed during a 6 mo-interval screening program for early diagnosis of HCC based upon ultrasound study of the liver and serum assay of AFP. After detection of the suspicious HCC nodule, all patients underwent characterization of the lesion using computed tomography (CT), magnetic resonance imaging (MRI), contrast enhanced ultrasound (CEUS), and/or fine-needle biopsy, in accordance to the current diagnostic guidelines; for this reason, most of the lesions diagnosed before 2001 were further evaluated using cyto-histological assessment, while lesions larger than 2 cm detected after 2001 were mainly diagnosed using two imaging studies showing coincident typical dynamic findings after contrast enhancement; a cytological and/or histological evaluation using fine-needle biopsy was reserved for lesions showing non-typical features on dynamic imaging study[7]. After 2005 this policy was extended to lesions less than 2 cm in size[11].

Patients were usually staged before treatment using CT scan. MRI was reserved for patients with contraindications to the administration of iodinated contrast media.

Among the 218 patients included in the study group, 48 were excluded because PEI was associated with other locoregional therapies as initial treatment, 19 because complete necrosis was not achieved after treatment completion, and 3 because of insufficient follow-up data. Hence, the study refers to 148 patients with a minimum post-treatment follow-up period of 6 mo. One hundred-three patients were treated during the period 1991-2000 while the remaining 45 patients underwent PEI during the period 2001-2008. All patients gave informed consent to all diagnostic investigations and therapeutic procedures and the study protocol conforms to the ethical guidelines of the of the 1975 Declaration of Helsinki as reflected in a priori approval by the institution's human research committee. For the whole population, the last follow-up data were recorded on February 2010.

The patients were 109 men and 39 women (mean age 67 ± 8 years, range 34-87 years). The cirrhosis etiology was post-hepatic C in 107 cases, post-hepatic B in 18 cases, post-hepatic C and B in 7 cases, alcoholic in 10 cases, and cryptogenic in 6 cases. According to the Child-Pugh scoring system[14], 130 patients were scored Class A, and 18 Class B. Fifteen patients had mild ascites, while 6 patients showed partial non-neoplastic thrombosis of the portal system according to dynamic imaging studies[15]. The mean HCC diameter was 2.8 cm (range 1.1-5.8 cm). Forty-seven patients (31.8%) had a single tumor < 2 cm, 57 (38.5%) between 2 and 3 cm, and 44 (27.0%) > 3 cm. In only 3 cases the tumor diameter exceeded 5 cm. Overall, a cytological and/or histological diagnosis of HCC was available in 63/148 HCCs (well differentiated in 52 cases, moderately differentiated in 10 cases, and poorly differentiated in 1 case).

All patients were excluded from resective surgery due to one or more of the following reasons: severe portal hypertension, impaired liver function, refusal of surgery, presence of severe comorbidities increasing the surgical risk, and severely impaired clotting parameters. Four patients were waiting for liver transplantation and PEI was used as a bridging treatment; all these patients were transplanted and the follow-up period ended at the moment of transplantation. For patients diagnosed after 2000, when RFA became available in our centre, the main reasons for choosing PEI for treatment were the following: impaired clotting parameters preventing the use of large bore needles, location of the tumor in a dangerous position for RFA (e.g. near the gall bladder, the glissonian capsule, or an intestinal loop adjacent to the liver edge), location of

Table 1 Clinical and biochemical risk factors influencing overall survival *n* (%)

Variables	Median (Interquartile range)	*P* value at Log-Rank[1]	Cox regression[2]
Pre-PEI			
Age (yr)	67 (61-72)	0.640	-
Male gender	109 (73.6)	0.640	-
HCV Pos	114 (77.0)	0.615	-
HBsAg Pos.	25 (16.9)	0.624	-
Child B	18	0.329	-
AST (IU/L)	66.5 (44.5-89.5)	0.794	-
ALT (IU/L)	64 (41.5-106.5)	0.877	-
TAP	79 (69-86)	0.248	-
Tot. Bilirubin	1.0 (0.80-1.45)	0.438	-
Albumin	3.6 (3.3-3.9)	0.116	-
PLT	100 (76.5-139.5)	0.342	-
AFP	11.1 (6-31)	0.330	-
Ascites	15 (10.1)	0.108	-
Portal thrombosis	6 (4.1)	0.133	-
Size < 2 cm	47 (31.8)	0.009	0.421 (0.216-0.821), *P* = 0.011
Post-PEI			
Portal invasion	14 (9.5)	0.003	-
Local recurrence	56 (37.8)	0.210	-
Distance recurrence	61 (41.2)	0.003	-
Global recurrence	86 (58.1)	0.180	-

Hepatocellular carcinoma size: 25, 5 (20-34). [1]*P* value at Log-Rank: Univariate: Results are expressed as *P* level at Log-Rank (Mentel-Cox); [2]Cox Regression: Results are expressed as HR (95% CI) and *P* level at Cox Regression analysis (backward wald). HCV: Hepatitis C virus; AFP: α-fetoprotein; PEI: Percutaneous ethanol injection; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; PLT: Platelets.

the tumor near a large vessel lowering the level of heating needed to induce tumor necrosis, and patient choice.

Patients were usually treated by multisession US-guided PEI on an out-patient basis. After local antisepsis and intradermal local anaesthesia at the site of the needle insertion, sterile 95% ethanol (Salf, Bergamo, Italy) was injected into the lesion using either a 20-cm-long Chiba needle with an inner calibre of 20 Gauge (Ekoject, Hospital Service, Italy) or a PEI dedicated 20-cm long multi-hole 21-Gauge needle (Peit Needle, Hospital Service, Italy) at a dosage of 1-8 mL per session. The Chiba needles were preferentially employed to treat small tumors while the multi-hole needles were usually reserved for lesions larger than 2-3 cm. Treatment was performed once or twice per week and the total amount of ethanol injected was calculated according to the numerical expression $V=(4/3)\pi(r+0.5)^3$, where V (in mL) is the volume of ethanol and r (in cm) is the radius of the lesion increased by 0.5 cm based on the concept that the volume of ethanol injected must overcome the theoretical volume of the lesion[16]. The number of PEI procedures needed to achieve tumor ablation was 6 on average (range 1-11) in the whole population and 2 (range 1-4) in the subgroup of patients with HCC of up to 2 cm. The amount of ethanol injected per session ranged between 2 and 9 mL, with no more than 15 min needed for each PEI session. The effectiveness of PEI was assessed by CT scan with contrast enhancement performed within one month after treatment completion. Complete necrosis was considered achieved when the lesion appeared as a non perfused area during the arterial phase of the study. In the case of intolerance to iodinated contrast media, MRI with gadolinium contrast enhancement was used. During the last 7 years, we added CEUS in the post-PEI assessment of the treated lesion; our policy was to perform CEUS about one month after PEI; in case of presence of residual arterial enhancement, suggesting the presence of a viable tumor, the lesion was immediately re-treated without further imaging evaluation while in the case of absence of contrast enhancement in the arterial phase, suggesting complete necrosis, we used CT or MRI as a confirmatory test[17].

After completion of treatment, the patients were followed by serum assay of AFP and US liver study performed every 3 mo. Local recurrence of the treated lesion was suspected on the basis of size increase and/or US pattern change; in this case, CEUS, CT scan or MRI were performed to assess the presence of arterial enhancement areas suggesting recurrent disease. Furthermore, each new lesion visualized by US in a liver segment different from that of the first neoplasm was characterized using CEUS, CT scan, MRI, and/or US-guided liver biopsy, following the current guidelines for HCC diagnosis[7,11].

The following clinical and biochemical parameters assessed before PEI were analyzed to examine their value as predictive factors for survival (Table 1): age, gender, hepatitis B surface antigen (HBsAg), hepatitis C virus (HCV) antibody, Child-Pugh class, aspartate aminotransferase (AST) level, alanine aminotransferase (ALT) level, prothrombin time ratio, serum bilirubin, serum albumin, platelet count, presence of ascites, presence of portal thrombosis, serum AFP and HCC size ≤ 2 cm. Finally, the impact on survival of local recurrence (HCC recurrence in the same liver segment), distant recurrence (HCC recurrence in a liver segment different from that of the

Table 2 Cumulative survival and disease free survival rates

Months	Overall survival		Disease free survival	
	%	*n*	%	*n*
0		148		148
6	100	147	89.2	132
12	95.8	135	71.0	99
24	86.7	99	48.5	51
36	73.4	66	33.5	27
48	64.1	42	32.1	23
60	55.9	33	24.1	9
Median (95% CI)	78 (54.38-101.62)		24 (16.90-31.09)	

first neoplasm), portal invasion (imaging detection or cytological diagnosis of neoplastic thrombosis of the portal tree), and disease free survival (DFS, defined as the interval in months between last PEI session until local HCC recurrence and/or appearance of new HCC lesions within or outside the liver) were also evaluated.

Statistical evaluation

Overall survival was defined as the interval in months between the first PEI session until death or the last recorded follow-up. Cumulative survival and recurrence curves were obtained using the Kaplan-Meier curves. For each variable taken into account, the differences between curves were assessed using the *post hoc* log-rank test. For age, ALT level, AST level, prothrombin time ratio, bilirubin level, albumin level, platelet count, and AFP, the patients were separated into groups: those $\leqslant$ the median and those > the median. For the purpose of the study, patients were split into two subgroups according to a HCC size smaller or larger than 2 cm. The parameters significant at univariate analysis were tested using the Cox's proportional hazard model. The post treatment parameters of neoplastic portal invasion, local recurrence, distant recurrence, and DFS were only tested by univariate analysis. Correlation was analyzed with the Spearman rank test. A *P* value of $\leqslant$ 0.05 was considered significant. All statistical analyses were performed using the SPSS™ 13.0 software package.

RESULTS

The mean follow-up period was 42 mo (range 8-166 mo), and was longer than 60 mo in 34 patients. In no case was the PEI procedure associated with mortality or complications requiring emergency treatment. Seeding of HCC to the abdominal wall was not recorded.

The 1-, 3-, and 5-year survival rate of the entire cohort was 95.8%, 73.4%, and 55.9%, respectively, and the estimated median survival rate was 78 mo [CI 95% 54.38-101.62] (Table 2). During the follow-up, 4 patients underwent OLT and 51 (34.5%) died. The causes of death were: liver failure due to progression of the neoplasm involving more than 50% of the liver parenchyma with or without invasion of the main intrahepatic vessels in 35 cases (68.6%), liver failure unrelated to tumor progression in 7 cases (13.7%), bleeding from esophageal or gastric varices in 2 cases (2.9%), and other extrahepatic diseases in 3 cases (sepsis, myocardial infarction, pulmonary embolism, 5.9%). The cause of death was unknown in 4 cases (7.8%).

Fifty-six patients showed local HCC recurrences, 61 distant recurrences, and 31 both local and distant recurrences. The median time elapsed between the PEI completion and the first detection of local or distant recurrence were 12 mo (CI 95% 7.0-16.5), and 21 mo (CI 95% 14.0-32.0), respectively. No cases of local recurrence were recorded after 36 mo from PEI completion. The overall DFS rate was 71.0%, 33.5%, and 24.1% at 1, 3, and 5 years, respectively (Table 2). Local recurrences were treated with PEI, RFA or transarterial chemoembolization (TACE) in 41 cases (73.2%). The recurrent lesions in segments different from that of the first neoplasm were treated with PEI, RFA, TACE or systemic therapy including tamoxifen, octreotide, or sorafenib in 34 cases (55.7%). In 15 cases of local recurrence and in 27 cases of distant recurrence, no treatment was applied due to one or more of the following reasons: end stage liver failure, multifocal or infiltrative pattern of recurrence with or without vascular invasion, and concomitant diagnosis of extrahepatic spreading.

Of the whole population, HCC size < 2 cm was the only pre-PEI parameter significantly linked to survival ($P = 0.009$). This parameter resulted to be an independent predictor of survival after multivariate analysis using the Cox regression model [HR 0.421 (0.216-0.821); $P = 0.011$]. Among the post-treatment parameters, only distant recurrence and portal invasion were significantly linked to survival ($P = 0.003$ for both parameters) (Table 1).

For further analysis, the 47 patients with a single HCC smaller than 2 cm were compared to the 108 patients with tumors larger than 2 cm. The clinical (age, sex, Child-Pugh class, ascites, portal thrombosis) and laboratory (HBsAg positive, anti-HCV positive, ALT, AST, prothrombin time ratio, bilirubin, albumin, platelet count, AFP) features were not significantly different between groups. As expected, the 1-, 3-, and 5- year survival rate of the patients with HCC < 2 cm (97.8%, 86.3%, and 73.0%) was significantly better than that of patients with larger tumors (94.8%, 67.5%, and 47.9%) ($P = 0.009$) (Figure 1). The estimated median survival of patients with HCC < 2 cm was longer than that of patients with HCC > 2 cm [93 mo (CI 95% 43.9-142.1) *vs* 60 mo (CI 95% 41.9-79.4)]. Furthermore, the cumulative 1-, 2- and 3-year local recurrence (13.2%, 21.6%, and 29.1% *vs* 28.2%, 43.7%, and 51.5%) of the patients with HCC < 2 cm was significantly lower than that of patients with larger tumors (log rank χ^2 0.825, $P = 0.011$). Likewise, the cumulative distant recurrence at 1-, 3-, and 5-years in patients with HCC < 2 cm was lower (2.3%, 27.2%, and 52.9% *vs* 11.0%, 49.6%, 62.2%); this difference demonstrated a clear trend towards statistical significance (log rank χ^2 5.338, $P = 0.054$). In order to analyze the possible influence of local on distant recurrence, we investigated the correlation between these 2 events. Interestingly, 62/92 (67.4%) of patients without local did not experience distant recurrence, while 31/56 (55.4%) of subjects with local developed distant recurrence of HCC.

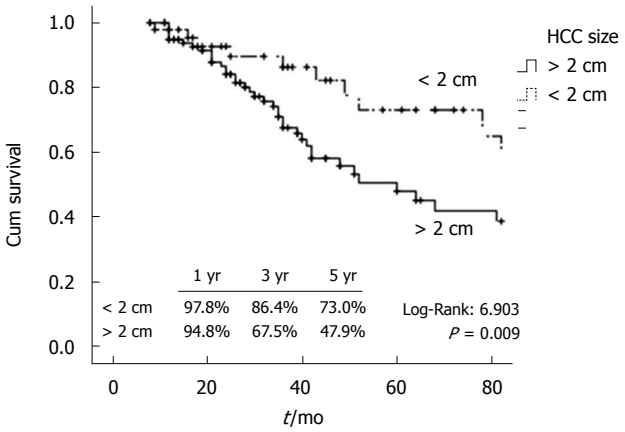

Figure 1 Comparison between cumulative survival rates in 47 patients with solitary hepatocellular carcinoma < 2 cm (dotted line) and 101 patients with solitary hepatocellular carcinoma > 2 cm (continuous line). HCC: Hepatocellular carcinoma.

On this ground, we found a positive correlation at Spearman-rho test (Coefficient: 0.224; $P = 0.006$) and calculated that subjects with local recurrence had an increased risk to develop distant recurrence with an OR of 1.698 (95% CI 1.165-2.473).

DISCUSSION

PEI has been the first ablation technique extensively used for the treatment of HCC and is usually indicated for lesions smaller than 3 cm in diameter since complete tumor necrosis may be achieved in 90%-100% of tumors smaller than 2 cm, 70%-80% of lesions between 2 and 3 cm, and about 50% of lesions between 3 and 5 cm[18]. Furthermore, PEI has been recently shown to achieve complete necrosis of the treated tumors at explant analysis in 38/59 patients (64.3%) undergoing liver transplantation[19]. However, PEI has been largely replaced by RFA during the last years, and this is due to the best predictability of the necrotic effect in all tumor sizes and to the best effectiveness in tumors larger than 2 cm[11]. According to some recent randomized trials involving tumors with a maximal size of 3 or 4 cm, RFA is more efficacious than PEI in terms of initial complete tumor necrosis rate (93%-100% *vs* 66%-100%), 3-year survival rate (63%-81% *vs* 48%-67%) and local tumor recurrence (8%-14% *vs* 22%-34%)[20-25]. Furthermore, a few recent meta-analytic studies support the superiority of RFA versus PEI in terms of patient survival and local disease recurrence[26-28]. However, when the analysis is restricted to lesions smaller than 2 cm, the superiority of RFA is questionable: a recent meta-analysis about the clinical outcomes of RFA, PEI and percutaneous acetic acid injection for HCC shows that for lesions smaller than 2 cm there is no significant difference between RFA and PEI for the proportion of patient mortality and for local recurrence[28].

Our long term cohort study confirms that PEI is still an effective treatment for compensated cirrhotics with a single HCC < 2 cm since the 5-year survival rate was as high as 73.0% and a tumor diameter up to this size was the only pre-treatment parameter independently linked to survival. This datum is comparable to that shown in eastern single center series. In a group of 270 cirrhotics with HCC treated by PEI, Ebara *et al*[13] described a subgroup of 96 Child-Pugh Class A patients with solitary HCC smaller than 2 cm with a 5-year survival of 78.3%. Omata *et al*[9] reported a survival rate of 70% in a series of 144 patients with a single HCC < 2 cm undergoing PEI. Less favourable results were shown by Arii *et al*[29] in a retrospective multicenter Japanese survey, showing a 54% survival rate in 767 patients with Stage I HCC < 2 cm submitted to PEI.

Available data from the literature do not provide unequivocally a survival benefit of RFA over PEI in these patients. A 5-year survival of 83.8% was reported by Tateishi *et al*[30] in a cohort of 87 patients with HCC lower than 2 cm treated by RFA. A similar rate of 5-year survival (83.8%) has been recently reported for Child-Pugh Class A patients with a single lesion of up to 2 cm treated with RFA and registered by the Liver Cancer Study Group of Japan[31]. However, in the recent western series by Livraghi *et al*[32] involving 218 patients with a single HCC < 2 cm undergoing ablation with RFA, the 5-year survival rate was 55% for the whole population and 68% for the subgroup of potentially operable patients.

In our cohort of patients, intrahepatic HCC progression was the cause of death in more than two thirds of the cases, and the 5-year survival of patients with a single nodule < 2 cm was significantly better than that of patients with HCC > 2 cm (73.0% *vs* 47.9%). This is not surprising, considering that in tumors > 2 cm the success of ethanol in achieving necrosis of the entire mass is limited by intratumoral septa, and that in most of these tumors PEI does not induce a peritumoral necrosis, preventing the persistence of peripheral minute neoplastic foci or extra-tumoral satellites[10]. Accordingly, in our group of patients, the cumulative local recurrence rate of tumors smaller than 2 cm was significantly lower than that of patients with larger HCC. The reappearance of a viable tumor in a lesion assessed as completely necrotic shortly after ablation implies, in most cases, the need for additional locoregional therapy and is negatively linked to survival. In a large study by Sala *et al*[33], among Child-Pugh Class A patients, the 5-year survival rate of patients achieving a sustained complete response at the end of follow-up was significantly higher than that of patients without a sustained complete response due to initial treatment failure, late local recurrence, or appearance of new HCC nodules outside the segment of the first neoplasm. Similarly, in our study, distant intrahepatic recurrence was linked to survival and occurred significantly more frequently in patients with tumors larger than 2 cm. This may be in part related to the insufficient local disease control obtained by PEI in tumors more than 2 cm large. Indeed, the reappearance of viable tumor tissue may promote the HCC spreading within the liver through microvessel invasion and satellitosis. The observation of a significantly increased risk of new HCC lesions within the liver in patients with local recurrence in this series reinforces this hypothesis.

In conclusion, our study shows that a tumor diameter of up to 2 cm is an independent predictor of survival in cirrhotics with single HCC treated by PEI. In this subgroup of patients, we observed a 5-year survival rate as high as 73.0%, significantly better than that observed in patients with solitary larger tumors. This datum supports the hypothesis that in compensated cirrhotic patients with small HCC up to 2 cm submitted to ablation the long term prognosis is excellent independently from the application of PEI or RFA as therapeutic procedure[10]. Although PEI could be less effective than RFA in providing complete necrosis of the tumor even in this subset of patients, this is counterbalanced by the universal feasibility of PEI in tumors located at risky sites for RFA, or in which a thermal ablation may be less efficient due to the proximity of large vessels. For these reasons, a large prospective randomized study, designed to assess the overall survival as primary end point according to the principles of the intention-to-treat analysis, taking into account the site of the tumor in the liver, and including a rigorous cost-effectiveness evaluation , is still needed.

COMMENTS

Background

Small hepatocellular carcinomas of up to 2 cm are increasingly being recognized due to the diffusion of the screening program for early diagnosis of this tumor in liver cirrhosis. The ideal treatment of such small lesions is a controversial issue.

Research frontiers

Prospective randomized studies comparing the available treatments for small hepatocellular carcinoma are lacking and should be planned.

Innovations and breakthroughs

This study shows that the survival rate of compensated cirrhotic patients with solitary hepatocellular carcinoma of up to 2 cm treated with percutaneous ethanol injection is higher than 70%. Furthermore, in the series of 148 patients with a single tumor up to 5.8 cm in size treated with this technique, a tumor diameter equal or lower than 2 cm was the only factor significantly linked to survival.

Applications

In authors' opinion, percutaneous ethanol injection is still a valuable treatment of small hepatocellular carcinoma of up to 2 cm in size emerging in liver cirrhosis. In this subset of patients, the demonstration of a better therapeutic performance of radiofrequency ablation in terms of overall survival and cost-effectiveness is still lacking.

Terminology

Percutaneous ethanol injection is a well standardized technique of chemical ablation of hepatocellular carcinoma. When introduced within the neoplasm through a fine-needle, ethanol shows a direct cytotoxic effect and produces coagulative necrosis, followed by fibrosis. In addition, due to the lesive effect of ethanol on the endothelial cells, tumor ischemic necrosis is elicited by thrombosis of the small tumor feeding vessels. The effectiveness of this technique has been questioned after the introduction in clinical practice of the thermal ablation techniques of liver tumors.

Peer review

This is an interesting series showing a continued role for PEI for single small HCC. However, my guess is that RFA has replaced PEI in most centers related to the number of procedures to achieve benefit. There are not many reports from western countries, making this report of interest and worthy of consideration for publication.

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BRIEF ARTICLE

Colorectal cancer screening behavior and willingness: An outpatient survey in China

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Abstract

AIM: To identity the factors influencing colorectal cancer (CRC) screening behavior and willingness among Chinese outpatients.

METHODS: An outpatient-based face-to-face survey was conducted from August 18 to September 7, 2010 in Changhai Hospital. A total of 1200 consecutive patients aged ⩾ 18 years were recruited for interview. The patient's knowledge about CRC and screening was pre-measured as a predictor variable, and other predictors included age, gender, educational level, monthly household income and health insurance status. The relationship between these predictors and screening behavior, screening willingness and screening approach were examined using Pearson's χ^2 test and logistic regression analyses.

RESULTS: Of these outpatients, 22.5% had undergone CRC screening prior to this study. Patients who had participated in the screening were more likely to have good knowledge about CRC and screening (OR: 5.299, 95% CI: 3.415-8.223), have health insurance (OR: 1.996, 95% CI: 1.426-2.794) and older in age. Higher income, however, was found to be a barrier to the screening (OR: 0.633, 95% CI: 0.467-0.858). An analysis of screening willingness showed that 37.5% of the patients would voluntarily participated in a screen at the recommended age, but 41.3% would do so under doctor's advice. Screening willingness was positively correlated with the patient's knowledge status. Patients with higher knowledge levels would like to participate in the screening (OR: 4.352, 95% CI: 3.008-6.298), and they would select colonoscopy as a screening approach (OR: 3.513, 95% CI: 2.290-5.389). However, higher income level was, again, a barrier to colonoscopic screening (OR: 0.667, 95% CI: 0.505-0.908).

CONCLUSION: Patient's level of knowledge and income should be taken into consideration when conducting a feasible CRC screening.

Key words: Colorectal cancer; Screening; Behavior; Willingness; Survey

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INTRODUCTION

Colorectal cancer (CRC) is one of the most common malignancies worldwide. The Asian Pacific Working Group on CRC has suggested that some Asian ethnic groups (e.g. Japanese, Korean and Chinese) are more susceptible to CRC than others, with an incidence similar to that of the West[1]. The incidence of CRC in China has increased rapidly since the 1980s[2,3]. CRC now ranks as the fifth leading cause of cancer-related deaths[4]. Screening is an effective tool for early diagnosis[5], but the compliance rates have been low in many countries[6-10]. A study of community-based CRC screening in Hangzhou, China, which involved a population of 34 726 individuals, revealed that the compliance rates for fecal occult blood test (FOBT) and colonoscopy were only 17.5% and 2.8%, respectively[11]. These figures are extremely low compared with those of the United States (overall screening rates were nearly 55% in 2008[12]).

Reasons for low compliance rates may vary among countries. A community-based screening among residents of Beijing revealed that busy work schedules and the complexity of screening procedures were the main barriers to CRC screening[13]. Lack of financial support, fear of pain and the necessity of bowel preparation were barriers to colonoscopic screening in a Hangzhou-based study[14]. Other researchers have doubted the feasibility of population screening in China, due to the requirements for a high awareness of the disease, sufficient medical resources and strong financial support[15]. Opportunistic screening (also called individual screening), which is performed on request from a physician or healthcare provider when a patient presents for consultation for other health reasons[16], has been widely used in most cancer screening protocols throughout the world[17] and may be also suitable for Chinese outpatients. Individuals with more personal experience with illness are more likely compliant with the CRC screening[18]. China has a large population but with an uneven distribution of health resources. Additionally, the general population has a low awareness of CRC and inadequate knowledge regarding CRC and screening[19,20].

Although CRC screening of outpatients might be effective, there have been few studies exploring its availability in China. The 2010 National Institutes of Health (NIH) State-of-the-Science Conference, which aimed to enhance the use and quality of CRC screening, recommended that studies should be carried out about patient screening preferences and other factors influencing informed, shared decision making regarding the choice of CRC screening modalities[12]. Therefore, our study was intended to explore outpatients' screening behavior and willingness as well as to identify influencing factors in Shanghai, China.

MATERIALS AND METHODS

Study population

From August 18 to September 7, 2010, 1200 consecutive outpatients were recruited for our survey from the Outpatient Department of Changhai Hospital, a tertiary care hospital in Shanghai, China. Both sporadic and hereditary cases of CRC were the target of early detection. All these outpatients were over 18 years of age, able to communicate properly and free of mental disorders. Patients with a medical emergency or incurable tumor were excluded. Health care workers including doctors, nurses, medical educators and medical students were not included as subjects of this study.

Study design

A self-designed questionnaire was developed after a literature review, and revised by epidemiologists and clinicians. The following contents were included: (1) Patient general information; (2) evaluation of CRC and screening knowledge; (3) previous screening behavior; (4) screening willingness; and (5) preferred approach. Available screening approaches in our hospital could be classified as fecal test (e.g. FOBT, stool DNA test), blood test (biomarkers in clinical research) and colonoscopy.

A pilot test was conducted in 50 outpatients by trained interviewers on August 18 to verify the feasibility of the survey. The questionnaire was distributed to the patients upon their arrival at the clinic, who were asked to answer the questions under the guidance of interviewers while waiting to see a doctor. To ensure the quality of the survey, additional information about screening was offered to guide the patient's choice of screening approach. This study was approved by the Ethics Committee of Changhai Hospital, and all patients gave written informed consent.

Comparing with other scoring system that evaluated the knowledge about CRC and screening[20,21], several simple factors were taken into account in the evaluation: (1) is the patient familiar with CRC; (2) does the patient understand at least one of the clinical manifestations of CRC; (3) has the patient ever heard of cancer screening; and (4) is the patient familiar with colonoscopy as an early detection method for CRC? Patients were classified as having a high level of knowledge (answered all the questions above), a low level (answered no more than 2 questions) and a moderate level (between high and low).

Statistical analysis

Data were managed using Microsoft Excel software, and duplicate questionnaires were excluded. The results were tabulated and analyzed with the PASW Statistics for Windows release 18.0 (SPSS, Inc., Chicago, Illinois). The primary outcomes were the patient's previous screening behavior, screening willingness and preferred screening approach. Pearson's χ^2 test was used to quantify the association between the outcomes and the predictor variables, which included gender, age, possession of health insurance and monthly household income. A bivariate logistic regression model was used to examine the association between the outcomes and levels of education and knowledge about CRC. Statistical significance was considered at $P < 0.05$, and odds ratios (OR) were given with 95% confidence intervals (CI).

Table 1 Characteristics of respondents

Patient characteristics	Number (n = 1001)	Percent (%)
Gender		
Female	510	50.9
Male	491	49.1
Age (yr)		
< 40	397	39.7
⩾ 40	604	60.3
Educational level		
Primary or no schooling	69	6.9
Secondary education	445	44.5
Higher education	487	48.7
Monthly household income		
< 4000 RMB (yuan)[1]	538	53.7
⩾ 4000 RMB (yuan)	463	46.3
Health insurance		
No[2]	358	35.8
Yes	643	64.2
Previous CRC screening		
No	775	77.4
Yes[3]	226	22.6
Screening willingness		
Voluntary attendance	375	37.5
Under recommendation	413	41.3
No attendance	213	21.3
Preferred screening approach		
Blood test	249	24.9
Fecal test	186	18.6
Colonoscopy	322	32.2
Not specified	244	24.4
Knowledge about CRC and CRC screening		
Low	288	28.8
Moderate	247	24.7
High	466	46.6

[1]Renminbi is the official currency of the People's Republic of China; [2]Including the status of health insurance application; [3]Including colonoscopy, fecal occult blood test (FOBT) and double contrast barium enema (DCBE). CRC: Colorectal cancer.

RESULTS

A total of 1200 consecutive patients were recruited for the survey. Of these, 1029 (85.75%) were successfully surveyed, and 171 (14.25%) did not respond to this survey. Among the 1029 respondents, 28 (2.72%) were found to have unfilled sections, but no duplicate data were detected. Ultimately, 1001 patients were included in our analysis. A total of 604 patients were not less than 40 years of age, which is the recommended minimal screening age in China for sporadic CRC[22]. The mean age of the patients was 45.25 years (range, 18-86 years). Patients were classified as having a high (n = 466, 46.6%), moderate (n = 247, 24.7%) or low (n = 288, 28.8%) levels of knowledge according to our definitions. Other predictor variables, such as educational level and monthly household income, are listed in Table 1.

Previous CRC screening behavior

Among the 1001 included patients, 22.5% (n = 226) had previously undergone CRC screening. The most common examination method used was colonoscopy (91.6%); other methods (FOBT or double contrast barium enema, DCBE) accounted for a small proportion (8.4%). Factors influencing the participation in the screening were age, possession of health insurance, monthly household income and status of CRC knowledge (Table 2). Patients who had been screened tended to have a good knowledge of CRC and screening (OR: 5.299, P < 0.001), have health insurance (OR: 1.996, P < 0.001) and are older in age (OR: 3.834, P < 0.001). High income, however, was found to be a barrier to the screening (OR: 0.633, P < 0.003).

Screening willingness

The analysis of screening willingness revealed that 37.5% of patients (n = 375) would voluntarily agree to be screened at the recommended age; 41.3% (n = 413) would need a physician's recommendation before attending the screening; and 21.3% (n = 213) refused to be screened (Table 1). We categorized the screening willingness into "attendance" (n = 788) and "rejection" (n = 213) and found that knowledge regarding CRC was the only factor influencing the screening willingness (Table 3). Patients with a high level of knowledge about CRC were more willing to attend the screening than those with a poor knowledge of CRC (OR: 4.352, P < 0.001).

Screening approach

The analysis of patients' preference in screening approach revealed that colonoscopy was the most commonly preferred approach (32.2%, n = 322), while blood testing ranked second (24.9%, n = 249), and a fecal test was the least popular option (18.6%, n = 186). However, 24.4% of patients (n = 244) expressed an equivalent preference for all screening approaches (Table 1).

Colonoscopy is the most precise screening approach for CRC. Thus, the screening approaches were characterized into "precise modes (colonoscopy)" and "normal modes (blood and fecal tests)", and factors influencing the patient's selection of screening approach were investigated. Both CRC-associated level of knowledge and monthly household income influenced the choice of screening approach (Table 4). With an increase in knowledge, the proportion of patients selecting a precise screening approach was increased from 25.4% to 54.4% (P < 0.001). Patients with higher incomes, however, prefer not to adopt precise screening approaches on average (P = 0.010).

DISCUSSION

In this outpatient-based study, we found that a high level of knowledge regarding CRC and screening techniques, possession of health insurance or advanced age were stimulus factors for prior CRC screening. Most of the patients were willing to participate in the screening, but 41.3% were willing to do so under doctor's recommendations before attendance. Level of knowledge was the only factor that influenced screening willingness. Outpatients with a higher level of knowledge were willing to participate in the screening and select colonoscopy as the screening approach. Higher income level, however, was a barrier to both the previous screening and the preference of colonoscopy as a screening methodology. These

Table 2 Factors associated with outpatients' previous screening behavior *n* (%)

Variable	Previously screened		OR (95% CI)	*P* value
	No (*n* = 775)	Yes (*n* = 226)		
Gender				
Female	401 (78.6)	109 (21.4)	1.000	0.365
Male	374 (76.2)	117 (23.8)	1.151 (0.856-1.548)	
Age (yr)				
< 40	356 (89.7)	41 (10.3)	1.000	< 0.001
⩾ 40	419 (69.4)	185 (30.6)	3.834 (2.657-5.532)	
Health insurance				
No	303 (84.6)	55 (15.4)	1.000	< 0.001
Yes	472 (73.4)	171 (26.6)	1.996 (1.426-2.794)	
Educational level				
Primary or no schooling	52 (75.4)	17 (24.6)	1.000	-
Secondary education	319 (71.7)	126 (28.3)	1.208 (0.673-2.169)	0.526
High education	404 (83.0)	83 (17.0)	0.628 (0.346-1.141)	0.127
Monthly household income, RMB (yuan)				
< 4000	397 (73.8)	141 (26.2)	1.000	0.003
⩾ 4000	378 (81.6)	85 (18.4)	0.633 (0.467-0.858)	
Level of knowledge				
Low	261 (90.6)	27 (9.4)	1.000	-
Moderate	213 (86.2)	34 (13.8)	1.543 (0.902-2.639)	0.113
High	301 (64.6)	165 (35.4)	5.299 (3.415-8.223)	< 0.001

OR: Odds ratio; CI: Confidence interval.

Table 3 Factors associated with outpatients' screening willingness *n* (%)

Variable	Screening willingness		OR (95% CI)	*P* value
	Rejection[1] (*n* = 213)	Attendance[2] (*n* = 788)		
Gender				
Female	120 (23.5)	390 (76.5)	1.000	0.089
Male	93 (18.9)	398 (81.1)	1.317 (0.971-1.786)	
Age (yr)				
< 40	76 (19.1)	321 (80.9)	1.000	0.207
⩾ 40	137 (22.7)	467 (77.3)	0.807 (0.589-1.105)	
Health insurance				
No	73 (20.4)	285 (79.6)	1.000	0.630
Yes	140 (21.8)	503 (78.2)	0.920 (0.670-1.265)	
Educational level				
Primary or no schooling	16 (23.2)	53 (76.8)	1.000	-
Secondary education	109 (24.5)	336 (75.5)	0.931 (0.511-1.695)	0.814
High education	88 (18.1)	399 (81.9)	1.369 (0.748-2.506)	0.309
Monthly household income, RMB (yuan)				
< 4000	123 (22.9)	415 (77.1)	1.000	0.189
⩾ 4000	90 (19.4)	373 (80.6)	1.228 (0.905-1.668)	
Level of knowledge				
Low	106 (36.8)	182 (63.2)	1.000	-
Moderate	52 (21.1)	195 (78.9)	2.184 (1.481-3.221)	< 0.001
High	55 (11.8)	411 (88.2)	4.352 (3.008-6.298)	< 0.001

[1]Patients rejected to attend screening; [2]Patients would attend screening voluntarily or under recommendation. OR: Odds ratio; CI: Confidence interval.

results indicated that patients' knowledge and income status should be considered when launching a screening program among outpatients in Shanghai.

To our knowledge, this is the first study to investigate outpatients' CRC screening behavior and to identity their screening preferences in China. The advantages of this study are the use of a prospective face-to-face survey of consecutive outpatients and a relatively large sample size. We attempted to establish a simple method to rapidly evaluate patients' levels of knowledge regarding CRC and screening techniques. This method differs from other scoring systems. Our method allows the physician to evaluate the patient's level of knowledge through asking several simple questions, and an appropriate screening approach can be offered immediately following the evaluation.

Our results have several similarities to those of previous population-based studies that explored factors influencing CRC screening[20,23-26] and analyzed CRC screening willingness in Malaysia[21] and Taiwan[27]; however, there

Table 4 Factors associated with outpatients' choice of screening approach *n* (%)

Variable	Screening approach		OR (95% CI)	*P* value
	Normal[1] (*n* = 435)	Precise[2] (*n* = 322)		
Gender				
Female	233 (59.1)	161 (40.9)	1.000	0.340
Male	202 (55.6)	161 (44.4)	1.153 (0.864-1.539)	
Age (yr)				
< 40	163 (58.8)	114 (41.2)	1.000	0.593
⩾ 40	272 (56.7)	208 (43.3)	1.093 (0.810-1.476)	
Health insurance				
No	149 (59.1)	103 (40.9)	1.000	0.533
Yes	286 (56.6)	219 (43.4)	1.108 (0.815-1.505)	
Educational level				
Primary or no schooling	27 (54.0)	23 (46.0)	1.000	-
Secondary education	197 (57.9)	143 (42.1)	0.852 (0.469-1.547)	0.599
High education	211 (57.5)	156 (42.5)	0.868 (0.479-1.571)	0.640
Monthly household income, RMB (yuan)				
< 4000	226 (53.3)	198 (46.7)	1.000	0.010
⩾ 4000	209 (62.8)	124 (37.2)	0.677 (0.505-0.908)	
Level of knowledge				
Low	103 (74.6)	35 (25.4)	1.000	-
Moderate	136 (72.0)	53 (28.0)	1.147 (0.697-1.887)	0.590
High	196 (45.6)	234 (54.4)	3.513 (2.290-5.389)	< 0.001

[1]Blood and feces test; [2]Colonoscopy. OR: Odds ratio; CI: Confidence interval.

have also been some inconsistent results.

As shown in the previous studies, a better knowledge of CRC and screening is related to a higher participation rate in population-based screening[20,23-26]. Among our patients, better knowledge was associated with the previous screening. This association is consistent with qualitative evidence in which lack of knowledge about CRC and screening has been cited as a barrier to screening participation in the United States, Canada and China[28].

Lack of health insurance is an important barrier to the screening participation among ethnic groups with all levels of education[29,30]. The US-based 2005 National Health Interview Survey (NHIS) showed that 19% of respondents with no insurance reported having CRC screening (FOBT or endoscopy), compared with over 39% of those who had insurance[29]. In our study, health insurance status was positively associated with the screening behavior. This is an important finding for outpatient screening because more than half of the patients (64.2%) were covered by health insurance. Their compliance with CRC screening may be relatively easy to promote if appropriate screening advice is offered.

Factors that could enhance the screening willingness in previous studies included the followings: being a close relative of a CRC patient[31], perceived susceptibility, perceived less barriers to screening, doctor's recommendation and personal contact with friends or relatives having CRC[21]. In Taiwan, factors related to intentions to have FOBT were influenced by the inconvenience and the unpleasantness of the screening procedure. Participants' gastrointestinal symptoms or family histories and physicians' recommendation or patients' health conditions were relevant to the intentions for a flexible sigmoidoscopic or colonoscopic screening[27]. Additionally, a knowledge of CRC symptoms was associated with willingness to be screened in Malaysia on univariate analysis but not on multivariate analysis[21]. Among the patients in our survey, the knowledge regarding CRC and screening was an important factor that influenced screening willingness, meanwhile 41.3% patients expressed that they would need doctor's recommendation before attending the screening. So interventional studies which intend to increase the patients' knowledge regarding CRC and screening would help enhance the screening willingness.

Income level is another important factor affecting an individual's decision to be screened. Patients with more affluent socioeconomic status have been shown to have a higher average rate of screening than the less affluent[8,32,33]. However, in our study, the high-income patients were found to have a lower rate of screening and the reluctance of colonoscopic screening. This opposite phenomenon might be related to some cultural reasons. High-income patients live in better conditions and tend to get good treatment, so they are less concerned about using the preventive screening because they are more "healthy". Similar trend was detected in a Hong Kong population who perceived their health status to be good and had a less concern about contracting CRC than those who perceived a fair or poor health status[19]. The reluctance of high-income patients to take colonoscopic screening may also be influenced by the complexity of bowel preparation and the uncomfortable feeling caused by colonoscopy.

There are several limitations in this study. First, it was based in a single center. Our preliminary results on outpatient behavior and willingness cannot represent all the outpatients in Shanghai. Second, some patients (14.25%) did not respond to our survey, although great efforts were made to publicize the significance of the survey.

This may cause some patient selection bias, and a multicenter survey may be needed to confirm our results. However, our hospital, which is the largest endoscopy center in Shanghai, attracts many patients for this procedure. Therefore, our results are fairly representative of urban outpatient clinics.

In conclusion, most of the outpatients are willing to participate in CRC screening. A better knowledge about CRC and screening techniques is positively correlated with previous screenings, higher willingness to participate in the screening and a preference for colonoscopy as a screening methodology. However, a higher income level is a barrier to the screening behavior and the selection of colonoscopy. These results may have some implications for outpatient CRC screening and may help guide the further interventional studies.

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COMMENTS

Background

The incidence of colorectal cancer (CRC) in China has increased since the 1980s. Screening is an effective method for early detection of CRC.

Research frontiers

Opportunistic screening which screened CRC among outpatients might be more effective in China, but has not been well illustrated. Studies exploring patient's screening preferences and factors influencing the choice of colorectal cancer screening modalities are needed before the screening is started. In this study, the authors demonstrated the factors influencing outpatients' screening behavior and willingness in Shanghai, China.

Innovations and breakthroughs

This is the first study to report outpatients' screening behavior and willingness as well as to identify influencing factors in Shanghai, China. The results indicate that patients' levels of knowledge and income should be considered when launching a screening program among outpatients.

Applications

By understanding what factors will influence colorectal cancer screening behavior and willingness among Chinese outpatients, this study has provided some implications for screening practice and may help guide further interventional studies.

Peer review

It is a very interesting research for the readers, the conclusions are very valuable and it should be accepted for publication in the journal.

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BRIEF ARTICLE

High incidence of biliary complications in rat liver transplantation: Can we avoid it?

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Abstract

AIM: To investigate how to reduce the incidence of biliary complications in rat orthotopic liver transplantation.

METHODS: A total of 165 male Wistar rats were randomly divided into three groups: Group A, orthotropic liver transplantation with modified "two-cuff" technique; Group B, bile duct was cut and reconstructed without transplantation; and Group C, only laparotomy was performed. Based on the approaches used for biliary reconstruction, Group A was divided into two sub-groups: A1 (n = 30), duct-duct reconstruction, and A2 (n = 30), duct-duodenum reconstruction. To study the influence of artery reconstruction on bile duct complication, Group B was divided into four sub-groups: B1 (n = 10), duct-duct reconstruction with hepatic artery ligation, B2 (n = 10), duct-duct reconstruction without hepatic artery ligation, B3 (n = 10), duct-duodenum reconstruction with hepatic artery ligation, and B4 (n = 10), duct-duodenum reconstruction without hepatic artery ligation. The samples were harvested 14 d after operation or at the time when significant biliary complication was found.

RESULTS: In Group A, the anhepatic phase was 13.7 ± 1.06 min, and cold ischemia time was 50.5 ± 8.6 min. There was no significant difference between A1 and A2 in the operation duration. The time for biliary reconstruction was almost the same among all groups. The success rate for transplantation was 98.3% (59/60). Significant differences were found in the incidence of biliary complications in Groups A (41.7%), B (27.5%) and C (0%). A2 was more likely to have biliary complications than A1 (50% *vs* 33.3%). B3 had the highest incidence of biliary complications in Group B.

CONCLUSION: Biliary complications are almost inevitable using the classical "two cuff" techniques, and duct-duodenum reconstruction is not an ideal option in rat orthotopic liver transplantation.

Key words: Rat; Liver transplantation; Biliary complication; Animal model

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INTRODUCTION

The occurrence of biliary complications (BC) remains one of the most critical challenges in clinical liver transplantation. According to the literature, the incidence of biliary complication for living-donor liver transplantations (LDLT) is as high as 64%[1-9]. Biliary complications have made biliary reconstruction the "Achilles heel" of liver transplantation. In rat liver transplantation, the occurrence of biliary complications has become a confounding factor in the judgment of experimental results and an obstacle to the practice of transplantion. Unfortunately, few researches could be found to report the incidence of biliary complications in rat liver transplantation. In this study, we investigated the biliary complications after rat orthotopic liver transplantation (ROLT), trying to find a better approach to biliary reconstruction and reduce the incidence of biliary complications after transplantation.

MATERIALS AND METHODS

Animals

Male Wistar rats weighing 200-250 g, were purchased from the Experimental Animal Center of Sun-Yat Sen University and fed in the specific pathogen free (SPF) animal lab. The weight of the recipient was similar to that of the donor. All animals had free access to food and water except the recipients, which had been fasted for 12 h before operation.

Technique

ROLTs were performed using the "two-cuff" technique established by Kamada[10,11]. All surgical procedures were performed by a single operator under naked eye. Napental was used for the anesthesia (40 mg/kg). All experiments were performed in compliance with the standards for animal use and care set by institutional animal care and use Committee.

Donor operation

After laparotomy, the left subphrenic vein was ligated and all the perihepatic ligaments were divided. The bile duct was incised and a stent (0.9-mm inner diameter, 4-mm length) was introduced and tied firmly. The right renal vein was dissociated and the right adrenal venous plexus was ligated. After 150 units of heparin was injected to form systemic heparinization, the liver was irrigated with physiological saline containing heparin (20 U/mL) through the aorta distal to the celiac artery. At the same time, infrahepatic inferior vena cava (IHIVC) and suprahepatic inferior vena cava (SHIVC) were dissected to allow outflow of the perfusate. When the liver turned khaki color, SHIVC was divided along the diaphragm (without the phrenic ring) , and the right renal vein, the portal vein (PV) and IHIVC were skeletonized and divided. The liver was then harvested and the graft was preserved at 4℃ in physiological saline with 20 U/mL heparin.

The PV was induced through the cuff (2-mm inner diameter, 3.5-mm length) and the distal end of the vein was completely reversed and fixed onto the cuff with a 5-0 silk ligation. The same method was used to prepare the cuff (3-mm inner diameter, 4-mm length) for IHIVC. The SHIVC was treated with two 8-0 silk sutures pierced via the two corners of the vein.

Recipient operation

After laparotomy, the self-made retractor was used to expose the operative area, and the left subphrenic vein. The transport vessels between the left liver and esophagus, hepatic artery and the right adrenal venous plexus were ligated orderly. One necessary step was to put a rubber under the SHIVC for the purpose of traction when removing the liver. Then IHIVC and PV were clamped to the anhepatic phase. SHIVC was blocked after exsanguination and the liver removed quickly. SHIVC was sutured by an end to end anastomosis (8-0, nylon suture), PV was reconstructed by means of cuff technique and the anhepatic phase was ended. The same method was used to reconstruct IHIVC. Based on the experimental design, the bile duct was reconstructed differently.

The recipient rats were fasted for at least 12 h after operation but water was permitted.

Biliary reconstruction and hepatic artery ligation

There were two ways to rebuild the biliary tract in this experiment: (1) end-to-end anastomosis with the stent; and (2) end-to-side anastomosis between bile duct and duodenum (1-2 cm away from the pylorus) with the stent.

Experimental design

One hundred and sixty-five male Wistar rats were randomly divided into three groups: Group A, orthotropic liver transplantation by modified two-cuff method; Group B, bile duct was cut and reconstructed without orthotopic liver transplantation; and Group C, sham-operation group. Based on the approaches of biliary reconstruction, Group A was divided into two sub-groups: A1 (n = 30), duct-duct reconstruction, and A2 (n = 30), duct-duodenum reconstruction. To study the influence of hepatic artery on bile duct complication, Group B was divided into four subgroups: B1 (n = 10), duct-duct reconstruction with hepatic artery ligation; B2 (n = 10), duct-duct reconstruction without hepatic artery ligation; B3 (n = 10), duct-duodenum reconstruction with hepatic artery ligation, and B4 (n = 10), duct-duodenum reconstruction without hepatic artery ligation. In Group C (n = 5), only laparotomy was performed.

Samples were harvested 14 d after operation or at the time when any significant biliary complication was found. Serologic samples were collected to test the levels of aspartate transaminase (AST), alanine transaminase (ALT), total bilirubin (TBIL), direct bilirubin (DBIL), gummaglutamyl transpeptidase (GGT) and alkaline phosphatase (ALP). Biliary complications were determined by pathologic examination and serologic analysis.

Statistical analysis

Data were expressed as mean ± SD. Statistical differences

Table 1 Incidence rates of biliary complications in different groups

Groups	*n*	Abscess	Sludge	EBD	IBD	Total	
Group A							41.7%[e] (25/60)
A1	30	26.70% (8/30)	13.30% (4/30)	26.70% (8/30)	13.30% (4/30)	33.30% (10/30)	
A2	30	43.30% (13/30)	20% (6/30)	26.70% (8/30)	10% (3/30)	50%[a] (15/30)	
Group B							32.50% (13/40)
B1	10	10% (1/10)	0	30% (3/10)	0	30% (3/10)	
B2	10	10% (1/10)	0	20% (2/10)	10% (1/10)	20% (2/10)	
B3	10	30% (3/10)	20% (2/10)	40% (4/10)	10% (1/10)	50%[c] (5/10)	
B4	10	20% (2/10)	10% (1/10)	30% (3/10)	10%*t* (1/10)	30% (3/10)	
Group C	5	0	0	0	0	0	0
Total	105	26.70% (28/105)	12.40% (13/105)	26.70% (28/105)	9.52% (10/105)	36.20% (38/105)	

Some rats had two (or more) kinds of biliary complications at the same time. [a]$P < 0.05$ *vs* A1; [c]$P < 0.05$ *vs* B1, B2 and B4; [e]$P < 0.05$ *vs* groups B and C EBD: Extrahepatic biliary dilatation; IBD: Intrahepatic biliary dilatation.

between the control and the experimental groups were analyzed using analysis of variance. $P < 0.05$ was considered statistically significant.

RESULTS

Operation time and success rate

In transplantation groups, the anhepatic phase was 13.7 ± 1.06 min, and cold ischemia time was 50.5 ± 8.6 min. There was no significant difference between A1 and A2 in operating time ($P > 0.05$). And the time for biliary reconstruction was almost the same among all groups ($P > 0.05$).

Recipients surviving at least 24 h were considered as success. The success rate of ROLT was 98.3% (59/60). Only one case in A1 died from bleeding at SHIVC 8 h after operation.

Biliary complications

The incidence rate of biliary complications was 41.7% in Group A, which was much higher than that in Group B (32.5%) and Group C (0%), with significant differences among the three groups ($P < 0.05$). After transplantation, A2 had a higher incidence of biliary complications than A1 (50% *vs* 33.3%, $P < 0.05$). B3 had the highest incidence of biliary complications among the groups without orthotopic liver transplantation ($P < 0.05$). No biliary complication was found in the sham-operation group.

General observation

The color of urine turned yellow in the rats with biliary complications. Other complications were dried hair, reaction retardation, reduced appetite and activities, and eye bleeding in some rats.

Gross anatomy

Biliary complications consisted of abscess, intrahepatic and extrahepatic biliary dilatation and biliary sludge. Abscess (35%) was most frequently seen in Group A, compared with dilatation of extrahepatic bile duct (25%) in Group B. B3 had the highest incidence of biliary complications in non-transplantation groups (50% *vs* 30%, 20% and 30%) and extrahepatic biliary dilatation and abscess were two of the most important complications in this group (Table 1).

Histopathology

In the samples with biliary complications, infiltration of a large number of mononuclear cells in the portal area was the most common change, followed by dilatation of bile ducts. Cellular infiltration of biliary wall and degeneration of epithelial cells, dilatation of the central vein could also be seen. Some samples even showed vacuolar degeneration, necrosis and fibrous tissue hyperplasia. In samples with severe biliary dilatation, the normal bile duct structure had completely disappeared, with a large number of infiltrated inflammatory cells.

Serology

The serum levels of AST, ALT, TBIL, DBIL, GGT and ALP in Group A and Group B were significantly higher than in Group C, particularly AST and TBIL, with significant difference among the three groups ($P < 0.05$). Significant difference could be easily observed between duct-duodenum reconstruction groups and duct-duct reconstruction groups (A2 *vs* A1, B3 *vs* B1, and B4 *vs* B2, $P < 0.05$). Among non-transplantation groups, the serological changes, especially the bilirubin level, were more remarkable in groups with hepatic artery ligation than in those without (B1 *vs* B2 and B3 *vs* B4, $P < 0.05$) (Table 2).

DISCUSSION

Biliary complication is the second most common cause of graft dysfunction in liver transplantation with an incidence rate of as high as 64%[1-9]. Biliary complications may be related to various factors, including hepatic artery thrombosis or stenosis, technical reasons, as well as ischemia-reperfusion injury and immunological injury. High incidence of biliary complication has made biliary reconstruction the "Achilles heel" of liver transplantation.

It has been proved that hepatic artery plays an important role in blood supply of bile duct, the artery must be reconstructed when injured or cut during operation. In the early 90s, Engermann *et al*[12] showed that reconstruction of the hepatic artery can significantly reduce the incidence of biliary complications such as biliary fistula and biliary obstruction after ROLT. But with the cuff method introduced in 1973, ROLT without hepatic artery (HA) recon-

Table 2 Serological values in different groups

	n	AST (U/L)	ALT (U/L)	TBIL (μ mol/L)	DBIL (μ mol/L)	GGT (U/L)	ALP (U/L)
A1	30	452.2 ± 296.9	223.7 ± 194.7	17.1 ± 26.0	14.8 ± 24.1	10.5 ± 6.2	366.5 ± 173.0
A2	30	534.2 ± 373.3	272.8 ± 274.7	11.6 ± 21.8	10.2 ± 20.2	8.0 ± 7.3	282.8 ± 154.9
B1	10	146.6 ± 43.6	75 ± 17.7	0.79 ± 1.59	0.54 ± 1.26	4.6 ± 3.8	208.6 ± 76.4
B2	10	108.7 ± 26.4	70.0 ± 6.6	0.33 ± 0.25	0.09 ± 0.12	2.5 ± 0.97	190.2 ± 44.2
B3	10	359.3 ± 452.4	126.6 ± 131.9	15.1 ± 31.3	11.5 ± 24.1	7.3 ± 5.1	240 ± 152.0
B4	10	271.2 ± 275.2	119.3 ± 138.2	9.7 ± 27.3	7.42 ± 21.5	5.7 ± 2.8	236.3 ± 158.3
Group C	5	81.7 ± 13.2	60.7 ± 4.5	0.13 ± 0.15	0 ± 0	2.7 ± 0.58	281.7 ± 139.3

ALT: Alanine aminotransferase; ALP: Alkaline phosphatase; AST: Aspartate aminotransferase; TBIL: Total bilirubin; DBIL: Direct bilirubin; GGT: Gumma-glutamyl transpeptidase.

struction became globally accepted [13-18]. It is reasonable to speculate that biliary complications after liver transplantation might be more common in rats than that in human beings. Unfortunately, few studies can be found to investigate and report the incidence of biliary complications in ROLT. In our study, we simulated the biliary processes of ROLT in Group B, and found that the incidence of complications were significantly higher in groups with hepatic artery ligation than those without, which meant that hepatic artery might play an important role in the occurrence and development of biliary complications, but further investigations are needed to verify the definite mechanism.

Bile duct reconstruction by stent has been well accepted since Kamada introduced the "two-cuff" technique in ROLT[12-18]. But in view of our experience in over 600 cases of rat orthotopic liver transplantation, it seems that biliary complications after orthotopic liver transplantation are almost inevitable in the classical model. Several reasons might be contributed to this: Firstly, the wall of bile duct will become thicker as the rat grows up, constant inner diameter of the stent will definitely make the bile duct relatively narrow. Secondly, the stent would become a foreign body in bile, which will make the eddy come into being at the proximal stent and induce the sludge. So it is a great challenge to seek a new approach to modify the ROLT model. This new approach should reduce the incidence of biliary complications, and be easier to achieve.

Choledochojejunostomy has been proved to be an effective surgery to reconstruct the bile duct in clinical liver transplantation, which does not increase the incidence of biliary complications compared with the end-to-end anastomosis[19-22]. In our study, we simulated the method and designed the duct-duodenum reconstruction model. Unfortunately, this method did not show ideal results, the incidence of biliary complications was significantly higher in A2 (duct-duodenum reconstruction) than that in A2 (duct-duct reconstruction) due to the following reasons: in ROLT, the stent is directly driven into the upper part of the duodenum, which makes the stent easily obstructed by chime and then more likely to have biliary complications. Based on our study, although choledochojejunostomy has been widely used in liver transplantation, duct-duodenum reconstruction is obviously not an ideal choice in ROLT.

In conclusion, biliary complications are almost inevitable using the classical "two-cuff" technique. More attention should be paid to the occurrence of biliary complications in ROLT model. The established mode of hepatic artery and biliary reconstruction should be modified.

COMMENTS

Background

Biliary complication is the second most common cause of graft dysfunction in liver transplantation with an incidence rate of as high as 64%. Unfortunately, few researches could be found to report the incidence of biliary complications in rat liver transplantation. In this study, the authors investigated the biliary complications after rat orthotopic liver transplantation, trying to find a better way to perform biliary reconstruction and reduce the incidence of biliary complications after transplantation

Research frontiers

Classical rat liver transplantation model using the "two-cuff" technique introduced by Kamada has been well accepted since 1983, but the incidence of biliary complications remains extremely high, there is an urgent need to improve the technology for biliary reconstruction.

Innovations and breakthroughs

Most previous studies used the "two-cuff" technique to establish rat liver transplant models, but few focused on the high incidence of biliary complications. In view of the experience in over 600 cases of rat orthotopic liver transplantation in this study, biliary complications are almost inevitable by using the classical "two-cuff" technique.

Applications

More attention should be paid to the occurrence of biliary complications in ROLT model. The established mode of hepatic artery and biliary reconstruction should be modified.

Terminology

Biliary complications: often include hilar abscess, intrahepatic and extrahepatic biliary dilatation, biliary sludge. "Two-cuff" technique: A classical method used in rat liver transplantation, which was first introduced by Kamada *et al*. The key steps are: Portal vein and infrahepatic inferior vena cava are induced through the cuffs and the distal end of the vein is completely reversed and fixed onto the cuff with a 5-0 silk ligation.

Peer review

The paper is well designed and the experience is valuable to be published.

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BRIEF ARTICLE

Evaluation of transarterial chemoembolization combined with percutaneous ethanol ablation for large hepatocellular carcinoma

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Abstract

AIM: To assess the effects of combined transcatheter arterial chemoembolization (TACE) and percutaneous ethanol ablation (PEA) in patients with large hepatocellular carcinoma (HCC).

METHODS: A total of 63 patients with unresectable large HCC were treated with TACE followed by PEA. The largest dimension of the tumors ranged from 5.3 cm to 17.8 cm. The survival rates, acute effects, toxicity and prognostic factors were analyzed.

RESULTS: The cumulative survival rates at 1, 3 and 5 years were 59.4%, 28.4% and 15.8%, respectively (a median survival of 27.7 mo). Tumor area was reduced by more than 50% in 30 (47.6%) cases. In 56 cases with increased α-fetoprotein (AFP) values, AFP level was declined by more than 75%. The combined therapy was generally well tolerated. Only two patients died from variceal bleeding associated with the therapy. The Cox proportional hazards model showed that the number of tumors, the tumor margin and the ethanol dose were independent prognostic factors.

CONCLUSION: The combined TACE and PEA therapy is a promising approach for unresectable large HCC.

Key words: Hepatocellular carcinoma; Chemoembolization; Ethanol ablation; Combination therapy

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INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common malignancies in the world, responsible for an estimated one million deaths annually. It has a poor prognosis due to its rapid infiltrating growth and complicating liver cirrhosis. Surgery is the only potential cure, but the resection rate for HCC is only 10%-30%. The remaining patients are subjected to various modes of non-surgical therapy. Transcatheter arterial chemoembolization (TACE) has become one of the most popular approaches of non-surgical treatment, being effective in reducing tumor size in HCC and improving survival[1-4]. However, tumor cells remain viable in and

around the capsule, which is supplied by both arterial and portal blood, and these cells are often responsible for later recurrence and spread[5-10]. Further treatment is needed to eradicate residual tumor cells. We used TACE combined with percutaneous ethanol ablation (PEA) to treat 63 patients with large HCC and retrospectively evaluated the effects of this combined therapy and the prognostic factors.

MATERIALS AND METHODS

Ethics

This study was approved ethically by the Sun Yat-Sen University Cancer Center. All patients provided informed written consent. This work was carried out in accordance with the Declaration of Helsinki (2000) of the World Medical Association.

Patients

From November 2001 to January 2009, 63 consecutive patients with large unresectable HCC were enrolled to this study. In all the patients, the diagnosis of HCC was made based on the histologic or angiographic findings combined with serum α-fetoprotein (AFP) levels. In 41 (65.1%) patients, the diagnosis of HCC was confirmed by histologic examination. The remaining 22 patients were diagnosed according to the findings on ultrasound, CT and angiography, and serum AFP levels. The enrolling criteria were as follows: (1) lesions detectable on ultrasound and CT; (2) tumor/liver volume ratio not above 0.7:1; (3) serum transaminase level under 80 IU/L; and (4) no evidence of extrahepatic metastasis or ascites. Patients who had ascites, extrahepatic metastasis, severe cirrhosis (class C according to Child's classification), or Karnofsky performance score < 70 were excluded. The baseline characteristics of patients are shown in Table 1.

Table 1 Baseline data of the patients

Variables	Values
Mean age (yr)	57.2
Cases of HBV-related liver disease	61.0
Cases of HCV-related liver disease	0.0
Mean AST (U/L)	43.4
Mean ALT (U/L)	49.5
Mean total bilirubin (μmol/L)	26.2
Mean AFP (ng/mL)	963.9
Mean tumor size (cm)	8.3

HBV: Hepatitis B virus; HCV: Hepatitis C virus; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; AFP: α-fetoprotein.

Methods

TACE was performed in the following processes: a 5.0 French catheter (Terumo, Tokyo, Japan) was inserted into the femoral artery by the Seldinger's method. Celiac angiography and selective hepatic arterial angiography were routinely performed to observe the tumorous blood supply, distribution of hepatic arteries and collateral circulation routes. The tip of the catheter was placed at the feeding artery of the tumor, and embolization was performed using emulsionized mixture of lipiodol ultra-fluid (Guerbet, France), Perarubicin (50 mg/m^2) and DDP (80 mg/m^2). The maximum dose for the embolization depended on the size of the tumor, blood supply and hepatic function of the patient. When the tumor was filled well with emulsifier, the embolization ended.

After 1-2 times of TACE, PEA was performed using an ethanol solution (99% concentration, mixed with lipiodol, 9:1 volume ratio) slowly injected into the tumor through a 15-cm 21 gauge Chiba needle (Cook, Bloomington, IN) guided by CT scan. The size of needle, the amount of ethanol injected per procedure and the number of procedures for the entire treatment, were planned depending on the volume of the tumor and the extent of the transient high-density zones induced by ethanol diffusion on CT scans. The procedure was completed when the entire targeted tumor appeared with a high density. PEA was performed 2-5 times for each tumor. The amount of ethanol injected per procedure and per tumor was 3-20 mL (mean ± SD, 8.2 ± 3.4 mL) and per patient 5-40 mL (mean ± SD, 30.5 ± 6.6 mL).

The follow-up protocol after the initial combined therapy was planned according to the volume of the tumor, tumor blood supply and the extent of the high-density zones on CT scans. The standard TACE for a 8.0-10.0 cm HCC needs two steps (3 wk for each step) when a good tumor blood supply was displayed on enhanced CT scan, and the standard PEA protocol for a 5.0-6.0 cm HCC needs three steps (1.5 wk for each step) when tumor blood supply was obviously decreased on enhanced CT scan. The ethanol treatment was ended when the entire targeted tumor appeared with a high density.

The therapeutic efficacy was evaluated by CT scan two mo after the combined treatment.

Prognostic factors

Factors thought to influence survival were selected and classified to obtain survival rates using the Kaplan-Meier method. The significance of the differences was evaluated by the log-rank test with univariate analysis. The following prognostic factors were investigated: sex, age, number of lesions, tumor size, tumor extension, tumor margin (the tumor margin is the edge of the tumor, and the boundary between the tumor tissues and normal tissues was determined based on hepatobiliary phase images), AFP, portal thrombosis, ascites, Child grade, Okuda stage, times of TACE and PEA and the total ethanol dose. Variables with possible prognostic significance were selected, and each variable was divided into 2-4 classes (Table 2). Factors related to the survival rate were used as variables, and stepwise multivariate analysis was performed. Multiple regression analysis was performed using the Cox proportional-hazard model to calculate the relative-risk ratio between each factor and the survival rate.

RESULTS

Recent results, survival and prognostic factors

Tumor area was reduced by more than 50% in 30 (47.6%) cases. In 56 cases with increased AFP, AFP level was declined by more than 75%.

At the end of this study, 11 patients remained alive,

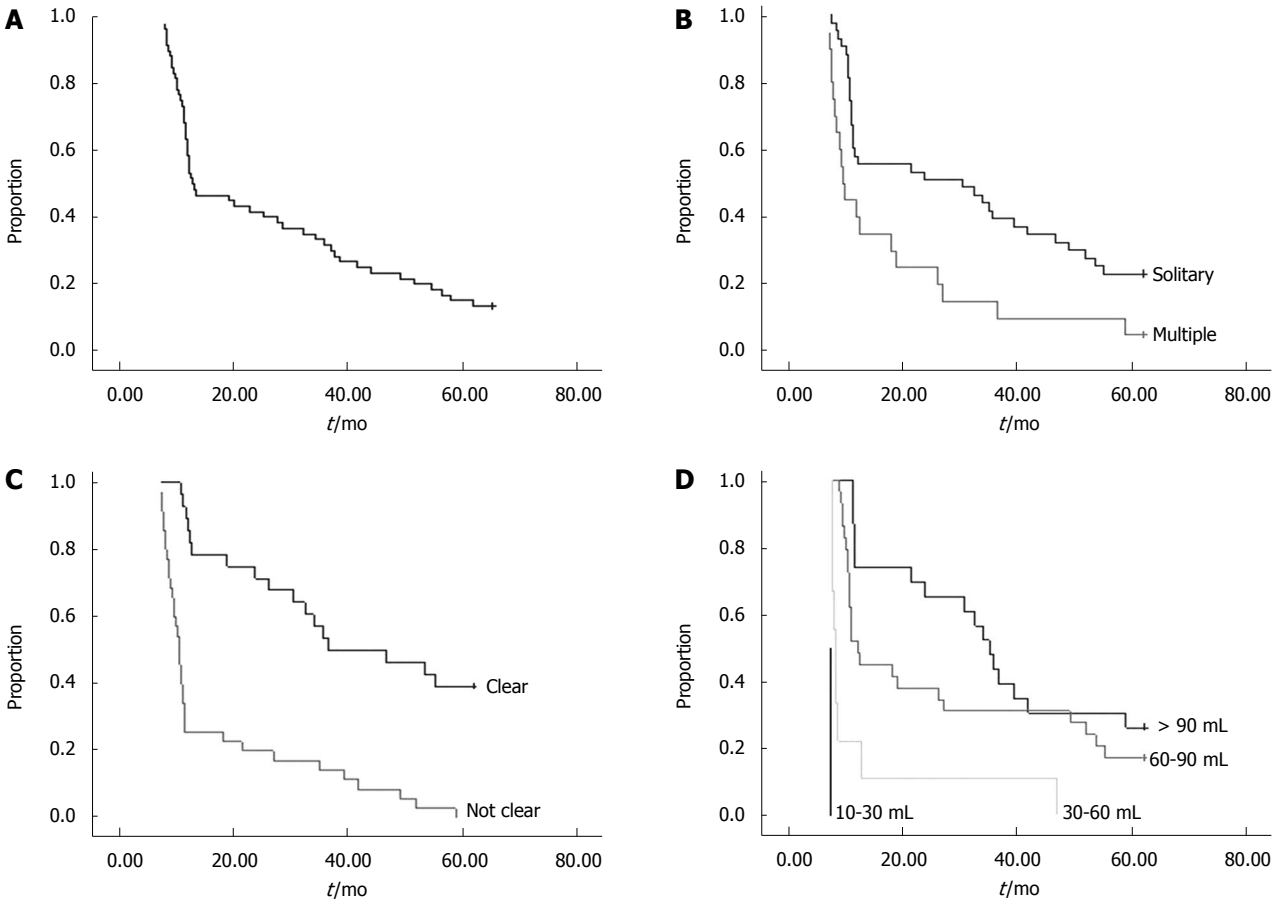

Figure 1 Overall cumulative survival curve and cumulative survival curves in patients based on the number of lesions, tumor margin and percutaneous ethanol ablation dose. A: Overall cumulative survival curve in 63 hepatocellular carcinoma (HCC) patients receiving combined therapy of transcatheter arterial chemoembolization (TACE) and percutaneous ethanol ablation (PEA); B: Cumulative survival curves in patients based on the number of lesions; C: Cumulative survival curves in patients based on the tumor margin; D: Cumulative survival curves in patients based on PEA dose.

Table 2 Variables and classes by univariate and multivariate analyses

Variables	Classes			
	A	B	C	D
Sex	M (52)	F (11)		
Age (yr)	< 55 (36)	> 55 (27)		
No. of lesions	Solitary (43)	Multiple (20)		
Tumor size (cm)	5-10 (41)	> 10 (22)		
Tumor extension	1 lobe (46)	2 lobe (17)		
Tumor margin	Clear (28)	Not clear (35)		
AFP	< 400 (22)	> 400 (41)		
Portal thrombosis	Absent (51)	Present (12)		
Ascites	Absent (56)	Present (7)		
Child grade	A (38)	B (25)		
Okuda stage	Ⅰ (29)	Ⅱ (34)		
TACE (number of times)	1 (11)	2 (26)	3 (20)	4 (6)
PEA (number of times)	1 (3)	2 (6)	3 (31)	> 4 (23)
Total ethanol dose	10-30 (2)	- 60 (9)	- 90 (29)	> 90 (23)

In the parenthesis are numbers of patients. AFP: α-fetoprotein; TACE: Transcatheter arterial chemoembolization; PEA: Percutaneous ethanol ablation.

and 52 patients had succumbed. The survival curve is shown in Figure 1A. Overall survival rates at one, three, and five years were 54.0%, 31.7% and 17.5%, respectively (median survival 27.7 mo).

Univariate analysis indicated that 11 factors significantly influence the survival. Sex, age and TACE times were not significant ($P > 0.05$), (Table 3).

The Cox proportional hazards model showed that only the number of tumors, tumor margin and the total ethanol dose were independent factors predicting survival (Table 4).

The overall survival rates at one, three and five years in the 43 patients with a solitary lesion were 58.1%, 39.5% and 23.3%, respectively, and were 45.0%, 15.0% and 5.0%, respectively in the 20 patients with multiple lesions. The mean survival of patients with a solitary lesion was significantly longer ($P = 0.0145$) than that of patients with multiple lesions (Figure 1B). In the patients with clear tumor margin ($n = 28$), the 1, 3, and 5-year survival rates were 89.3%, 53.6% and 39.3%, respectively, and these figures were significantly higher ($P = 0.0052$) than in the patients without clear tumor margin ($n = 35$), who had survival rates of 25.7% at one year, 14.3% at three years, and 0 at five years (Figure 1C). The mean 1-, 3- and 5-year survival rates were estimated to be 0%, 0% and 0%, respectively, in the 2 patients who received 10-30 mL total ethanol dose; 33.3%, 16.7% and 0% in the 9 patients who received 30-60 mL total ethanol dose; 51.7%, 31.0% and 16.1% in the 29 patients who received 60-90 mL total ethanol dose; and 73.9%, 43.5%, and 26.1% in the 23 patients who received

Table 3 Factors affecting survival by univariate analysis *n* (%)

Variables	Class	Survival			*P* value
		1 yr	3 yr	5 yr	
Sex	A	27 (51.9)	16 (30.8)	9 (17.3)	
	B	7 (63.6)	4 (36.4)	2 (18.2)	0.924[1]
Age (yr)	A	21 (58.3)	11 (30.6)	5 (13.9)	
	B	13 (48.1)	9 (33.3)	6 (22.2)	0.223[1]
No. of lesions	A	25 (58.1)	17 (39.5)	10 (23.3)	
	B	9 (45.0)	3 (15.0)	1 (5.0)	0.0145[1]
Tumor size	A	28 (68.3)	18 (43.9)	11 (26.8)	
	B	6 (27.3)	2 (9.1)	0 (0)	0.0041[1]
Tumor extension	A	29 (63.0)	18 (39.1)	11 (23.9)	
	B	5 (29.4)	2 (11.8)	0 (0)	0.0054[1]
Tumor margin	A	25 (89.3)	15 (53.6)	11 (39.3)	
	B	9 (25.7)	5 (14.3)	0 (0)	0.0052[1]
AFP	A	16 (72.7)	10 (45.5)	9 (40.9)	
	B	18 (43.9)	10 (24.4)	2 (4.9)	0.0030[1]
Portal thrombosis	A	31 (60.8)	18 (35.3)	11 (21.6)	
	B	3 (25.0)	2 (16.7)	0 (0)	0.0111[1]
Ascites	A	32 (57.1)	19 (33.9)	11 (19.6)	
	B	2 (28.6)	1 (14.3)	0 (0)	0.0115[1]
Child grade	A	28 (73.7)	17 (44.7)	11 (28.9)	
	B	6 (24.0)	3 (12.0)	0 (0)	0.0132[1]
Okuda stage	A	22 (75.9)	14 (48.3)	10 (34.5)	
	B	12 (35.3)	6 (17.6)	1 (2.9)	0.0150[1]
TACE (No. of times)	A	3 (27.2)	2 (18.2)	1 (9.1)	
	B	13 (50.0)	7 (26.9)	3 (11.5)	
	C	13 (65.0)	8 (40.0)	5 (25.0)	
	D	5 (83.3)	3 (50.0)	2 (33.3)	0.1719[2]
PEA (No. of times)	A	1 (33.3)	0 (0)	0 (0)	
	B	2 (33.3)	1 (16.7)	0 (0)	
	C	16 (48.5)	10 (32.3)	5 (16.1)	
	D	15 (65.2)	9 (39.1)	6 (26.1)	< 0.0001[2]
Total ethanol dose	A	0 (0)	0 (0)	0 (0)	
	B	2 (33.3)	1 (16.7)	0 (0)	
	C	15 (51.7)	9 (31.0)	5 (16.1)	
	D	17 (73.9)	10 (43.5)	6 (26.1)	< 0.0001[2]

[1]*P* value: B *vs* A; [2]*P* value: D *vs* A. AFP: α-fetoprotein; TACE: Transcatheter arterial chemoembolization; PEA: Percutaneous ethanol ablation.

Table 4 Significant factors predicting survival found using Cox proportional hazards model

Variables	Hazard ratio	*P* value
No. of lesions		
Multiple *vs* single solitary lesions	2.626	0.001
Tumor margin		
Not clear *vs* clear	2.439	0.000
Total ethanol dose		
30-60 mL *vs* 10-30 mL	0.386	0.000
60-90 mL *vs* 10-30 mL	0.202	0.000
> 90 mL *vs* 10-30 mL	0.116	0.000

over 90 mL total ethanol dose. Statistically significant difference was found between the high-dose group and low-dose group ($P < 0.0001$), (Figure 1D).

Side effects

Fever, abdominal pain, nausea and vomiting occurred in most of the patients after TACE and PEA. These symptoms were self-limiting in almost all the patients, lasting less than one week. A slight increase in serum bilirubin (37 cases), elevated serum transaminase level (59 cases), ascites (6 cases), leucopenia (15 cases) and thrombocytopenia (11 cases) were associated with the combined therapy. These side effects were transitory or easily controlled with medication in most of the patients. Two patients died of variceal bleeding because of the increased portal vein pressure caused by deterioration of liver cirrhosis after repeated TACE-PEA, which had an impact on liver function.

DISCUSSION

The rationale for combined therapy of TACE and PEA relies on the fact that after TACE, tumor blood supply is markedly decreased and intratumoral septa are usually destroyed as a result of the necrosis induced by the procedure. These histopathologic changes make subsequent PEA treatment easier as they can provide enhanced ethanol diffusion within the tumor. Consequently, treatment with PEA is facilitated by the TACE-derived fibrous wall around the lesion, which favors a better retention of the injected ethanol within the tumor[11-15]. Tanaka *et al*[14] first reported the effectiveness of TACE combined with PEA for large (> 3.0 cm in diameter) primary HCC compared with that of TACE alone. His study found that a partial

response of the tumor was seen in only 10% of the patients, and the 1-, 2-, and 3-year survival rates were 68%, 37% and 0%, respectively with TACE alone, and histologic examinations showed that TACE alone caused complete necrosis in only 20% of the tumors. In contrast, PEA combined with TACE significantly increased the partial response rate (45%), prolonged the 1-, 2-, and 3-year survival rates (100%, 85% and 85%), and achieved complete histologic necrosis in 83% of the tumors. Dohmen *et al*[15] proved that the combined TACE and PEA treatment had a lower incidence of local recurrence than TACE alone which resulted in an increased survival of the patients with unresectable large HCC.

Ethanol in PEA diffused within the cells, causing immediate dehydration of cytoplasmic proteins with consequent coagulation necrosis followed by fibrosis, and entered the circulation, inducing necrosis of endothelial cells and platelet aggregation with consequent thrombosis of small vessels followed by ischemia of the neoplastic tissues. Advantages of PEA were[16-18]: no remarkable damage to the remaining parenchyma, being safe, easy to be repeated when new lesions appear, low in cost, easy to operate, and possessing good long-term results. PEA can be carried out either in patients with HCC who have a poor liver function or in elderly patients (age $\geqslant$ 70 years)[19,20]. Our results proved that higher doses of ethanol can be injected, which can achieve complete and homogeneous perfusion even in large lesions.

It is necessary to analyze prognostic factors in a large number of patients in sufficient detail and to evaluate the result of each method of treatment between groups with similar prognostic factors. Our study showed that only the number of tumors, tumor margin and the total ethanol dose were independent factors predicting survival. Although various prognostic factors have been reported[21-23], no conclusion has been drawn as to which factor is significant. In this study, the significant factors for better prognosis included the number of tumors, tumor margin and the total ethanol dose. The prognostic factors identified in this study suggested that, therapeutic results in patients with solitary tumors and clear tumor margin treated at a higher total ethanol dose should be better than those in patients with multiple tumors, without clear tumor margin treated at a lower total ethanol dose. It is worth noting the tumor margin is one of the important prognostic factors. It is determined based on hepatobiliary phase images and represents the growth pattern of tumor to some extent. The tumor margin imaging can predict microscopic portal vein invasion, intrahepatic metastasis and early recurrence after hepatectomy in HCC patients[24].

Ebara *et al*[25] and Vilana *et al*[26] proposed tumors < 30 mm in size and < 3 in number as indications for PEA, mainly because of technical limitation such as the inability to inject an effective volume of ethanol into the whole area of the tumor. Our results suggested that some tumors > 50 mm in size could be treated by PEA because the therapeutic results of PEA were also good for large HCC patients with solitary tumors and clear tumor margin at a higher total ethanol dose after TACE.

Long-term survival rates of PEA-treated patients are similar to those obtained in matched patients undergoing partial hepatectomy[27,28]. However, the long-term prognosis remains disappointing because of the high recurrence rate among patients with HCC after PEA, especially in those with multiple lesions, cirrhosis and a high level of AFP and those without a clear tumor margin and peritumoral capsule[29,30]. In fact, histological examination of HCC after PEA reveals that residual tumor tissues remain in portions isolated by septa or with extracapsular or intracapsular invasion. It has been demonstrated that the high vascularity of HCC promotes an early wash-out of injected ethanol, so that PEA for patients with hypervascular tumors may be less effective than for patients with hypovascular tumors[31,32].

COMMENTS

Background

The incidence of large hepatocellular carcinoma (HCC) is increasing in China and HCC has a poor prognosis due to its rapid infiltration and complicating liver cirrhosis. The results in this study indicated that combined transcatheter arterial chemoembolization (TACE) with percutaneous ethanol ablation (PEA) is a promising therapeutic approach for large unresectable HCC.

Research frontiers

The authors analyzed the prognostic factors in a large number of patients in detail and evaluated the result of each method of treatment between groups with similar prognostic factors. This study showed that the number of tumors, tumor margin and the total ethanol dose were independent factors predicting survival.

Innovations and breakthroughs

This is the first study to report that the number of tumors, tumor margin and the total ethanol dose were independent factors predicting survival of large HCC. The combined TACE and PEA therapy is a promising approach for large unresectable HCC.

Applications

By understanding the independent prognostic factors, this study may represent a future strategy in the treatment of patients with large unresectable HCC.

Terminology

TACE has become one of the most popular approaches of non-surgical treatment, with good results in reducing the tumor size of HCC and improving the survival of the patients. PEA is facilitated by the TACE-derived fibrous wall around the lesion, which favors a better retention of the injected ethanol within the tumor.

Peer review

This is a constructive study to report that the number of tumors, tumor margin and the total ethanol dose were independent factors predicting survival of large HCC, which is expected to improve the therapeutic effects for large unresectable HCC.

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BRIEF ARTICLE

Lactobacillus species shift in distal esophagus of high-fat-diet-fed rats

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Abstract

AIM: To analyze the microbiota shift in the distal esophagus of Sprague-Dawley rats fed a high-fat diet.

METHODS: Twenty Sprague-Dawley rats were divided into high-fat diet and normal control groups of 10 rats each. The composition of microbiota in the mucosa from the distal esophagus was analyzed based on selective culture. A variety of *Lactobacillus* species were identified by molecular biological techniques. Bacterial DNA from *Lactobacillus* colonies was extracted, and 16S rDNA was amplified by PCR using bacterial universal primers. The amplified 16S rDNA products were separated by denaturing gradient gel electrophoresis (DGGE). Every single band was purified from the gel and sent to be sequenced.

RESULTS: Based on mucosal bacterial culturing in the distal esophagus, *Staphylococcus aureus* was absent, and total anaerobes and *Lactobacillus* species were decreased significantly in the high-fat diet group compared with the normal control group ($P < 0.01$). Detailed DGGE analysis on the composition of *Lactobacillus* species in the distal esophagus revealed that *Lactobacillus crispatus*, *Lactobacillus gasseri* (*L. gasseri*) and *Lactobacillus reuteri* (*L. reuteri*) comprised the *Lactobacillus* species in the high-fat diet group, while the composition of *Lactobacillus* species in the normal control group consisted of *L. gasseri*, *Lactobacillus jensenii* and *L. reuteri*.

CONCLUSION: High-fat diet led to a mucosal microflora shift in the distal esophagus in rats, especially the composition of *Lactobacillus* species.

Key words: Obesity; *Lactobacillus*; Sprague-Dawley rats; Distal esophagus; Denaturing gradient gel electrophoresis

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INTRODUCTION

Gastroesophageal reflux disease (GERD) is one of the most common clinical disorders, and its incidence

has been steadily increasing around the world in recent years[1-4]. Numerous studies have demonstrated that obesity is a risk factor for GERD. The incidence of GERD and its complications in subjects who are overweight or obese is higher than in those with normal weight[1-4]. With obesity gradually increasing worldwide, obesity-associated GERD is becoming a more complicated clinical problem. Unfortunately, to date, the pathogenesis of obesity-associated GERD has not been fully understood. Thus, the usual management of GERD is merely to inhibit acid production and improve motility of the upper gastrointestinal tract to ameliorate symptoms, which can seriously affect quality of life[5-8].

It is well accepted that GERD is mainly due to abnormality of the gastroesophageal junction or a decrease in esophageal clearance capacity. Current data have demonstrated that the association of obesity with GERD is mainly related to changes in lower esophageal sphincter motility or a pressure imbalance between the distal esophagus and gastric fundus[1-8]. However, the detailed mechanism needs to be further elucidated.

Recent studies have found that the composition of intestinal flora in obese individuals differs from that in normal individuals. Bacterial diversity, either different composition or quantity of bacteria in the gastrointestinal tract, is associated with gastrointestinal smooth muscle motility[9-13]. In clinical practice, many bacterial agents have been used for treatment of diarrhea, irritable bowel syndrome and other gastrointestinal motility diseases, and have achieved a positive response. Based upon the published findings, it can be estimated that microflora in the distal esophagus, to some extent, might play a role in regulating smooth muscle motility. However, the features of colonization and functionality of the microflora in the distal esophagus remain obscure. Some evidence has shown that the esophageal bacteria are transitory, and other studies have demonstrated a complex, residential microflora in the distal esophagus. Several studies also have confirmed the presence of a residential bacterial population in the distal esophagus in patients with esophageal-reflux-related disorders[14,15]. More detailed research has shown a significant alteration in the number of lactobacilli in the intestine of obese rats[11,16-19]. Thus, it is reasonable that obesity, a passive risk factor for GERD, might alter the composition of microflora in the distal esophagus, and lead to the formation of GERD.

Therefore, we hypothesize that potential variations in microbial composition in the distal esophagus of obese individuals might influence the distal esophageal motility and lead to GERD. Thus, we focused on the variation in microflora, especially *Lactobacillus* species, in the distal esophagus in obese Sprague-Dawley rats induced by high-fat diet, compared with normal rats, to illustrate the role of obesity or high-fat diet in the microfloral composition shift.

MATERIALS AND METHODS

Animals

Twenty-five healthy male Sprague-Dawley rats (purchased from the Experimental Animal Co., Hayes Lake, Hunan, China) aged 3 wk, weighing 50-70 g, were used. The animal experiments were approved by the Animal Experiment Ethics Committee of Central South University, Changsha, China.

Table 1 Comparison of the components of each diet: high-fat diet and common diet (per 0.1 kg)

Diet	Protein (g)	Fat (g)	Carbohydrate (g)	Energy (KJ)
Common diet	17.53	6.08	59.98	1250
High-fat diet	16.52	25.17	56.66	1810

Establishment of animal model

Sprague-Dawley rats were housed individually in cages at constant room temperature of 18-22℃, 50% humidity, in a 12-h light/dark cycle, and had free access to common diet and water. After 1 wk of adaptive feeding, five rats were killed and the other 20 were randomly divided into two groups of 10. One group was fed a high-fat diet (Dongchuang Nursery, Hunan, China; Table 1) for 6 wk, and the normal control group was fed a common diet. The daily diet was sterilized by Co^{60} irradiation and water by autoclave before feeding to the rats. Rats were killed at the end of 7 wk. Body weight, body length and feed consumption were recorded weekly and daily[20-22].

Histopathological examination of esophageal mucosa

The esophagus of each rat was removed and dissected longitudinally. Esophageal specimens were obtained from 1.5 cm above the gastroesophageal junction. Then 0.5 cm of each longitudinal strip was fixed in 10% formalin-buffered saline, embedded in paraffin, and processed for histopathological analysis. Two sections were cut from each paraffin block and stained with hematoxylin-eosin (HE) for evaluation of inflammation.

Determination of serum lipid levels

After fasting for 10 h, venous blood was obtained at the end of 7 wk. Three hundred microliters of serum for each sample was extracted and stored at -20℃. Serum triglyceride and total cholesterol were detected by GPO-PAP and CHOD-PAP methods, respectively.

Bacterial culture

The esophagus of each rat was removed and dissected longitudinally. Esophageal mucosa samples were obtained from 1 cm above the gastroesophageal junction. After being weighed on an electronic balance, the samples were homogenized immediately, and the homogenates were diluted at 10^{-7} in sterile normal saline. Ten microliters was spread thoroughly over the surfaces of specific medium plates. The media utilized were as follows: Mannitol Salt Agar for *Staphylococcus aureus* (*S. aureus*); Actinomyces Agar for *Actinomyces*; TTC Agar for *Enterococcus*; KF Streptococcus Agar for *Streptococcus*; EMB Agar for *Enterobacter*; Anaerobic Agar for total anaerobes; Clostridium Agar for

Clostridium; PY Agar for *Clostridium perfringens*; LBS Agar for *Lactobacillus* and *Lactobacillus crispatus* (*L. crispatus*); and BL Agar for *Bifidobacterium*. All aerobic plates were incubated at 37℃ for 24 h, followed by incubation for 12 h at room temperature. The LBS plates were incubated in a candle-jar atmosphere of 10% CO_2. Anaerobes were grown in an anaerobic jar at 37℃ for 72 h. The number of colonies from each plate was recorded.

Amplification of Lactobacillus 16S rDNA for denaturing gradient gel electrophoresis

DNA from *Lactobacillus* cultures was extracted according to the protocol of BioTeke Corporation (Beijing, China) and stored at -20℃. Universal bacterial primers (Invitrogen Biotechnology, Shanghai, China) HAD1-GC (CGCCCGGGGCGCGCCCCGGGCGGGGCGGGGGCACGGGGGGGACTCCTACGGGAGGCAGCAGT-3') and HAD2(5'-GTATTACCGCGGCTGCTGGCAC-3')[23] were used to amplify V2 to V3 regions of the bacterial 16S rDNA. PCR reactions were run at 95℃ for 5 min, followed by 38 cycles of amplification at 94℃ for 40 s, 52℃ for 40 s, and 72℃ for 50 s, and a 7-min extension at 72℃.

Denaturing gradient gel electrophoresis

Denaturing gradient gel electrophoresis (DGGE) was performed with the DCode™ Universal Detection System (Bio-Rad) that utilized 16 cm × 16 cm × 1 cm gels. Eight percent polyacrylamide gels were prepared and run with 1 × TAE buffer (2 mol/mL Tris base, 1 mol/mL glacial acetic acid, and 50 mmol/mL EDTA) diluted from 50 × TAE buffer (Sigma, Beijing, China). The denaturing gradient was formed with two 8% acrylamide (acrylamide/bis, 37.5:1) stock solutions (Bio-Rad). The gels contained a 30%-70% gradient of urea and formamide that increased in the direction of electrophoresis. A 100% denaturing solution contained 40% (v/v) formamide and 7.0 mol/mL urea. The electrophoresis was conducted with a constant voltage of 110 V at 60℃ for 12 h. Gels were stained with ethidium bromide solution (5 μg/mL) for 30 min, washed with deionized water, and viewed by UV transillumination (Bio-Rad).

Amplification and sequencing of the 16S V2-V3 region

Single predominant bands of the DGGE gel were selected by cutting with a sterile scalpel, and added to 50 μL deionized sterile water (Fermentas Life Sciences, Shenzhen, China). The gel pieces were placed at 4℃ for 24 h, centrifuged at 12000 × *g* for 10 min, and the supernatants were used as templates for PCR amplification. Universal bacterial primers (Invitrogen Biotechnology) HAD1(5'-TCCTACGGGAGGCAGCAGT-3') and HAD2(5'-GTATTACCGCGGCTGCTGGCAC-3')[23] were used for amplification. For each PCR amplification, 5 μL template was added to 45 μL PCR reaction mixture (Fermentas Life Sciences) that contained 5 μL 10 × PCR buffer, 5 μL 25 mmol/L $MgCl_2$, 1.5 μL 10 mmol/L dNTPs, 1.5 μL 100 ng/μL each primer, and 2.5 U Taq DNA polymerase. Reactions were run at 95℃ for 5 min, followed by 36 cycles of amplification at 94℃ for 30 s, 56℃ for 40 s, and 72℃ for 40 s, and a 10-min extension at 72℃. Wizard PCR Preps DNA Purification System (Promega, Beijing, China) were used to purify the PCR products. The purified products were sent to Beijing Genomics Institute of Technology for sequencing. Blast sequences were obtained through GenBank BLAST searches (http://www.ncbi.nlm.nih.gov/blast).

Statistical analysis

Statistical analysis was performed using SPSS for Windows version 15.0. Results were expressed as measurement and enumeration data. Data are presented as means and SDs. For statistical comparisons, the *t* test and χ^2 test were performed and $P < 0.05$ was considered statistically significant.

RESULTS

High-fat diet resulted in elevated body weight and serum lipid with no significant histological change in vivo

There was no significant change in body weight in the high-fat diet and normal control groups at the end of the first week. Since the second week, the body weight and Lee's index increased weekly (Figure 1A and B). Accordingly, serum triglyceride and total cholesterol in the high-fat diet group were significantly increased compared with the normal control group (Figure 1C). HE staining of esophageal mucosa showed no musculature damage or mucosal injury in the distal esophagus in the high-fat diet or normal control group (Figure 1D).

High-fat diet led to microfloral shift in distal esophagus based on bacterial culture

Bacteria in the distal esophagus were cultured on selective media. In the high-fat diet group, *S. aureus* was absent, and the numbers of total anaerobes and lactobacilli were reduced significantly in the distal esophagus compared with the normal control group. There was no obvious difference between the common and adaptive feeding groups for composition of cultivable bacteria in the distal esophagus of Sprague-Dawley rats (Table 2).

Determination of Lactobacillus species shift in distal esophagus of high-fat diet-fed rats based on DGGE and sequencing

16S rDNA of cultivable *Lactobacillus* from the high-fat and common diet groups was amplified by PCR using universal bacteria primers HAD1-GC and HAD2 (Figure 2A). The amplified products of 16S rDNA were separated by DGGE. The two groups shared dramatically different bands, with bands A, C, D and E in the high-fat diet group, and bands B, C, D and E in the common diet group (Figure 2B). The purified bands were sequenced and BLASTed online with the V2-V3 region. The composition of *Lactobacillus* species in the distal esophagus in the common diet group was *Lactobacillus gasseri* (*L. gasseri*), *Lac-*

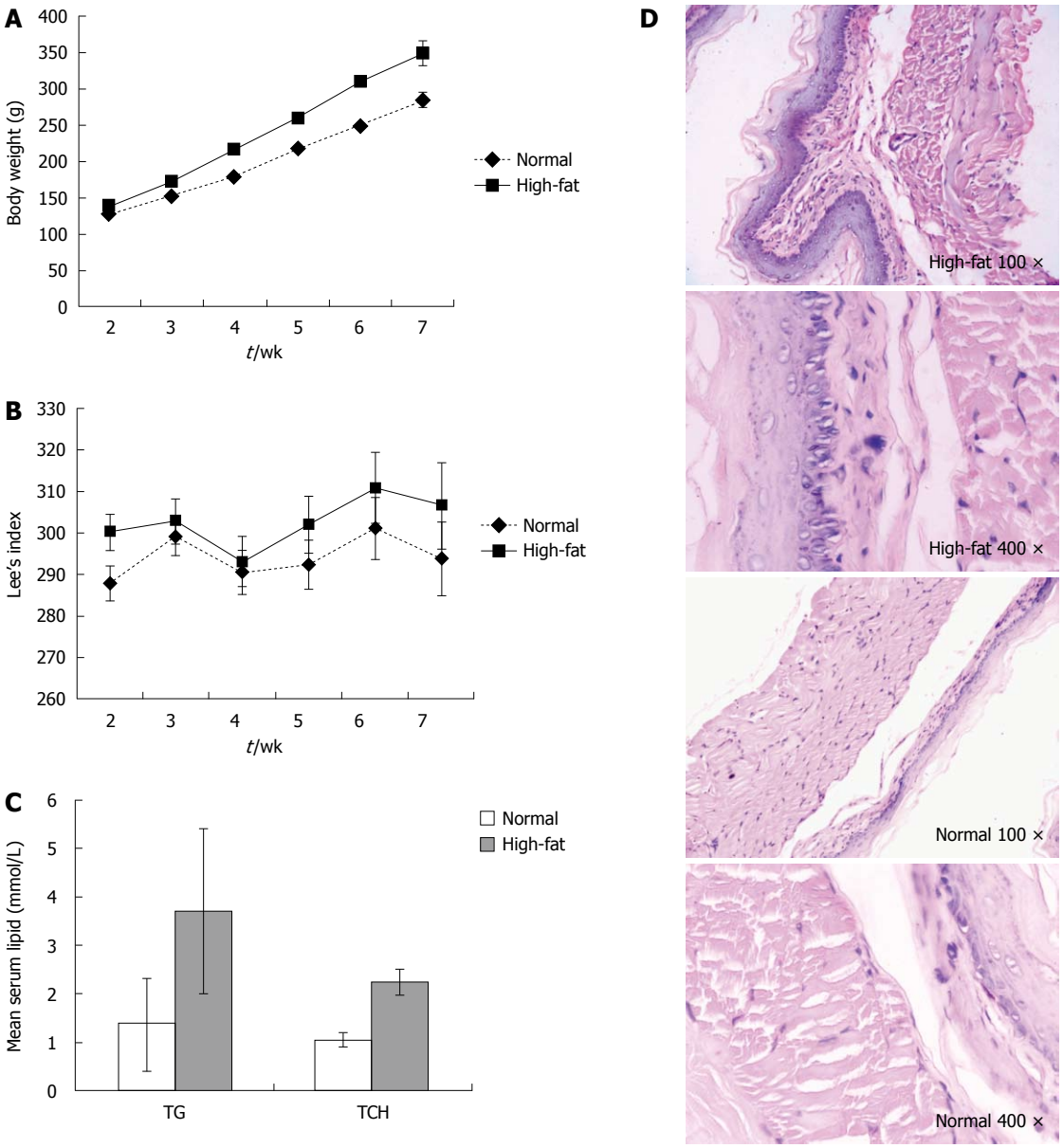


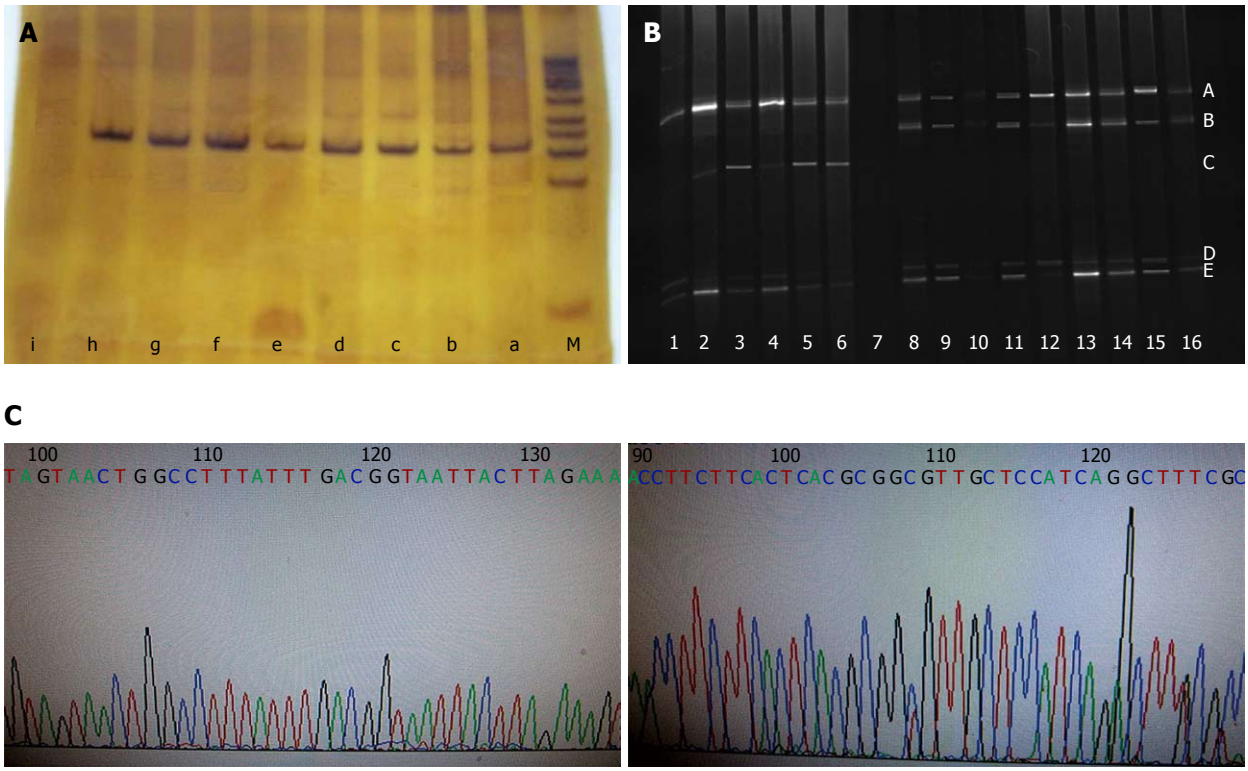
Figure 1 No significant change in distal esophageal mucosa in Sprague-Dawley rats fed a high-fat diet. A: Body weight changes of age- and sex-matched rats in the high-fat diet and normal control groups (n = 10 each); B: Lees index changes for the rats in (A) (n = 10); C: Mean serum lipid for the rats in (A); D: Histological analysis of representative distal esophagus from the rats in (A) (Original magnification, hematoxylin-eosin staining, 100 × or 400 ×).

tobacillus jensenii (*L. jensenii*) and *Lactobacillus reuteri* (*L. reuteri*), whereas the composition shifted to *L. crispatus*, *L. gasseri* and *L. reuteri* in the high-fat diet group (Figure 2C and D).

DISCUSSION

Accumulating evidence has shown that obesity is a risk factor in the development of GERD[1-8,24,25]. However, is not well established how obesity affects the incidence of GERD. According to the published experimental data, no musculature damage has been reported in the distal esophagus of obese rats or mice. However, different components of the microbiota have been identified between obese and lean animals/humans. Based on this evidence, the purpose of this study was to analyze the microbiota composition and focus on how the shift in microflora occurred in the distal esophagus of rats fed a high-fat diet.

To date, the role of microbiota in the distal esophagus under conditions of weight gain remains obscure. Some studies have shown that high-fat/high-sugar chow diet-fed, germ-free mice gain significantly less weight than their control littermates. The absence of microbiota has a protective role against weight gain in mice that consume a Western-style diet. Thus, body weight gain could be associated with a shift in the microbiota in mice fed a western-style diet. On the other hand, current research has demonstrated that there is a complex residential microbiota shift in the distal esophagus of patients with esophageal-reflux-related disorders, such as GERD, reflux esophagitis and Barrett's esophagus[14,15,26]. The possible explanations are that the reflux content might contain gastric bac-



D

Code	Sequence length (bp)	The most similar bacteria (species)	%Identity
A	253	Lactobacillus gasseri 224-1 (gb|ADFT01000001.1|)	98
B	250	Lactobacillus jensenii SJ-7A-US (gb|ACQD01000066.1|)	98
C	168	Lactobacillus crispatus 214-1 (gb|ADGR01000076.1|)	96
D	259	Lactobacillus reuteri MM2-3 (gb|ACLB01000138.1|)	99
E	231	Lactobacillus reuteri MM2-3 (gb|ACLB01000138.1|)	98

Figure 2 Determination of *Lactobacillus* species shift in distal esophagus of high-fat diet-fed Sprague-Dawley rats based on the combination of denaturing gradient gel electrophoresis and sequencing. A: 16S rDNA of cultivable *Lactobacillus* was amplified by polymerase chain reaction using universal bacteria primers HAD1-GC and HAD2 in the high-fat diet and common diet groups. a: *Lactobacillus crispatus* (*L. crispatus*) for positive control, b-d: High-fat diet group; e-h: Common diet group; i: Negative control; M: DNA marker; B: Amplified products of 16S rDNA were separated by denaturing gradient gel electrophoresis (DGGE). Lanes 1-6: High-fat diet group; lane 7: Negative control; lanes 8-16: Normal diet group. The bands were marked with A, B, C, D and E, respectively; C: Purified bands were purified and sequenced; D: BLASTed online with V2-V3 region. Bands A, B, C, D and E represented *Lactobacillus gasseri*, *Lactobacillus jensenii*, *L. crispatus*, *Lactobacillus reuteri*, and *Lactobacillus reuteri*, respectively.

Table 2 Analysis of cultivable microbiota of distal esophagus from high-fat diet group and common diet group (n = 10) (mean ± SE, lg CFU/g)

	Adaptive group (n = 5)	High-fat diet (n = 10)	Common diet (n = 10)
Total aerobic bacteria	4.92 ± 0.83	3.81 ± 1.83	4.45 ± 1.46
S. aureus	4.80 ± 1.42	0	3.80 ± 2.83[a]
Actinomycetes	4.52 ± 1.61	4.58 ± 0.47	4.12 ± 1.51
Enterococcus	3.21 ± 1.64	2.02 ± 1.82	2.89 ± 2.47
Streptococcus	4.14 ± 1.91	2.82 ± 3.55	3.74 ± 2.19
Enterobacter	3.22 ± 1.47	1.45 ± 2.91	3.05 ± 1.32
Total anaerobes	4.61 ± 0.42	2.11 ± 0.80[a]	4.48 ± 0.38[a]
Clostridium	4.47 ± 1.67	5.28 ± 1.60	4.77 ± 1.86
Clostridium perfringens	4.97 ± 1.49	3.59 ± 2.49	5.11 ± 1.58
Lactobacillus	5.31 ± 1.62	2.44 ± 0.97	5.44 ± 1.54[a]
Bifidobacterium	4.24 ± 2.97	2.53 ± 2.68	4.32 ± 3.21

[a]$P < 0.05$, indicates high-fat diet group *vs* common group. *S. aureus*: *Staphylococcus aureus*.

teria or damage the esophageal mucosa and lead to an abnormal inhabitation niche for the distal esophageal microbiota. Thus, the abnormal interactions among distal esophageal residential microbiota, gastric bacteria, and distal esophageal mucosa could lead to a composition shift in the microbiota in the distal esophagus.

Based on analysis of cultivable microbiota of the distal esophagus, we found a certain microbiota compositional shift in the distal esophagus of obese rats. The major microbiota shift in the distal esophagus involved the loss of *S. aureus* and decrease in anaerobes and *Lactobacillus* species. The focus in this research was diet-associated obesity, with integrated esophageal mucosal changes, and no obvious histological changes. This means that the microbiota shift in the distal esophagus was not due to the reflux of gastric content or alteration of the inhabitable niche of microbiota in the distal esophagus. Thus, a possible explanation for this microbiota shift in mice fed a western-style diet, compared with their control littermates, could be the

components of high-energy materials in the distal esophageal mucosa. These high-energy components might alter the mucosal microenvironment and help the local bacteria to colonize the distal esophageal mucosa.

GERD is an obvious motility disorder of the distal esophagus. Recent research has shown that several *Lactobacillus* or other bacterial species can alter the motility of smooth muscle in the distal esophagus[9-12]. It has been shown that different composition of *Lactobacillus* species can lead to different influences on smooth muscle motility in the distal esophagus[9-12]. However, the details are still unclear. Some evidence has shown increased smooth muscle contraction in certain GERD cases, whereas other studies have shown opposite results. The probable reason is that analysis of the shift in *Lactobacillus* was restricted to the level of the *Lactobacillus* genus. Fortunately, with the development of modern molecular techniques, analysis of *Lactobacillus* at the species level has become possible.

In our study, the combination of bacterial culture and DGGE was used to analyze the composition of *Lactobacillus* in the distal esophagus. It was clearly demonstrated that three cultivable *Lactobacillus* species, *L. gasseri*, *L. jensenii* and *L. reuteri*, colonized the distal esophagus in normal control rats. However, the composition of *Lactobacillus* species was shifted to *L. crispatus*, *L. gasseri* and *L. reuteri* in the distal esophagus in high-fat diet-fed rats. Four *Lactobacillus* species constituted a specific group in the distal esophagus of normal control rats as well as in obese rats. To our surprise, *L. jensenii* disappeared from the mucosa of the distal esophagus, which was replaced by *L. crispatus* in obese rats. It is possible that the disappearance of *L. jensenii* and re-colonization with *L. crispatus* results in alteration of smooth muscle motility in the distal esophagus. Therefore, this could probably be involved in the etiology of GERD, because of the variations in cultivable *Lactobacillus* species in the distal esophagus in obese rats. Another reasonable explanation is that different *Lactobacillus* species could have different metabolic characteristics, and influence the inhabitation niche of the microbiota in the distal esophagus. Yet another possibility is that the composition of cultivable *Lactobacillus* species in the distal esophagus of obese rats could be beneficial in preventing esophageal reflux.

Clearly, many unresolved questions remain to be elucidated. Our research will next focus on the effects of the composition shift in *Lactobacillus* on smooth muscle motility in the distal esophagus. Then, we will distinguish between the promoting or inhibitory properties of ingredients or metabolites of different *Lactobacillus* species. Therefore, the data could result in potential therapeutic targets in GERD.

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COMMENTS

Background

Obesity is a risk factor for gastroesophageal reflux disease (GERD), but how obesity affects the incidence of GERD is not well established. Current research has demonstrated that there is a complex residential microbiota shift in distal esophagus of esophageal-reflux-related disorders in humans. The different components of the microbiota are identified between obese animals/humans and lean animals/humans. Thus, it is reasonable that obesity, a passive risk factor of GERD, might alter the composition of microflora in the distal esophagus, and lead to development of GERD.

Research frontiers

Bacterial diversity is associated with gastrointestinal smooth muscle motility, and is also identified between obese animals/humans and lean animals/humans. However, the microbiota shift in the distal esophagus in obesity has not been fully addressed. In this study, we demonstrated the composition of cultivable *Lactobacillus* species shift in the distal esophagus of obese rats.

Innovations and breakthroughs

Published data show that different constitution of *Lactobacillus* species may exert different influences on smooth muscle motility in the distal esophagus. However, the details are still unclear. Analysis on the shift in *Lactobacillus* is restrained at the genus level. This study analyzed the composition of *Lactobacillus* in the distal esophagus, and found that the composition of cultivable *Lactobacillus* species shifted in the distal esophagus in obese rats. Therefore, potential variations in microbial composition in the distal esophagus in obesity may influence the distal esophageal motility and lead to GERD.

Applications

This study proved that the composition of cultivable *Lactobacillus* species shifted in the distal esophagus in obese rats, therefore, it may represent a further study between the microbial composition shift in the distal esophagus and GERD. This could lead to the development of a potential therapeutic target in GERD.

Terminology

GERD is a more serious form of gastroesophageal reflux, which is common. Persistent reflux that occurs more than twice weekly is considered GERD, and it can affect people of all ages. The main symptom of GERD in adults is frequent heartburn, also called acid indigestion - burning-type pain in the lower part of the mid-chest, behind the breast bone, and in the mid-abdomen.

Peer review

This study considers the investigation of microbiota composition in the distal esophagus of high-fat-diet-fed rats. The authors hypothesize that potential variations in microbial composition in the distal esophagus of obese individuals may influence distal esophageal motility and lead to GERD. In this study, the composition of *Lactobacillus* spp. was analyzed using the combination of bacterial culturing, denaturing gradient gel electrophoresis and sequencing in a rat model. The interesting and important finding of this study was the fact that high-fat diet led to a shift in mucosal microflora (especially *Lactobacillus* species) in the distal esophagus in rats, which resulted in alteration of smooth muscle motility. This study makes an additional contribution to studies of the etiology of GERD.

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BRIEF ARTICLE

Hemihepatic *versus* total hepatic inflow occlusion during hepatectomy: A systematic review and meta-analysis

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Abstract

AIM: To evaluate the clinical outcomes of patients undergoing hepatectomy with hemihepatic vascular occlusion (HHO) compared with total hepatic inflow occlusion (THO).

METHODS: Randomized controlled trials (RCTs) comparing hemihepatic vascular occlusion and total hepatic inflow occlusion were included by a systematic literature search. Two authors independently assessed the trials for inclusion and extracted the data. A meta-analysis was conducted to estimate blood loss, transfusion requirement, and liver injury based on the levels of aspartate aminotransferase (AST) and alanine aminotransferase (ALT). Either the fixed effects model or random effects model was used.

RESULTS: Four RCTs including 338 patients met the predefined inclusion criteria. A total of 167 patients were treated with THO and 171 with HHO. Meta-analysis of AST levels on postoperative day 1 indicated higher levels in the THO group with weighted mean difference (WMD) 342.27; 95% confidence intervals (CI) 217.28-467.26; P = 0.00001; I^2 = 16%. Meta-analysis showed no significant difference between THO group and HHO group on blood loss, transfusion requirement, mortality, morbidity, operating time, ischemic duration, hospital stay, ALT levels on postoperative day 1, 3 and 7 and AST levels on postoperative day 3 and 7.

CONCLUSION: Hemihepatic vascular occlusion does not offer satisfying benefit to the patients undergoing hepatic resection. However, they have less liver injury after liver resections.

Key words: Inflow occlusion; Hemihepatic; Vascular occlusion; Hepatectomy; Pringle maneuver

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INTRODUCTION

Liver resection is performed mainly for benign and malignant liver tumors, especially for hepatocellular carcinoma. It is a potential curative treatment option in patients with early stage carcinoma[1]. Intraoperative bleeding is a main concern during liver resections, and mortality and morbidity are clearly correlated with the amount of blood loss

and the subsequent blood transfusions[2]. Many methods of hepatic vascular control have been introduced to control intraoperative blood loss. In 1908, Pringle applied inflow vascular occlusion technique (the Pringle maneuver) at the hepatic hilar for the first time. It is a technique of total compression of the hepatoduodenal ligament and the most commonly used and relatively easy method for controlling afferent blood flow[3]. However, the Pringle maneuver also carries the risk of global ischemic damage to the liver and intestinal congestion, especially in patients with chronic liver diseases, the degree of which is likely to be accentuated by a prolonged period of vascular inflow occlusion[4,5]. In 1987, Bismuth and Makuuchi proposed a hemihepatic vascular occlusion (HHO) technique to reduce the severity of visceral congestion and total liver ischemia, especially for the remaining liver[6,7]. By this method, visceral congestion is considered to be limited, because considerable portal blood flow is preserved and only portions of the liver are rendered anoxic[8]. The technique with occlusion of vessels supplying the hemiliver containing the tumor, has been suggested to reduce intraoperative bleeding and postoperative liver functional disturbances because of the interruption of blood flow to the liver[9]. But, portal vein and artery dissection to perform selective clamping is time consuming and may result in another blood loss[10]. Many prospective randomized controlled trials (RCTs) and retrospective clinical trials have evaluated the feasibility, safety and efficacy of HHO and total hepatic inflow occlusion (THO), however, the clinical significance between the two vascular control methods remain inconsistent. So, the optimal method of vascular control during hepatic resection continues to be debated.

Up to now, a meta-analysis including all available RCTs is still insufficient. We conducted a systematic review and meta-analysis to evaluate the feasibility, safety and efficacy of HHO and THO in patients undergoing hepatectomy.

MATERIALS AND METHODS

Systematic literature search

A systematic literature search was independently conducted by two authors. They systematically searched the Medline, Embase, Science Citation Index, PubMed and CNKI (China National Knowledge Infrastructure Whole Article Database). The following keywords were used: hemihepatic vascular occlusion, hemihepatic occlusion, selective inflow occlusion, selective clamping or selective portal clamping. The literature search was performed with restriction in languages of English or Chinese and types of randomized controlled trial or controlled clinical trial. The last search was done on November 2, 2010.

Inclusion and exclusion criteria

Type of studies: Only RCTs were considered for this review. Quasi-randomized studies, cohort studies, and case-control studies were excluded.

Type of participants: Patients who were about to undergo selective liver resection for benign or malignant liver tumor were included, irrespective of age, gender, cirrhosis, tumor size and nodule numbers. Trials in which patients required contralateral hepatic resection or had distant metastasis or synchronous malignancy in other organs were excluded in the study.

Types of interventions: We included trials comparing total hepatic inflow occlusion with hemihepatic vascular occlusion in hepatectomy, irrespective of ischemic preconditioning before vascular occlusion. Trials only comparing other types of vascular occlusion were excluded.

Type of outcome measures: Primary outcomes: Operative blood loss, biochemical markers of liver injury, aspartate aminotransferase (AST) and alanine aminotransferase (ALT), and transfusion requirement. Secondary outcomes: Peri-operative mortality, peri-operative morbidity, operating time , ischemic duration and hospital stay.

Selection of studies

Two authors identified and evaluated independently the trials for inclusion in form of abstracts or full text if necessary. Any disagreement in study selection and data extraction was resolved by discussion.

Data extraction

Two authors extracted the data on a standard form that included population characteristics (sex, age, percentage of major liver resections, methods of ischemic preconditioning and the presence of chronic liver disease) the co-interventions and information on the outcome measures in each trial.

Quality assessment

We assessed the methodological quality of the trials independently. The assessment was made based on sample size calculation; sequence generation; allocation concealment; whether blinding method was adopted for the participants of patients and those who performed the trial and evaluate the outcome; efficacy of randomization; deviations, withdrawals and dropouts; and definition of outcome parameters[11,12].

Statistical analysis

We pooled the synchronized extraction results as estimates of overall therapeutic effects in a meta-analysis using Review Manager Version 5.0 for Windows. The estimated effect measures were odds ratio (OR) for dichotomous data and weighted mean difference (WMD) for continuous data, both reported with 95% confidence intervals (CI). We checked all results for clinical and statistical heterogeneity. Clinical heterogeneity was evaluated based on the study populations and interventions, definition of outcome measures, concomitant treatment, and perioperative management. Heterogeneity was determined by Chi-squared test. P value of 0.10 was considered significant difference and I^2 values were used for the evaluation of statistical heterogeneity (I^2 of 50% or more indicating presence of heterogeneity)[13]. We used a fixed-effects model to synthesize

Table 1 Characteristics of randomized controlled trials comparing total hepatic inflow occlusion with hemihepatic vascular occlusion

Author (yr)	Design	Sample size (*n*)	THO (*n*)	HHO (*n*)	Journal	Comparison
Figueras *et al* (2005)	RCT	80	39	41	*Annals of Surgery*	Complete *vs* selective portal triad clamping
Wu *et al* (2002)	RCT	58	28	30	*Arch Surg*	Hemihepatic *vs* total hepatic occlusion techniques
Yuan *et al* (2010)	RCT	120	60	60	*The American Journal of Surgery*	Pringle maneuver *vs* hemihepatic vascular occlusion
Liang *et al* (2009)	RCT	80	40	40	*Hepato-Gastroen-terology*	Continuous hemihepatic with intermittent total hepatic inflow occlusion
Total	--	338	167	171	--	--

THO: Total hepatic inflow occlusion; HHO: Hemihepatic vascular occlusion; RCT: Randomized controlled trials.

Table 2 Characteristics of patients in randomized controlled trials comparing total hepatic inflow occlusion with hemihepatic vascular occlusion

Author (yr)	Age (mean yr) THO/HHO	Sex (male:female) THO/HHO	Cirrhosis (*n*:N) THO/HHO	Ischaemic preconditioning	Resection margin (≤ 1 segments:≥ 2 segments) THO/HHO	Diseases HCC: Others THO/HHO
Figueras *et al* (2005)	61.8/62	31:8/28:13	18:39/21:41	IC	25:14/29:12	16:23/17:24
Wu *et al* (2002)	57.5/53.2	23:5/25:5	28:28/30:30	IC	5:23/7:23	25:3/26:4
Yuan *et al* (2010)	48.6/49.3	46:14/41:19	39:60/35:60	IC if transaction time > 30 min or CC	5:55/5:55	44:16/43:17
Liang *et al* (2009)	49.4/49.55	27:13/31:9	17:40/19:40	IC or CC	6:34/10:30	20:20/21:19
Total	--	127:40/125:46	102:167/105:171	--	41:126/51:120	105:62/107:64

IC: Intermittent clamping; CC: Continuous clamping; HCC: Hepatocellular carcinoma; N: The number of all patients in one trial; *n*: The number of patients with cirrhosis; THO: Total hepatic inflow occlusion; HHO: Hemihepatic vascular occlusion.

Table 3 Outcomes of randomized controlled trials comparing total hepatic inflow occlusion with hemihepatic vascular occlusion

Author (yr)	Operative time (min) THO/HHO	Ischemic duration (min) THO/HHO	Blood loss (mL) THO/HHO	Transfusion requirements THO/HHO	Complications total (*n*) THO/HHO	In-hospital stay (d) THO/HHO	In-hospital death (*n*) THO/HHO
Figueras *et al* (2005)	207 ± 48/219 ± 45	41 ± 14/47 ± 18	671 ± 533/735 ±397	4:39/6:41	15:39/ 12:41	9.38 ± 4.9/8.15 ± 3.8	0:39/1:41
Wu *et al* (2002)	409 ± 19.2/ 399 ± 15.6	96.0 ± 10.9/ 94.2 ± 9.9	1685 ± 170/ 1159 ± 221	12:28/5:30	8:28/10:30	14.8 ± 1.4/16.4 ± 1.4	0:28/0:30
Yuan *et al* (2010)	114.2 ± 37.2/ 133.5 ± 44.6	16.6 ± 8.7/ 14.9 ± 4.5	339.5 ± 205.1/ 354.4 ± 240.3	6:60/4:60	19:60/12:60	13.7 ± 5.2/10.2 ±4.1	1:60/0:60
Liang *et al* (2009)	203.98 ± 38.36/ 236.15 ± 49.2	40.17 ± 13.30/ 42.38 ± 12.79	569.8 ± 285.56/ 649.35 ± 279.05	14:40/15:40	8:40/9:40	9.85 ± 3.55/10.12 ± 2.41	0:40/0:40
Total	--	--	--	36:167/30:171	50:167/43:171	--	1:167/1:171

THO: Total hepatic inflow occlusion; HHO: Hemihepatic vascular occlusion.

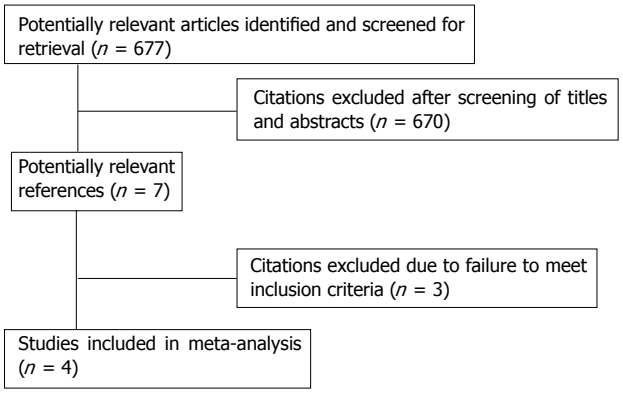

Figure 1 Reference flow chart.

data when heterogeneity was absent, otherwise a random-effects model would be used. Data were presented as forest plot and funnel plot was used to assess publication bias.

RESULTS

We searched a total of 677 references published between 2002 and 2010. Four RCTs[14-17] including 338 patients met the predefined inclusion criteria (Figure 1). All the trials (Table 1) compared HHO (*n* = 171) with THO (*n* = 167). Three trials enrolled cirrhotic and non-cirrhotic patients[14,16,17] and one trial enrolled only cirrhotic patients[15]. In all trials, both major (> 2 segments) and minor (≤ 1 segments) hepatic resections were performed, but one trial exclusively included patients undergoing complex central liver resections. Tables 2-4 summarize the baseline characteristics and outcomes of the trials. The potential bias of included trials are shown in Table 5. Only one of the trials reported the blinding methods used and the generation of allocation sequence[16].

Effects of interventions

Blood loss. Information on intraoperative blood loss was

Table 4 Postoperative aspartate aminotransferase and alanine aminotransferase levels of patients in randomized controlled trials comparing total hepatic inflow occlusion with hemihepatic vascular occlusion

Author (yr)	AST (U/L) Day 1 THO/HHO	AST (U/L) Day 3 THO/HHO	AST (U/L) Day 7 THO/HHO	ALT (U/L) Day 1 THO/HHO	ALT (U/L) Day 3 THO/HHO	ALT (U/L) Day 7 THO/HHO
Wu *et al* (2002)	420 ± 790/290 ± 770	180 ± 320/190 ± 510	50 ± 40/30 ± 20	370 ± 490/480 ± 510	330 ± 320/320 ± 270	90 ± 20/70 ± 20
Yuan *et al* (2010)	812.6 ± 475.3/ 447.6 ± 210.3	423.7 ± 265.4/ 207.5 ± 79.3	143.6 ± 87.5/ 64.2 ± 29.4	1013.6 ± 654.4/ 369.4 ± 347.2	592.2 ± 416.4/ 218.4 ± 185.3	172.4 ± 125.8/ 79.6 ± 55.3
Figueras *et al* (2005)	NS	NS	NS	402 ± 258/372 ± 234	NS	NS

THO: Total hepatic inflow occlusion; HHO: Hemihepatic vascular occlusion.

Table 5 Assessment the methodological quality of included studies

Author (yr)	Sample size calculation	Generation of allocation sequence	Allocation concealment	Deviations, withdrawals and dropouts	Efficacy of randomization	Blinding	Definition of outcome parameters
Figueras *et al* (2005)	Yes	No description	Sealed envelope	Yes	Yes	No description	Yes
Wu *et al* (2002)	No description	No description	Sealed envelope	No description	Yes	No description	Yes
Yuan *et al* (2010)	Yes	Yes	Sealed envelope	Yes	Yes	Single-blinded	No description
Liang *et al* (2009)	No description	No description	No description	Yes	Yes	No description	Yes

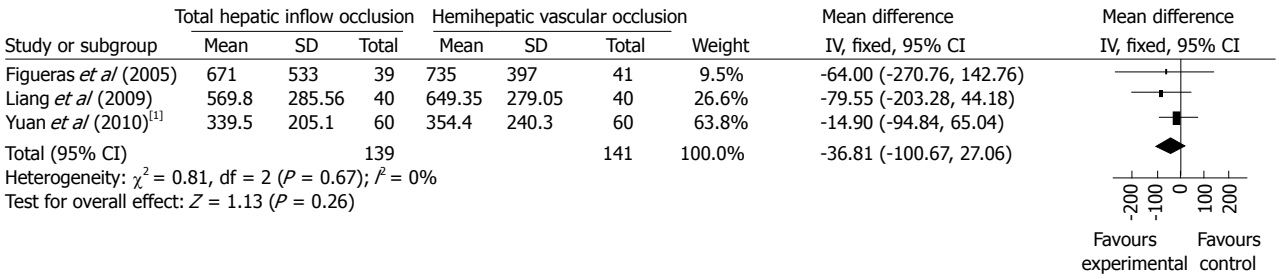

Figure 2 Meta-analysis of blood loss in randomized controlled trials comparing total hepatic inflow occlusion with hemihepatic vascular occlusion. [1]Blood loss.

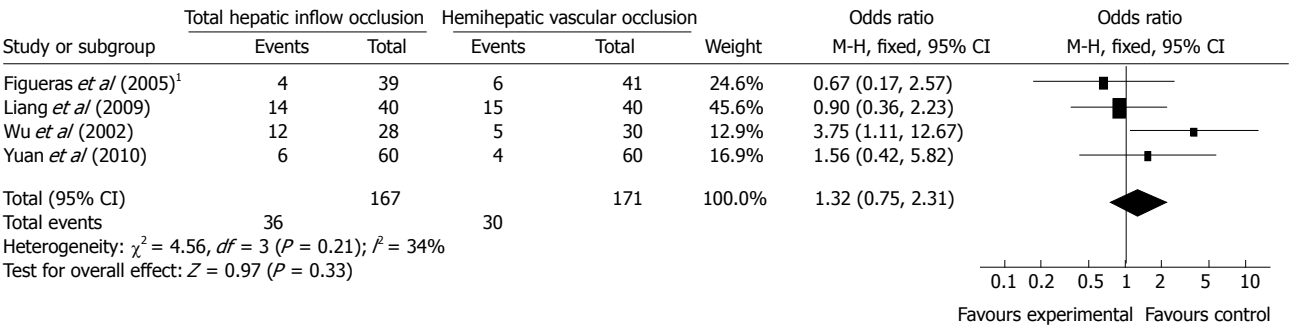

Figure 3 Meta-analysis of aspartate aminotransferase levels on postoperative 1st d. [1]Transfusion requirements (*n*).

available in all analyzed trials. The trial by Wu *et al*[15] reported significantly more blood loss in patients of both groups. Statistical heterogeneity was presented and $P = 0.000\,001$. Funnel plot to evaluate publication bias for outcome of blood loss demonstrated a strong asymmetry, suggesting the existence of severe publication bias. Clinical heterogeneity analysis found that complex central liver resections were performed on cirrhotic patients, and the cut surface area was wider and would increase intraoperative blood loss. Meta-analysis of the other three trials showed no significant difference between THO group and HHO group (WMD -36.81; 95% CI -100.67 to 27.06, $P = 0.26$, $I^2 = 0\%$) (Figure 2).

Transfusion requirement. All trials reported the number of patients who needed transfusion in both groups. Funnel plot did not demonstrate a strong asymmetry. Meta-analysis (Figure 3) indicated no difference in postoperative transfusion requirement between the groups (OR 1.32 95% CI 0.75-2.31, $P = 0.33$, $I^2 = 34\%$). Since there was no uniform definition of the average transfusion volume in the trials, we did not compare the transfusion volume in the study.

Biochemical markers of liver injury. All the four tri-

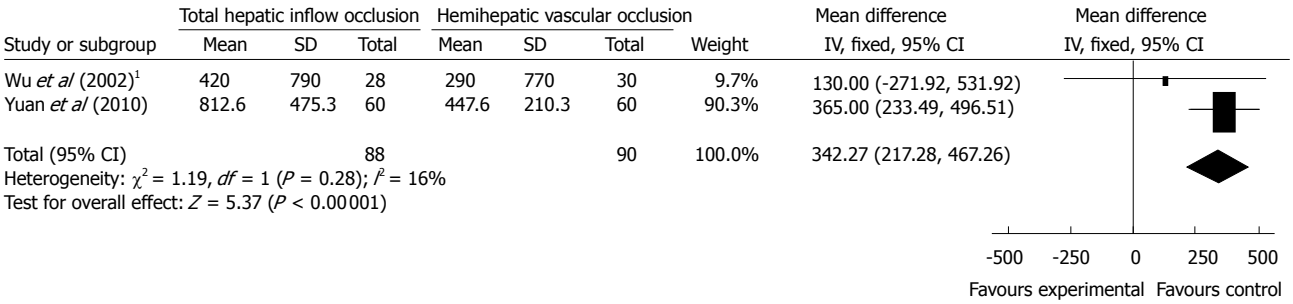

Figure 4 Meta-analysis of aspartate aminotransferase levels on postoperative Day 1. [1]Aspartate aminotransferase (D1).

als provided AST and ALT levels on postoperative days. However, data on AST and ALT were available in only two studies. We did not draw funnel plots to examine the potential publication bias in this review, because the number of the included trials was small. Wu *et al*[15] provided the data of ALT and AST levels on postoperative days 1, 3, 5 and 7, and Yuan *et al*[16] gave the information on postoperative days 1, 3 and 7. The ALT levels on postoperative day 1 in Figueras's study[14] were also available. Meta-analysis of ALT levels on postoperative days 1, 3 and 7 showed no significant difference between the two groups (WMD on day 1/191.03, 95% CI -239.04 to 621.10, $P = 0.38$, $I^2 = 94\%$; WMD on day 3/192.86, 95% CI -163.66 to 549.37, $P = 0.29$, $I^2 = 94\%$, and WMD on day 7/54.43, 95% CI -16.81 to 125.67, $P = 0.13$, $I^2 = 94\%$). Meta-analysis of AST levels on postoperative days 3 and 7 in the two studies showed no significant difference between THO group and HHO group (WMD on day 3/127.52, 95% CI -88.92 to 343.96, $P = 0.25$, $I^2 = 73\%$; WMD on day 7/49.10, 95% CI -9.1 to 107.3, $P = 0.10$, $I^2 = 94\%$). Meta-analysis of AST levels on postoperative day 1 indicated higher postoperative AST levels in the THO group (WMD 342.27; 95% CI 217.28 to 467.26; $P = 0.00001$, $I^2 = 16\%$) (Figure 4).

Peri-operative mortality and morbidity. Four studies provided data on peri-operative mortality and morbidity. In total, two patients died in the four trials. Both died from liver failure, one in THO group and the other in HHO group. Meta-analysis of these studies revealed neither of the two groups showed superiority in overall morbidity (OR 1.28, 95% CI 0.79-2.07, $P = 0.31$, $I^2 = 0\%$) and mortality (OR 1.03, 95% CI 0.14-7.44, $P = 0.98$, $I^2 = 0\%$). Meta-analysis of bile leak (OR 0.92, 95% CI 0.35 -2.44, $P = 0.87$, $I^2 = 0\%$) and hepatic insufficiency (OR 1.02, 95% CI 0.29 - 3.60, $P = 0.97$, $I^2 = 35\%$) showed no statistically significant difference.

Operating time, ischemic duration and hospital stay. There was no statistically significant difference in operating time (WMD -12.44, 95% CI -32.88 to 8.00, $P = 0.23$, $I^2 = 86\%$) between the two groups, also in hospital stay (WMD 0.63, 95% CI -1.60 to 2.85, $P = 0.58$, $I^2 = 91\%$) and in ischemic duration (WMD 0.61, 95% CI -1.40 to 2.61, $P = 0.55$, $I^2 = 43\%$)

DISCUSSION

The key points in hepatectomy are to control intraoperative bleeding and prevent postoperative complications such as liver failure and bile leakage[18]. Intraoperative blood loss has been shown to significantly influence the short-term prognosis of patients undergoing liver resection[19,20]. Hemihepatic vascular clamping selectively interrupts the arterial and venous inflow to the right or left hemiliver and therefore avoids both splanchnic blood stasis and ischemia or ischemia-reperfusion injury to the whole liver[9,21]. A retrospective study[22] indicated that the average bleeding volume and transfusion requirements were less in hemihepatic vascular occlusion group compared with Pringle maneuver group. But, other retrospective studies[8,23] showed no difference between the two groups. Our meta-analysis showed no significant difference in blood loss and transfusion requirements between the two groups. Three[14,16,17] of the four trials in the review showed no difference in the amount of hemorrhage and blood transfusion requirements, but one study[15] reported that the amount of operative blood loss and the incidence of blood transfusion were significantly higher in group THO patients (1685 mL *vs* 1159 mL, $P = 0.049$) and the volume of blood loss was much higher than in other studies. It could be explained by the fact that the patients in the study had cirrhosis and underwent complex central liver resections, while other trials included both cirrhotic and non-cirrhotic patients. The procedures presented herein were more difficult and time-consuming than conventional major hepatectomy and transected plane was also wider[15,24-26]. Both factors induced massive bleeding and difficulties in hemostasia.

Liver injury due to ischemia and subsequent reperfusion are major concerns in inflow vascular occlusion[27-29] and are usually monitored after surgery by measuring aminotransferase levels[30]. We found no significant difference on ALT levels on postoperative days 1, 3 and 7 in the two groups, also on AST levels on postoperative days 3 and 7. Three RCTs[14,15,17] and one retrospective study[18] drew the same conclusion. Theoretically, the blood flow in one lobe of the liver in group HHO is preserved and the liver function damage may be less than that in group THO[31].Yuan *et al*[16] indicated that the Pringle maneuver group was associated with a significantly higher peak in ALT and AST levels ($P = 0.01$). Meta-analysis showed that AST levels on postoperative day 1 were also higher in the THO group (WMD 342.27, 95% CI 217.28-467.26, $P = 0.00001$, $I^2 = 16\%$). Chau *et al*[23] concluded that patients subjected to HHO responded better than those subjected to the Prin-

gle maneuver in terms of earlier recovery of postoperative liver function. Therefore, HHO resulted in less liver injury and was advantageous in the recovery of postoperative liver function.

Unfortunately, only two trials in our analysis included data on ALT and AST levels. There were no significant differences in patients' general characteristics, resection margin, and ratio of cirrhotic to non-cirrhotic patients ($P = 0.05$). However, intermittent clamping was used in the trial by Wu *et al*[15], whereas Yuan *et al*[16] did continuous clamping if transaction time was ≤ 30min, otherwise intermittent clamping would be used. A RCT[32] comparing intermittent portal triad clamping with continuous clamping showed no statistically significant differences, although the peak AST level was lower in the intermittent portal triad clamping. Belghiti *et al*[33] suggested that in chronic patients, the transaminase levels were significantly higher in the continuous portal triad clamping than in the intermittent portal triad clamping. Cirrhotic liver and pre-existing liver were less able to tolerate ischemia than normal liver in clinical observations or animal experiments[28,34,35]. The proportion of chronic patients in the two RCTs were 100% and 61.7% respectively, which may influence the ALT and AST levels in HHO and THO groups and account for the lack of difference between the two groups on postoperative days 3 and 7. Due to the limited number and non-available data in the trials, no subgroup analysis was performed in patients with cirrhosis, which is known to increase the sensitivity of the livers to ischemia[30].

There was one death in Figueras' trials[14] in HHO group as a result of hepatic insufficiency in a patient with hepatitis C virus (HCV) cirrhosis . His blood loss during the operation was 2120 mL and 5 units of red blood cell transfusion were required. Yuan *et al*[16] reported one patient in the Pringle maneuver group who died of liver failure on the 26th d after a right hepatectomy. The total mortality was 0.51% and total peri-operative morbidity was 27.51%. But no statistically significant difference was found in the peri-operative mortality, peri-operative morbidity, operating time, ischemic duration and hospital stay. Complications included ascites, bile leak, hepatic insufficiency, portal thrombosis, pleural effusion, wound infection, hemorrhage and so on. Meta-analysis of bile leak and hepatic insufficiency showed no significant difference between THO group and HHO group.

This review has some limitations. First, our literature search might have not detected all relevant evidences and the number of RCTs included in this review is small. Second, incomplete reporting of important methodological issues, such as sample size calculation, randomization process and blinding assessment of trial quality, might raise doubts on the adequate power of these studies[36]. Third, the heterogeneity of the patients in the included trials may influence the conclusions as some trials included major and complex central liver resections and some included normal and cirrhotic livers.

In conclusion, the current evidence shows no advantage of hemihepatic vascular occlusion over the total hepatic inflow occlusion in terms of blood loss, transfusion requirement, mortality and morbidity, operating time and hospital stay. However, HHO results in less liver injury after liver resections. Further trials are required to assess optimal technique of hepatic vascular control for the patients hepatectomy especially for the patients with chronic cirrhosis.

COMMENTS

Background

Possibility of life-threatening hemorrhage always exists in patients with liver resection, so liver vascular control to reduce blood loss is important. Since the Pringle maneuver technique was successfully applied by Pringle in 1908, many methods of hepatic vascular control have been introduced to accelerate the development of hepatic surgery. Bismuth and Makuuchi proposed the hemihepatic vascular occlusion technique, which attracted much attention among surgeons.

Research frontiers

Both Pringle maneuver and hemihepatic vascular occlusion techniques can reduce blood loss during transaction of the hepatic parenchyma but Pringle maneuver produces ischemic injury to the remaining liver and intestinal congestion. Hemihepatic vascular occlusion technique has become very popular in recent years, because it is thought to limit visceral congestion and can protect the remaining liver. Many studies including randomized controlled trials (RCTs) have been designed to evaluate the safety, feasibility and efficiency of the two methods.

Innovations and breakthroughs

The authors searched and assessed all the RCTs comparing the two techniques and drew a conclusion by a systematic review and meta-analysis. They found that hemihepatic vascular occlusion did not offer benefit to the patients except for reducing ischemic liver injury after liver resections.

Applications

Hemihepatic vascular occlusion technique should be recommended for hepatectomy to reduce peri-operative blood loss and protect the remaining liver after the surgery.

Terminology

Hemihepatic vascular occlusion is a method which selectively interrupts the arterial and portal inflow to the part of the liver (right or left hemiliver) ipsilateral to the lesion that requires resection. It can be achieved after placing a curved renal pedicle clamp across the right or left portal structures. And Pringle maneuver involves compression of the hepatoduodenal ligament to interrupt all arterial and portal inflow to the whole liver.

Peer review

The manuscript is methodologically well designed and is concise in its data and conclusion. However, it should be subjected to linguistic revision to improve several mistakes in grammar and style.

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CASE REPORT

Cure of alopecia areata after eradication of *Helicobacter pylori*: A new association?

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Abstract

Alopecia areata is a disease of the hair follicles, with strong evidence supporting autoimmune etiology. Alopecia areata is frequently associated with immune-mediated diseases with skin manifestations such as psoriasis and lichen planus, or without skin manifestations such as autoimmune thyroiditis and idiopathic thrombocytopenic purpura. *Helicobacter pylori* (*H. pylori*) infection is present in around 50% of the world's population and has been associated with a variety of immune-mediated extra-digestive disorders including autoimmune thyroiditis, idiopathic thrombocytopenic purpura, and psoriasis. A case of a 43-year old man with an 8-mo history of alopecia areata of the scalp and beard is presented. The patient was being treated by a dermatologist and had psychiatric support, without any improvement. He had a history of dyspepsia and the urea breath test confirmed *H. pylori* infection. The patient went into remission from alopecia areata after *H. pylori* eradication. If such an association is confirmed by epidemiological studies designed for this purpose, new therapeutic options could be available for these patients, especially in areas where infection with *H. pylori* is highly prevalent.

INTRODUCTION

Alopecia areata is a disease of the hair follicles, with strong evidence supporting an autoimmune origin[1], although the exact pathogenesis of the disease is not clear. Alopecia areata has a frequency ranging from 0.7% to 3.8% in patients attending dermatology clinics, affects both sexes[2], and a familial occurrence is often reported[3,4]. The pattern of hair loss can vary and can affect any part of the body. Alopecia areata frequently occurs in association with other autoimmune diseases, including autoimmune thyroiditis[5], psoriasis[6-8] and Sjögren syndrome[9], among others.

Helicobacter pylori (*H. pylori*) is a microaerophilic Gram-negative bacterium that colonizes the gastric mucosa[10] and is present in around 50% of the world's population[11], with varying prevalence rates between 7% in the Czech Republic and 87% in a South African population[12]. In the case of Medellín, Colombia, prevalence of *H. pylori* infection in children under 12 years is 60.9%[13] and in adults, it is 77.2%[14]. *H. pylori* infection has been associated with the pathogenesis of gastric disorders such as gastritis, duodenal and gastric ulcers, gastric cancer, mucosa-associated lymphoid tissue lymphoma[10], and a variety of

extra-digestive disorders, many of them clearly identified as immune-mediated[15], such as idiopathic thrombocytopenic purpura[16,17], autoimmune thyroiditis[18,19], Sjögren's syndrome[20,21], rosacea[22] and psoriasis[23,24].

A case of a 43-year-old man with patchy alopecia areata and *H. pylori* infection is presented. The patient had hair regrowth after bacterial eradication.

CASE REPORT

A 43-year-old man presented with an 8-mo history of patchy hair loss in the scalp and beard (Figure 1A-C). He had consulted a dermatologist who prescribed 0.25% desoximetasone and 5% minoxidil, according to the guidelines for the management of alopecia[25], and had psychiatric support with escitalopram 5 mg/d, without any response other than progression of the condition.

The patient had a history of dyspepsia, therefore, he underwent analysis to determine *H. pylori* status. Urea breath test (^{13}C-UBT) (6.95 $\delta^{13}CO_2$; negative, < 1)[26], and *H. pylori* IgG antibodies (IgG index: 52.4; negative, < 9) were positive. Subsequent laboratory evaluation included normal values of ultrasensitive thyroid stimulating hormone, free thyroxine and free tri-iodothyronine; and negative antinuclear, antithyroid peroxidase and intrinsic factor antibodies. The patient was prescribed first line *H. pylori* eradication with proton pump inhibitor (omeprazole) 20 mg twice daily, amoxicillin 1000 mg twice daily, and clarithromycin 500 mg twice daily for 14 d, according to recommendations from the Maastricht Ⅲ Consensus Report[27], and was followed photographically every 2 wk. He was instructed not to take or apply any medications for alopecia areata. *H. pylori* eradication was confirmed 6 wk after treatment with a negative result of the ^{13}C-UBT (0.81 $\delta^{13}CO_2$).

Figure 1 shows the photographic sequence of the lesions before and after *H. pylori* eradication. From week 4, there was evidence of hair regrowth in the scalp and beard (Figure 1D-F). To date, the patient continues in complete remission from alopecia areata, as shown in Figure 1M-O.

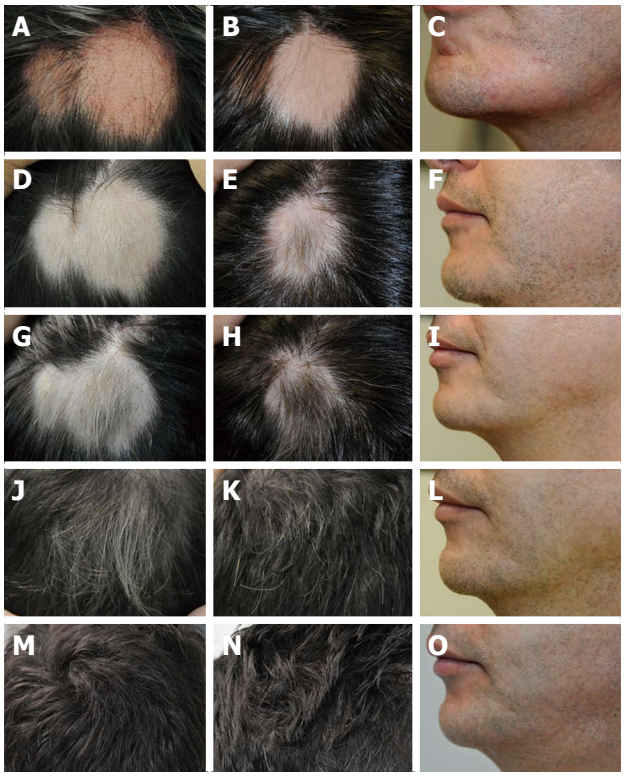

Figure 1 Photographic sequence of lesions before and after *Helicobacter pylori* eradication. A-C: Alopecia areata of the scalp (A and B) and beard (C) at baseline visit (week 0) before *Helicobacter pylori* (*H. pylori*) eradication. Positive ^{13}C-UBT (6.95 $\delta^{13}CO_2$); D-F: Evidence of hair regrowth at week 4; G-I: Hair regrowth at week 8. Negative ^{13}C-UBT (0.81 $\delta^{13}CO_2$); J-L: Hair regrowth at week 16; M-O: Hair regrowth at week 44. Negative ^{13}C-UBT (0.67 $\delta^{13}CO_2$).

DISCUSSION

H. pylori infection has been associated with numerous immune and non-immune disorders including dermatological conditions, such as chronic urticaria[28-30], rosacea[22,28,31-39], psoriasis[23,24], Schönlein-Henoch purpura[40-46], Behçet's disease[47,48], prurigo nodularis[49], chronic cutaneous pruritus[50], progressive systemic sclerosis[51-54], Sjögren's syndrome[20,21,55-57], and Sweet's syndrome[58]; many of them improving or going into remission after eradication of *H. pylori* infection[24,30,49,59-61]. Several mechanisms have been suggested to mediate the systemic effects of *H. pylori* infection, including the development of antigen-antibody complexes and cross-reactive antibodies (by molecular mimicry)[61-63], where antibodies developed against *H. pylori* cross-react with autoantigens to cause tissue damage, as has been reported in atrophic gastritis[62,64], chronic gastritis[65-67], chronic idiopathic thrombocytopenic purpura[16,17,68-70], Hashimoto's thyroiditis[19], atherosclerosis[71], arterial hypertension[72], unstable angina pectoris[73], ischemic heart disease[74,75], Alzheimer's disease[76], systemic sclerosis[77,78], central serous chorioretinopathy[79], iron deficiency[80,81], autoimmune pancreatitis[82-86], and chronic urticaria[87].

Alopecia areata has been described to be of autoimmune origin[88], with the presence of inflammatory cells around and within the human hair follicles. Alopecia areata has been associated with other autoimmune disorders including thyroid disease[89-93], psoriasis[6,7], and celiac disease[94-97]; conditions that have also been associated with *H. pylori* infection.

In the literature, there is ample evidence to suggest an association between *H. pylori* and alopecia areata that could explain the cure in this patient after eradication of infection. There is concurrent alopecia areata with immune diseases that are also concurrent with *H. pylori* infection. There are three different scenarios: immune-mediated skin diseases associated with *H. pylori* infection and alopecia areata, including psoriasis[6,7,23,24,98-103] and lichen planus[101,104-109]; immune-mediated non-skin conditions associated with *H. pylori* infection and alopecia areata, including autoimmune thyroiditis[18,19,110-115], celiac disease[94-97,116-118], idiopathic thrombocytopenic purpura[119,120], and autoimmune pancreatitis[82,84,85,121-124]; and laboratory findings that show the immunological nature of the conditions that are found in *H. pylori*-infected patients as well as in alopecia areata patients, including parietal cell antibodies[117,125-127] and thyroid antibodies[90,128].

After reviewing the medical literature, an association between *H. pylori* infection and alopecia areata has not been clearly demonstrated; only three reports have explored such association and had different results[129-131]. Abdel Hafez *et al*[131] have compared 31 patients with alopecia areata with 24 healthy controls and have found no significant difference in the *H. pylori* status, as determined by an antigen stool test. Rigopoulos *et al*[130] have compared *H. pylori* seroprevalence in 30 patients with alopecia areata and 30 healthy controls, and found no significant difference between the groups, whereas Tosti *et al*[129] have found, in a group of 68 patients with alopecia areata, that the seroprevalence of *H. pylori* infection was higher than in matched controls. It is of note that the presence of IgG antibodies against *H. pylori* does not confirm current infection and is only an indicator of previous exposure to the bacterium[132]. However, none of the studies tried to eradicate the infection and evaluate posterior hair regrowth.

Here, I have described the case of one patient who had patchy hair loss of the scalp and beard. The patient's condition started to improve within 4 wk of completing *H. pylori* eradication (Figure 1D-F). By week 16 (Figure 1J-L), the patient had completely reversed the hair loss, and by week 44 (Figure 1M-O), he remained *H. pylori*-negative and completely cured of alopecia areata. Although prior studies have only reported the prevalence of *H. pylori* infection in alopecia areata patients, to the best of my knowledge, this is the first documented case of reversed hair loss after *H. pylori* eradication.

There have been a few early studies in which antibiotic treatment was used in an attempt to cure alopecia areata, but in no case was there information on whether the patients were infected with *H. pylori*. Dapsone was used unsuccessfully[133,134]. There was one case of a 13-year-old girl with multiple autoimmune diseases who was successfully treated for alopecia areata with co-trimoxazole, a drug with antibiotic properties and immunomodulatory effects that could have been responsible for hair regrowth. Finally, there was one case in the literature describing the occurrence of alopecia areata after antibiotic treatment with rifampicin[135]. However, further case-control studies could be useful to rule out this possibility completely.

Hence, a common denominator in various autoimmune diseases is *H. pylori* infection; therefore, *H. pylori* status could be determined in several autoimmune conditions, and if positive, eradication treatment could follow as an initial step. More studies are needed to clarify the reality of the proposed association.

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Gut-liver axis plays a role in hepatocarcinogenesis of patients with Crohn's disease

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Abstract

The development of hepatocellular carcinoma (HCC) is attributed to several factors, including chronic viral infection, alcohol consumption, exposure to aflatoxin B1 and metabolic disorders. Several recent reports have shown that HCC can occur in patients with long-standing Crohn's disease (CD) in the absence of other underlying high-risk liver diseases. There may be an association between CD and hepatocarcinogenesis, however, the precise mechanism for this requires further investigations.

Key words: Inflammatory bowel disease; Crohn's disease; Hepatocellular carcinoma; Intestinal flora; Azathioprine; Enterohepatic circulation

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TO THE EDITOR

We have read with great interest the article by Ishida *et al*[1], which was recently published in *World J Gastroenterology*, issue No. 25, 2010. The authors reported a case of hepatocellular carcinoma (HCC) that occurred in a patient with Crohn's disease (CD) in the absence of chronic hepatitis or liver cirrhosis. This suggested that Azathioprine treatment could be related to hepatocarcinogenesis in CD patients. Upon reading this interesting case report, we wondered whether the risk factor for the development of HCC in the setting of CD was CD itself or its treatment.

Azathioprine is currently the most common immunosuppressive drug for the treatment of inflammatory bowel disease (IBD), particularly for maintaining the remission of the patients with a complex clinical course. There is little doubt that, once this drug is indicated, its treatment should be continued for an extended period of time. Whether or not immunosuppressive therapy increases the risk of malignancy in IBD patients is controversial. Although the prolonged use of Azathioprine is considered theoretically to increase the occurrence of cancer, studies aimed at elucidating the risk of neoplasia in IBD patients treated with Azathioprine have concluded that Azathioprine does not substantially increase the risk of cancer development[2-4]. A global consensus about the association between immunosuppressants and malignancies has suggested a favorable risk/benefit ratio in the long-term use of Azathioprine[5].

Although the causes of IBD remain incompletely understood, the prevailing consensus is that the intestinal flora drives an unmitigated intestinal immune response and inflammation in the genetically susceptible host. CD is considered to be a systemic disorder that often involves multiple organs including the gastrointestinal tract. A meta-analysis[6] that assessed the relative risk of all types of cancers occurring outside the gastrointestinal tract found an increased risk in CD patients; and a potential correlation between long-standing CD and the development of HCC may therefore exist. A recent study[7]

revealed an intimate cross-talk between gut microbes, the lower bowel and liver in the evolution of HCC, and demonstrated that gut microbes could promote HCC. Several mechanisms could be involved. Firstly, bacteria may alter the colonic mucosal integrity and/or receptor activation, permitting the passive or facilitated the entry of harmful bacteria or their products into the circulation. Secondly, the microbial colonization of the bowel may invoke the release of numerous cytokines from the intestine and/or mesenteric lymph nodes that act upon the liver. Finally, intestinal bacteria may disrupt enterohepatic feedback loops, such as those associated with bile acid recirculation.

Recently, enterohepatic *Helicobacter* species, such as *H. hepaticus*, *H. bilis*, *Helicobacter sp. flexispira* and *H. cinaedi*, which belong to a rare phyla of luminal flora, have been identified in the lower intestinal and biliary tract of animals, and their overgrowth may cause chronic inflammatory bowel and liver diseases in rodents, poultry and primates. These bacteria have also been implicated in gastroenteritis, cholecystitis and certain liver diseases, including HCC in humans[8-11]. Several clinical observations have indicated that the modulation of the gut-liver axis using probiotics may play a therapeutic role, especially in the pathophysiological conditions where intestinal microflora may be involved as a cofactor of chronic liver damage.

In summary, it is possible that several factors related to CD may directly or indirectly affect the development of HCC in patients with CD. We consider that altered gut microbes would more likely disrupt enterohepatic homeostasis and promote the development of liver cancer than medication. A study of cumulative cases and further researches may unmask the intestinal bacteria that are associated with the increased risk of HCC in humans. Such microbes may represent attractive therapeutic targets.

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Events Calendar 2011

January 14-15, 2011
AGA Clinical Congress of Gastroenterology and Hepatology: Best Practices in 2011 Miami, FL 33101, United States

January 20-22, 2011
Gastrointestinal Cancers Symposium 2011, San Francisco, CA 94143, United States

January 27-28, 2011
Falk Workshop, Liver and Immunology, Medical University, Franz-Josef-Strauss-Allee 11, 93053 Regensburg, Germany

January 28-29, 2011
9. Gastro Forum München, Munich, Germany

February 4-5, 2011
13th Duesseldorf International Endoscopy Symposium, Duesseldorf, Germany

February 13-27, 2011
Gastroenterology: New Zealand CME Cruise Conference, Sydney, NSW, Australia

February 17-20, 2011
APASL 2011-The 21st Conference of the Asian Pacific Association for the Study of the Liver
Bangkok, Thailand

February 22, 2011-March 04, 2011
Canadian Digestive Diseases Week 2011, Vancouver, BC, Canada

February 24-26, 2011
Inflammatory Bowel Diseases 2011-6th Congress of the European Crohn's and Colitis Organisation, Dublin, Ireland

February 24-26, 2011
2nd International Congress on Abdominal Obesity, Buenos Aires, Brazil

February 24-26, 2011
International Colorectal Disease Symposium 2011, Hong Kong, China

February 26-March 1, 2011
Canadian Digestive Diseases Week, Westin Bayshore, Vancouver, British Columbia, Canada

February 28-March 1, 2011
Childhood & Adolescent Obesity: A whole-system strategic approach, Abu Dhabi, United Arab Emirates

March 3-5, 2011
42nd Annual Topics in Internal Medicine, Gainesville, FL 32614, United States

March 7-11, 2011
Infectious Diseases: Adult Issues in the Outpatient and Inpatient Settings, Sarasota, FL 34234, United States

March 14-17, 2011
British Society of Gastroenterology Annual Meeting 2011, Birmingham, England, United Kingdom

March 17-19, 2011
41. Kongress der Deutschen Gesellschaft für Endoskopie und Bildgebende Verfahren e.V., Munich, Germany

March 17-20, 2011
Mayo Clinic Gastroenterology & Hepatology 2011, Jacksonville, FL 34234, United States

March 18, 2011
UC Davis Health Informatics: Change Management and Health Informatics, The Keys to Health Reform, Sacramento, CA 94143, United States

March 25-27, 2011
MedicReS IC 2011 Good Medical Research, Istanbul, Turkey

March 26-27, 2011
26th Annual New Treatments in Chronic Liver Disease, San Diego, CA 94143, United States

April 6-7, 2011
IBS-A Global Perspective, Pfister Hotel, 424 East Wisconsin Avenue, Milwaukee, WI 53202, United States

April 7-9, 2011
International and Interdisciplinary Conference Excellence in Female Surgery, Florence, Italy

April 15-16, 2011
Falk Symposium 177, Endoscopy Live Berlin 2011 Intestinal Disease Meeting, Stauffenbergstr. 26, 10785 Berlin, Germany

April 18-22, 2011
Pediatric Emergency Medicine: Detection, Diagnosis and Developing Treatment Plans, Sarasota, FL 34234, United States

April 20-23, 2011
9th International Gastric Cancer Congress, COEX, World Trade Center, Samseong-dong, Gangnam-gu, Seoul 135-731, South Korea

April 25-27, 2011
The Second International Conference of the Saudi Society of Pediatric Gastroenterology, Hepatology & Nutrition, Riyadh, Saudi Arabia

April 25-29, 2011
Neurology Updates for Primary Care, Sarasota, FL 34230-6947, United States

April 28-30, 2011
4th Central European Congress of Surgery, Budapest, Hungary

May 7-10, 2011
Digestive Disease Week, Chicago, IL 60446, United States

May 12-13, 2011
2nd National Conference Clinical Advances in Cystic Fibrosis, London, England, United Kingdom

May 19-22, 2011
1st World Congress on Controversies in the Management of Viral Hepatitis (C-Hep), Palau de Congressos de Catalunya, Av. Diagonal, 661-671 Barcelona 08028, Spain

May 21-24, 2011
22nd European Society of Gastrointestinal and Abdominal Radiology Annual Meeting and Postgraduate Course, Venise, Italy

May 25-28, 2011
4th Congress of the Gastroenterology Association of Bosnia and Herzegovina with international participation, Hotel Holiday Inn, Sarajevo, Bosnia and Herzegovina

June 11-12, 2011
The International Digestive Disease Forum 2011, Hong Kong, China

June 13-16, 2011
Surgery and Disillusion XXIV SPIGC, II ESYS, Napoli, Italy

June 14-16, 2011
International Scientific Conference on Probiotics and Prebiotics-IPC2011, Kosice, Slovakia

June 22-25, 2011
ESMO Conference: 13th World Congress on Gastrointestinal Cancer, Barcelona, Spain

June 29-2, 2011
XI Congreso Interamericano de Pediatria "Monterrey 2011", Monterrey, Mexico

September 2-3, 2011 Falk Symposium 178, Diverticular Disease, A Fresh Approach to a Neglected Disease, Gürzenich Cologne, Martinstr. 29-37, 50667 Cologne, Germany

September 10-11, 2011
New Advances in Inflammatory Bowel Disease, La Jolla, CA 92093, United States

September 10-14, 2011
ICE 2011-International Congress of Endoscopy, Los Angeles Convention Center, 1201 South Figueroa Street Los Angeles, CA 90015, United States

September 30-October 1, 2011
Falk Symposium 179, Revisiting IBD Management: Dogmas to be Challenged, Sheraton Brussels Hotel, Place Rogier 3, 1210 Brussels, Belgium

October 19-29, 2011
Cardiology & Gastroenterology | Tahiti 10 night CME Cruise, Papeete, French Polynesia

October 22-26, 2011
19th United European Gastroenterology Week, Stockholm, Sweden

October 28-November 2, 2011
ACG Annual Scientific Meeting & Postgraduate Course, Washington, DC 20001, United States

November 11-12, 2011
Falk Symposium 180, IBD 2011: Progress and Future for Lifelong Management, ANA Interconti Hotel, 1-12-33 Akasaka, Minato-ku, Tokyo 107-0052, Japan

December 1-4, 2011
2011 Advances in Inflammatory Bowel Diseases/Crohn's & Colitis Foundation's Clinical & Research Conference, Hollywood, FL 34234, United States

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3 **Tian D**, Araki H, Stahl E, Bergelson J, Kreitman M. Signature of balancing selection in Arabidopsis. *Proc Natl Acad Sci USA* 2006; In press

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4 **Diabetes Prevention Program Research Group**. Hypertension, insulin, and proinsulin in participants with impaired glucose tolerance. *Hypertension* 2002; **40**: 679-686 [PMID: 12411462 PMCID:2516377 DOI:10.1161/01.HYP.0000035706.28494.09]

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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 DOI:10.1097/01.ju.0000067940.76090.73]

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6 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303 DOI:10.1136/bmj.325.7357.184]

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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 DOI:10.1046/j.1526-4610.42.s2.7.x]

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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 DOI:10.1097/00003086-200208000-00026]

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9 Outreach: Bringing HIV-positive individuals into care. *HRSA Careaction* 2002; 1-6 [PMID: 12154804]

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Personal author(s)

10 **Sherlock S**, Dooley J. Diseases of the liver and billiary system. 9th ed. Oxford: Blackwell Sci Pub, 1993: 258-296

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11 **Lam SK**. Academic investigator's perspectives of medical treatment for peptic ulcer. In: Swabb EA, Azabo S. Ulcer disease: investigation and basis for therapy. New York: Marcel Dekker, 1991: 431-450

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13 **Harnden P**, Joffe JK, Jones WG, editors. Germ cell tumours V. Proceedings of the 5th Germ cell tumours Conference; 2001 Sep 13-15; Leeds, UK. New York: Springer, 2002: 30-56

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14 **Christensen S**, Oppacher F. An analysis of Koza's computational effort statistic for genetic programming. In: Foster JA, Lutton E, Miller J, Ryan C, Tettamanzi AG, editors. Genetic programming. EuroGP 2002: Proceedings of the 5th European Conference on Genetic Programming; 2002 Apr 3-5; Kinsdale, Ireland. Berlin: Springer, 2002: 182-191

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15 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/index.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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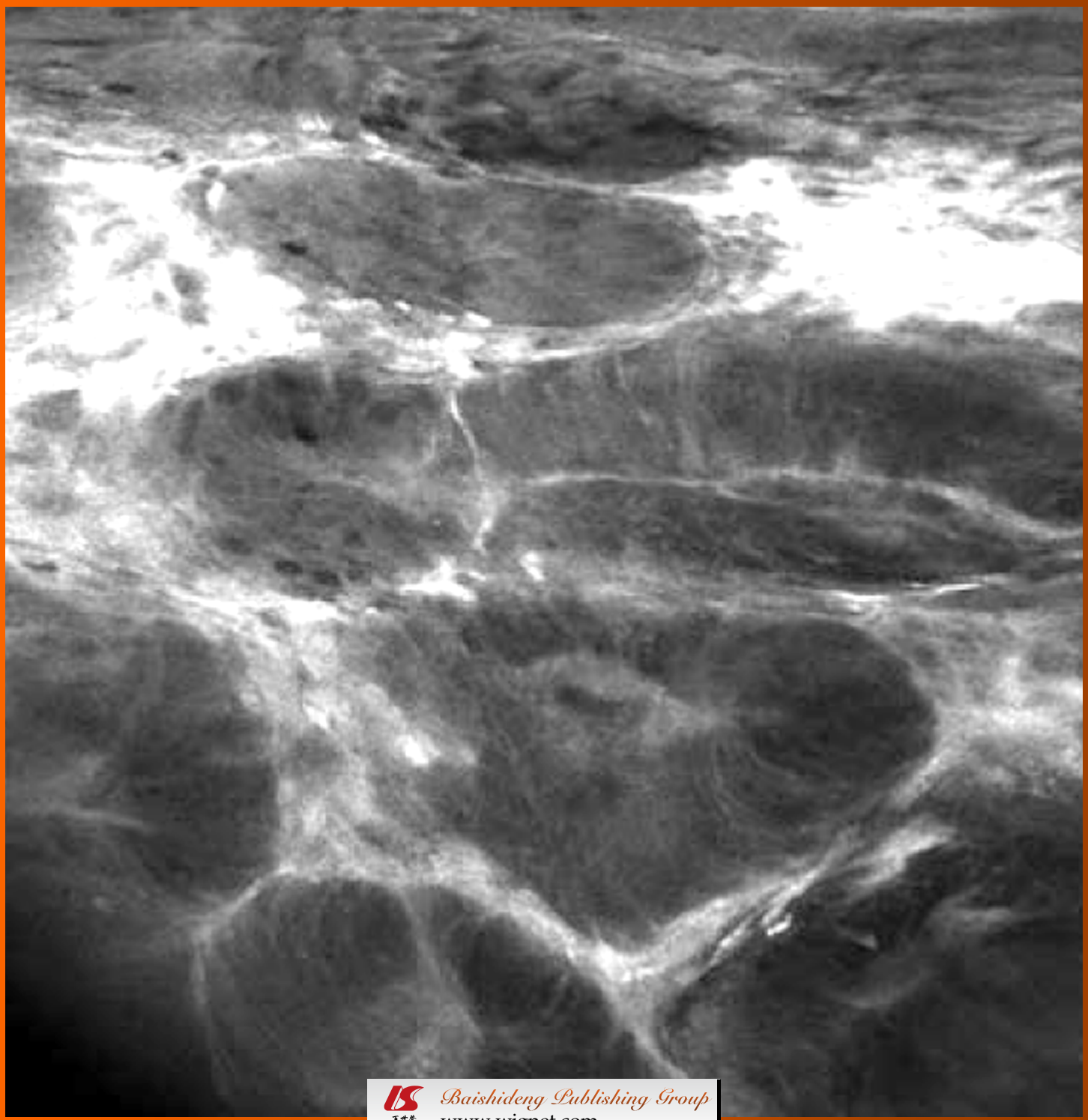

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EDITORIAL

Infected pancreatic necrosis: Not necessarily a late event in acute pancreatitis

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Abstract

It is widely believed that infection of pancreatic necrosis is a late event in the natural course of acute pancreatitis. This paper discusses the available data on the timing of pancreatic infection. It appears that infected pancreatic necrosis occurs early in almost a quarter of patients. This has practical implications for the type, timing and duration of preventive strategies used in these patients. There are also implications for the classification of severity in patients with acute pancreatitis. Given that the main determinants of severity are both local and systemic complications and that they can occur both early and late in the course of acute pancreatitis, the classification of severity should be based on their presence or absence rather than on when they occur. To do otherwise, and in particular overlook early infected pancreatic necrosis, may lead to a misclassification error and fallacies of clinical studies in patients with acute pancreatitis.

Key words: Acute pancreatitis; Classification; Enteral nutrition; Infected pancreatic necrosis; Pancreatic infection

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INTRODUCTION

Mortality in patients with acute pancreatitis is determined by both local and systemic factors[1,2]. The local factor is infection of pancreatic and peripancreatic necrosis/collections. The systemic factor is organ dysfunction, especially when it persists and/or when multiple organ systems are involved. The timing of these local and systemic factors is thought to be important, and thus acute pancreatitis is generally regarded as having an early and late phase[3,4]. Infected pancreatic necrosis (IPN) is considered the cardinal feature of the late phase[5-7]. This view is, however, challenged by a body of evidence that demonstrates that IPN also occurs early in some patients with acute pancreatitis. The present editorial will examine the time course of IPN and consider the clinical implications of the timing of pancreatic infection.

EARLIER SURGICAL STUDIES

The incidence and significance of early IPN can be reliably examined as there are published series that include operations performed during the first and second week. In 1986, Beger and colleagues published a seminal prospective clinical study from Germany that evaluated the bacteriological status of pancreatic necrosis in relation to the timing of surgery for acute pancreatitis[8]. Overall, 39% (45/114) of the consecutive series of patients had a

Table 1 Timing of pancreatic infection in the referred clinical studies *n* (%)

Study ID	Setting	No. of patients with confirmed IPN	Duration of disease at the time of diagnosing IPN		
			Day 1-7	Day 8-14	Day 15 +
Beger *et al*[8], 1986	Germany	45	5 (11)	8 (18)	32 (71)
Rattner *et al*[9], 1992	USA	44	2 (5)	10 (23)	32 (72)
Gerzof *et al*[10], 1987	USA	36	8 (22)	12 (33)	16 (45)
Tsui *et al*[16], 2009	China	65	1 (2)	15 (23)	49 (75)
Besselink *et al*[17], 2009	Netherlands	98	5 (5)	13 (13)	80 (82)
Overall		288	21 (7)	58 (20)	209 (73)

IPN: Infected pancreatic necrosis.

positive bacteriological culture of the debrided necrosis. Although pancreatic infection was most often detected after the second week, it is pertinent to note that 11% and 29% of the patients developed IPN within the first 7 and 14 d after onset of acute pancreatitis, respectively (Table 1). Another study, from the Warshaw group, looked back at 44 patients with proven IPN and demonstrated a similar incidence of early IPN with 5% and 28% within the first 7 and 14 d, respectively[9].

FNA STUDIES

Further evidence regarding the timing of development of IPN comes from studies that evaluated the utility of fine-needle aspiration (FNA) for the diagnosis of pancreatic infection. The first rigorous study was reported in 1987 by Gerzof and colleagues who performed computed tomography (CT)-guided percutaneous FNA and Gram staining in 60 patients with suspected pancreatic infection[10]. Overall, 60% (36/60) had pancreatic infection confirmed, with 22% (8/36) within 7 d of the onset of acute pancreatitis and 56% (20/36) within 14 d (Table 1). Similarly, in a study from Germany (1988-1996) on the utility of ultrasound-guided FNA in 98 patients with CT-proven pancreatic necrosis, it was shown that the overall incidence of IPN was 34%. During the first week, 21% (7/33) of patients had a positive FNA, and this was confirmed by bacteriological culture of the debrided necrosis[11].

BIOMARKER STUDIES

Another potential source of evidence regarding the timing of IPN in acute pancreatitis comes from studies that evaluated different serological markers of infection, for instance procalcitonin[12,13]. These studies used FNA as the gold standard to diagnose IPN but they did not formally report on the timing of the onset of IPN. However, it is interesting to note that in some cases FNA yielded a positive result as early as day 2[14] and day 3[15].

MOST RECENT STUDIES

The timing of diagnosing of IPN has been specifically examined in two recent studies, both published in 2009. In a study from China (2000-2008) there were 336 patients with predicted severe acute pancreatitis and all received intravenous antibiotic prophylaxis for 14 d from admission[16]. Infected pancreatic necrosis was confirmed by FNA in 19% (66/336) of patients overall and 25% (16/66) of these patients had proven IPN within the first 14 d (Table 1). In a study from the Netherlands (2004-2007) there were 154 patients with pancreatic necrosis and all received enteral nutrition (EN)[17]. Infected pancreatic necrosis was confirmed in 64% (98/154) of patients overall. In 5% (5/98) of these patients, IPN was proven within the first 7 d and in 18% (18/98) within the first 14 d (Table 1). These modern studies are in accordance with earlier studies, which showed that IPN occurs early in a notable proportion of patients. Furthermore, the two most recent studies may have underestimated the incidence of early IPN for three reasons. The first is that they were carried out in an era when there was a waning enthusiasm for the liberal use of FNA[18-20] and it is probable that some patients with early IPN were overlooked. The second is that there is a reported false negative rate for FNA of up to 10%[9,11]. The third reason is that the use of antibiotics and EN in the two studies may have prevented or postponed the clinical manifestation of IPN beyond the first two weeks after onset[21-23].

TRENDS IN THE INCIDENCE OF PANCREATIC INFECTION

Comparison of earlier studies with more recent studies reveals an apparent reduction in the incidence of early IPN from 29% in the 1980s[8] to 18% in the 2000s[17], but not in the overall incidence of IPN during the same time period. The explanation for this reduction is a matter of speculation, but it is worth noting that both studies[8,17] included patients with pancreatic necrosis and all patients had intra-operative confirmation of IPN. What was different between the two studies is the employed management strategies: "nil-by-mouth" and early surgery in the 1980s[8] in contrast to EN and late surgery in the 2000s[17]. The observation that there is a reduction in the early incidence, but not overall incidence, of IPN raises the questions as to whether standard EN is only able to prevent early IPN, whether it is delivered for long enough to prevent late IPN, and which criteria ought

Table 2 The new classification of severity of acute pancreatitis (Modified from[2])

Severity category	Local determinants		Systemic determinants
Mild	No (peri)pancreatic necrosis	and	No organ failure
Moderate[1]	Sterile (peri)pancreatic necrosis	or	Transient organ failure
Severe[1]	Infected (peri)pancreatic necrosis	or	Persistent organ failure
Critical	Infected (peri)pancreatic necrosis	and	Persistent organ failure

[1]Severity is graded on the basis of more severe local or systemic determinants (e.g. sterile pancreatic necrosis without organ failure has to be graded as moderate; sterile pancreatic necrosis with persistent organ failure has to be graded as severe).

to be used to stop EN. If standard EN cannot prevent late IPN then it is important to evaluate more advanced enteral formulations, including those supplemented with glutamine, antioxidants, and/or other targeted treatments[24-27].

CONCLUSION

With the main focus on the diagnosis and treatment of late IPN it appears that early IPN may have been overlooked. There are two important practical clinical implications that derive from giving due recognition to the importance of early IPN. The first is that more effective prophylactic strategies are required, as it would appear that the overall incidence of IPN has not decreased, and may even be increasing. The cornerstone of this prophylactic strategy must be EN[22,23,26], but there remain questions about when to start, what to give and when to stop. The second implication relates to the importance of IPN in determining the severity of acute pancreatitis and how this might be reflected in any classification of severity. More specifically, the severity of acute pancreatitis relates to the presence or absence of IPN rather than whether it occurs early or late in the disease course. The timing of IPN varies widely between patients and, as discussed above, occurs during the first two weeks after onset of acute pancreatitis in almost a quarter of patients. The recently proposed classification of the severity of acute pancreatitis (Table 2) takes this into account as it is based on the presence or absence of local and systemic complications[2]. It also recognizes the dynamic nature of these complications allowing for the transition from sterile to infected pancreatic and peripancreatic necrosis, and transient to persistent organ dysfunction. Furthermore, the new classification of severity takes into account the interaction between the local and systemic determinants as it has been shown that mortality rate is significantly worse in patients with both IPN and organ failure, than either alone[1]. This new severity classification system is based on actual determinants of severity and will prove useful to practicing clinicians managing individual patients through the early and late phases of acute pancreatitis and will provide a more reliable way of selecting and matching groups of patients for clinical trials, including those seeking to prevent or treat IPN in patients with acute pancreatitis.

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EDITORIAL

What's new about inflammatory bowel diseases in 2011

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Abstract

Inflammatory bowel diseases (IBD) are chronic disorders of the intestine with increasing incidence in Europe, Northern America and asiatic countries such as china. Thus, we have putted together these topic highlight articles to give insights into the current understanding of IBD pathogenesis, diagnostics and treatment.

Key words: Inflammatory bowel disease; Endoscopy; Endomicroscopy; Confocal laser endomicroscopy; Cytokines; Immune system; Colorectal cancer; Postoperative recurrence

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FROM THE EDITOR

Inflammatory bowel diseases (IBD) are chronic disorders of the intestine with increasing incidence in Europe, Northern America and Asiatic countries such as china. Thus, we have putted together these topic highlight articles to give insights into the current understanding of IBD pathogenesis, diagnostics and treatment. The articles[1-6] aim at informing both expert gastroenterologists and physicians who are not very familiar with IBD. We are focusing mainly on topics that have undergone basic changes within the last decade. In view of diagnostic, modern high resolution ultrasound has become an important instrument for clinical monitoring of disease activity in some countries. Detection of intraepithelial neoplasia (IEN) and the therapeutic algorithm of IEN management in ulcerative colitis have undergone changes. Furthermore, the change of paradigm in Crohn`s disease surgery towards a very cautious extent of bowel resection combined with minimal invasive (endoscopic) surgery has ameliorated the outcome. In this context, some new data about the postoperative recurrence of Crohn's disease and its management are pointed out. Even in this field, prognostic parameters and data about the adequate therapeutic option in the case of recurrence are lacking. Thus, we give overview concerning the main issues in surgery and disease recurrence as well as current treatment options. To get insights into IBD pathogenesis, we inform about the mucosal interface and the immune system also highlighting the link between inflammation and carcinogenesis.

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TOPIC HIGHLIGHT

Dr. Jonas Mudter, MD, *Series Editor*

Innate and adaptive immunity in inflammatory bowel disease

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Abstract

Inflammatory bowel diseases are the consequence of a dysregulated mucosal immune system. The mucosal immune system consists of two arms, innate and adaptive immunity, that have been studied separately for a long time. Functional studies from *in vivo* models of intestinal inflammation as well as results from genome-wide association studies strongly suggest a cross-regulation of both arms. The present review will illustrate this interaction by selecting examples from innate immunity and adaptive immunity, and their direct impact on each other. Broadening our view by focusing on the cross-regulated areas of the mucosal immune system will not only facilitate our understanding of disease, but furthermore will allow identification of future therapeutic targets.

Key words: Inflammatory bowel diseases; Immune system

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INTRODUCTION

The pathogenesis of inflammatory bowel diseases (IBD) has not been completely resolved. However, the general hypothesis that in genetically predisposed people the exposure to distinct environmental factors results in a dysregulation of the mucosal immune system is still valid. Before genetics started to dominate the field, immunology identified various factors as critical in initiating intestinal inflammation. These studies included the functional characterization of T cell subpopulations and the relevance of the specific cytokines released. While the effector T cell response reflects one arm of the immune system, genetic work as well as studies on barrier function shed light on the second arm of the immune system. These two forms of immunity can be described as: (1) a more simplistic innate form, recognizing evolutionary conserved bacterial and viral patterns, resulting in a rapid but limited response; and (2) the adaptive form develops a highly specific immune response, that requires more time to evolve but provides immunological memory.

Each component plays a key role in the pathogenesis of IBD. Thus research in the field could be divided into groups focusing on the adaptive immune system, while other groups revealed innate factors at the site of epithelial cells or within the lamina propria which play a critical role. Cross-regulation of the innate and adaptive immune system was widely ignored.

Genome-wide association studies (GWAS) shed new light into the understanding of the pathogenesis[1,2].

The results obtained from GWAS not only confirmed the relevance of earlier characterized pathways, but also opened novel avenues and, in particular, provided strong evidence for a close link between the innate and the adaptive immune system in regulating the sensitive balance of the mucosal immune system.

Probably even more remarkable is the fact that GWAS have identified a large number of major loci, with many associations shared between various autoimmune diseases. These associations highlight key roles for lymphocyte activation, and prioritize specific cytokine pathways and mechanisms of host-microbe recognition[3]. Thus the close link and cross-regulation between the innate and adaptive immune system plays a critical role not only in IBD, but also in other autoimmune diseases. Interestingly, similarities of association patterns between various autoimmune diseases are particularly intriguing. Thus the question occurs as to which cells belong to the innate and which to the adaptive immune system.

In the intestine, innate immunity includes the epithelial barrier and phagocytic cells within the lamina propria (e.g. macrophages, dendritic cells, and neutrophils). Notably, patients exhibiting genetic defects in innate immunity (e.g. chronic granulomatous disease, Hermansky Pudlak syndrome) have an increase incidence of IBD[4,5]. This correlation has led to the development of agents used to boost innate immunity (e.g. granulocyte macrophage colony-stimulating factor) as therapeutic agents in IBD[6].

T lymphocytes represent the key cell population of the adaptive immunity arm. T cells become activated, secrete cytokines and affect all other cell types within a local environment (macrophages, dendritic cells, neutrophils, epithelium, endothelial cells, stromal elements). Both human and murine studies have led to the recognition that different T cell subpopulations are aberrantly activated in Crohn's disease (CD) *versus* ulcerative colitis (UC)[7,8]. T helper cell type 1 (Th1)-mediated immune responses are typically evoked in response to an intracellular pathogen presented by an antigen-presenting cell in the presence of interleukin (IL)-12. The coordinated immune response is elicited to localize the infectious agent and to secrete factors that either promote apoptosis [e.g. interferon (IFN)-γ, tumor necrosis factor-α] or induce the differentiation of cytotoxic T lymphocytes. The hallmark of a Th1 response is granuloma[9]. Recently, an additional Th subset has been described, the so-called Th17 cells[10]. These cells produce IL-17 and IL-22, both of which are pro-inflammatory cytokines capable of promoting local tissue destruction. Th17 cells are activated by the combination of IL-6 and transforming growth factor (TGF)-β and are induced to further differentiate to mature IL-17-secreting cells by IL-23[11]. IL-23 belongs to the IL-12 family and shares the p40 subunit with IL-12. IL-17 as well as IL-22 are found at increased levels in inflamed CD mucosa suggesting that these cytokines play a role in disease pathogenesis[12,13]. Functional data from mice and men are in support of this hypothesis as described in more detail below. An additional Th subset consists of Th2 cells. These secrete IL-4, IL-5, and IL-13[14]. Th2 cells promote atopy with induction of IgE responses, and eosinophil and mast cell activation. UC was thought to represent a Th2-driven disease, but the absence of IL-4 in colonic tissue from UC patients and the observation that both IL-13 and IFN-γ are found at elevated levels in UC mucosa changed this dogma[15]. Recent data suggest that IL-13 originates from a natural killer T cell, and targets the epithelial cell to become dysfunctional[7,16]. Consequently, UC may be more of a superficial epithelial injury disorder.

Last, there are regulatory T cell subpopulations. This is of particular importance since the immunological defense of the intestine, in contrast to the systemic immune system, is one of suppression. Healthy individuals generally do not develop systemic immune responses against commensal flora or dietary antigens. Regulatory cells are responsible for the immunologically suppressed milieu in the intestinal mucosa. One group of regulatory cells contributing to this milieu are regulatory T cells[17,18]. Various subsets have been described as being involved in suppressing mucosal responses, but the detailed description is beyond the scope of this review. Tr1 secrete IL-10[19], a potent anti-inflammatory cytokine. The impact in intestinal inflammation is underlined by the fact that IL-10-deficient mice develop spontaneous intestinal inflammation[20]. Th3 cells produce TGF-β, another potent immunosuppressant cytokine that promotes IgA production while suppressing T and B cell activation[21]. In addition, there are $CD4^+$ $CD25^+$ Treg requiring the transcription factor FoxP3[17,18]. Remarkably, the absence of FoxP3 in humans (IPEX syndrome)[22,23] and mouse (Scurfy) is followed by an autoimmune endocrine disease, immunodeficiency, and an enteropathy mainly affecting the small bowel.

Although there are specific T cell subpopulations, exclusive activation of a single T cell subpopulation during the course of an immune response is impossible. There is additional experimental evidence suggesting that the classification of T cell subpopulations on the basis of cytokine secretion profiles are helpful but not absolute. It seems to be more important to underscore that a dysregulated immune response of any type is poorly tolerated by the gastrointestinal tracts and thus results in intestinal inflammation.

Under which conditions does dysregulation of innate and adaptive immunity occur? Luminal antigens cross the epithelial barrier, and this process is significantly increased during intestinal inflammation. The role of epithelial and Paneth cells within the first line of defense is discussed within this issue by others. The antigens reaching the lamina propria will first activate the innate immune system via pattern recognition receptors and second, be presented by professional antigen-presenting cells resulting in an effector T cell response as described above.

The present review will serve to illustrate the cross-regulation of the innate and adaptive immune system within this process and the impact on the pathogenesis of IBD. The cross-regulation will be described by selecting two examples. First, NOD2 a pattern recognition receptor, thus belonging primarily to the innate immune system. However, we will demonstrate that NOD2 ex-

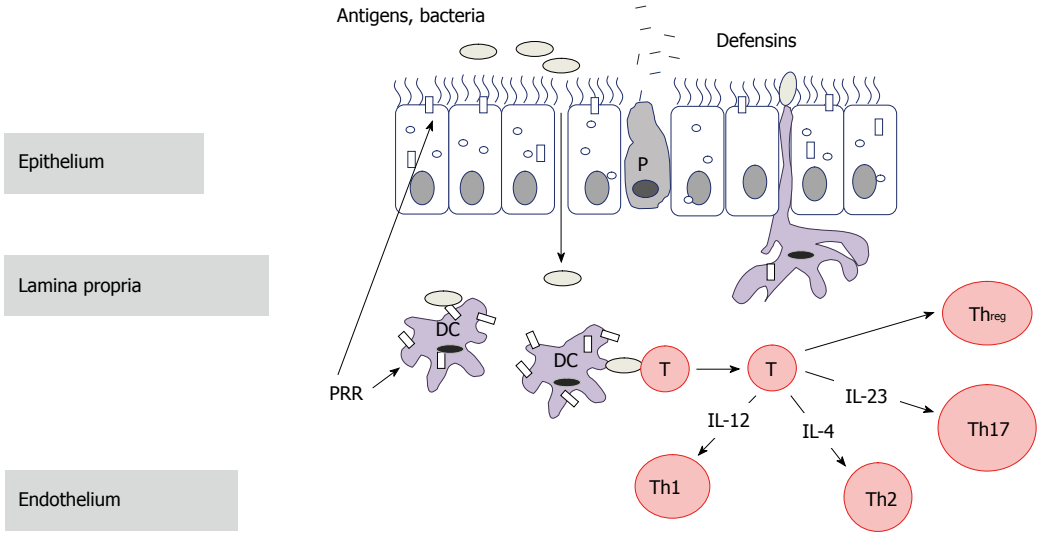

Figure 1 Cell populations primarily belonging to the innate immune system are marked blue, and cells primarily categorized as cells of the adaptive immune system are marked red. Purple marked cells identify the cross-link between both systems. IL: Interleukin.

erts a direct impact on the regulation of the adaptive immune system. This cross-regulation leads to the second example that is directly linked to NOD2, namely the IL-12 family (for illustration see Figure 1).

NOD2

NOD2 (also designated *CARD15*) was identified by fine mapping of the IBD1 locus by positional cloning and candidate gene analysis as the first gene to be firmly associated with CD susceptibility in North American and European populations[24-26]. Three main variants, *R702W*, *G908R*, and *1007fs*, exhibit the strongest CD association[27]. All three variants alter the C-terminal third of the gene product, and are within or close to a region of leucin-rich repeats thought to be involved in ligand recognition. Individuals with one of the three major disease-associated alleles have a 2-4-fold increased risk of developing CD, whereas homozygous or compound heterozygous carriers have a 15-40-fold increase in risk[28]. NOD2 is a cytoplasmic protein that serves as a microbial sensor for muramyl dipeptide (MDP), a peptidoglycan motif present in the cell wall of Gram-positive and Gram-negative bacteria[29,30]. There are currently three mechanistic explanations for the apparent inflammation-promoting functions of the variant NOD2 proteins in the literature. These hypotheses are not mutually exclusive and may remain valid in combination.

Transfection of wild-type, but not mutant *NOD2* into intestinal epithelial cells inhibited uptake or growth of invasive bacteria[31]. Accordingly, mice lacking *NOD2* showed a defect in intestinal innate defence against oral infection with *L. monocytogenes*, which was accompanied by diminished expression of at least two Paneth cell-derived antimicrobial peptides, Defcr4 and Decfcr-rs10[32]. CD patients with mutant *NOD2* were shown to have decreased expression of the human Paneth cell α-defensins HD-5 and HD-6 in the small intestine[33], suggesting that CD-associated *NOD2* mutations may be functionally equivalent to the loss of the protein in knockout mice. This model would initially restrict the function of NOD2 to the innate system, however when considering that a decrease in antimicrobial peptides results in an increase of bacterial translocation and thus activation of the adaptive immune system, the link becomes apparent.

Incubation of normal murine splenic macrophages with MDP resulted in suppression of IL-12p40 and IL-12p70 secretion induced by stimulation with Toll-like receptor (TLR) 2 ligands, such as peptidoglycan[34]. This suppression did not occur in cells lacking *NOD2*, or in cells expressing a mutant form of *NOD2* after transfection. A similar mechanism appeared to act *in vivo*, as systemic administration of peptidoglycan to *NOD2*$^{-/-}$ mice induced more serum IL-12p40 and IL-12p70 compared to wild-type mice. The IL-12-enhancing effects occurred only *via* TLR2, but not other TLRs[34]. Thus TLR2 would be expected to be uniquely capable of promoting colonic inflammation under conditions of NOD2 deficiency. In this model NOD2 is activated as an innate receptor, however the effector response directly activates the adaptive immune system. This is further underlined by the findings described below for the IL-12 family.

Macrophages of mice homozygous for a mutant *NOD2* allele (*NOD*^{2939iC}) equivalent to the most common CD-associated allele (*3020insC*) were shown to secrete higher levels of the mature form of IL-1β and have elevated IL-1β mRNA levels, as well as increased IkB kinase and nuclear factor-κB activities, upon stimulation with MDP relative to wild-type macrophages but retain normal responses to TLR ligands[35]. Simultaneously, these mice exhibited greater colonic inflammation upon experimental challenge with dextran sulfate sodium, a phenotype that was attenuated by treatment with an IL-1 receptor antagonist[35]. These data suggest that the variant NOD2 protein expressed in these mice promotes processing of proIL-1β to mature, biologically active, IL-1β. Consistent with a

role of IL-1β in regulating colitis, mice deficient in IL-1β converting enzyme have reduced inflammation in an experimental colitis model[36].

IL-12 FAMILY

A key role for the IL-12 family in the pathogenesis of IBD was initially suggested over a decade ago. In this study neutralizing antibodies targeting "IL-12" resulted in an amelioration of TNBS-induced colitis in mice[37]. In 2004, anti-IL-12 treatment showed efficacy in patients with CD, although the primary endpoint of this study was safety and not clinical efficacy[38]. In a more recent study investigating an anti-IL-12 antibody in patients with CD, the primary endpoint, namely the clinical response at week 8 was not achieved[39]. At this time, since IL-12 is the key Th1 cytokine, CD was classified as a Th1-mediated disease. These findings additionally supported the concept that CD is driven by the acquired immune system. In 2006, another member of the IL-12 family, namely IL-23, came into the focus in IBD. GWAS revealed a highly significant association between CD and the *IL-23R* gene on chromosome 1p31, which encodes a subunit of the IL-23 receptor. An uncommon coding variant confers strong protection against CD[40,41].

IL-12 and IL-23 share the subunit p40 that builds a heterodimer with p35, in the case of IL-12, and with the subunit p19, in the case of IL-23. IL-23 caused some confusion in the field, since the antibody administered in the clinical study published in 2004 targeted the IL-12 subunit p40, thus it suddenly became unclear whether the beneficial effect observed had been mediated by neutralizing IL-12 or IL-23. Several animal studies added valuable information to this controversy. In a model of CD40-induced colitis, neutralizing either the subunit p40 or the subunit p19 was followed by a significant reduction of the macroscopic and histologic inflammation score, indicating that in this model, IL-23 represents a critical pro-inflammatory mediator[42]. However, from this data one could not draw a conclusion on the relevance of IL-12. This question been answered in the following by applying the transfer model of colitis. In this model the disease-inducing population of naïve T cells isolated from wild-type mice is transferred to immunodeficient *Rag*$^{-/-}$ mice. Transfer of naïve T cells in either *p40*$^{-/-}$ or *p19*$^{-/-}$ *Rag*$^{-/-}$ mice failed to induce colitis, while no difference between the transfer of naïve T cells into *Rag*$^{-/-}$ versus *Rag*$^{-/-}$ *p35*$^{-/-}$ mice was observed. Consequently, at least in this model, IL-23 but not IL-12 represents the key pro-inflammatory cytokine[43]. However, selective neutralization of IL-23 may not always be beneficial. *p19*$^{-/-}$ mice are highly susceptible to T cell-mediated colitis induced by hapten. In the absence of IL-23, gut dendritic cells produced excessive amounts of IL-12 as a result of the missing regulatory effect of IL-23[44]. Thus, in the absence of IL-23, mice developed enhanced IL-12-driven mucosal immunopathology; whether such cross-regulation is relevant in patients with IBD remains to be determined.

The IL-12 family is closely linked to the innate immune system. IL-12 and IL-23 within the lamina propria are predominantly produced by macrophages and dendritic cells. Both cell populations can be activated via innate receptors on one hand and via the development of an adaptive immune response on the other hand. NOD2 is probably the best example for this central function. As described above, a mutation in *NOD2* is followed by an increase in IL-12p70[34], thus directly linking a receptor of the innate immune system with the effector cascade of the adaptive immune system.

PERSPECTIVE

The aim of this review was to illustrate the tight cross-regulation between the innate and the adaptive immune system. The study of either system alone will be complemented by focusing on pathways directly linking both systems. This is not only of interest for the understanding of disease pathogenesis but also may have an impact on future therapeutic directions.

CONFLICT OF INTEREST

B. Siegmund has served on Advisory Boards for Abbott and Nycomed, and has received lecture fees from Abbott, Essex, Shire, Falk and Merckle Recordati, and M. Zeitz has served on Advisory Boards of Abbott, Essex, Shire and UCB, he has received lecture fees from Abbott, Essex, Falk, and Merckle Recordati.

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TOPIC HIGHLIGHT

Dr. Jonas Mudter, MD, *Series Editor*

Cancer risk in IBD: How to diagnose and how to manage DALM and ALM

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Abstract

The risk of developing neoplasia leading to colorectal cancer is significantly increased in ulcerative colitis (UC) and most likely in Crohn's disease. Several endoscopic surveillance strategies have been implemented to identify these lesions. The main issue is that colitis-associated neoplasms often occurs in flat mucosa, often being detected on taking random biopsies rather than by identification of these lesions *via* endoscopic imaging. The standard diagnostic procedure in long lasting UC is to take four biopsies every 10 cm. Image enhancement methods, such as chromoendoscopy and virtual histology using endomicroscopy, have greatly improved neoplasia detection rates and may contribute to reduced random biopsies by taking targeted "smart" biopsies. Chromoendoscopy may effectively be performed by experienced endoscopists for routine screening of UC patients. By contrast, endomicroscopy is often only available in selected specialized endoscopic centers. Importantly, advanced endoscopic imaging has the potential to increase the detection rate of neoplasia whereas the interplay between endoscopic experience and interpretation of histological biopsy evaluation allows the physician to make a proper diagnosis and to find the appropriate therapeutic approach. Colitis-associated intraepithelial neoplasms may occur in flat mucosa of endoscopically normal appearance or may arise as dysplasia-associated lesion or mass (DALM), which may be indistinguishable from sporadic adenomas in healthy or non-colitis mucosa [adenoma-like mass (ALM)]. The aim of this review was to summarize endoscopic and histological characteristics of DALM and ALM in the context of therapeutic procedures.

Key words: Inflammatory bowel disease; Crohn's disease; Endoscopy; Colitis; Dysplasia-associated lesion or mass; Adenoma-like mass; Endomicroscopy; Ulcerative colitis; Endomicroscopy; Confocal laser endomicroscopy; Probe-based confocal laser endomicroscopy; Integrated confocal laser endomicroscopy; Endoscope-based confocal laser endomicroscopy; Narrow band imaging; Chromoendoscopy; Cancer; Dysplasia

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INTRODUCTION

Inflammatory bowel disease (IBD) encompasses two major forms of chronic intestinal disorders, Crohn's disease and ulcerative colitis (UC). Evidence suggests that IBD results from an inappropriate inflammatory response to intestinal microbes in a genetically susceptible host environment[1]. Increased production of proinflammatory cytokines and increased resistance to apoptosis finally lead to an uncontrolled chronic activation of the mucosal immune system[2]. Previously, it was shown that proinflammatory cytokines, such as Interleukin-6 produced by lamina propria mononuclear cells, directly contribute to tumor growth and to cancer development in UC[3,4]. Although the association between IBD and colorectal cancer (CRC) is well-established, there are still concerns regarding adequate diagnosis and treatment of early preneoplastic and neoplastic lesions.

The overall prevalence of CRC in UC patients was recently analyzed in a large meta-analysis and estimated to be 3.7%[5]. In this context, the duration and anatomical extent are well-established risk factors for cancer development. Patients with a course of disease longer than 10 years and with pancolitis are at the highest risk. Additionally, patients with left-sided UC or those with more proximal disease are considered to be at high risk for cancer development[6]. Therefore, surveillance colonoscopy is considered to be the gold standard in diagnosing intraepithelial neoplasia (formerly termed dysplasia) and cancer in IBD patients. Nevertheless, to date, no randomized controlled studies have shown a reduced risk of CRC development by surveillance colonoscopy in IBD patients.

The main aim of surveillance programs in IBD is to detect early dysplastic alterations. Dysplastic alterations of the intestinal mucosa in IBD patients may occur in flat or raised mucosal lesions, and are differentiated by the terms dysplasia-associated lesion or mass (DALM, Figure 1) and adenoma-like mass (ALM, Figure 2)[6-8].

The differential diagnosis between colitis associated intraepithelial neoplasms and sporadic adenoma in patients with UC may be difficult, especially if only biopsy specimens are obtained[9,10]. Even carcinomas are difficult to diagnose because of their commonly well-differentiated morphology, and accurate diagnosis may only be possible in a resection specimen showing unequivocal invasion into the submucosal layer. Thus, in approximately 40% of cases with high-grade intraepithelial neoplasia on biopsy, diagnosis of the corresponding resection specimen shows invasive adenocarcinoma[11-13]. In low-grade intraepithelial neoplasia, the prevalence of carcinoma in the resection specimen has historically been believed to be up to 19%[14]. More recent data, however, indicate a significantly lower prevalence, indicating that a diagnosis of low-grade intraepithelial neoplasia does not justify prophylactic colectomy and local endoscopic treatment should not be ruled out[15].

ENDOSCOPIC DIAGNOSIS

Often the macroscopic endoscopic appearance of a lesion does not render the crucial clue to differential diagnosis, because colitis associated neoplasms may show subtle macroscopic features mimicking a broad range of lesions, ranging from inflammatory appearance to suspicion of carcinoma. Data from the literature indicate that in 50%-80% of cases with colitis-associated neoplasms, the lesions are not visible upon endoscopy[11].

Adenomas are mainly sharply delineated, with or without a stalk, and often show a smooth surface with Kudo surface pit pattern ⅢS (small roundish or tubular pits), ⅢL (large roundish or tubular pits), or Ⅳ (branch-like or gyrus-like pits)[16,17]. The gross appearance of colitis-associated neoplasms varies from case to case. These lesions can be endoscopically invisible or may be encountered as irregularly delineated, plaque-like, or irregularly elevated, lesions or verrucous structures. Some cases even show a combination of different types (Figures 1 and 2).

Previously, it was reported that to exclude dysplasia in colonic mucosal biopsies, at least 56 or 33 non-targeted jumbo-forceps biopsies have to be taken (95% and 90% confidence, respectively)[6]. In 2005, an international consensus conference agreed that a minimum of 32 biopsies should be performed at each surveillance colonoscopy by obtaining four-quadrant biopsies every 10 cm. In addition, separate jars should be used for each quartet and suspicious lesions[18].

To avoid sampling error in IBD-cancer surveillance, new endoscopic imaging techniques were introduced, including chromoendoscopy, magnification endoscopy, and confocal laser endomicroscopy (Table 1, Figures 3-6)[19].

In 2003, Kiesslich and colleagues conducted the first study of chromoendoscopy in IBD patients[20]. One hundred and sixty-five patients with long standing UC were randomized in a 1:1 ratio to undergo conventional colonoscopy or colonoscopy with chromoendoscopy using 0.1% methylene blue. In the chromoendoscopy group, significantly more intraepithelial neoplasms were detected compared to the conventional colonoscopy group (32 *vs* 10, $P = 0.003$). Another "back-to-back" colonoscopy study included 100 patients with longstanding UC[21]. Patients received both random and directed biopsies, followed by spraying of the entire mucosa with 0.1% indigo carmine, and subsequent biopsy of any additional visible abnormality. There was a strong trend towards statistically increased dysplasia detection following dye spraying (7/100 patients *vs* 2/100 patients, $P = 0.06$). The targeted biopsy protocol detected dysplasia in significantly more patients than the non-targeted protocol (7/100 patients *vs* 0/100 patients, $P = 0.02$). Additionally, the targeted biopsy protocol with pancolonic chromoendoscopy required fewer biopsies than taking multiple non-targeted biopsies (157 biopsies *vs* 2904 biopsies).

However, dye-based chromoendoscopy has some potential limitations, as it harbors additional costs for the equipment needed for dye spraying and is a time consuming procedure. Additionally, the dye often does not coat the entire surface and it does not allow for a detailed analysis of subepithelial capillary network, which is an

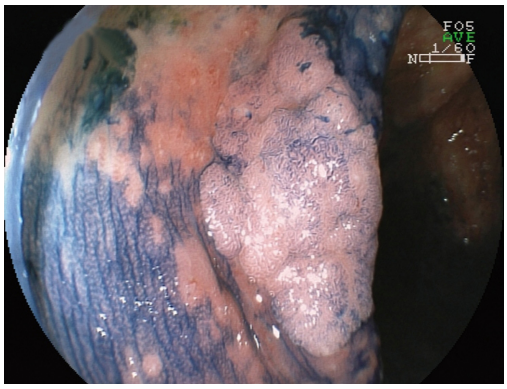

Figure 1 Visualization of a dysplasia-associated lesion or mass after topical application of indigo carmine. Surface analysis revealed a Kudo pit pattern 3L.

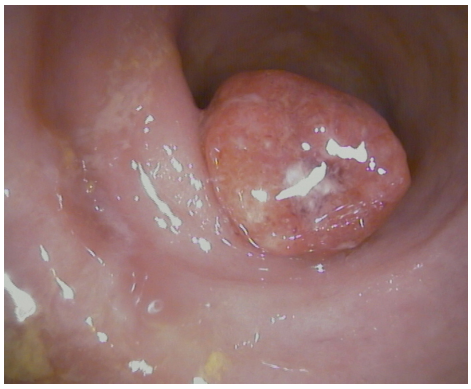

Figure 2 High-resolution standard white light endoscopic image of an adenoma-like mass in a patient with long standing ulcerative colitis. Surface of the polyp is irregular and shows fibrin plaques. Histopathological analysis revealed high-grade intraepithelial neoplasia.

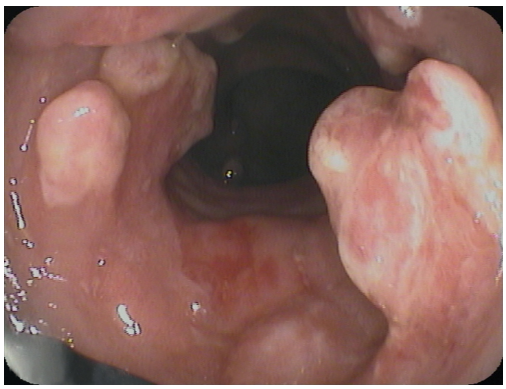

Figure 3 Multiple inflammatory polyps in chronic ulcerative colitis. Image was recorded using the Pentax endomicroscope (EC-3870CIFK). Note the confocal lens at the 7 o'clock position.

important feature in the early diagnosis of gastrointestinal neoplasia (Figure 4).

Therefore, dye-less chromoendoscopy (also called virtual chromoendoscopy) has been developed, including narrow band imaging (NBI; Olympus, Tokyo, Japan), Fujinon intelligent color enhancement (FICE; Fujinon, Tokyo, Japan), and i-Scan (Pentax, Tokyo, Japan). NBI

Figure 4 Chromoendoscopy with indigo carmine allows distinct surface analysis and demarcation of subtle lesions in long standing ulcerative colitis.

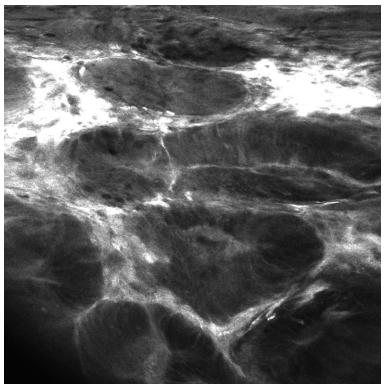

Figure 5 Fluorescein-guided confocal laser endomicroscopy (iCLE, Pentax, Tokyo, Japan) of dysplasia-associated lesion or mass. Endomicroscopy visualizes tubular architecture and enlarged cells with depletion of goblet cells. The shape and size of the crypts is irregular, and leakage, demonstrated by the extravasation of fluorescein, is visible.

is based on optical filters within the light source of the endoscope, which narrow the bandwidth of spectral transmittance such that the blood vessels are enhanced and are thus seen more easily. FICE and i-Scan use an endoscopic image from the video processor and reconstruct virtual images in real time, resulting in an improved contrast of the capillary patterns and enhancement of the mucosal surface[19,22].

The first pilot study on NBI in patients with UC included 46 patients. Suspicious lesions were observed by magnifying NBI-colonoscopy and were subsequently classified based on their surface appearance into honeycomb-like, villous, or tortuous patterns. The tortuous pattern determined by NBI-colonoscopy may be a clue for the identification of dysplasia during surveillance for UC[23].

One recently published study assessed the value of endoscopic tri-modal imaging, incorporating white light endoscopy (WLE), autofluorescence imaging (AFI), and NBI for the detection and classification of neoplasia in 50 patients with longstanding UC[24]. Neoplasia miss-rates for AFI and WLE were 0% and 50%, respectively (P = 0.036). The Kudo classification by NBI had a sensitivity and specificity of 75% and 81%, respectively. Thus, in

Table 1 Studies evaluating advanced endoscopic imaging in adenoma-like mass and dysplasia-associated lesion or mass

Author and reference	Study design	Patients	Technique	Results
Kiesslich *et al*[20]	Prospective	165	Chromoendoscopy (methylene blue)	Chromoendoscopy detects significantly more IEN compared to conventional colonoscopy
Rutter *et al*[21]	Prospective	100	Chromoendoscopy (indigo carmine)	Targeted mucosal biopsies detect significantly more IEN than non-targeted biopsies
van den Broek *et al*[24]	Prospective	50	AFI + NBI + WLE	AFI improves detection of colitis-associated neoplasia.
Hurlstone *et al*[25]	Prospective	162	MCE (indigo carmine)	MCE significantly increases diagnostic yield of IEN and number of flat lesions with IEN
Hurlstone *et al*[26]	Prospective	350	MCE (indigo carmine)	MCE significantly increases diagnostic yield of IEN and number of flat lesions with IEN
Kiesslich *et al*[29]	Prospective	153	Chromoendoscopy (methylene blue) + endomicroscopy	Combination of both techniques detects 4.75-fold more neoplasms and requires 50% less biopsy specimen
Hurlstone *et al*[30]	Prospective	36	Endomicroscopy	Endomicroscopy can differentiate ALM and DALM *in vivo* with high accuracy (97%)

IEN: Intraepithelial neoplasia; AFI: Autofluorescence imaging; NBI: Narrow band imaging; WLE: White light endoscopy; MCE: Magnifying chromoendoscopy; ALM: Adenoma-like mass; DALM: Dysplasia-associated lesion or mass.

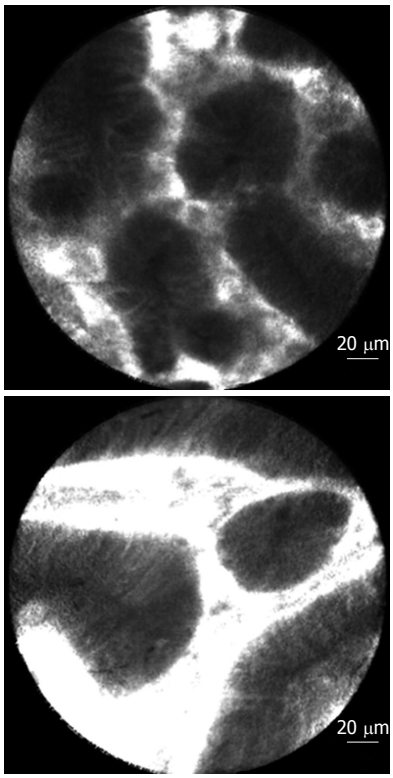

Figure 6 Fluorescein guided confocal laser endomicroscopy of adenoma-like mass (ALM; pCLE, Cellvizio, Mauna Kea Technologies, Paris, France). Endomicroscopy shows villous transformation of colonic architecture and depletion of goblet cells indicating adenomatous tissue.

this study, AFI improved the detection of neoplasia in patients with UC and decreased the yield of random biopsies, while pit pattern analysis by NBI had a moderate accuracy for the prediction of histology.

While both vital and virtual chromoendoscopy offer the identification of subtle lesions and their borderlines, subsequent magnification endoscopy has the capability to enable detailed surface analysis and pit pattern classification of those lesions.

Indeed, Hurlstone and colleagues prospectively analyzed 162 patients with longstanding UC using magnifying chromoendoscopy with 0.5% indigo carmine[25]. Selective chromoendoscopy was used, following detection of subtle mucosal changes. Magnification chromoendoscopy with targeted biopsies significantly increased diagnostic yield for intraepithelial neoplasia (42 lesions *vs* 11 lesions, $P < 0.001$) and the number of flat lesions with intraepithelial neoplasia as compared to conventional colonoscopy (31 lesions *vs* 6 lesions, $P < 0.001$). The overall sensitivity and specificity of magnification chromoendoscopy in predicting neoplasia was 97% and 93%, respectively. These data were also confirmed in a larger cohort of patients[26]. A total of 350 patients with longstanding UC underwent surveillance colonoscopy using high-magnification chromoendoscopy. Quadrantic biopsies at 10-cm intervals were taken on extubation, in addition to targeted biopsies of abnormal mucosal areas, and data were compared to 350 disease duration- and disease extent-matched control patients who had undergone conventional colonoscopic surveillance. Magnification chromoendoscopy detected significantly more intraepithelial neoplastic lesions compared with controls (69 lesions *vs* 24 lesions, $P < 0.0001$). In addition, chromoendoscopy detected more flat lesions with intraepithelial neoplasia compared with controls (53 lesions *vs* 14 lesions, $P < 0.001$).

Taken together, this study indicates that magnification chromoendoscopy has the capability to predict neoplastic and non-neoplastic mucosal changes with a high overall accuracy.

Although high-magnification endoscopy enables detailed surface analysis and pit pattern classification, it cannot visualize cellular and subcellular details. Certainly, diagnosis of intraepithelial neoplasia is mostly based on alterations of cellular and subcellular structures. Additionally, magnifying chromoendoscopy cannot differentiate between colitis-associated intraepithelial neoplasms and adenoma, because the staining pattern of both entities is similar[27].

Recently, confocal laser endomicroscopy was intro-

duced, allowing real-time *in vivo* imaging of the gastrointestinal mucosa at 1000-fold magnification, thereby providing an optical biopsy (Figures 5 and 6)[28].

One randomized controlled trial assessed the value of combined chromoendoscopy (0.1% methylene blue) and endomicroscopy for the *in vivo* diagnosis of intraepithelial neoplasia in patients with UC[29]. One hundred and fifty-three patients with long-term UC in clinical remission were randomized in a 1:1 ratio to undergo conventional colonoscopy or chromoendoscopy with endomicroscopy. In the combined group, 4.75-fold more neoplasms were detected compared to conventional colonoscopy (P = 0.005) and 50% less biopsy specimens were required (P = 0.008). Moreover, if only circumscribed lesions would have been biopsied, the total number of biopsy specimens could have been reduced by more than 90%. Overall, endomicroscopy predicted the presence of neoplastic changes with high sensitivity, specificity, and accuracy (94.7%, 98.3%, and 97.8%, respectively).

Recently, a study by Hurlstone and colleagues evaluated the clinical applicability and predictive power of endomicroscopy for the *in vivo* differentiation of DALM and ALM. Thirty-six patients with 36 lesions were prospectively included. The accuracy of endomicroscopy was 97%, and there was an excellent agreement between endomicroscopy and histopathological diagnosis (κ = 0.91)[30].

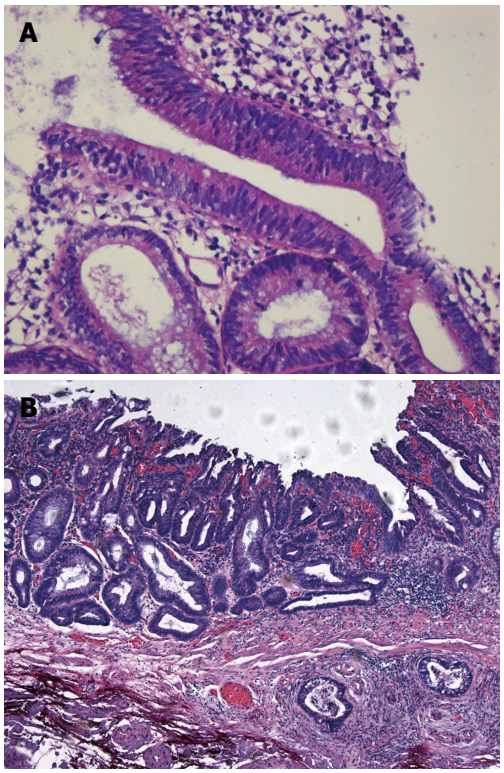

Figure 7 Histopathological image of dysplasia-associated lesion or mass with low-grade intraepithelial neoplasia in a patient with quiescent ulcerative colitis (A). Panel B illustrates colitis-associated cancer with submucosal invasion.

HISTOPATHOLOGICAL DIAGNOSIS

The histopathological differential diagnosis between colitis-associated neoplasms and sporadic adenoma is difficult (Figure 7)[31]. Both entities are defined as unequivocal intraepithelial neoplasms that can show either low-grade or high-grade intraepithelial neoplasia. The lesions are mainly differentiated by combined analysis of histological growth pattern and gross appearance[32]. Clinically, accurate pathological diagnosis is very important with respect to different therapeutic consequences: endoscopic polypectomy *vs* potential surgical proctocolectomy.

Clinically-based criteria for the differentiation of patients with sporadic adenoma from those with colitis-associated neoplasia have been known from the early 1990s. Thus, patients with adenomas differ from those with colitis-associated neoplasms with respect to age, onset age of colitis, number of lesions and extent of colitis[33].

Glands

In sporadic adenomas, the glands are round or oval shaped with regular structure, equal configuration, and similar diameter. Furthermore, sporadic adenomas show a "top-down" morphology, whereas colitis associated neoplasms show a "down-top" morphology, with glands that are irregular in size, shape, configuration, and diameter.

Proliferation zone

The proliferation zone in sporadic adenomas starts from the apical/luminal part of the mucosa ("top-down"). In contrast, the proliferation zone of colitis-associated neoplasms starts from the base of the mucosa ("down-top"); the neoplastic epithelium ultimately covers the whole length of the crypts, finally reaching the mucosal surface. Horizontal tubular proliferations above the muscularis mucosae are typically found in colitis-associated neoplasms.

Mucin vacuoles

In adenomas, mucin vacuoles are evenly distributed within the neoplastic epithelium and are mostly found in the apical part of the cytoplasm. Colitis-associated neoplasms show irregularly distributed mucin vacuoles. Often so-called "dystrophic" goblet cells are found, which are characterized by mucin vacuoles located in the basal parts of the cytoplasm, i.e. between nucleus and basal membrane.

Nuclei

Sporadic adenomas show palisading of elongated hyperchromatic nuclei, which are regular in shape and diameter. In colitis-associated neoplasms, nuclei are oval to round, less densely packed, and vary in diameter and configuration. These nuclei are often more irregularly arranged within the glands compared with sporadic adenomas, and may show greater variation in chromatin content.

Stroma

In between the glands of a sporadic adenoma, the stromal tissue is loosely and evenly arranged, whereas in colitis-associated intraepithelial neoplasms, the stromal tissue is often irregularly packed between the glands, with bands of connective tissue largely varying in shape and

thickness. Depending on the activity of the underlying disease, mixed inflammatory infiltrates, with cryptitis and crypt abscess formation, may be observed.

Delineation of the lesion towards the adjacent mucosa

Sporadic adenomas commonly show sharp and abrupt delineation between neoplastic glands and adjacent non-neoplastic epithelium. In colitis-associated neoplasms, this delineation is more irregular and less well defined.

The question of whether a lesion is a sporadic adenoma or a colitis-associated neoplasm depends on all of the above named criteria. Making a definite diagnosis based upon a single criterion is largely unreliable in this respect. In addition, other factors should be taken into account, such as the endoscopic appearance of a particular lesion, the age of the patient, duration and extent of the underlying disease, and the localization within or above the segment of colon affected by colitis. The use of immunohistochemistry is not very helpful in differentiating the two entities and consequently cannot be recommended[31].

DNA-cytometry

This tool may help to differentiate sporadic adenomas from colitis-associated neoplasms in selected cases. A small study assessing 19 patients showed that all sporadic adenomas evaluated were euploid, in contrast to only four out of 19 colitis-associated neoplasms. Fourteen of the remaining 15 lesions were unequivocally aneuploid[34].

Molecular analysis

While APC-mutations (adenomatous polyposis coli protein) and shift from cytoplasmic to nuclear β-catenin expression are common findings in sporadic adenomas, they are infrequent in colitis-associated neoplasms[35-37]. In contrast, p53 mutations are less common in sporadic adenomas compared with colitis-associated neoplasms (4% *vs* 40%), where they have been identified as the most common initiating mutation within micro-dissected individual neoplastic crypts[31,38]. Similarly, abnormalities of the p16 gene locus are far more common in colitis-associated neoplasms compared with sporadic adenomas[39]. Cyclin-D1 upregulation and p21 (WAF/CIP1) downregulation occur early in colitis-associated carcinogenesis. However, cyclin-D1 upregulation in colitis-associated cancers is less common than in sporadic colon cancers[40]. Finally, LOH analyses (loss of heterozygosity) have shown more frequent deletions on chromosome 3p within the von-Hippel-Lindau gene locus in colitis-associated neoplasms compared with sporadic adenomas in colitis patients[41].

These molecular features may only give a first insight into ongoing research work. However, nuclear β catenin expression appears to be a promising marker, especially if used in combination with ki67 immunohistochemistry, which may facilitate detection of "down-top" or "top-down" morphology as mentioned above[42]. Of note, however, no molecular marker, including those mentioned above, has yet entered daily practice to aid the differential diagnosis between sporadic adenomas and colitis-associated intraepithelial neoplasms. The skills of an experienced histopathologist remain the golden standard. In critical cases, obtaining a second opinion may be helpful to ensure the diagnosis and subsequent therapeutic approaches.

MANAGEMENT OF DALM AND ALM

Macroscopically flat or raised lesions without proper delineation to the surrounding mucosa that occur in long standing IBD are mostly diagnosed as DALM and harbor a high risk of progression to CRC (Figure 1). Furthermore, the occurrence of DALM is frequently associated with synchronic or metachronic neoplasia. Therefore, patients with DALM are recommended to undergo prophylactic proctocolectomy with ileoanal pouch. In contrast, the term ALM describes sporadic adenomas that are similar to those observed in non-IBD patients and which are treated by standard polypectomy (Figure 2).

From the clinical perspective, the endoscopic resectability of a lesion is more important than whether it is thought to be a sporadic adenoma (ALM) or a DALM. One essential point is that the lesion, whether DALM or ALM, should be removed in its entirety.

Early data support the hypothesis that ALMs can be successfully removed using standard polypectomy techniques, with little risk of subsequent malignancy on follow-up[43-45].

In a retrospective study, including 525 patients with UC, a total of 110 neoplastic areas were detected in 56 patients. Eighty-five (77.3%) of the lesions were macroscopically visible at colonoscopy. Fifty patients (89.3%) had macroscopically detectable neoplasia, and six (10.7%) had macroscopically invisible lesions. Importantly, the frequency of cancer in patients who had endoscopic resection of neoplasia did not differ from that of the surveillance population as a whole, irrespective of whether the lesion was thought to be an adenoma or a DALM. Conversely, a high proportion of unresectable lesions harbored cancer[46].

Additionally, in a large retrospective study Vieth and coworkers showed that endoscopic resection of ALMs represents an adequate treatment for those lesions[47]. Furthermore, it was shown that an adenoma based on biopsy material from a patient with UC must be subjected to endoscopic resection, both to confirm the biopsy-based adenoma diagnosis and to exclude colitis-associated intraepithelial neoplasms. In this large retrospective cohort, 2.3% of patients developed a colitis-associated carcinoma (follow up 6 years). Importantly, these carcinomas were located in a segment of the colon other than that bearing the primarily endoscopically resected adenoma.

CONCLUSION

Considerable progress has been made recently in both the diagnosis and treatment of adenomatous and non-ad-

enomatous lesions in UC. New and emerging endoscopic imaging techniques, like chromoendoscopy, magnification endoscopy, and confocal laser endomicroscopy, offer the potential for real time *in vivo* diagnosis of surface, vascular, and cellular patterns, and to perform tactical and targeted biopsies thereby increasing the diagnostic yield for intraepithelial neoplasia.

The following seven basic rules for the detection of neoplasia should be taken into account and applied in accordance with international guidelines, respectively. (1) Experienced gastroenterologist; (2) Endoscopic and bioptic control in remission phase; (3) Examination outside routine schedule without time limitation; (4) Ileocolonoscopy with special focus on the detection of DALMs and step (quadrant) biopsies from the rectum to the cecum in 10 cm intervals (sigmoid and rectum: quadrant biopsies at 5 cm intervals); (5) ALMs with low-grade intraepithelial neoplasia and clear cut margins can be resected endoscopically; (6) Experienced histopathologist who has all clinical and endoscopy data readily available; and (7) Second opinion recommended in cases of histological diagnosis of neoplasia.

In any event, patient follow-up will disclose whether the histopathological diagnosis of sporadic adenoma or colitis-associated neoplasms was justified. Nevertheless, the biopsy-based diagnosis of adenoma is uncertain. Therefore, endoscopic resection of suspicious lesions should be preferred. Several studies are available indicating that polypectomy of sporadic adenomas is an adequate and curative treatment. Of note, all patients with UC should undergo regular surveillance colonoscopies 8 years after the first appearance of symptoms (in the case of pancolitis); repeat colonoscopy with random biopsies in 10 cm intervals should be recommended every 1 to 2 years. Alternatively, chromoendoscopy with targeted biopsies may be applied in specialized centers. Patients that have been diagnosed with left-sided colitis should undergo colonoscopy in year eight after the first appearance of symptoms. If the colitis is still restricted to the distal colon (left-sided) surveillance may start at year 15. If colitis has progressed to pancolitis surveillance colonoscopies should be performed every 1 to 2 years. In general, if intraepithelial neoplasia is present in random biopsy specimens, colectomy should be recommended. If an adenomatous lesion with intraepithelial neoplasia was resected endoscopically control colonoscopy should be performed within 6 mo.

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TOPIC HIGHLIGHT

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Diagnostics in inflammatory bowel disease: Ultrasound

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Abstract

Diagnosis of chronic inflammatory bowel diseases (IBD) is based on a combination of clinical symptoms, laboratory tests and imaging data. Imaging of the morphological characteristics of IBD includes the assessment of mucosal alterations, transmural involvement and extraintestinal manifestations. No single imaging technique serves as a diagnostic gold standard to encompass all disease manifestations. Ultrasound, computed tomography (CT) or magnetic resonance imaging (MRI) allow cross-sectional imaging of the transmural alterations and extraintestinal manifestations. While in the USA the technique of choice is CT, in Europe the focus is more on MRI and ultrasound (US). Most patients with chronic IBD are diagnosed at a young age. After baseline diagnosis many of these young patients have to undergo repetitive imaging procedures during the variable clinical course of the disease, characterized by alternate periods of remission and active disease, and in monitoring the response to treatment. US has the advantage of being noninvasive, less costly, and easily repeatable, and thus can be very useful in following up patients with IBD. In addition, rising concern about radiation exposure in young adults indicates the demand for radiation-sparing techniques like US and MRI. This article focuses on the current clinical practice of US in IBD, describing the current technologies used in transabdominal intestinal US and the characteristic sonographic findings in Crohn´s disease and ulcerative colitis.

Key words: High-frequency waves; Ultrasound; Clinical practice; Inflammatory bowel disease; Color Doppler; Contrast agents

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ULTRASOUND TECHNOLOGY

Ultrasound (US) for inflammatory bowel diseases (IBD) requires high-frequency (5-17 MHz) linear array probes. High-frequency linear-array probes provide increased spatial resolution of the intestinal wall, which is essential for the assessment of wall diameter and wall layer discrimination. In modern high-frequency probes the creation of special modulated US pulses in transmission results in greater penetration of high frequency US. Compounding technology allows image reconstruction using signal responses from different frequencies or from viewing indifferent directions that results in an increase in contrast resolution and border definition of bowel wall architecture. Color or power Doppler imaging and contrast-enhanced US (CEUS) provide detailed information on mural and extraintestinal vascularity, which reflect inflammatory disease activity.

US EXAMINATION

Conventional transabdominal US with a conventional 3.5-5 MHz convex probe is recommended prior to high-frequency US of the intestinal tract so as not to overlook

underlying extraintestinal causes of abdominal discomfort. Special attention should be paid to the lower abdomen (urogenital tract) and the individual patient's pain location. High-frequency US of the intestinal tract requires extra time and patience of the examiner. With the exception of emergency situations the standard US examination should be performed preprandial in the morning or at least after 4 h fasting to diminish peristaltic movements and the amount of intraluminal air. Gradual compression of the bowel with the US probe helps to reduce intraluminal air. The application of intraluminal fluid as used in bowel preparation for colonoscopy or the use of enteral contrast medium[1-4] has been shown to improve the delineation of the wall architecture and the detection of jejunal and colonic lesions in patients with IBD, but these more sophisticated techniques have not been transferred into routine clinical practice. For US diagnosis of IBD, an understanding of the anatomical location of Crohn's disease (CD) and ulcerative colitis (UC), and of the more difficult or non accessible parts of the small and large bowel is essential for a systemic US approach to the patient. Transabdominal high-frequency US does not provide a continuous and complete examination of the small and large bowel. The ileocecal region and the sigmoid colon can be identified in all patients. The left and right colon can be adequately evaluated in most of patients. The colonic flexures (especially the left flexure) are more difficult to visualize due to their cranial position and ligamentous fixation to the diaphragm. The colon transversum can be identified in most patients, but complete examination is not easy to achieve because of its variable anatomy. The rectum and anal region cannot be visualized accurately by the transabdominal route due to their pelvic location. Transperineal US is useful in the evaluation of the perianal region and the distal rectum[5]. A proposal for a systematic approach in IBD patients could be to start in the left lower abdomen with transverse scans using the left iliac artery and vein as landmarks to visualize the sigmoid colon. The sigmoid colon can be easily identified by its prominent hypoechoic muscle layer (muscularis propria). Examination of the left colon can then be adequately performed by continuous scanning from the rectosigmoid transition along the colon descendens upwards to the left costal arch. Gradual compression is recommended to follow the left-sided colon along. The next step of a systematic examination could be visualization of the ileocecal region with transverse scans in the right lower abdomen using the right iliac artery and vein as landmarks and gradual compression. A variable location of ileocecal region in the right middle abdomen (also a frequent location of the neoterminal ileum after surgical resection) can be identified after manual palpation of the right spina iliaca anterior superior as a landmark. Moving the US probe in transverse sections from the right spina iliaca anterior superior upwards to the right costal arch with graded compression helps to find the lumen of the colon ascendens with its characteristic broad lumen (hyperechoic air filled lumen or hypoechoic fluid filled lumen). The cecum can easily be found by turning the probe towards a longitudinal position to follow the colon ascendens downwards until the broad luminal echo disappears. The distal part of the ileum can also be identified by transverse scanning from the cecum towards the middle and lower parts of the abdomen, again using the right iliac artery and vein and the urinary bladder as landmarks. Whereas the distal part of the small intestine (terminal ileum) can be evaluated in all patients, a complete and continuous evaluation of the proximal parts of the ileum and jejunum is not possible due to multiple overlying bowel loops. However, for a systematic approach to the proximal small intestine, four scanning positions in the upper and lower, right and left abdominal quadrants are recommended as final steps of a systematic approach to search for thickened intestinal wall segments.

Table 1 Sonoanatomy of the normal intestinal wall

Layer echogenicity	Anatomic structure
Hypoechoic (fluid) or hyperechoic (air) lumen	
Hyperechoic entrance	Transition lumen/mucosa
Hypoechoic	Mucosa
Hyperechoic	Submucosa
Hypoechoic	Muscularis propria
Hyperechoic	Transition muscularis propria/serosa, surrounding structures (fat, peritoneal wall)

SONOMORPHOLOGY OF THE INTESTINAL WALL

With the use of high US frequencies in the range from 7.5 MHz to 17 MHz, the wall of the intestine usually exhibits five different layers (Table 1). The small and large bowel can usually be distinguished in various stages of filling during movement by scanning the haustra of the colon and/or the circular folds of Kerckring in the small intestine. Measurement of the wall thickness is crucial for the diagnosis of IBD. Discrepancies in the measurements are mainly due to the presence or absence of graded compression during the examination by the operator in addition to various technical causes (US frequency, equipment). With modern high-frequency linear array probes the normal intestinal wall thickness is generally $\leqslant$ 3 mm (using mild compression) ranging from small diameters in the jejunum, ileum and proximal colon to larger diameters in the sigmoid colon (due to the hypertensive function of the sigmoid zone). Physiological contraction of the intestine leading to a thickened wall segment may cause misinterpretation, therefore bowel motions have to be taken into account before measurements are performed. In addition to wall thickening, echomorphology (integrity of wall architecture) and surrounding structures have also to be considered in the interpretation of the intestinal wall diameter.

CROHN'S DISEASE

CD can be localized in any part of the gastrointestinal tract, although the main location is the terminal ileum.

Small intestinal localization of the disease is found in 30%-40% of patients with CD (with involvement of the terminal ileum in 90%), and 40%-55% of the patients show an ileum and colonic localization. Only in a minority of patients (15%-25%) is colonic localization only observed. A systematic examination in patients with IBD should include complete scanning of the ileocecal region and sigmoid colon as well as the remaining parts of the colon, and evaluation of the small intestine (in sections) and surrounding mesenteric structures.

Assessment of bowel wall involvement

The most widely used diagnostic criterion for the diagnosis of IBD is bowel wall thickening with increased vascularization (with maintenance or loss of wall stratification).

In most studies, the bowel is considered to be thickened when the wall diameter exceeds 3 mm (Figure 1). The diagnostic accuracy of different cut-off values in CD were compared in a metaanalysis by Fraquelli *et al*[6]. Sensitivity and specificity of 88% and 93%, respectively, were achieved when a bowel wall thickness threshold greater than 3 mm was used, and sensitivity and specificity of 75% and 97%, respectively, were achieved when a threshold greater than 4 mm was used. Several studies report a relation between bowel wall thickness and clinical disease activity using the Crohn´s Disease Activity Index (CDAI) or Harvey Bradshaw Index (HBI) at initial diagnosis and during the clinical course of CD and in relation to endoscopic findings[7-9].

Bowel ultrasonographic signs used in CD can be standardized as most show a fair to good reproducibility. In particular, bowel wall thickness, the most relevant parameter for CD detection, showed an excellent reproducibility[10]. Color or power Doppler imaging of the vascularity of thickened wall segments using semiquantitative scores has proved useful in the distinction between remission or the active phase in CD and correlated with the clinical and endoscopic activity scores in adults[9,11] and children[12]. A reduction in wall thickness and mural vascularity (color Doppler imaging) could be shown in patients with a positive clinical-biological response to anti-tumor necrosis factor-α induction therapy in a small study in 24 patients. Maintenance of wall thickness and increased mural vascularity were seen in all non-responders ($n = 7$). Sonographic normality (defined as wall thickness ⩽ 3 mm and Doppler flow grade = 0) was only seen in 5 of 17 patients with a positive clinical-biological therapeutic response (29%)[13]. Most studies on Doppler US include a subjective semiquantitative assessment of mural vascularity, e.g. grade 0 = no vascular signal, grade 1 = barely visibly signals, grade 2 = moderate vascularity, grade 3 = marked vascularity. Currently there is no objective scale to determine the degree of disease activity on Doppler US.

CEUS is the most sensitive technique to visualize microperfusion and has been shown to be superior to conventional color or power Doppler imaging in determining tumor vascularity[14]. Currently, the clinical value of CEUS in IBD is not well defined. Most studies on CEUS have been feasibility or pilot studies.

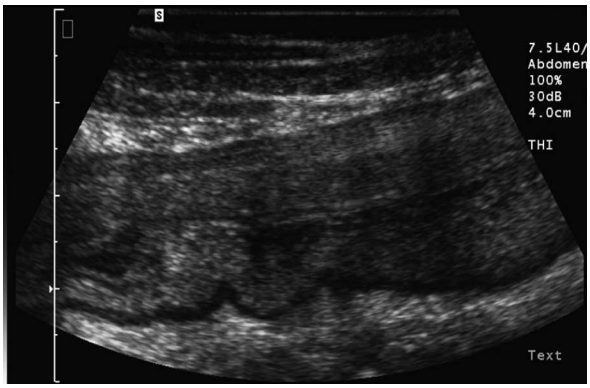

Figure 1 Sonographic appearance of an inflamed colon segment in Crohn's disease. Characteristic appearance: thickened wall diameter (almost 1 cm), partial loss of wall stratification, prominent submucosal layer, narrowed lumen and mesenteric fat hypertrophy.

In a small study (21 patients) with histologically confirmed CD and bowel wall diameters > 5 mm, contrast enhancement was observed after a mean of 13.4 s (± 4.2 s; range, 7-19 s) with a maximum vascularity after 30 s[15]. In addition, the length of contrast-enhanced bowel segments in US correlated significantly with the length of thickened bowel segments in magnetic resonance imaging (MRI)[16]. In a retrospective analysis, the assessment of the bowel wall vascularization in CD was performed using quantification software, indicating that CEUS data can not only be analyzed in a semi-quantitative way, but also in a reproducible, quantitative manner[17]. Using time-intensity curves, patients with CD showed a maximum enhancement 36 s after injection with 9 dB (range, 5.9-13.2 dB), while healthy volunteers reached the maximum level of 2.8 dB (range, 2-3.8 dB) after 23 s ($P < 0.05$)[18].

Correlation of CEUS with disease activity (endoscopy, histology, CDAI) indicate that active disease can be identified by CEUS)[19,20]. Characterization of the enhancement pattern in relation to wall thickness was shown to distinguish CD patients with active and inactive disease[21]. Lower levels of bowel contrast enhancement were observed in some patients who responded to anti-inflammatory treatment[22,23], as was shown in power Doppler imaging a few years ago. CEUS may be a useful method to assess the therapeutic effectiveness of specific medical anti-inflammatory treatment in patients with CD, or to differentiate inflammatory from fibrotic bowel wall changes. In addition, CEUS was suggested to be helpful in surgical management, in deciding upon conservative *vs* surgical treatment[24,25]. However, the role of CEUS in addition to conventional Doppler imaging is not yet well defined in clinical studies. Pilot studies on CEUS are promising, but studies on larger patient numbers, including the objective contrast enhancement score are needed before CEUS can be considered a clinical useful tool.

Mesenteric blood flow parameters

Patients with CD had significantly higher portal vein and mesenteric flow and a lower resistance index than con-

trols[26]. Blood flow in the superior mesenteric artery (SMA) has shown an increase in the postprandial pulsatility index (PI) in remission[27] in Doppler US. A decrease in PI predicted a non responder to azathioprine therapy and clinical relapse in a 12-mo follow-up[28]. Contrast enhancement in the SMA and vein, and calculation of splanchnic transit time showed a reduction in transit time (4.0 s *vs* 6.9 s) in patients with active CD[29]. However, in our own experience splanchnic transit time measurement in CEUS was not correlated with clinical disease activity (HBI) in mild stages of CD.

Extraintestinal findings

In addition to the assessment of bowel thickness and increased mural vascularity, the surrounding structures (fat, lymph nodes, free fluid accumulation) may indicate a peritestinal inflammatory reaction. Mesenteric fat hypertrophy correlated with biochemical and clinical activity of CD and with internal fistulas and increased bowel wall thickness. In quiescent CD, mesenteric hypertrophy does not appear to be a risk factor for relapse[30]. US is very sensitive for the detection of free fluid in CD[31]. The presence of regional lymph nodes shows only a weak correlation with clinical and biochemical CD activity[32]. Furthermore, the finding of mesenteric lymph nodes is non-specific and may reflect disease activity, but infectious intestinal diseases have to be excluded by stool and serologic tests.

Since US can find both intraluminal and peri-intestinal pathological features, it is a particularly valuable tool for the detection of complications of CD, such as stenosis, fistulas, and abscesses. Sensitivity and specificity for detecting fistulae in transabdominal US have been reported as 50%-89% and 90%-95%, respectively[33]. Sensitivity and specificity for detecting abscesses in transabdominal US is even higher with sensitivities of 71%-100% and specificities of 77%-94%[33-36]. In a series of 58 patients with CD, including 28 patients with bowel stenosis, 23 patients with fistulas and 10 patients with abscesses, high-resolution US showed a high diagnostic accuracy in comparison to clinical, endoscopic, radiological and operative findings[37]. The sensitivity, specificity, positive predictive and negative predictive values for US were 86%, 90%, 83% and 92%, respectively, for stenosis and 78%, 95%, 86% and 91%, respectively, for fistulas. The highest diagnostic accuracy was found for abscesses with sensitivity, specificity, positive predictive and negative predictive values 90%, 99%, 90% and 99%, respectively.

ULCERATIVE COLITIS

UC exclusively affects the colon with a predictable way of spreading from distal to proximal colon in a continuous manner. UC is classified by disease extent into proctitis, left-sided colitis and extensive colitis beyond the splenic flexure. A solitary rectal location of UC cannot be visualized accurately due to the pelvic location of the rectum. Mural stratification is preserved in most UC patients due to the superficial pattern of inflammation. The spatial resolution of US is not high enough to allow detection of mucosal pathology but bowel wall thickening is also a characteristic feature of UC[38].

The clinical role of US in UC is less well established as compared with CD. In contrast to CD, bowel thickening in UC could not be correlated with clinical disease activity in some studies[39,40]. However, compared with endoscopic findings, with an overall accuracy of 89% for US (bowel wall thickness > 3 mm and increased Doppler signal) and 73% for MRI (contrast enhancement in bowel wall) in identifying active IBD, the diagnostic accuracy was better in patients with UC than in patients with CD for both US and MRI[41].

Mucosal healing (MH) after short-term medical treatment is being considered as an important step in the therapeutic work-up of IBD patients due to the potential prognostic role of MH in predicting disease outcome. However, IBD patients are reluctant to be re-endoscopes during follow-up; therefore, there is a need for a non-invasive alternative index of MH which can replace endoscopy in clinical practice. In a prospective trial in 83 patients with UC with a follow-up of 15 mo, a high and consistent concordance between endoscopic and US scores was shown. In patients with UC, moderate-to-severe endoscopic and US scores at 3 mo were associated with a high risk of endoscopic activity at 15 mo, indicating that bowel US may be used as a surrogate of colonoscopy in assessing the short-term response of severe forms of UC to therapy. In addition, the US score and endoscopic score after 3 mo of steroid therapy predicted the outcome of disease at 15 mo[42,43].

Splanchnic flow measurements in the inferior mesenteric artery have been shown to be closely related to clinical and endoscopic disease activity in patients with UC[44,45]. In a small trial, CEUS showed entire bowel wall vascularity in correlation to clinical nonresponders to cytapheresis for steroid-refractory or -dependent UC[46]. So far CEUS is not routinely used in UC.

CONCLUSION

Transabdominal US is currently accepted as a clinically important first-line imaging technique in IBD in initial diagnosis and during the clinical course of the disease.

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TOPIC HIGHLIGHT

Dr. Jonas Mudter, MD, *Series Editor*

From intestinal stem cells to inflammatory bowel diseases

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Abstract

The pathogenesis of both entities of inflammatory bowel disease (IBD), namely Crohn's disease (CD) and ulcerative colitis (UC), is still complex and under investigation. The importance of the microbial flora in developing IBD is beyond debate. In the last few years, the focus has changed from adaptive towards innate immunity. Crohn's ileitis is associated with a deficiency of the antimicrobial shield, as shown by a reduced expression and secretion of the Paneth cell defensin HD5 and HD6, which is related to a Paneth cell differentiation defect mediated by a diminished expression of the Wnt transcription factor TCF4. In UC, the protective mucus layer, acting as a physical and chemical barrier between the gut epithelium and the luminal microbes, is thinner and in part denuded as compared to controls. This could be caused by a missing induction of the goblet cell differentiation factors Hath1 and KLF4 leading to immature goblet cells. This defective Paneth and goblet cell differentiation in Crohn's ileitis and UC may enable the luminal microbes to invade the mucosa and trigger the inflammation. The exact molecular mechanisms behind ileal CD and also UC must be further clarified, but these observations could give rise to new therapeutic strategies based on a stimulation of the protective innate immune system.

Key words: Inflammatory bowel disease; Paneth cells; Goblet cells; Cell differentiation; TCF4; Hath1; KLF4

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INTRODUCTION

Crohn's disease (CD) and ulcerative colitis (UC) are the 2 main entities of inflammatory bowel disease (IBD). Whereas CD mostly involves the distal ileum and/or the colon, the inflammation in UC is restricted to the colon. Since both disease locations are characterized by a high concentration of intestinal bacteria (10^7-10^8 organisms/g luminal content in the distal ileum and 10^{11}-10^{12} in the colon), it is not surprisingly that these luminal microbes are playing an important role in the development of IBD (see our review in the *World Journal of Gastroenterology*[1]). In healthy mucosa, these microbes are sufficiently con-

trolled by an adequate secretion of antimicrobial peptides and by the mucus layer, acting as a physical and chemical barrier (Figure 1A). Even if the pathogenesis of both forms of IBD is still under investigation, the last few years have revealed evidence that the differentiation from the intestinal stem cell towards the Paneth cell in ileal CD and towards the goblet cell in UC may be disturbed. This may result in a defective antimicrobial and mucus barrier, which enables the intestinal bacteria to invade the mucosa and trigger the inflammation. In this review, we focus on the intestinal stem cells, their differentiation towards Paneth and goblet cells, and the defective antimicrobial shield in ileal CD and mucus layer in UC as a consequence of a stem cell differentiation defect.

INTESTINAL STEM CELLS AND THEIR MARKERS

The whole intestinal tract is a rapidly self-renewing tissue maintained by a population of intestinal stem cells. For many decades, scientists tried to identify these relatively undifferentiated enigmatic cells - unfortunately with limited success due to a lack of good cellular markers. Nevertheless, about 30 years ago, 2 models were established concerning intestinal stem cell location in the human gut, which are still debated. The first hypothesis is called the "+4 position model". It assumes the intestinal stem cells to be located above the Paneth cells at position +4 relative to the crypt base[2]. The second hypothesis, also called the "stem cell zone model", proposed that the crypt base columnar cells, a cell population at the bottom of the crypt, represent the intestinal stem cells[2]. Both models suggest that about 6 intestinal stem cells are found in each crypt[2], surrounded by epithelial and mesenchymal cells regulating stem cell behavior[3].

The intestinal stem cells differentiate into 4 epithelial cell types[3]. The most abundant cells in the epithelium are the columnar cells. Their main function is the absorption of nutrients by apical microvilli. Goblet cells produce and secrete mucins in order to form a protective luminal mucus layer. Neuroendocrine cells release hormones in an endocrine and paracrine fashion. Finally, the Paneth cells, located in the crypt base of the small intestine and ascending colon secrete defensins and other antimicrobial peptides to keep the crypts sterile[4].

In 2007, Hans Clevers and his group found the Wnt target gene leucine-rich-repeat-containing G-protein-coupled receptor 5 (Lgr5) to be expressed in cells at the crypt base[5]. Furthermore, irreversible labeling of these cells revealed, that all 4 epithelial cell types (columnar cells, goblet cells, Paneth cells and neuroendocrine cells) arise from these cells. Since LGR5-positive cells are located at the crypt base, are pluripotent and also self-renewing, they concluded that LGR5 is a good marker for the intestinal stem cells[5]. In 2009, the same group found olfactomedin 4 (also known as OLFM4, hGC1 or GW112), a protein with unknown function, to be a robust and specific marker for these LGR5 stem cells in the human intestine[6].

Intestinal stem cells are regulated by several cell signaling pathways, including the Wnt and Notch pathway[7,8]. Defects in these pathways are known to be related to the development of intestinal cancer[3,8,9]. The role of stem cell differentiation towards Paneth cells in ileal CD and towards goblet cells in UC is a new and promising field of science which could contribute to a better understanding of these 2 diseases[10,11].

DEFECTIVE PANETH CELL DIFFERENTIATION IN ILEAL CD IS RELATED TO α-DEFENSIN DEFIENCY

Paneth cells were first described more than 100 years ago as granular cells at the base of the intestinal crypts of Lieberkühn[12]. About 5-12 Paneth cells can be found in each small intestinal crypt and, in contrast to columnar cells, goblet cells and neuroendocrine cells, they do not migrate upwards in the crypt but remain beneath or between the stem cells in the crypt base[13]. Their numerous apical cytoplasmic granula are filled with antimicrobial peptides, especially the α-defensins HD5 and HD6, but also other antimicrobials such as lysozyme and phospholipase A2[14]. These Paneth cell defensins, which are active against a large number of microbes, including Gram-positive and Gram-negative bacteria, fungi and viruses[15], can be released into the crypt lumen in order to regulate the microbial density and therefore protect the intestinal epithelium against bacterial invasion and inflammation.

Paneth cells derive from intestinal stem cells under the control of the Wnt signaling pathway. In particular, the Wnt transcription factor TCF4 is essential in Paneth cell differentiation, as shown in the embryonic mouse intestine[16]. After the activation of Wnt signaling, an intracellular β-catenin-TCF4-complex is formed and translocates into the nucleus, where the compound acts as a transcription factor controlling the expression of several downstream target genes, such as Paneth cell defensins. This was shown in TCF4 null mice, where TCF4 regulates the expression of the cryptids (the mouse homologs to the human α-defensins)[16].

This Paneth cell differentiation factor TCF4 was found to be reduced in ileal CD as compared to colonic CD and colonic UC[10]. Interestingly, this observation was independent of the degree of inflammation and independent of the NOD2 genotype[10]. In human samples HD5 and HD6 expression correlated well with TCF4, thus it seems that not only in animal models, but also in humans, Paneth cell defensins are regulated by TCF4[10]. The functional relevance of this TCF4 decrease was shown in TCF4 knockout mice: the reduced TCF4 expression apparently resulted in a decreased HD5 and HD6 expression[10]. Moreover, we found a high affinity binding site in the HD5 and HD6 promotor for TCF4, and notably, we detected genetic variants in the putative promoter region

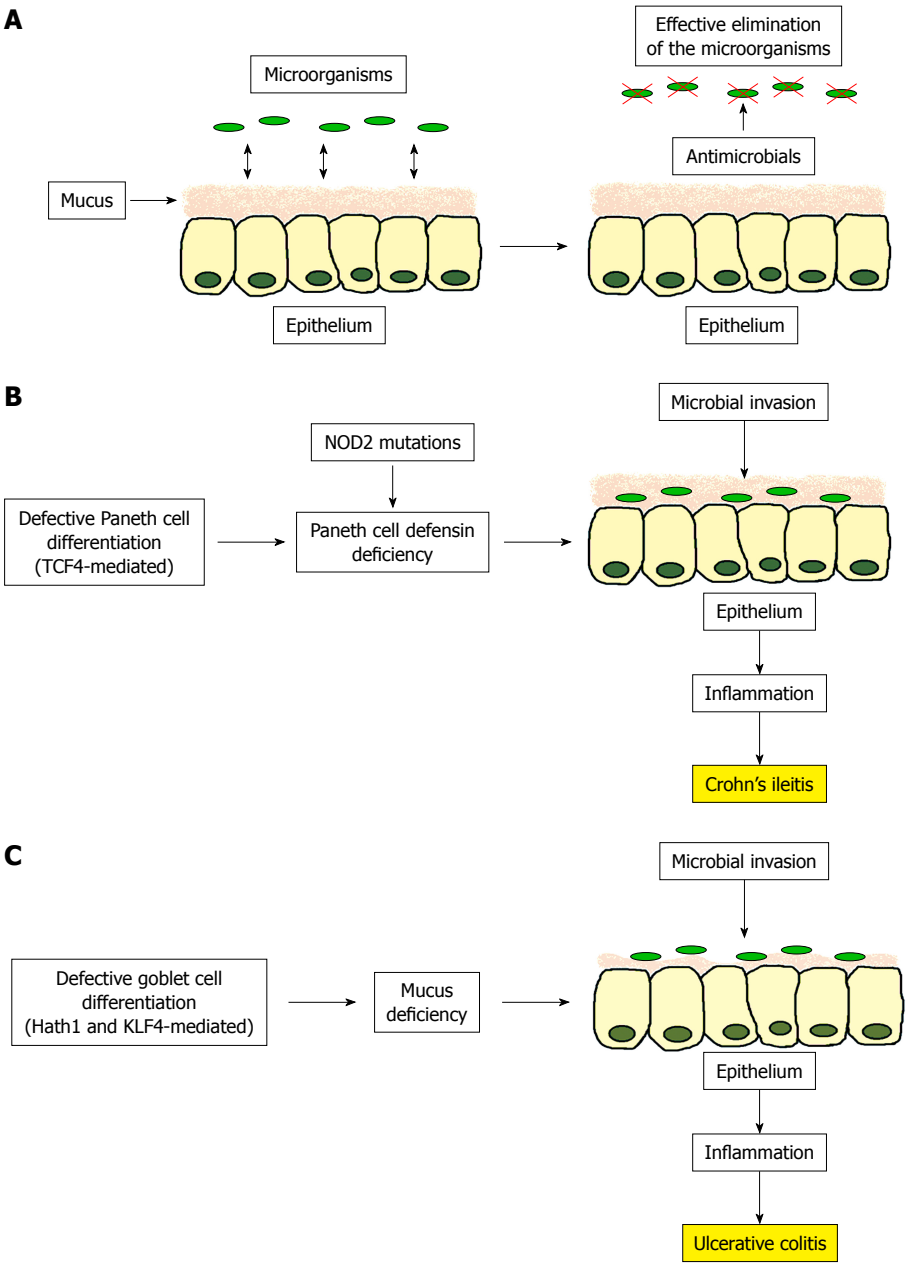

Figure 1 Proposed model for the pathogenesis of ileal Crohn's disease and ulcerative colitis. A: In the healthy ileal and colonic mucosa, luminal microbes are sufficiently controlled by an adequate secretion of antimicrobials peptides and a sufficient mucus layer; B: In ileal Crohn's disease, defective Paneth cell differentiation mediated by a reduction of the Wnt transcription factor TCF4 leads to a decreased Paneth cell defensin secretion, especially in case of NOD2 mutations. This allows the luminal microbes to attach and invade in the mucosa causing inflammation; C: In ulcerative colitis, a defective goblet cell differentiation based on a missing induction of the transcription factors Hath1 and KLF4 results in mucus deficiency enabling the luminal microbes to invade the mucosa and trigger inflammation.

of the Wnt transcription factor TCF4 (TCF7L2) to be associated with ileal CD[17]. This genetic finding emphasizes the primary role of the TCF4 defect in CD.

This also explains in part why in ileal CD, but not in colonic CD or UC, and also not in pouchitis samples, we had found a decreased expression of the α-defensins HD5 and HD6[14,18]. The other Paneth cell products were unchanged or even increased as compared with control samples[14]. This observation was independent of the degree of inflammation[14,18], and even more pronounced in patients with NOD2 mutations[14,18], which are clearly associated with ileal CD[19,20]. This is interesting, since Kobayashi *et al*[21] found NOD2-deficient mice to be susceptible to bacterial infection and, in addition, NOD2 seems to be essential for the expression of cryptdins. Elphick *et al*[22] confirmed this low defensin formation in ileal CD especially in those with a NOD2 mutation. Another study showed the same low defensin synthesis in CD but linked it to inflammation and loss of Paneth cells[23]. In contrast to this observation, Kelly *et al*[24] and also our group found an unaltered number of Paneth cells in ileal CD[14]. We were also able to demonstrate a reduced antibacterial activity in mucosal extracts from patients with ileal CD suggesting that the missing expression of Paneth

cell defensins in humans leads to a defective antimicrobial shield[14]. Since transgenic mice with human HD5 expression had an abnormal composition of the luminal microbial flora, it seems plausible that Paneth cell defensins can influence the makeup of the bacterial flora[14].

Taken together, the defective differentiation from the intestinal stem cell towards the Paneth cell is mediated by a diminished expression of the Wnt signaling transcription factor TCF4 resulting in a specific deficiency of the Paneth cell defensins in humans, especially in case of NOD2 mutations. This leads to a dysfunction of the mucosal barrier, which enables the luminal microbes to invade the mucosa and cause inflammation (Figure 1B). Besides the genetic link of TCF4 to ileal CD, other molecules, such as NOD2, ATG16L1, XBP1, KCNN4 and HD5 are genetically defective in ileal CD. As all these molecules are necessary for Paneth cell function, it seems to be plausible that the decrease in Paneth cell α-defensins is a primary (genetic) factor in disease pathogenesis[17,25].

IMPAIRED GOBLET CELL DIFFERENTIATION IN UC IS LINKED TO A DEFECTIVE MUCUS LAYER

Goblet cells are glandular epithelial cells scattered among the absorptive cells in the epithelium. The cytoplasm of these cells is filled with granula containing mucins, which are secreted into the intestinal lumen. These mucins form the mucus layer, acting as a barrier between the luminal contents and the epithelial surface[26].

Goblet cells derive from intestinal stem and progenitor cells located in the lower part of the crypt. Their differentiation is controlled by several transcription factors, especially by the basic helix-loop-helix transcription factor Hath1, the zinc-finger transcription factor KLF4 and the Notch target gene Hes1. For example, Math1 null mice (Math1 is the mouse homologe of human Hath1) are not able to develop goblet cells, whereas the columnar cells are intact[27]. In accordance to Hath1, goblet cell differentiation was also defective in KLF4 null mice as shown by a decrease of about 90% of colonic goblet cells in these mice[28]. Hath1 seems to be involved in an early stage of goblet cell differentiation, KLF4 appears to play a crucial role especially in the terminal stage of goblet cell differentiation[28]. In contrast, the genetic knockout of Hes1 gives rise to an increased number of goblet cells accompanied by a reduced number of nonsecretory cells[27,29,30], suggesting that Hes1 is a antagonist of Hath1[31]. In particular, the balance between Hath1 and Hes1 seems to be essential in controlling goblet cell differentiation in the epithelium, at least in the small intestine[31].

In 2009, we investigated these 3 crucial goblet cell differentiation factors in IBD. We found a significant induction of the goblet cell differentiation factors Hath1 and KLF4 in noninflamed *vs* inflamed CD, but not in UC[11]. Particularly in inflamed CD, the expression of Hath1 and KLF4 was about twice as high as that in inflamed UC samples[11]. For Hath1, these mRNA data were confirmed on the protein level by immunohistochemistry and Western blotting. Interestingly, this attenuated induction of Hath1 and KLF4 in UC was independent of the degree of inflammation and seems not to result from a down-regulation by increased Notch activity[11]. Hes1 expression was also augmented in inflamed CD as compared to inflamed UC, but without reaching significance[11].

In contrast to CD, the expression of the colonic antimicrobials is increased in inflamed UC suggesting that the antimicrobial shield is intact in these patients[32-34]. Nevertheless, the colonic mucus layer, which cover the whole gastrointestinal tract and act as a physical and chemical barrier against the luminal microbes, is altered and even deficient in UC[35-37]. The thickness of the mucus layer in the healthy colon was measured between 100 and 300 μm, increasing from the ascending colon to the rectum[36,38,39], whereas in UC, this mucus layer is thinner, more variable and in part denuded[35,36].

Since the "raison d'être" of goblet cells is to secrete mucus, it is not surprising that a decrease of the mature goblet cells, located in the upper part of the crypts, was found in UC compared with controls[11]. Moreover, in our samples the expression of the main colonic mucins (Muc1, 2 and 4) tended to increase in inflamed IBD, whereas this induction was clearly less pronounced in inflamed UC compared with inflamed CD[11]. Since Hath1 and KLF4 were also highly correlated with these mucins, it is possible that the defective mucus layer in UC is related to these 2 differentiation factors[11].

Overall, the defective differentiation from intestinal stem cells towards goblet cells, which is mediated by the 2 crucial transcription factors Hath1 and KLF4, may lead to goblet cell depletion and deficient mucin induction in active UC. This could explain the defective mucus barrier in UC, resulting in a collapse of the physical barrier, which enables the luminal microbes to invade the mucosa and trigger inflammation (Figure 1C). The role of mucins in host defense was shown by MUC2-deficient mice which spontaneously develop colitis[40]. As Swidsinski *et al*[41] found high concentrations of mucosal bacteria in patients with IBD, and as kationic defensins bind to the negatively charged mucins[42], we hypothesize that in UC the mucosa is not capable of holding back the fecal bacteria based on a defective mucus layer which is unable to bind the adequate amounts of secreted defensins.

CONCLUSION

The pathogenesis of IBD is certainly complex and still under investigation. Nevertheless, ileal CD and colonic UC are associated with defects in stem cell differentiation either to protective Paneth cells or goblet cells. Diminished Paneth cell defensins result in a defective antimicrobial barrier in ileal CD, whereas in UC, an insufficient induction of goblet cell mucins can lead to a disturbed

mucosal barrier. In both cases, luminal microbes are allowed to invade the mucosa and cause inflammation. New therapeutic strategies should focus on a stimulation of the antimicrobial shield in ileal CD and stabilization of the mucus layer in UC.

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TOPIC HIGHLIGHT

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Current treatment of ulcerative colitis

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Abstract

Ulcerative colitis (UC) is a chronic disease featuring recurrent inflammation of the colonic mucosa. The goal of medical treatment is to rapidly induce a steroid-free remission while at the same time preventing complications of the disease itself and its treatment. The choice of treatment depends on severity, localization and the course of the disease. For proctitis, topical therapy with 5-aminosalicylic acid (5-ASA) compounds is used. More extensive or severe disease should be treated with oral and local 5-ASA compounds and corticosteroids to induce remission. Patients who do not respond to this treatment require hospitalization. Intravenous steroids or, when refractory, calcineurin inhibitors (cyclosporine, tacrolimus), tumor necrosis factor-α antibodies (infliximab) or immunomodulators (azathioprine, 6-mercaptopurine) are then called for. Indications for emergency surgery include refractory toxic megacolon, perforation, and continuous severe colorectal bleeding. Close collaboration between gastroenterologist and surgeon is mandatory in order not to delay surgical therapy when needed. This article is intended to give a general, practice-orientated overview of the key issues in ulcerative colitis treatment. Recommendations are based on published consensus guidelines derived from national and international guidelines on the treatment of ulcerative colitis.

INTRODUCTION

Ulcerative colitis (UC) is a chronic disease with recurrent uncontrolled inflammation of the colon. The rectum is always affected with inflammation spreading from the distal to the proximal colonic segments. The terminal ileum is typically not involved but some patients with extensive disease may show endoscopic signs of "backwash ileitis". As the course of disease and extent vary considerably among patients, an individualized diagnostic and therapeutic approach is necessary.

The purpose of clinical practice guidelines is to indicate the best approaches to medical problems based on scientific findings. However, in the case of UC, if we consider just 3 different distribution patterns (proctitis, left-sided, pancolitis), 4 disease activities (remission, mild, moderate, severe), and 4 possible disease courses (asymptomatic after initial flare, increase in severity over time, chronic continuous symptoms, chronic relapsing symptoms), 48 different situations have to be evaluated before giving scientific advice. With the addition of further important factors such as the patient's extra-intestinal manifestations, age, concomitant diseases, previous operations, medical intolerances, lifestyles and personal wishes this number exceeds a thousand possible regimes.

So guidelines can only aim to indicate the preferable but not necessarily the only acceptable therapeutic approach and are meant to be used flexibly in a manner best suited to the individual patient. Therefore, the therapeutic approaches described in this article are meant to provide a general, practice-orientated overview of the important issues on UC treatment. Recommendations are based on published consensus guidelines of the national German [Deutsche Gesellschaft für Verdauungs- und Stoffwechselkrankheiten (DGVS)][1,2] and international societies [American College of Gastroenterology (ACG)[3], European Crohn's and Colitis Organisation (ECCO)][4-6] as well as on the authors' experience. Evidence levels (EL) and recommendation grades (RG) are given according to the Oxford Centre for Evidence-Based Medicine, EL 1 being the highest evidence level and RG A the strongest recommendation. ACG and DGVS guideline recommendations are graded from A (highest) to D (lowest).

The goal of medical treatment in UC is the rapid induction of a steroid-free remission and the prevention of complications of the disease itself and its treatment. In Crohn's disease experts are currently debating the usefulness of a "top-down" strategy, giving highly potent drugs in the early stages of the disease in order to prevent complications. In contrast, guidelines on UC, a putatively curable disease (by means of colectomy), still favor a pyramidal step-up approach where 5-aminosalicylic acid (5-ASA) is considered the baseline medication, steroids and immunomodulators function to intensify the treatment, while infliximab (IFX), calcineurin inhibitors [cyclosporine A (CsA), tacrolimus] or surgery are considered as rescue therapy.

MANAGEMENT OF ACTIVE UC

Symptoms of new onset UC or recurrent flare-ups usually consist of abdominal pain, bloody and/or mucous diarrhea. Severe cases present with weight loss, tachycardia, fever, anemia and bowel distension. Before starting medical treatment other etiologies of colitis/enteritis such as infections [*Clostridium difficile*, cytomegalovirus (CMV)], toxic reactions (e.g. antibiotics, NSAID colitis), mesenteric ischemia or intestinal malignancies should be ruled out. Opportunistic infections (e.g. CMV infection) need to be excluded prior to medical therapy escalation, especially in patients under immunosuppressive therapy with a corticosteroid-refractory course.

Although there is no gold standard, minimal diagnostic workup for UC includes medical history, clinical evaluation (focusing on extraintestinal manifestations), full blood count, erythrocyte sedimentation rate (ESR), C-reactive protein (CRP), stool microbiology, ultrasound and endoscopy with mucosal biopsies[6]. If there is any doubt about the diagnosis in the acute setting, endoscopic and histological confirmation should be repeated after a period of time has passed (ECCO EL 5, RG D; DGVS C).

The choice of treatment depends on the degree of activity, distribution (proctitis, left-sided or extensive colitis), course of disease, frequency of relapses, extraintestinal manifestations, previous medications, side-effect profile and the patient´s individual wishes.

The degree of activity can be classified according to the Montreal classification as: Remission (S0): 3 or less stools per day without any presence of blood or increased urgency of defecation; Mild (S1): up to 4 stools per day, possibly bloody. Pulse, temperature, hemoglobin concentration and ESR are normal; Moderate (S2): 4 to 6 bloody stools daily, no signs of systemic involvement; Severe (S3): more than 6 bloody stools daily, signs of systemic involvement (temperature above 37.5°C, heart rate above 90/min, hemoglobin concentration below 10.5 g/dL, or ESR above 30 mm/h).

Distribution patterns depend on the part of the colon involved and are designated according to the Montreal classification as proctitis (E1), left-sided colitis (E2, limited to the sigmoid and descending colon) or extensive colitis (E3, also referred to as pancolitis). A graphical treatment algorithm is shown in Figure 1.

Proctitis/distal colitis

Colitis limited to the rectum with mild or moderate activity should be initially treated topically[7]. A 5-ASA suppository (e.g. mesalazine 1 g/d) is the drug of first choice (ECCO EL 1b, RG B; DGVS EL A) and induces remission in 31-80% of patients compared to 7-11% in the placebo-treated group[8]. There is no dose response to topical therapy above 1 g mesalazine daily. 5-ASA foam enemas are an alternative, but suppositories deliver the drug more effectively to the rectum and are often better tolerated by patients due to their smaller volume[9].

Topical corticoids (budesonide 2-8 mg/d, hydrocortisone 100 mg/d) are less effective than topical mesalazine[10]. If no therapeutic effect is observed, treatment escalation using a combination of oral mesalazine (2-6 g/d for induction), together with topical mesalazine and/or a topical steroid is recommended as second-line therapy (ECCO EL 1b, RG B; DGVS A). If symptoms do not resolve within 2-4 wk, the patient's adherence to medical treatment should be evaluated. Repeated exclusion of infectious colitis and endoscopic reconfirmation of persisting inflammatory proctitis might be helpful to guide the subsequent therapeutic approach, as an unrecognized co-existing irritable bowel syndrome or infectious colitis may be the reason for the refractory course. CMV infection is best diagnosed using immunohistochemical staining of viral proteins in mucosal biopsies, while conventional staining often gives false negative results. Quantitative CMV PCR presents the dilemma that positive results cannot distinguish between the presence of CMV as an innocent bystander in inflamed mucosa and its causative role in inflammation[11].

Confirmed persistent proctitis, in spite of combined local and topical therapy, is best treated as if it were more extensive or severe colitis.

Left-sided UC

Left-sided active UC of mild-to-moderate severity should be initially treated with topical aminosalicylates (ECCO

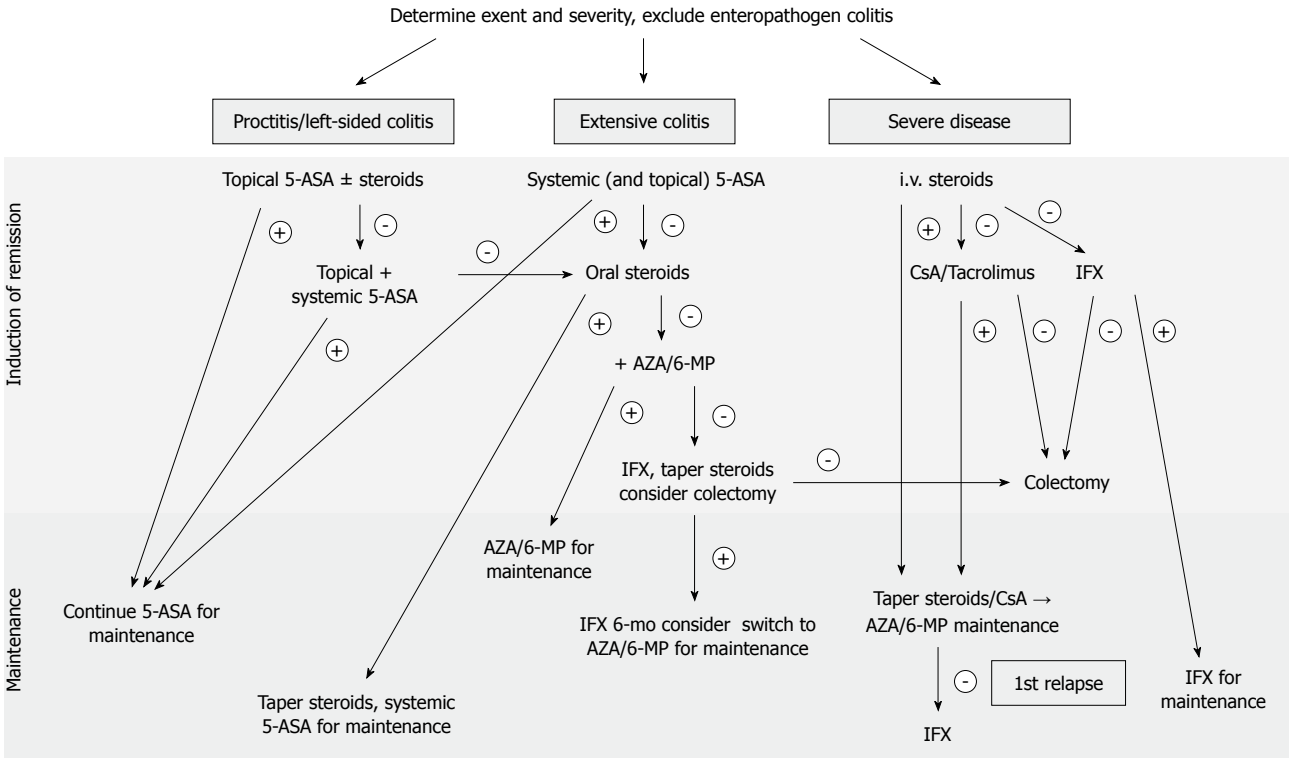

Figure 1 Treatment algorithm for ulcerative colitis. 5-ASA: 5-Aminosalicylic acid; AZA: Azathioprine; CsA: Cyclosporine A; IFX: Infliximab; 6-MP: 6-Mercaptopurine.

EL1b, RG B; ACG EL A) combined with > 2 g/d oral mesalazine. Comparative trials revealed a dose-response of oral mesalazine (< 2.4 g *vs* 4.8 g/d) with more rapid clinical improvement and cessation of rectal bleeding in patients taking a higher dose (16 d *vs* 9 d, $P < 0.05$), but failed to show significant differences in remission rates 20.2% *vs* 17.7% (not significant)[12,13]. Again, treatment escalation by a combination of topical mesalazine with oral 5-ASA and/or topical steroids is possible (ECCO EL 1b, RG B). If rectal bleeding persists after 10-14 d despite combined treatment, systemic steroids should be introduced (ECCO EL 1b, RG C; DGVS EL B; ACG EL C). The steroid starting dose is 40-60 mg orally once daily. Marked differences between 40 and 60 mg starting doses have not been found (DGVS EL A)[14], and steroid regimes differ depending on country and hospital. Without proven superiority, common regimes start with 40 mg prednisolone daily for 1 wk, followed by 30 mg/d for another week and 20 mg/d for 1 mo, before decreasing the dose by 5 mg/d per week. Concerns about possible steroid side effects have led to a more restrictive introduction of steroids in the US compared with European countries and the development of promising new oral steroid formulas with mainly colonic release and low systemic bioavailability (e.g. beclomethasone dipropionate, budesonide)[15,16].

Severe left-sided colitis is usually an indication for hospital admission and systemic therapy (ECCO EL 1b, RG B).

Extensive UC

Extensive UC of mild-to-moderate severity should initially be treated with oral sulfasalazine at a dose titrated up to 4-6 g/d (ACG EL A) or a combination of oral and topical mesalazine (ECCO EL 1a, RG A; DGVS EL A). However, oral 5-ASA formulas induce remission in only approximately 20% of patients[17]. Patients who do not respond to this treatment within 10-14 d or who are already taking appropriate maintenance therapy should be treated additionally with a course of oral steroids (ECCO EL 1b, RG C; ACG EL B). In the case of steroid-dependency (ECCO EL 1a, RG A) or steroid refractory course (ECCO EL 1a, RG B, ACG A), azathioprine (2.5 mg/kg per day) or 6-mercaptopurine (1.5 mg/kg per day) should be introduced for induction of remission and remission maintenance.

Severe UC

Severe UC is defined as more than 6 bloody stools per day and signs of systemic involvement (fever, tachycardia, anemia). These patients should be hospitalized for intensive treatment and surveillance (ECCO EL 5, RG D) as the development of a toxic megacolon and perforation is a potentially life-threatening condition. Intravenous steroids (e.g. methylprednisolone 60 mg/d or hydrocortisone 400 mg/d) remain the mainstay of conventional therapy to induce remission (ECCO EL 1b, RG D; DGVS C). Patients refractory to maximal oral treatment with prednisolone and 5-ASA can be given the tumor necrosis factor (TNF)-α blocker IFX at 5 mg/kg (ACG EL A).

Nevertheless, colectomy rates are as high as 29% in patients with severe UC and who need intravenous corticosteroids[18]. They should therefore be presented to the colorectal surgeon on the day of admission. It is crucial

that gastroenterologists and surgeons provide joint daily care in order to avoid delaying the necessary surgical therapy. In the case of a worsening condition or a lack of amelioration after 3 d of steroid therapy, colectomy should be discussed, since extending steroid therapy beyond 7 d without clinical effect carries no benefit[18], but causes otherwise preventable postoperative wound-healing disorders[19]. The response to intravenous steroids is best assessed by stool frequency, CRP and abdominal radiography on day 3 (ECCO EL 2b, RG B). If drug therapy fails, either proctocolectomy (DGVS EL C, ACG EL B) or rescue therapy with CsA (ACG EL A) is recommended.

In order to prevent immediate surgical therapy in corticoid resistant cases calcineurin inhibitors (CsA, tacrolimus) and IFX are available as second-line therapies, as detailed below.

Continuous intravenous CsA monotherapy with 4 mg/kg per day is effective and can be an alternative for patients with contraindications for corticosteroid therapy (e.g. a history of steroid psychosis, DGVS EL A). After successful induction of remission, an immunosuppressant such as azathioprine (2.5 mg/kg per day) should soon be added, CsA switched to oral therapy with tacrolimus and tapered over a period of 3-6 mo (DGVS C). Note that it may take up to 3 mo for the therapeutic effects of azathioprine and 6-mercaptopurine to develop. Neither CsA nor tacrolimus are indicated for maintenance therapy. Intravenous CsA achieves marked short-term responses in 50%-80% of patients receiving CsA as rescue therapy[20,21]. However, studies on long-term outcomes indicated that 58%-88% of these patients underwent colectomy within the following 7 years[21,22]. One major advantage of CsA over IFX in rescue therapy is its short half-life. If it proves ineffective it is cleared within a few hours, whereas IFX will circulate for weeks.

Tacrolimus is another calcineurin inhibitor given in an oral dose of 0.1-0.2 mg/kg per day or 0.01-0.02 mg/kg per day intravenously to achieve trough concentrations of 10-15 ng/mL[23]. A retrospective uncontrolled study indicated that lower trough levels of 4-8 ng/mL are also effective and are associated with fewer side effects[24].

Due to the elevated risk of opportunistic infection with *Pneumocystis jiroveci*, chemoprophylaxis is recommended in patients under triple immunosuppressive therapy (DGVS EL B). Possible regimes are trimethoprim-sulfamethoxazole 160/800 mg twice a week or, in case of intolerance, inhalation of 300 mg pentamidine once per month.

According to the ECCO and the newer ACG guidelines, IFX *may* be effective in the prevention of colectomy. In clinical practice it is widely considered a second choice due to its long half-life compared to CsA. Infliximab is given intravenously at a single dose of 5 mg/kg followed by scheduled infusions on weeks 2 and 6, and every 8 wk thereafter. One controlled trial on 45 patients with severe corticoid-refractory colitis compared IFX *versus* continued intravenous betamethasone. A significantly lower number of patients, 7/24 *vs* 14/21 ($P = 0.017$; odds ratio 4.9; 95% confidence interval (CI), 1.4-17), proceeded to colectomy within 3 mo with IFX[25]. In a recent trial of infliximab IFX as rescue therapy in tacrolimus-refractory patients with active UC, about a quarter of patients (6 of 24) responded to IFX[26]. Nevertheless, effectiveness is not yet proven, as the present number of case series of infliximab IFX rescue therapy in steroid-refractory severe extended colitis is small, with wide differences concerning colectomy rates (20% to 75%)[27,28]. Patients who required IFX to induce remission should receive regular maintenance therapy with IFX for at least 6 mo. Adalimumab, a fully humanized TNF-α blocker, is not yet available for the treatment of UC.

Selective physical apheresis of activated immune cells involved in the inflammatory process of UC (leukocytapheresis) is an alternative strategy proposed for the treatment of active UC, but its role remains controversial. Although trials in Japan showed leukocytapheresis to be equal to corticoid treatment for inducing remission while displaying fewer side effects[29,30], the most recent study of an international cohort did not show significant differences in clinical outcome between the apheresis- and sham-treatment groups[31].

Only a small amount of data is available on methotrexate (MTX) for induction of remission. The only randomized placebo-controlled study did not show any effect in UC. Neither did a comparative study of 6-mercaptopurine, oral MTX or 5-ASA additional to prednisolone in 34 steroid-dependent UC patients, with remission rates of 58.3% in the MTX group compared with 35% in the 5-ASA group ($P > 0.5$). This disappointing effect may result from the very low doses of MTX (15 mg/kg per week) administered orally in both studies. Although existing guidelines do not generally recommended MTX, in individual cases therapy with an initial dose of 25 mg/wk followed by dose reduction to 15 mg/wk after achieving remission can be tried. Significant side effects (hepatotoxicity, bone marrow depression, MTX-induced lung injury) should be monitored and strict contraception performed (risk of teratogenicity). In order to reduce side effects 5 mg oral folic acid given on the morning after MTX administration is effective and safe[32].

Adjuvant therapeutic considerations in severe UC

Antibiotic therapy is only recommended if infection is considered (DGVS EL A). Application of metronidazole or tobramycin has not shown consistent benefit in severe UC[33-35]. Patients should be given enteral nutrition if tolerated and subileus/ileus are absent (DGVS EL B/C) since bowel rest in acute colitis did not alter the outcome and enteral nutrition was shown to be associated with significantly fewer complications (9% *vs* 35%)[36].

Prediction of outcome and surgical therapy

In a recent population-based European study, the global risk of colectomy in UC was 8.7% over 10 years. Efforts have been made to identify patients who are at high risk of not responding adequately to pharmacological therapy. In Crohn's disease, factors such as young age at first di-

agnosis, early steroid use, ileal disease, mucosal healing, and smoking were identified as important for developing disabling disease or for major abdominal surgery[37]. Much less is known in UC. According to data from different population-based studies, including the recent 10-year data from the Norwegian IBSEN cohort, initial extensive colitis, elevated ESR (> or = 30 mm/h) and sclerosing cholangitis were associated with an increased risk of colectomy[38-40]. In contrast, older age at disease onset (> or = 50 years)[40] and smoking[41,42] reduced the risk of subsequent colectomy. In a prospective study evaluating 49 hospitalized patients with severe UC, patients treated with steroids and/or CsA, a stool frequency of > 8/d or 3-8 stools/d, and increased CRP (> 0.45 mg/L) on day 3 predicted the need for colectomy with 85% certainty[43]. Further work to identify predictive parameters of refractory courses should help to prevent a delay in inevitable surgical therapy.

Emergency indications for surgery includes refractory toxic megacolon, perforation and continuous severe colorectal bleeding (ACG EL C)[44,45]. In this situation the recommended operation is colectomy and ileostomy, leaving the rectum *in situ*, since reconstruction is not an option in the acute setting (ECCO EL 4, RG C).

Elective surgery is indicated in chronic continuous colitis refractory to immunosuppressive treatment, detection of dysplasia or malignancy, and stricturing disease causing partial or total intestinal obstruction. In elective surgery common surgical therapy is total proctocolectomy with ileal J-pouch anal anastomosis (IPAA). Although with IPAA a curative therapy for UC is available, high rates (up to 20%) of postoperative complications with abscesses, sepsis, fistulas[46], and postoperative impaired fertility and sexual function are unsolved problems[47,48]. Ileorectal anastomosis is a temporary alternative in selected cases (e.g. young women who have not had children), but harbors the risk of disease recurrence and/or cancer development in the remaining rectal segment[45].

MAINTENANCE OF REMISSION

Remission is clinically defined by 3 or less stools per day without any presence of blood or increased urgency of defecation. The major goal of maintenance therapy is a steroid-free remission to avoid severe and partially disabling long-term side effects of corticoid treatment. Continuing medical therapy that does not achieve this goal is therefore not recommended and should be changed (ECCO EL 5, RG D). More than half of patients with UC have a relapse in the year following a flare. In a recent population-based outcome survey conducted in Copenhagen with a cohort of 1575 patients with newly diagnosed UC, 13% had no relapse within the following 5 years, 74% had less than 5 relapses and 13% suffered an aggressive course with more than one relapse per year[49]. Maintenance treatment is therefore recommended for all patients (ECCO EL 1a, RG A), but intermittent therapy is also acceptable for a few patients with an indolent course of the disease.

First line therapy for maintenance of remission is 5-ASA administered orally or (in the case of left-sided colitis) rectally[13,50]. All the available different 5-ASA preparations are effective and no convincing data are available favoring any specific preparation. Sulfasalazine, an azo-bound combination of mesalazine and sulfapyridine, is equally or even slightly more effective. While the ACG guidelines recommend it for induction as well as remission therapy, the European guidelines reserve its use for induction and maintenance therapy in patients with additional joint manifestations due to its higher toxicity (ECCO EL 1a, RG A). First-line medical therapy for proctitis and left-sided colitis consists of topical 5-ASA with a minimum dose of 1 g 3 times a week (ECCO EL 1b, RG B; ACG EL A). Oral mesalazine can be added as second-line therapy and has been shown to be superior compared with monotherapy (ECCO EL 1b, RG B), or it can be given alone if long-term rectal treatment is not accepted by the patient. For extensive disease, oral mesalazine is the therapy of first choice. It is effective and well tolerated at doses > 800 mg/d for maintenance of remission[13], although a clear dose-response effect has yet to be established.

Compliance is a key factor in disease control and maintenance of remission. In an internet-based survey of 1595 UC patients receiving 5-ASA therapy, major reasons for poor compliance were identified as 'too many pills' and 'dosing required too many times each day'[51]. In a prospective survey in Michigan only 71% of the originally prescribed medical therapy was finally taken by the patients included in the study[52]. A new generation of aminosalicylates with prolonged release formulations has been engineered over the last few decades (e.g. Eudragit-S-coated, pH-dependent mesalamine, ethylcellulose-coated mesalamine, and multimatrix-release mesalamine). All three currently available trials comparing a once *versus* a twice daily dose of prolonged release mesalamine for maintenance of remission in mild-to-moderate UC did show non-inferiority or even superiority of a once daily medication, in part due to increased compliance[53-55]. Once daily dosing of a prolonged release formulation could therefore be a promising approach to further reduce recurrent flares in maintenance therapy.

In case of side effects of the 5-ASA treatment with the probiotic strain *Escherichia coli Nissle* is an alternative for maintenance of remission with comparable efficacy (ECCO EL 1b, RG A)[56,57].

Azathioprine and 6-mercaptopurine are indicated as steroid-sparing agents for steroid-dependent patients or for patients not adequately sustained and with frequent relapses under aminosalicylate treatment (ECCO EL 5, RG D; ECCO EL A). The optimal dose (1.5-2.5 mg/kg per day) can be taken once daily. Therapy should be monitored by a leucocyte count of lower than 4.5×10^9/L but higher than 2.5×10^9/L. If remission is successfully induced it is recommended to continue maintenance therapy for at least 3-5 years[58,59], although there is no hard evidence on the optimal duration of treatment. Side effects such as bone marrow suppression, progressive elevation of lever enzymes, toxic pancreatitis may

occur (usually within the first weeks) and require immediate termination of azathioprine treatment. A 3-fold increased risk of opportunistic infection is estimated under azathioprine therapy, especially when used in conjunction with IFX and steroids[60].

IFX is effective in maintaining improvement and remission and is therefore recommended for those patients who initially respond to the IFX induction regime (ECCO EL 1b, RG A)[61]. The standard IFX dose is 5 mg/kg. Higher initial treatment doses have not been shown to be of any benefit. As shown for Crohn's disease, 25%-40% of patients with initial response to IFX develop loss of response and benefit from dose escalation to 10 mg/kg or shortening dosing intervals during further therapy[62,63].

For maintenance therapy, scheduled intravenous administration every 8 wk has been proven to be more effective and safer than periodic application, probably due to a reduced formation of antibodies (ABs) against anti-TNF agents[64,65]. Most infusion reactions are mild-to-moderate and consist of flushing, headaches, dizziness, chest pain, dyspnea, fever or pruritus. Halting or lowering the infusion rate often provides relief. In order to prevent adverse reactions and AB formation, premedication with steroids prior to IFX administration is recommended (ECCO EL 2, RG C)[66]. Serious infection occurred in approximately 3% of patients treated with IFX in the ACT 1 and ACT 2 trials[61]. Although the information available from meta-analyses, from IFX safety registries in Crohn's disease, and from IFX therapy in rheumatoid arthritis differ widely, a 3-fold higher rate of opportunistic infectious under IFX therapy is estimated[67-70]. In order to prevent reactivation of latent infection, exclusion of latent tuberculosis and hepatitis B should be performed by chest radiography, serologic testing, skin test and/or lymphocyte stimulation test (QuantiFERON-TB Gold®).

There is no existing recommendation on the duration of IFX treatment in stable remission. In stable long-term remission, interruption of IFX treatment while continuing 5-ASA or switching maintenance therapy to azathioprine/6-mercaptopurine are possible de-escalation approaches.

MTX for maintenance therapy can be considered in individual cases, especially when other immunosuppressants are not tolerated. Furthermore, patients with refractory arthropathy may benefit. Because the available data are restricted to one randomized prospective study[71] and several retrospective series with a total of only 91 patients[72-74], no consensus recommendation for MTX in UC is given[6].

ALTERNATIVE AND FUTURE TREATMENTS

Several alternative therapies have emerged for the treatment of UC. Ova of the non-pathogenic helminth *trichiuris suis* taken orally has shown initial success in a double-blind placebo-controlled trial, inducing remission in 43% of patients taking ova compared with 16.7% in the placebo group[75]. Transdermal administration of nicotine was proposed as being effective in active UC. A systematic review and analysis of 5 relevant studies demonstrated its effectiveness in achieving remission compared with placebo. However, direct comparative trials with 5-ASA are still missing. Omega-3 fatty acids, which are largely present in fish oil, have shown anti-inflammatory properties by reducing the production of leukotriene B_4[76]. However, in a meta-analysis of the 3 available studies on 138 UC patients in remission, no evidence was found to support the use of omega-3 fatty acids for maintenance of remission as similar relapse rates were found in the study group and the placebo group (relative risk, 1.02; 95% CI, 0.51-2.03; $P = 0.96$)[77]. Taken together, due to a lack of data on efficacy, safety and adverse events, no recommendation is given for the therapies mentioned above.

Advances in the field of biological therapy focus on novel target molecules and alternative means of administration, some of which have already been approved for the treatment of Crohn's disease. Further TNF-α AB preparations include certolizumab, etanercept and adalimumab - approval of the latter for the treatment of UC could be expected this year. Other biologicals such as natalizumab (anti-α4-integrin AB), visilizumab (anti-CD3 receptor AB), fontolizumab (anti-interferon gamma AB), alicaforsen (anti-sense oligonucleotide to human ICAM1), basiliximab (IL-2 receptor AB), anti-IL12 ABs and anti-IL-6 ABs have in part been tested in acute steroid-refractory UC but data on maintenance of remission are not available as yet.

RECOMMENDATIONS FOR CANCER SURVEILLANCE

Patient with UC have an elevated risk of developing colon cancer. After 8-10 years of colitis, annual or biannual surveillance colonoscopy with multiple biopsies at regular intervals should be performed (ACG EL B; ECCO EL5, RGD). Detection of high grade dysplasia in flat mucosa has to be confirmed by a second pathologist and is an indication for colectomy (ACG EL B; ECCO EL2, RG B).

The sensitivity of random biopsies is a matter of debate and uncertainty as dysplasia in flat mucosa can be easily overlooked. Implementation of advanced endoscopic imaging techniques such as high-resolution white-light endoscopy, autofluorescence and narrow-band imaging may help to better identify pathological lesions and optimize cancer surveillance in inflammatory bowel disease in the future[78-80].

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TOPIC HIGHLIGHT

Dr. Jonas Mudter, MD, *Series Editor*

Risk of postoperative recurrence and postoperative management of Crohn's disease

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Abstract

Crohn's disease (CD) is a chronic inflammatory disease of the digestive tract with systemic manifestations. Etiology is unknown, even if immunological, genetic and environmental factors are involved. The majority of CD patients require surgery during their lifetime due to progressive bowel damage, but, even when all macroscopic lesions have been removed by surgery, the disease recurs in most cases. Postoperative management represents therefore a crucial mean for preventing recurrence. Several drugs and approaches have been proposed to achieve this aim. Endoscopic inspection of the ileocolic anastomosis within 1 year from surgery is widely encouraged, given that endoscopic recurrence is one of the greatest predictors for clinical recurrence. A strategy should be planned only after stratifying patients according to their individual risk of recurrence, avoiding unnecessary therapies when possible benefits are reduced, and selecting high-risk patients for more aggressive intervention.

Key words: Crohn's disease; Recurrence; Postoperative treatment; Surgery; Surveillance

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INTRODUCTION

Crohn's disease (CD) is a chronic inflammatory disease of the digestive tract with associated several systemic manifestations. Etiology of CD seems to be multifactorial, which results from an interaction of genetic susceptibility, immunological dysregulation of the mucosal immune system, gut microflora and environmental factors, but the final cause remains still unknown. Pathologically, CD is characterized by non-caseating granulomas and transmural inflammation, which can affect all the digestive tract from the mouth to the anus[1]. The most commonly affected sites are the ileum and ascending colon and the disease recurs in most cases after surgical resection of macroscopically affected segments[1]. For this reason, almost all studies on postoperative recurrence are primarily focused on ileocolic disease. CD patients have a high likelihood of undergoing repeated surgery for disease recurrence during their lifetime, with risk for short bowel syndrome: surgery in these patients is as conservative as possible in order to reduce this risk. Two distinct approaches are possible in clinical practice for postoperative CD management: starting prophylactic medical therapy after surgery, which aims to avoid or postpone recurrence;

or waiting for endoscopic recurrence, and eventually starting or adapting medical therapy before the patient becomes symptomatic. Both these strategies are rational and can be indicated in specific situations. Recurrence risk assessment after surgery is critical for correct decision making about medical prophylaxis and surveillance, and has to consider many different variables. Regueiro *et al*[2] have considered three grades of recurrence risk: (1) very low, defined as long-standing, first surgery, short stricture; (2) low-moderate, i.e. < 10-year-long history of CD, presence of a long stricture or any case of inflammatory CD; and (3) high risk, defined as presence of penetrating disease or patients with repeated intestinal resection (more than twice). Those patients may require a more aggressive approach[2]. This review provides an overview on this relevant and debated clinical topic.

NATURAL HISTORY

Schofield introduced the definition of "natural history" for ulcerative colitis first and later for regional enteritis[3]. The natural history of CD is characterized by the progression of bowel injury, which leads to surgery in up to 80% of patients during their lifetime[4]. Surgery in CD is not curative, even when all of the macroscopic disease has been removed. Disease recurrence typically presents at an anastomotic site, mostly in the preterminal ileum in patients with previous ileal involvement[4]. Endoscopic surveillance programs for operated CD patients has led to identification of endoscopic lesions (endoscopic recurrence) that, in most cases, precede clinical symptoms (clinical recurrence). Clinical recurrence rates and reoperation rates increase over the years: about 80% of patients will experience clinical recurrence within 20 years from surgery, and about 50% will undergo repeated surgery during the same time in most series[4,5].

Regarding location, CD tends to be stable, since only about 6.5% of the patients will experience a change of site in disease involvement over time[4]. Henriksen has investigated the changes in disease location in the Inflammatory Bowel South-Eastern Norway (IBSEN) population-based study. Of 200 CD patients, only 14% changed affected site after 5 years[6].

It has to be underlined that biological therapies are relatively new in the management of CD, and most of the studies on CD natural history refer to an era when the efficacy of medication was lower compared to the present. Anti-tumor necrosis factor (TNF)-α antibodies like infliximab and adalimumab show strong efficacy in inducing mucosal healing: they could possibly modify the natural history of the disease, but data on reduction of the need for repeated surgery in the long-term are still not available. Data from clinical trials in inflammatory bowel diseases (IBD) patients treated by anti TNF-α show that scheduled therapy significantly reduces the risk of hospitalization and surgery related to CD up to 55 mo on average[7,8]. There is then a reasonable perspective that scheduled therapy by infliximab or adalimumab might prevent or delay postoperative recurrence in high-risk patients, with a significant change in the natural history of the disease, although further large prospective studies focused on this topic are needed.

Today, surgery remains part of the clinical history of CD patients[9,10]. In recent years, laparoscopic surgery is gaining a place in the surgical treatment of CD, especially for ileocecal disease[4].

The assessment of activity, severity and complications of CD that may require surgical procedures remains a big challenge for physicians. Different kinds of objective measures of those parameters are available, but none of them is comprehensive for all aspects of the disease. The Crohn's disease activity index (CDAI)[11] and the Harvey-Bradshaw index[12] are commonly used to assess the activity and severity of the disease, but they can be affected by other non-CD gastrointestinal or extraintestinal disorders that may affect the correct evaluation of the disease. These scores do not include the endoscopic features of the disease that can be measured using two other different scores: the Crohn's Disease Endoscopic Index of Severity[13] and the Simplified Endoscopic Score-Crohn's Disease[14]. Other methods to assess disease activity include the level of fecal markers, such as fecal calprotectin, fecal lactoferrin and polymorphonuclear neutrophil elastase, which are produced in the course of bowel inflammation, and that can be detected in fecal samples. They have been shown to be more sensitive, specific and accurate than C-reactive protein in differentiating active from inactive disease, and to distinguish between CD-related symptoms and irritable bowel syndrome[15]. These non-invasive biomarkers can also be used in the postoperative follow-up in order to distinguish bowel symptoms related to mechanical causes (such as shortening of the small bowel, colonic resection, bile salt diarrhea) from a clinical recurrence of CD. None of these tools is comprehensive for all aspects of CD; therefore, a new global score that considers clinical, endoscopic, radiological parameters and surgical history to assess the disease activity, severity and organ damage is urgently needed.

CD RECURRENCE

Definition

Recurrence is defined as reappearance of lesions after complete surgical resection[16]. Recurrence can be assessed by endoscopy, radiology or surgery. Clinical recurrence is defined as the appearance, after complete resection of macroscopic disease, of CD symptoms, which confirms recurrence of the lesions[16]. This confirmation is important because postoperative recurrence-like symptoms may be due to causes other than CD, such as motility disturbances or bile malabsorption.

Endoscopic assessment

The severity of recurrence assessed by endoscopy is the best predictor of clinical outcome in postoperative CD[17-21]. In order to correlate the endoscopic findings with the risk of clinical recurrence, Rutgeerts has validated an endoscopic scoring system to be assessed at the first ileocolonoscopy, 6-12 mo after surgery: it divides patients into

Table 1 Endoscopic recurrence score as reported by Rutgeerts *et al*[17]

Endoscopic score	Definition
i0	No lesions
i1	≤ 5 aphthous lesions
i2	> 5 aphthous lesions with normal mucosa between the lesions, or skip areas of larger lesions, or lesions confined to the ileocolic anastomosis
i3	Diffuse aphthous ileitis with diffusely inflamed mucosa
i4	Diffuse inflammation with already large ulcers, nodules, and/or narrowing

Remission: Endoscopic score i0 or i1; Recurrence: Endoscopic score of i2-i4.

five groups (i0-i4, see Table 1)[17], according to the severity of endoscopic lesions; in the i0-i1 group, there is < 5% recurrence at 3 years; in i2, about 15%; in i3, 40%; and i4, > 90%[17]. The most common endoscopic lesions are aphthous ulcerations that are located in the neoterminal ileum near to the anastomotic area[17]. In the absence of treatment, such lesions can evolve into large ulcerations or tight strictures: Rutgeerts has reported that only 20% of these patients become clinically symptomatic within 1 year after surgery[17]. Symptoms develop only after a lesion has been established, and it is not uncommon to find endoscopic severe lesions in an asymptomatic patient. Clinical scoring systems such as the CDAI may therefore be of little help in identifying patients with postoperative recurrence[22].

Risk factors

Most patients experience disease recurrence over time, but long-term medical strategies for prevention of recurrence can carry various risks: risk-benefit analysis should be individualized to each patient. Identification of risk factors for CD recurrence is therefore extremely important in order to identify high-risk patients, who are more likely to benefit from an aggressive therapeutic strategy.

Several factors have been studied as possible contributors to CD recurrence, including age, sex, cigarette smoking, age at onset of disease, duration of disease, anatomical site, extent and severity of disease, previous surgery, and disease phenotype, disease-free margins, anastomotic technique, blood transfusion, and occurrence of complications.

Among these many factors, the following have been shown to predict early recurrence in the majority of studies: smoking[23], prior intestinal surgery (including appendectomy)[24], penetrating disease behavior[25], perianal involvement, and extended small bowel resection (> 100 cm)[26]. Absence of prophylactic therapy is associated with early relapse (defined as a flare of symptoms)[27].

Reese *et al*[28] in a recent meta-analysis have reported that clinical relapse occurred in 58.3% of smoking patients, compared with 39% of non-smokers ($P < 0.005$). The 5-year reoperation rate was higher among smokers, but not significantly (34.2% *vs* 31.1%); however, this difference becomes significant when considering the 10-year reoperation rates: 55.5% in smokers and 17.5% in non-smokers ($P < 0.04$)[28]. No differences were noted between ex-smokers and non-smokers[28].

Data about sex, age at onset of the disease, duration of the disease, resection margins, type of surgery are discordant and inconclusive[27].

POSTOPERATIVE MANAGEMENT STRATEGIES

General management

Postoperative management of CD represents a hard challenge. Stratifying patients according to their risk is essential: not all the patients benefit from maintenance therapy, and a patient at high risk of recurrence requires a different, stronger therapy than others. Cigarette smoking is consistently correlated with CD recurrence and, among the known related risk factors, it is the only potentially modifiable factor[29]. Therefore, as a first step for preventing recurrence, it should be stressed to every patient the importance to stop smoking after surgery for CD[29]. Smoking cessation decreases risk to the level of that of non-smokers[28].

Drug prophylaxis of recurrence

Prophylactic drugs have considerable costs and risks, with limited efficacy[4]. It has to be underlined that there is currently no evidence that, in terms of reduction of hospitalization or need for surgery, prophylactic therapy gives any advantage over a strategy of surveillance with prompt treatment of recurrence[4]. An overview of the most important drugs used to prevent recurrence follows.

5-Aminosalicylic acid: 5-Aminosalicylic acid (5-ASA) has been extensively studied in postoperative management of CD. Lochs *et al*[30] and Florent *et al*[31] have treated patients with 5-ASA (mesalazine 4 g/d) or placebo for > 18 mo postoperatively, and have shown that clinical recurrence rates are lower in the 5-ASA group (24.5% *vs* 31.4%), although the difference was not statistically significant. In a study by Florent *et al*, mesalazine (2.4 g/d) did not significantly reduce endoscopic recurrence rates at 12 wk (50% *vs* 63%)[27]. Ewe *et al*[32] have demonstrated that sulfasalazine (3 g/d) significantly reduces clinical recurrence rates at 1 year (16% *vs* 28%, $P < 0.01$), with effects maintained at 2 years. Endoscopic recurrence has been shown to occur more rarely after 12 mo mesalazine treatment (3 g/d); however the clinical recurrence rates were similar in the two groups[33]. In a recent double-blind trial, two groups were randomized to receive 4 g or 2.4 g/d of mesalazine at 2 wk after surgery. At 12 mo, endoscopic recurrence was higher in the second group, although clinical recurrence rates were similar in both groups[12]. The current evidence seems to indicate that 5-ASA is generally safe in postoperative CD prophylaxis, even if it seems to provide, at best, only a small reduction in clinical and endoscopic recurrence[1].

Antibiotics: Two randomized clinical trials[34,35] have

evaluated antibiotic therapy in the prevention of CD recurrence after surgery. In the first study, patients treated with metronidazole (20 mg/kg per day, for 3 mo after surgery) had a reduced incidence of severe endoscopic recurrence after a follow-up of 1 year (4% *vs* 25%), but experienced a three times higher incidence of side effects than the placebo group (23.3% *vs* 6.7%)[36]. Similar results were obtained by another study by the same group on another nitroimidazole antibiotic, ornidazole (1 g/d), administered for 1 year: recurrence rates were reduced at 1 year, but not maintained at 2 and 3 years. The therapy in these studies was not well tolerated. Nitroimidazole antibiotics have shown efficacy in reducing severe endoscopic recurrence in the short- and medium-term and can be eventually associated with other treatments in the first postoperative period[1]. The possible use of other more tolerable antibiotics such as rifaximin or ciprofloxacin has still to be evaluated.

Budesonide: Two large randomized clinical trials have found that oral budesonide is ineffective in reducing postoperative recurrence rates after surgery for CD[37]. Ewe *et al*[32] have found that endoscopic and clinical recurrences after 1 year were not significantly different when comparing budesonide to placebo treatment; Hellers *et al*[38] have not found differences between the two groups at 3 and 12 mo regarding endoscopic recurrence rates.

Immunomodulators: The thiopurines azathioprine (AZA)/6-mercaptopurine (6-MP) have proven efficacy and are widely recommended for postoperative prophylaxis of recurrence after surgery for CD. Ardizzone *et al* have demonstrated that patients after surgical resection benefit more from AZA than mesalazine for prevention of clinical recurrence, despite the side effects experienced by patients in the AZA group (22% *vs* 8% compared to the mesalazine group)[1]. Hanauer *et al*[36] have found that clinical recurrence at 2 years was 50% in the 6-MP group, 58% in the mesalazine, and 77% in the placebo group. In this study, only 69% of patients assigned to 6-MP was able to complete the treatment, because of the significant side effects. A recent meta-analysis of four controlled studies of AZA for recurrence prohylaxis has demonstrated its efficacy for reducing the incidence of overall recurrence at 12 mo. On the other hand, the rate of adverse events leading to therapy withdrawal was higher in the AZA group than in the control groups[39]. Thiopurines have been shown to be more effective than mesalazine for prevention of postoperative recurrence, despite the higher rate of side effects reported[1].

Anti-TNF agents: There is only one randomized, double-blind, placebo-controlled trial that has assessed the efficacy of infliximab for reducing postoperative recurrence rates after ileocolic resection[2]: 24 patients were randomized to receive 5 mg/kg infliximab with a standard three-dose induction and a maintenance dose once every 8 wk, for 1 year, versus placebo. In the infliximab group, 9.1% developed endoscopic recurrence compared with 84.6% in the placebo group (P = 0.0006). Infliximab patients experienced a significantly lower risk of endoscopic, histological and clinical recurrence at 1 year[2]. No data are available about other anti-TNF agents for preventing CD recurrence after surgery.

Other therapies

Different types of probiotics have been evaluated, but none of them has showed a significant effect over placebo in preventing recurrence. There is no evidence for the use of other agents such as synbiotics or interleukin-10 for preventing CD recurrence after surgery[40-44].

Endoscopic surveillance strategy

Surgery for CD treats complications such as strictures, abscesses or fistulae, but it cannot interrupt the course of the disease. Postoperative recurrence rate in absence of treatment reaches 65%-90% within 12 mo and 80%-100% within 3 years from surgery[27], with an increase of 22%-25% per year[19,45]. Moreover, about 50% of the patients will need reoperation within 10 years after the first bowel resection[9,16,24-26,46,47]. It is therefore important to plan a surveillance of the patient after having assessed their risk of recurrence and chosen the most indicated treatment.

The CDAI can be used to assume the possible activity of the disease, but in postoperative settings it does not reach a sufficient sensitivity to predict disease recurrence accurately: patients with a high CDAI may be completely asymptomatic[22]. Fecal markers can play a role, but their use for this particular indication needs further investigation.

Rutgeerts *et al*[47] have developed a validated endoscopic score to measure the postoperative recurrence and stratify patients, according to their endoscopic findings, in five categories, already described (see Table 1 for details). The severity of endoscopic lesions correlates with the risk of disease progression. Endoscopic signs of recurrence do appear before symptomatic disease, which allows early treatment that may be effective in modifying the natural course of the disease, preventing clinical recurrence and possibly, the need for new surgery.

Patients presenting with no or minimal endoscopic lesions (endoscopic score i0-2) may not benefit from therapy; on the contrary, severe endoscopic recurrence (endoscopic score i3-4) should promote aggressive therapy. Based on correlation data between endoscopic lesions and clinical recurrence, and on data about risk factors for postoperative CD recurrence, patients can be considered at low risk if their disease is long-standing, mainly fibrostenotic, and involves a limited bowel segment; on the other hand, all other patients, especially active smokers, with prior intestinal surgery, perianal disease, perforating and inflammatory disease, who have undergone extensive bowel resection should be considered at high risk for recurrence. It can be a rational strategy to wait to treat low-risk patients until performing the first ileocolonoscopy, 6 mo to 1 year postoperatively, and reserving the immediate start of postoperative prophylactic therapy (with thiopurines or anti-TNF) for high-risk patients. For low-risk patients, if there is endoscopic recurrence at ileocolonoscopy, im-

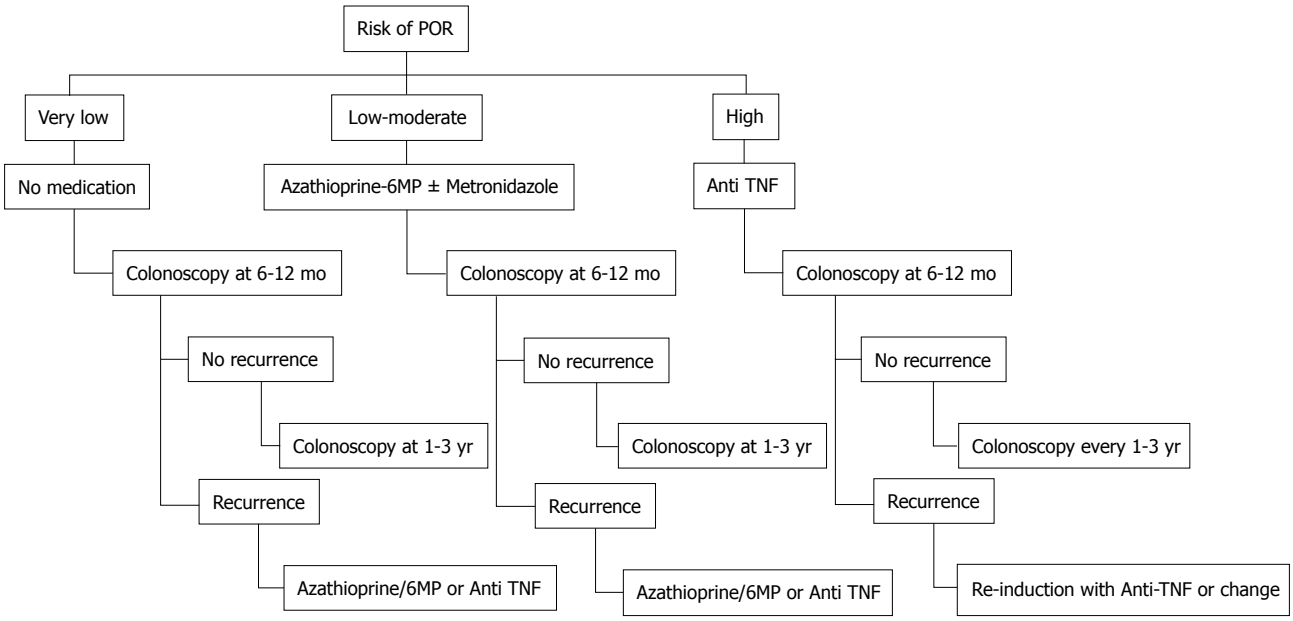

Figure 1 Flow-chart for treatment of postoperative recurrence of CD (adapted from Regueiro[29]). 6-MP: 6-mercaptopurine; TNF: Tumor necrosis factor; POR: postoperative recurrence.

munomodulatory or anti-TNF therapy could be started; if negative, only a new endoscopic surveillance can be performed 1-3 years later. Further optimization or modification of therapy could be performed according to the severity of endoscopic lesions, repeating ileocolonoscopy after another 6-12 mo. For patients at high risk of recurrence, thiopurines or anti-TNF treatment is recommended, starting a few weeks after surgery; if endoscopic controls during surveillance reveal recurrence of disease, it is possible to intensify the dose or switch to another agent[29].

Surgical strictureplasty represents a valid treatment for most patients with small bowel strictures, while its role in duodenal and colonic disease remains debated[48]. A recent meta-analysis by Yamamoto *et al*[48] has shown that younger age, short duration of the disease, and short interval from previous resection can increase the risk of recurrence. Differently form what previously reported[49], the most recent evidence has shown that the number of strictures and strictureplasties do not affect the risk of recurrence[48]. Except for the duodenum and the last ileal loop, small bowel is difficult to be explored endoscopically; therefore, data regarding the CD recurrence are focused on ileocecal resection, and there is no evidence that medical management or timeline for surveillance after strictureplasty is different than in patients undergoing ileocecal resection.

CONCLUSION

CD recurrence after surgery is very frequent. Identification of risk factors for recurrence is extremely important in order to stratify patients according to their risk, identifying those who can benefit from an aggressive medication regimen, possibly modifying the natural course of the disease. If postoperative prophylaxis of recurrence is indicated, then thiopurines (eventually with metronidazole) and anti-TNF agents are the most effective choices. Ileocolonoscopy within 1 year of surgery, regardless of the eventual postoperative treatment, is widely recognized as the best tool for early detection of CD recurrence, allowing tailoring the appropriate therapy to the individual patient. A summary of the suggested management of postoperative recurrence is shown in Figure 1. Cooperation between gastroenterologists, endoscopists and surgeons is desirable in order to assess the risk of recurrence, to plan postoperative surveillance (not limited to symptomatic flares), and to offer the most appropriate treatment strategy to the patient. Which approach would be better for treating and preventing postoperative recurrence of CD remains debatable.

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ORIGINAL ARTICLE

Relationship between LYVE-1, VEGFR-3 and CD44 gene expressions and lymphatic metastasis in gastric cancer

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Abstract

AIM: To investigate the expression levels of lymphatic vessel endothelial hyaluronan receptor-1 (LYVE-1), vascular endothelial growth factor receptor-3 (VEGFR-3) and CD44 genes and the relationship between their levels and clinicopathological parameters in gastric cancer.

METHODS: Tissue samples were obtained from 33 patients (8 females) with gastric cancer. mRNA levels of LYVE-1, VEGFR-3 and CD44 in normal and tumor tissues were quantitatively measured using real time polymerase chain reaction. The results were correlated with lymph node metastasis, histological type and differentiation of the tumor, T-stage, and presence of vascular, perineural and lymphatic invasions. The distribution of molecules in the tissue was evaluated using immunohistochemistry.

RESULTS: LYVE-1, CD44 and VEGFR-3 gene expression levels were significantly higher in gastric cancer than in normal tissue. While there was no correlation between gene expressions and clinicopathologic features such as histologic type, differentiation and stage, gene expression levels were found to be increased in conjunction with positive lymph node/total lymph node ratio and the presence of perineural invasion. A significant correlation was also found between LYVE-1 and CD44 over-expressions and perineural invasion and lymph node positivity in gastric cancers. When the distribution of LYVE-1 antibody-stained lymphatic vessels in tissue was evaluated, lymphatic vessels were located intra-tumorally in 13% and peri-tumorally in 27% of the patients. Moreover, lymph node metastases were also positive in all patients with LYVE-1-staining.

CONCLUSION: LYVE-1, VEGFR-3 and CD44 all play an important role in lymphangiogenesis, invasion and metastasis. LYVE-1 is a perfectly reliable lymphatic vessel marker and useful for immunohistochemistry.

Key words: CD44; Gastric cancer; Lymphatic metastasis; Lymphatic vessel endothelial hyaluronan receptor-1; Metastasis; Vascular endothelial growth factor receptor-3

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INTRODUCTION

Gastric cancer is the second most common cause of cancer deaths worldwide[1]. As the stage of disease has definitive influence on survival, earlier diagnosis is highly critical. Lymphatic spread of gastric cancer cells to regional lymph nodes is one of the earliest events associated with distant metastasis and poor prognosis[2]. Depth of invasion, lymph node metastases and presence of distant metastases have all been found to be essential prognostic factors[3]. Vascular endothelial growth factor receptor-3 (VEGFR-3) is a tyrosine kinase receptor and is expressed in lymphatic endothelial cells. Increased VEGFR-3 expression correlates with regional lymph node metastasis in colorectal cancers[4-6]. In particular, VEGF-D and VEGFR-3 were recently reported to be independent prognostic markers in gastric cancer, and VEGF-D was found to be correlated with lymphatic metastasis[7]. Many factors have recently been proposed as markers for lymphatic endothelium. Lymphatic vessel endothelial hyaluronan receptor-1 (LYVE-1) is the lymphatic vessel endothelial hyaluronic acid (HA) receptor, located in lymph nodes and in the luminal and abluminal surfaces of lymphatic vessels. LYVE-1 is very effective in the passage of lymphocytes and tumor cells into the lymphatics[8,9]. CD44 is another cell surface receptor for HA, expressed by lymphocytes, macrophages and tumor cells. CD44 is also responsible for the passage of lymphocytes and tumor cells into the lymphatics, similar to LYVE-1[10]. LYVE-1 has 43% similarity with CD44 but its specificity to HA is higher than that of CD44[11]. Quantification of the lymphatic vessel density (LVD) in the tumor might also be important for the evaluation of lymphangiogenesis and lymphatic metastasis[11].

The present study aimed to investigate the expression levels of LYVE-1, VEGFR-3 and CD44 genes in human tissues with or without tumor using real-time polymerase chain reaction (RT-PCR), and to evaluate the relationship between these expression levels and clinicopathological parameters such as tumor type, stage, differentiation, and the presence of lymph node metastasis, vascular invasion and neural/perineural invasion in gastric cancer. The LYVE-1, CD44 and VEGFR-3 protein expressions in tissues were also demonstrated using immunohistochemical and Western blotting (WB) analysis.

MATERIALS AND METHODS

All tissue samples used in this study were obtained from patients who underwent radical surgery for gastric cancer in the 7th Surgical Clinic of the Ankara Numune Teaching and Research Hospital. All molecular studies were carried out in the research laboratories of Hacettepe University Institute of Oncology and Department of Pathology, Ankara Numune Hospital.

The study protocol was approved by the local ethics committee of Hacettepe University (04/12-19), in accordance with the Declaration of Helsinki, and all participants provided written informed consent.

Patients

Thirty-three patients [8 females; median age (range) 58 years (42-78)] with gastric cancer who underwent radical gastrectomy and D2α lymph node dissection were included in the study. All patients provided written informed consent for the procedure and to participate in the study. During and after surgery, paired tissue samples (tumoral and normal tissues) from all patients were obtained according to the protocol. During surgical removal of each tumor, an adjacent section of normal tissue was also removed following pathological confirmation that it was free from tumor deposits. Samples were then transferred to the laboratory and kept at either -20℃ (tissues in RNA-Later) or -80℃ (tissues in liquid nitrogen), for further testing.

Surgical sampling technique

During the operations, lymph nodes were sampled according to the Japanese Research Society of Gastric Cancer classification[12]. Macroscopically involved lymph nodes or lymph nodes of more than 1 cm in size were sampled. The same procedure was applied for the normal-appearing lymph nodes. After laparotomy, in parallel with the dissection protocol, paraaortic lymph nodes were sampled first, followed by perivascular (celiac trunk) and perigastric lymph nodes sampling. After completion of surgery and total or subtotal removal of the stomach, samples were obtained from the tumor and normal tissues. Routine pathological examination of surgical specimens was carried out to determine the lymph node status and for accurate staging.

RNA isolation

Total RNA was extracted from normal/tumoral tissues (200 mg) and normal/tumoral lymph node specimens (100 mg) using an RNeasy Midi Kit (Qiagen, CA, USA) according to the manufacturer's instructions. Tissue lysis was performed with Mini Bead Beater-8 (Biospec, OK, USA). Concentration and purity of RNAs were determined on the NanoDrop 1000 spectrophotometer (Thermo, DE, USA). All samples were run on denaturing agarose gel. cDNA was subsequently synthesized using the RevertAid First Strand cDNA Synthesis Kit (Fermentas, MD, USA) with 800 ng of total RNA isolated for each sample.

Table 1 Primer sequences for quantitative real-time polymerase chain reaction

Genes	Primer sequences (5'-3')	Chromosome	PCR products (bp)
LYVE-1	F: CCAGTGAGCCGACAGTTTGCAG		
	R: CAGGTATTGTAGAGTAAGGGGATGCC	11p15	184
VEGFR-3	F: ACGGCCTGGTGAGTGGC		
	R: CGTTTGACTCCTCCGTGATG	5q35.3	63
CD44	F: GCAACTCCTAGTAGTACAACGGAAGA		
	R: CGATATCCCTCATGCCATCTGA	11p13	80
GAPDH	F: GGCTGAGAACGGGAAGCTTGTCAT		
	R: CAGCCTTCTCCATGGTGGTGAAGA	12p13	143

PCR: Polymerase chain reaction; LYVE-1: Lymphatic vessel endothelial hyaluronan receptor-1; VEGFR-3: Vascular endothelial growth factor receptor-3.

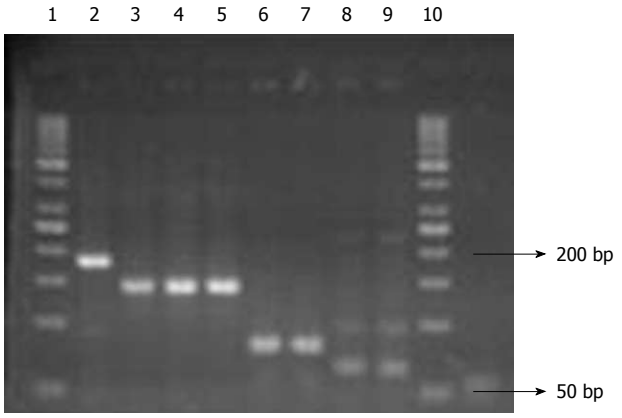

Figure 1 Polymerase chain reaction products of genes. 1: Ladder (50 bp); 2: Lymphatic vessel endothelial hyaluronan receptor-1 (184 bp), gastric tissue; 3-5: GAPDH (143 bp), in colon, stomach and pancreas, respectively; 6, 7: CD44 (80 bp) in stomach and colon; 8, 9: Vascular endothelial growth factor receptor-3 (63 bp) in stomach and colon; 10: ladder (50 bp).

PCR

Before RT-PCR, traditional PCR was performed in order to test the primers of LYVE-1, VEGFR-3, CD44 and GAPDH genes and the annealing degrees of the primers, as well as to optimize the levels of $MgCl_2$. The primers for the genes are given in Table 1. To ensure that the correct products were obtained, amplified products were separated by 3% agarose gel electrophoresis (Figure 1).

RT-PCR

The quantitative RT-PCR (qRT-PCR) assays were performed with the Corbet 6000 (Rotor-Gene, CA, USA) instrument using Lightcycler-DNA master SYBR Green I (Roche Diagnostics, Mannheim, Germany), and the expression levels of lymphangiogenic genes were quantified. The RT-PCR reactions were set up in a volume of 20 μL, containing 5 μL of sample cDNA, 1 × SYBR Green I dye, 1.5 mmol/L $MgCl_2$, and 5 pmol of LYVE-1, VEGFR-3, CD44 and GAPDH specific primers. The cycling conditions were as follows: 95℃ for 30 s, 60℃ for 30 s, 81℃ for 5 s and 72℃ for 30 s for 40 cycles, with initial melting at 95℃ for 5 min.

Relative expression levels were calculated using the PCR threshold cycle number (C_T) for each tissue and control sample using the formula $2^{-(\Delta CT\text{sample} - \Delta CT\text{control})}$[13,14]. ΔC_T represents the difference in C_T values between the target and *GAPDH* transcripts. RT-PCR was performed in duplicate for each sample and average C_T values were calculated.

Immunohistochemistry

Consecutive 4-μm-thick sections were cut from each paraffin sample. Sections were immunolabeled for LYVE-1, VEGFR-3 and CD44.

The stains were carried out using the Bond Max (Leica Microsystems - Biosystems Division, Wetzlar, Germany) with the Polymer Refine Detection Kit (Vision Biosystem, MA, USA). Immunohistochemical stains were performed with rabbit polyclonal antibodies LYVE-1 (Abcam, MA, USA) (1:100), VEGFR-3 (Gene-Tex, CA, USA) and CD44 (Gene-Tex, CA, USA).

LVD was determined from the counts of LYVE-1-positive vessels. Vessel density was assessed by light microscopy of the intratumoral region containing the greatest number of capillaries and small venules. Highly vascular areas were identified by scanning tumor sections at low power (× 40 and × 100). After the six areas of greatest neovascularization were identified, a vessel count was performed at X200, and the mean count of six fields was calculated. In slides immunolabeled for LVYE-1 and VEGFR-3, only vessels with typical morphology (including a lumen) were counted as lymphatic vessels[15]. CD44-positive cells were determined in tumor and normal tissue sections and compared.

Statistical analysis

Data from patients and laboratory studies were stored and evaluated using SPSS 15.00 for Windows (SPSS, Chicago, IL, USA). All descriptive data were expressed as median (range). The relationships between these and expression levels of LYVE-1, VEGFR-3 and CD44 were evaluated using the Fisher Exact Probability test. As the distribution of genes was not normal, the nonparametric tests were used for evaluation. The comparison between clinicopathological characteristics and gene expression levels was performed using Mann-Whitney *U* and Kruskal-Wallis tests. The correlation between clinicopathological features and gene expression levels was evaluated

Table 2 Clinicopathological features and expression levels of lymphatic vessel endothelial hyaluronan receptor-1, vascular endothelial growth factor receptor-3 and CD44 genes in patients with gastric cancer

Features	*n*	LYVE-1	*P*	CD44	*P*	VEGFR-3	*P*
Lauren type							
Intestinal	15	0.76 (0.04-2.66)	NS	1.14 (0.17-2.80)	NS	1.20 (0.02-3.70)	NS
Diffuse	18	0.81 (0.09-3.12)		1.14 (0.28-2.51)		0.87 (0.26-1.83)	
Differentiation							
Well	14	0.80 (0.04-2.66)	NS	1.14 (0.17-2.80)	NS	1.23 (0.02-3.70)	NS
Poor	19	0.78 (0.09-3.12)		1.14 (0.28-2.51)		0.86 (0.26-1.83)	
T-stage							
T2	2	0.54 (0.15-0.93)	NS	0.39 (0.17-0.60)	NS	0.6	NS
T3	17	0.69 (0.04-2.66)		1.17 (0.28-2.80)		0.99 (0.02-2.94)	
T4	14	1.0 (0.10-3.12)		1.24 (0.47-2.14)		1.0 (0.26-3.70)	
Vascular invasion							
Negative	15	0.82 (0.15-1.68)	NS	1.80 (0.60-2.80)	NS	1.06 (0.60-1.57)	NS
Positive	18	0.96 (0.10-3.12)		1.07 (0.28-2.14)		1.13 (0.26-3.70)	
Perineural invasion							
Negative	10	0.32 (0.10-0.93)	0.020	0.57 (0.17-0.76)	0.020	0.8 (0.26-2.21)	NS
Positive	23	1.0 (0.04-3.12)		1.40 (0.31-2.80)		1.13 (0.02-3.70)	
Neural invasion							
Negative	15	0.78 (0.04-3.12)	NS	0.95 (0.17-2.80)	NS	0.93 (0.02-2.94)	NS
Positive	18	0.84 (0.21-2.22)		1.48 (0.46-2.51)		1.24 (0.40-3.70)	
Lymph nodes							
Negative	5	0.66 (0.15-1.68)	NS	0.74 (0.17-1.61)	NS	0.86 (0.30-2.21)	NS
Positive	28	0.82 (0.04-3.12)		1.22 (0.28-2.80)		1.06 (0.02-3.70)	
PLN/TLN ratio							
≤ 0.4	25	0.53 (0.04-1.68)	NS	1.06 (0.17-2.80)	NS	0.83 (0.02-2.21)	NS
> 0.4	8	2.0 (0.69-3.12)	0.003	1.53 (0.47-2.40)	NS	1.97 (0.28-3.70)	0.050

PLN: Positive lymph nodes; TLN: Total lymph nodes; NS: Not significant; LYVE-1: Lymphatic vessel endothelial hyaluronan receptor-1; VEGFR-3: Vascular endothelial growth factor receptor-3.

using logistic regression analysis and Spearman's correlation coefficients. *P*-values less than 0.05 were considered significant.

RESULTS

Clinicopathological features and relative gene expression levels

All information on patients with gastric cancer, including age, sex, Lauren histological tumor type, degree of differentiation, T-stage, final stage, number of total lymph nodes (TLN) removed, number of positive lymph nodes (PLN), the PLN/TLN ratio, presence of vascular, neural and perineural invasion, and relative expression levels of LYVE-1, VEGFR-3 and CD44 calculated using qRT-PCR and the $2^{-\Delta\Delta CT}$ method[13,14], was stored and evaluated using SPSS 15.00 for Windows.

LYVE-1, VEGFR-3 and CD44 gene expression levels in relation to clinicopathological features are shown in Table 2.

Expression levels of LYVE-1, VEGFR-3 and CD44 genes were significantly higher in tumoral tissues than in the normal tissues in gastric cancer patients, which was found to be independent of the age and gender of the patients ($P < 0.001$) (Figure 2). There was a linear correlation between the expression levels of LYVE-1 and CD44 molecules, and the same correlation was also found between their over-expression levels (Spearman's rank correlation, $P = 0.025$ and $P = 0.033$).

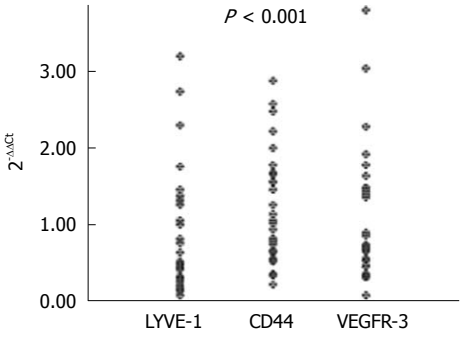

Figure 2 Lymphatic vessel endothelial hyaluronan receptor-1, CD44 and vascular endothelial growth factor receptor-3 expression levels are increased in tumor tissues as compared to normal tissues in patients with gastric cancer (*P* < 0.001). LYVE-1: Lymphatic vessel endothelial hyaluronan receptor-1; VEGFR-3: Vascular endothelial growth factor receptor-3.

The expression levels of the genes were higher in patients with lymph node metastases than patients without metastases, however, the difference did not reach the level of significance. When the PLN/TLN ratio was investigated, the expression levels of LYVE-1, VEGFR-3 and CD44 genes were significantly less in patients with a ratio of ≤ 0.20 than in patients with a PLN/TLN ratio of ≥ 0.4 ($P = 0.001$). Both expression levels and over-expression of the genes were increased with increased PLN/TLN ratio (Spearman's rank correlations; $P = 0.01$ for LYVE-1, $P = 0.036$ for CD44 and $P = 0.016$ for VEGFR-3). The value of significance was most appar-

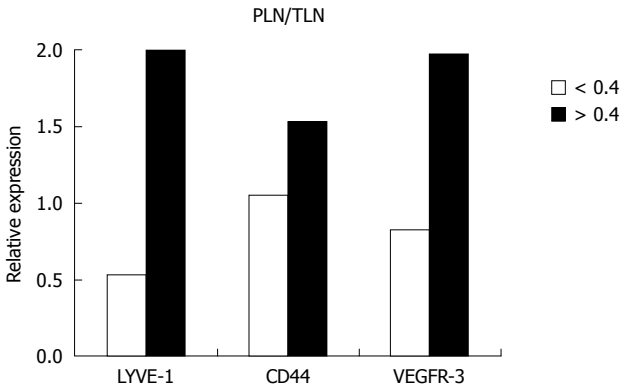

Figure 3 The positive lymph nodes/total lymph nodes ratios and the relative expression levels. The expression levels of lymphatic vessel endothelial hyaluronan receptor-1 (LYVE-1) are increased in conjunction with increased positive lymph nodes (PLN)/total lymph nodes (TLN) ratio (P = 0.003). VEGFR-3: Vascular endothelial growth factor receptor-3.

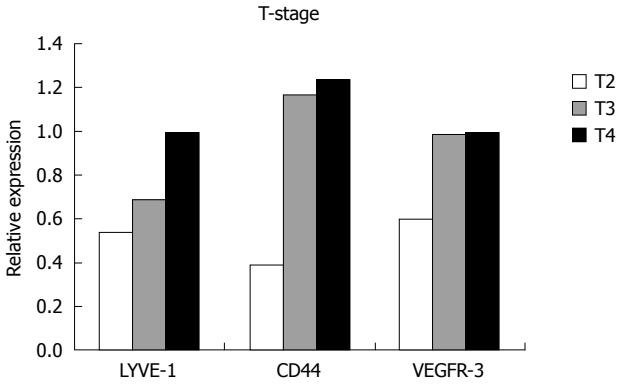

Figure 4 The relative expression levels and T-stage. No correlation was found between expression levels and T-stage. LYVE-1: Lymphatic vessel endothelial hyaluronan receptor-1; VEGFR-3: Vascular endothelial growth factor receptor-3.

ent when the PLN/TLN ratio was taken as ⩾ 0.4 or < 0.4 (Figure 3). There was also a significant correlation between the PLN/TLN ratio, tumor volume and number of PLN (P < 0.001). When the expression levels of the genes in the lymph nodes of the stations were evaluated, it was found that their levels increased in parallel with expression levels in the tumor, but the difference was not found to be statistically significant.

When the relationship between the T-stage of the tumor and relative expression levels of the genes was evaluated, we found that expression levels increased with increased T-stage, however, this increase was not statistically significant. Although the number of patients in earlier stages was too small, expression levels were much higher in T4-stage than T2, but the difference did not reach statistical significance, and no correlation was found between the T-stage and over-expressions (Figure 4).

On the other hand, there was a significant correlation between the final stage and T-stage, presence of lymph node metastasis, number of PLNs, and the PLN/TLN ratio.

There was also no correlation between over-expressions and neural invasion, whereas relative expression levels of LYVE-1, VEGFR-3 and CD44 were significantly increased with the presence of perineural invasion and reached statistical significance for LYVE-1 and CD44 (P < 0.001). All three genes were found to be over-expressed with perineural invasion and a statistically significant correlation was found between them (Figure 5).

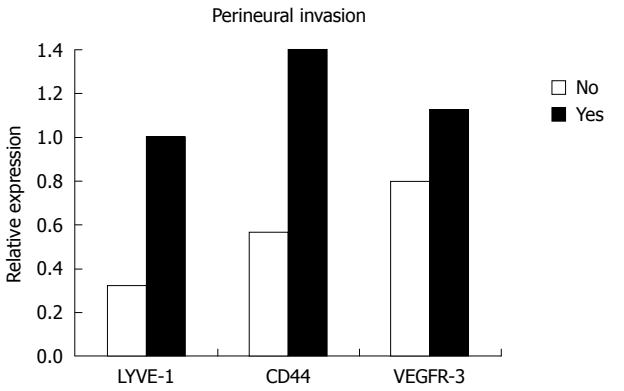

Figure 5 The relative expression levels and perineural invasion. The expression levels of lymphatic vessel endothelial hyaluronan receptor-1 (LYVE-1) and CD44 were significantly increased with the presence of perineural invasion. VEGFR-3: Vascular endothelial growth factor receptor-3.

Although the relative expression levels of all three genes were increased with the presence of vascular invasion, the difference was not statistically significant.

Immunohistochemistry

The expressions of LYVE-1, VGFR-3 and CD44 were all shown in tumor tissues using immunohistochemistry (Figure 6A-C). When the LVD at the central zone and periphery of the tumor was measured, 13% of patients had intratumoral staining and 27% had peritumoral staining for LYVE-1. Although the peritumoral distribution of LYVE-1$^+$ LVD was higher, the difference was not statistically significant (χ^2 test, 2.773, Df = 1, P = 0.09). Lymph node metastasis was positive in all patients with LYVE-1-stained lymphatic vessels, whether the LVD was peritumoral or intratumoral. However, we failed to find any correlation between LVD and lymph node metastasis (Spearman's rank correlation coefficient, 0.26, P = 0.92).

DISCUSSION

The present study was designed to investigate whether LYVE-1, which is a specific molecule for lymphatics, as well as CD44 and VEGFR-3 might be used as markers for lymph node metastases of gastric tumors. We chose gastric cancer since it is well known to exhibit early metastasis to lymph nodes.

We evaluated the expressions of LYVE-1, VEGFR-3 and CD44 in normal gastric tissues, tumoral gastric tissues and lymph nodes with or without metastases using RT-PCR. The levels were compared to those in normal tissues.

Using qRT-PCR, we were able to show significantly

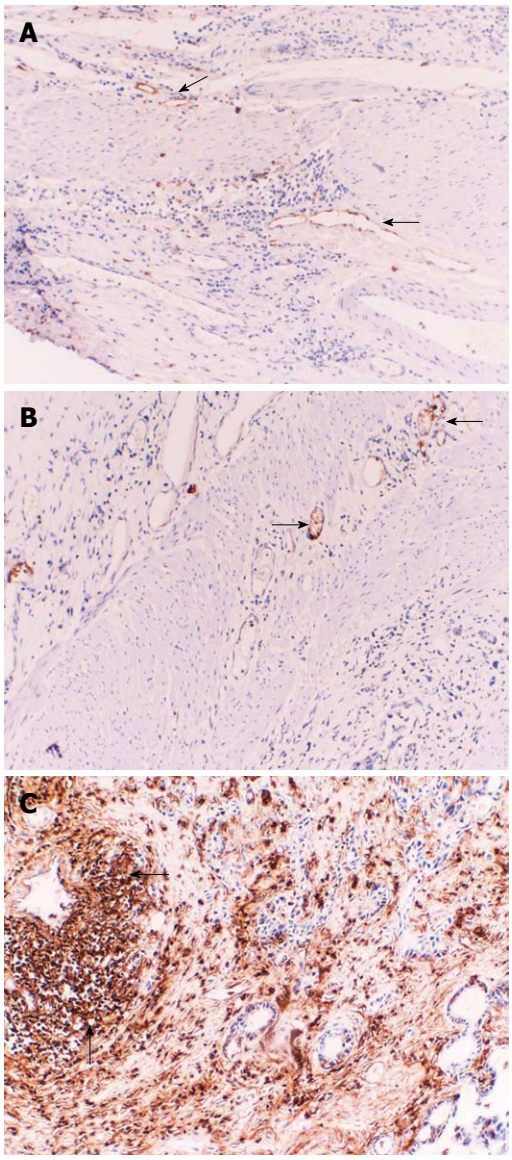

Figure 6 Expressions of lymphatic vessel endothelial hyaluronan receptor-1, vascular endothelial growth factor receptor-3 and CD44 by immunohistochemistry. A: Arrows indicate the lymphatic vessel endothelial hyaluronan receptor-1-positive lymphatic vessels in gastric tumor (× 100); B: Arrows indicate vascular endothelial growth factor receptor-3-3-positive lymphatic vessels in gastric tumor (× 100); C: Arrows indicate CD44-positive lymphoid cells in gastric tumor (× 100).

higher relative expression levels of all three genes in tumoral tissue than in the normal tissue counterparts. The expression levels of LYVE-1, VEGFR-3 and CD44 were higher in patients with lymph node metastasis than in those without metastasis, however, the difference was not statistically significant. On the other hand, when we measured the correlation between the PLN/TLN ratio and relative expression levels of LYVE-1 in tumors, levels were significantly higher in patients with a PLN/TLN ratio $\geq$ 0.4. There was no significant correlation for VEGFR-3 or CD44. Over-expressions of LYVE-1, VEGFR-3 and CD44 were all significantly increased with the increased number of involved lymph nodes.

The lymphatic metastatic process of tumor cells follows a series of complex biologic reactions. In the initial phase, tumor cells first invade the stroma and penetrate into lymphatic vessels and reach the lymph nodes *via* lymphatic flow. Lymphangiogenesis plays a very important role in this stage. The secretion of growth factors such as VEGF-C, platelet-derived growth factor (PDGF) and fibroblast growth factor (FGF) from tumor cells increases the expression of VEGFR-3, which triggers lymphangiogenesis followed by the development of new lymphatic capillaries and increases the number of LYVE-1-secreting cells[16]. The increase in VEGFR-3 and LYVE-1 expressions is highly critical because it reflects lymphangiogenesis, indicating how tumor cells might easily reach the lymphatic system[17]. Molecules such as LYVE-1 and VEGFR-3 provide information on the behavior of the tumor, as the increase in these molecules reflects the increase in the number of lymphatic endothelial cells.

LYVE-1, VEGFR-3 and CD44 expression and over-expression levels were increased with the presence of neural and perineural invasion; on the other hand, VEGFR-3 and LYVE-1 were both increased with vascular invasion. However, the increase in LYVE-1 and CD44 was only significant in the presence of perineural invasion, which might be explained partly by the size of the study, heterogeneity of the patients and variation in tumor behavior.

The present study also investigated the correlation between increased expression levels of the genes and poor prognostic factors. It was found that expression levels did not change with Lauren type or with the degree of differentiation. LYVE-1, VEGFR-3 and CD44 expression levels were increased in conjunction with T stage.

We evaluated the gene expression levels in metastatic lymph nodes and found that the expression levels of LYVE-1, VEGFR-3 and CD44 all increased in parallel with the expression levels in tumor tissue, which was most apparent in LYVE-1 levels. When the relationships between the gene expression levels were analyzed, it was found that CD44 levels increased in parallel with LYVE-1 expression in tumors.

Hyaluronic acid (HA) is an extracellular matrix glycosaminoglycan found in tissues and body fluids, and plays a role in inflammation, leukocyte extravasation, wound healing, and metastasis of tumor cells[18]. The CD44 molecule is one of the most well-defined HA cell surface receptors with a very important role in HA homeostasis and is expressed on the surface of endothelial, mesenchymal and lymphoid cells. However, CD44 is functionally silent in resting leukocytes and is activated only to bind HA[19].

Lymphatic vessel endothelial HA receptor (LYVE-1) is a receptor for hyaluronan and is expressed by the endothelial cells of the lymphatic vessels. LYVE-1 is located both intra- and extra-luminally in these vessels; therefore, it is thought that LYVE-1 plays an important role in the transportation of both lymphocytes and other similar cells into the lymphatic system. Lymphocytes are routed into the lymphatic flow and caught by lymph nodes with the help of CD44 cells integrated with HA and with LYVE-1. As it is specific to the lymphatic endothelium,

LYVE-1 might be a useful marker in identifying tissue lymphatics.

Vascular endothelial growth factor receptor-3 (VEGFR-3) is another lymphatic endothelial cell receptor that has an important role in lymphangiogenesis. Increased expression of VEGF-C during growth of the tumor increases the development of new lymphatic vessels so that VEGF-C may be considered as a marker of lymph node metastasis. VEGFR-3 might also be a marker for lymphangiogenesis; however, it appears to be inadequate for suggesting lymph node metastasis. Despite the definitions of LYVE-1, VEGFR-3 and CD44 and rapidly growing information on tumor biology, the molecular mechanisms regarding lymphatic invasion and the metastatic process to lymph nodes remain unclear.

Lymphatic vessels act not only in metastasis but also in the invasion of cancer cells. The molecules that are released from lymphatic endothelial cells such as matrix metalloproteinases and urokinase plasminogen activators increase invasion by helping tumor cell movement in adjacent tissues[20]. VEGF-C stimulates the secretion of CCL1 chemokine from endothelial cells, which interacts with CCR8 expressed by tumor cells and moves tumor cells towards lymphatic vessels[21]. This interaction is one of the most important steps in metastasis. All these molecules are secreted by both the endothelium of new lymphatic capillaries and that of other lymphatics already present around the tumor[22,23]. In addition to lymphatic markers such as LYVE-1 and VEGFR-3, we also used lymphatic involvement and perineural, neural and vascular invasion in order to better explain the process of tumor invasion and metastasis. Information gathered from this study supports this relationship.

CD44, which is secreted by tumor cells, binds HA, and during the transport of HA, it also intervenes in the transportation of tumor cells into the lymphatic flow[24]. Furthermore, over-secretion of CD44 by tumor cells might cause tumor cells to be perceived as lymphocytes by the immune system, which might trigger the occurrence of immune escape[25]. As we have shown, increased secretion of CD44 mRNA in gastric cancer might be an important indicator of the biologic process explained above.

We are not aware of any previous study investigating LYVE-1 mRNA levels in gastric cancer. In two previous studies, VEGFR-3 expressions were shown to be increased in tumor tissues and with the presence of lymph node involvement using RT-PCR[26,27]. It was recently shown that there was a correlation between increased VEGFR-3 levels and metastasis[11,28]. It has also been reported that increased expression levels of VEGFR-3 might be a prognostic indicator, especially in patients with advanced gastric cancer[7,29].

To our knowledge, there has only been one published study investigating the quantitatively measured relative expression levels of the CD44 gene in human gastric cancer[30]. Most of the previous studies on CD44 were based on immunohistochemical analysis and analyzed the correlation between poor prognosis and presence of CD44[31-33].

We found that 40% of cases (13/33) had lymphatic vessels in tumor tissue; 27% were located peritumorally and 13% were located intratumorally. However, we failed to find any correlation between LVD and lymph node metastasis, possibly due to the small study size.

There is still controversy regarding whether the intratumoral or the peritumoral lymphatics are functional, and which should be used for evaluation[34,35]. Although our study population was too small to comment, peritumoral lymphatics with wide lumens seem to be more functional and effective for the occurrence of metastasis as compared to the intratumoral lymphatics with either narrow or obstructed lumen. This hypothesis appears to be in accordance with previous findings obtained from animal studies[17,36,37]. It is also known that the lymphatic vessels are located at the submucosa and extend through the muscularis propria. When tumor cells reach this anatomic location, they can easily move to the lymphatic vessels, thereby increasing metastatic potential[17].

The question of whether the tumor uses new lymphatic capillaries occurring at its center due to lymphangiogenesis or lymphatic vessels already located peritumorally remains unanswered[36]. We believe that peritumoral lymphatics have a very important role in the metastatic process.

The present study also shows that immunohistochemical staining using LYVE-1 antibodies is more sensitive than staining using VEGFR-3 antibodies, which is in parallel with previous findings by other researchers[38]. VEGFR-3 antibodies stain venous capillaries as well as lymphatic vessels, which makes differentiation difficult. The presence of VEGFR-3 proteins in both normal and tumor tissues was shown by Western blotting, which correlated with the findings of Yonemura *et al*[26].

A previous study in patients with colorectal cancers revealed that the expression levels of VEGFR-3 are increased, whereas LYVE-1 levels were decreased in tumor tissue[39]. LYVE-1 levels were found to be increased in another study using immunohistochemistry, but the authors failed to show any increase in quantitatively measured relative expression levels[40]. Yuanming *et al*[28] investigated the expression levels of LYVE-1 and VEGFR-3 in gastroenteric tumors and evaluated their relationships with lymphatic metastasis and tumor progression. They concluded that VEGFR-3 expression was increased when lymph node metastasis was present, but they failed to show any correlation with LYVE-1. By using LYVE-1 antibody and immunohistochemistry, they found that there was a correlation between LVD and lymph node metastasis[28]. Although we were unable to show this correlation using immunohistochemistry, we were able to determine that relative expression levels of LYVE-1 increased with lymph node involvement in parallel with PLN/TLN ratios. Therefore, the findings of Chen *et al*[41] using immunohistochemistry support our study based on quantitative measurements. In the literature, there are

only a few immunohistochemical studies on the quantitative evaluation of CD44 in gastric cancer and they did not reach a definitive conclusion[41,42].

In conclusion, we found that the expression levels of LYVE-1, CD44 and VEGFR-3 genes in gastric tumors were significantly increased when compared to levels in normal tissue. We failed to show any correlation between these expression levels and clinicopathological features such as histological type, differentiation and stage, however, a significant correlation was found between relative expression levels of LYVE-1 and CD44 genes and perineural invasion and lymphatic involvement, which supports the hypothesis that those genes play an important role during the process of lymphangiogenesis, invasion and lymph node metastasis in gastric cancer.

Although it is not possible to make definitive comments based on our findings regarding the relationships between clinicopathological features and peritumoral/intratumoral LVD and their effects on prognosis, LYVE-1 appears to be an excellent lymphatic vessel marker and LVD can be evaluated using immunohistochemical techniques. We believe that increased expression levels of these genes in biopsy specimens can be used as a predictor of metastasis, which might further facilitate disease staging, treatment planning and prognosis estimation.

COMMENTS

Background

Lymph node metastasis is one of the most important prognostic factors in gastric cancer. Quantification of the lymphatic vessel density in the tumor may be important for the evaluation of lymphangiogenesis and lymphatic metastasis. Lymphatic metastasis involves a series of complex reactions. Tumor cells first invade the stroma, penetrate the lymphatic vessels and reach the lymph nodes *via* lymphatic flow. Lymphangiogenesis is involved in this stage. The secretion of growth factors from the tumor cells increases the expression of vascular endothelial growth factor receptor-3 (VEGFR-3) which triggers lymphangiogenesis followed by the development of new lymphatic capillaries and an increase in the number of lymphatic vessel endothelial hyaluronan receptor-1 (LYVE-1) secreting cells. The increase in VEGFR-3 and LYVE-1 expressions is very important as it reflects lymphangiogenesis which allows tumor cells to reach the lymphatic system easily. Molecules such as LYVE-1 and VEGFR-3 provide information on the behaviour of the tumor, as an increase in these molecules reflects the increased number of lymphatic endothelial cells. Furthermore, over-secretion of CD44 by tumor cells might cause tumor cells to be perceived as lymphocytes by the immune system which might trigger the occurrence of immune escape. The present study aimed to investigate the expression levels of LYVE-1, VEGFR-3, CD44 genes and the relationship between these levels and clinicopathological parameters in gastric cancer.

Research frontiers

To briefly introduce important areas in the research field related to this article.

Innovations and breakthroughs

Using quantitative real-time polymerase chain reaction, the authors were able to show significantly higher relative expression levels of all three genes in tumoral tissue than in the normal tissue counterparts. They failed to show any correlation between expression levels and clinicopathological features such as histological type, differentiation and stage, however, a significant correlation was found between relative expression levels of LYVE-1 and CD44 genes and perineural invasion and lymphatic involvement, which supports the hypothesis that these genes play an important role during the process of lymphangiogenesis, invasion and lymph node metastasis in gastric cancer.

Applications

Increased expression levels of these genes in biopsy specimens can be used as a predictor of metastasis, which might further facilitate disease staging, treatment planning and prognosis estimation.

Peer review

This paper investigates the importance of LYVE1, VEGFR-3 and CD44 expression levels in gastric cancer and its relation to the metastasis.

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ORIGINAL ARTICLE

Oncologic outcomes of primary and post-irradiated early stage rectal cancer: A retrospective cohort study

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Abstract

AIM: To evaluate the oncologic outcomes of primary and post-irradiated early stage rectal cancer and the effectiveness of adjuvant chemotherapy for rectal cancer patients.

METHODS: Eighty-four patients with stage I rectal cancer after radical surgery were studied retrospectively and divided into ypstage I group (n = 45) and pstage I group (n = 39), according to their preoperative radiation, and compared by univariate and multivariate analysis.

RESULTS: The median follow-up time of patients was 70 mo. No significant difference was observed in disease progression between the two groups. The 5-year disease-free survival rate was 84.4% and 92.3%, respectively (P = 0.327) and the 5-year overall survival rate was 88.9% and 92.3%, respectively, for the two groups (P = 0.692). The disease progression was not significantly associated with the pretreatment clinical stage in ypstage I group. The 5-year disease progression rate was 10.5% and 19.2%, respectively, for the patients who received adjuvant chemotherapy and for those who rejected chemotherapy in the ypstage I group (P = 0.681).

CONCLUSION: The oncologic outcomes of primary and post-irradiated early stage rectal cancer are similar. Patients with ypstage I rectal cancer may slightly benefit from adjuvant chemotherapy.

Key words: Rectal cancer; Neoadjuvant radiotherapy; Total mesorectal excision

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INTRODUCTION

Rectal cancer is a worldwide health concern[1,2]. It is the fifth leading cause of cancer-related death and its incidence is increasing at a rate of 4.2% per year in China[2].

Currently, the management of rectal cancer has become multidisciplinary[3-5]. Neoadjuvant therapy, including long- or short-course chemo- and radiotherapy, can control locally advanced rectal cancer and increase the sphincter preservation rate[6-8].

Neoadjuvant therapy can decrease the tumor size and histopathological stage in a considerable number of rectal cancer patients, depending on the dose of radiation, chemotherapy regimen, and many other factors[9-12]. The pathologic stage of tumor after neoadjuvant therapy (ypstage) is one of the most important factors for oncologic outcome, and the clinical and pathologic meanings of ypstage are different from those of primary pathologic TNM stage (pstage)[13,14]. For example, patients with early stage rectal cancer (pT1-2N0M0) have a low risk of progression and no indication for adjuvant chemotherapy. Nevertheless, patients with ypstage I rectal cancer should undergo postoperative chemotherapy according to the National comprehensive cancer network guidelines[15], since some studies suggested that adjuvant chemotherapy may further decrease the risk of rectal cancer progression in patients who have received preoperative radiation[13-16]. However, to date, no worldwide consensus has been reached on whether adjuvant chemotherapy is proper for patients with ypstage I rectal cancer. Few studies have neither specifically compared the prognostic difference in ypstage I and pstage I rectal cancer, nor investigated the effectiveness of adjuvant chemotherapy for post-irradiated early stage rectal cancer. Thus, this study was to compare the long-term outcomes of ypstage I and pstage I rectal cancer patients after radical resection, and the outcomes of ypstage I patients who received post-operative chemotherapy with those who did not receive chemotherapy.

MATERIALS AND METHODS

Data were collected from all patients with pathologic stage I rectal cancer admitted to Peking University Cancer Hospital from February 1998 to February 2005. The inclusion criteria were those with histologically identified primary adenocarcinoma of the rectum before treatment, resectable rectal cancer 12 cm or less from the anal verge, evaluated by endorectal ultrasound (ERUS) or magnetic resonance imaging (MRI) before treatment, no clinical evidence of synchronous distant metastases, transabdominal radical resection based on the principle of total mesorectal excision (TME), and R0 resection.

The exclusion criteria were those with transanal excision, pathologic complete response to neoadjuvant radiotherapy, multiple primary malignancy or history of other malignant tumors within 5 years, familial adenomatous polyposis and hereditary non-polyposis colorectal carcinoma, and those who died of complications or due to other non-cancer related reasons.

Finally, 84 eligible patients were included in this study and divided into ypstage I group (n = 45) and pstage I group (n = 39). Demographic and clinical data of the patients are presented in Table 1.

Table 1 Characteristics of patients included in this study

Characteristic	Group		*P* value
	Ypstage I (n = 45)	Pstage I (n = 39)	
Gender			
Male	28	21	0.437
Female	17	18	
Age (yr)			
< 60	19	10	0.111
> 60	26	29	
Median	62	67	
Distance from anal verge (cm)			
≤ 5	20	9	0.040
> 5	25	30	
Pretreatment serum CEA (ng/mL)			
≤ 5	29	32	0.016
> 5	13	3	
unknown	3	4	
Surgery			
APR	14	6	0.091
LAR	31	33	
Clinical and pathologic stage			
cT1-2N0	0	28	< 0.001
cT3-4N0	15	6	
cTanyN+	30	5	
pT1N0	5	14	0.007
pT2N0	40	25	
Histological differentiation			
High	3	15	< 0.001
Moderate	34	23	
Poor	8	1	
Lymphovascular invasion			
Positive	0	1	0.464
Negative	45	38	
NELN			
< 12	27	23	0.924
≥ 12	18	16	

APR: Abdominoperineal resection; LAR: Low anterior resection; NELN: Number of examined lymph nodes; CEA: Carcinoembryonic antigen.

Neoadjuvant radiotherapy

Neoadjuvant radiotherapy was indicated for patients with clinical T stage more than T2 (T3 or T4), or with nodes involved. We adopted the regimen recommended by the Chinese Anti-Cancer Association[17]. The patients were irradiated with a 10 MV dual photon linear accelerator using a 3-field box technique (posteroanterior and bilateral fields). The total radiation dosage was 3000 cGy in 10 fractions delivered within 2 wk, with a biological equivalent dose of 36 Gy. The radiation field was set at the upper margin 1.5 cm above the sacral promontory (L5 level), bilateral margin 1 cm outside the pelvic brim, and inferior margin 3 cm below the lower margin of the tumor, or at the anal verge in some lower rectal cancer cases. Surgery was performed 2-3 wk after radiotherapy.

Surgery

All included patients underwent radical resection according to the TME principles[18], irrespective as to whether they received abdominoperineal resection or low anterior resection. All surgeries were performed by sharp pelvic dissection under direct vision along the Holly plane. The

mesorectum was excised 4-5 cm from the distal inferior edge of upper rectal cancer, and TME was performed in mid-level and lower rectal cancer. The bowel wall was excised at least 2 cm from the distal inferior edge of the tumor. All surgeries were performed by the same surgeon. R0 resection was defined when no microscopic residual tumor cells were found at the distal and circumferential resection margins.

Pathologic evaluation

Pathologic evaluation was performed again by one senior pathologist who was blinded to the clinical and oncologic outcome of the patients. All resected specimens were stained with hematoxylin and eosin, and evaluated for tumor differentiation and invasion, lymph node metastases, and lymphovascular invasion (LVI). The pathologic stage of rectal cancer was evaluated according to the 6th UICC TNM Staging System after histopathological examination.

Adjuvant chemotherapy

Of the 45 patients in the ypstage I group who were recommended to receive postoperative chemotherapy, 19 accepted chemotherapy and 26 refused adjuvant chemotherapy because of lack of authoritative evidence and consensus in China. The patients underwent adjuvant chemotherapy with 5-FU or capecitabine in combination with FOLFOX and CapeOX or capecitabine alone, according to their condition for 8-12 cycles. Patients in the pstage I group had no indications for chemotherapy, and were thus observed after surgery with a regular follow-up.

Follow-up

All patients were followed up every 3 mo during the first 2 years after surgery, and then every 6 mo for 5 years. Clinical examination was performed and serum Carcinoembryonic antigen (CEA) was detected at each follow-up. Abdominal ultrasound, pelvic MRI, and chest radiograph were performed every 6 mo, and colonoscopy was performed annually. The follow-up time was 3-131 mo (mean 70 mo). The terminal time for evaluation of outcomes was 5 years. The follow-up rate was 89.3% with 9 inconclusive results (follow-up was lost in 2 patients after disease progression).

Statistical analysis

Statistical analysis was performed using SPSS 16.0 statistical software (SPSS Inc., Chicago, IL, USA). Categorical variables were analyzed by Pearson chi-squared or Fisher's exact test when appropriate. Kaplan-Meier survival curve was used to estimate the number of patients surviving or remaining disease-free at each time. Disease-free survival (DFS) and overall survival (OS) curves were compared between the two groups using the Wilcoxon test for time-to-event parameters. Multivariate Cox proportional hazards regression (forward stepwise selection) was used to analyze the major factors affecting DFS rate. All statistical tests were 2-sided, and $P < 0.05$ was considered statistically significant.

Table 2 Oncologic outcomes of patients in two groups *n* (%)

	Ypstage I	Pstage I	*P* value
Local recurrence rate	2 (4.4)	1 (2.6)	1.000[1]
Distant metastasis rate	6 (13.3)	3 (7.7)	0.494[1]
5-yr DFS rate	38 (84.4)	36 (92.3)	0.327
5-yr OS rate	40 (88.9)	36 (92.3)	0.692

[1]Fisher's exact test. DFS: Disease free survival; OS: Overall survival.

Table 3 Correlation between clinical stage and disease progression in ypstage I group

Clinical stage	Disease progression (*n*)		OR (95% CI)	*P* value
	Yes	No		
cT3-4N0	3	12	0.31-8.43	0.670[1]
cTanyN+	4	26		

[1]Fisher's exact test. OR: Odds ratio.

RESULTS

Characteristics of patients

Eighty-four patients (49 males and 35 females) were included in this study with a median age of 64 years (range, 28-80 years). Complete follow-up information about the patients was available except for 9 patients (5 in ypstage I group and 4 in pstage I group) after surgery. No statistically significant difference was observed in gender and age of the patients, surgery, number of examined lymph nodes and LVI between the two groups (Table 1). However, a significant difference was found in distal anal verge (DAV), preoperative serum CEA level, histological differentiation, and pathologic T stage between the two groups, indicating that the condition of patients is better in pstage I group than in ypstage I group (Table 1).

Disease progression

Local recurrence was noted in 3 patients (3.6%). The 5-year local recurrence rate was 4.4% in ypstage I group and 2.6% in pstage I group (Table 2). Distant metastasis was observed in 9 patients (10.7%), which initially occurred in the liver of 5 patients (55.6%), in the lung of 3 patients (33.3%), and in the ovary of 1 patient (11.1%). The 5-year distant metastasis rate was 13.3% in ypstage I group and 7.7% in pstage I group (Table 2).

Disease progression was observed in 7 patients of the ypstage I group. Of the 7 patients, 4 had lymph node involvement based on ERUS/MRI before treatment and 3 were staged as T3-4N0. Pretreatment clinical stage was not significantly associated with disease progression in ypstage I group [odds ratio (OR) = 0.31-8.43, $P = 0.670$, Table 3].

Five-year DFS and 5-year OS rate

The overall 5-year DFS and 5-year OS rate was 88.1%, and 90.5%, respectively for the two groups. The 5-year DFS rate was 84.4% and 92.3%, respectively ($P = 0.327$,

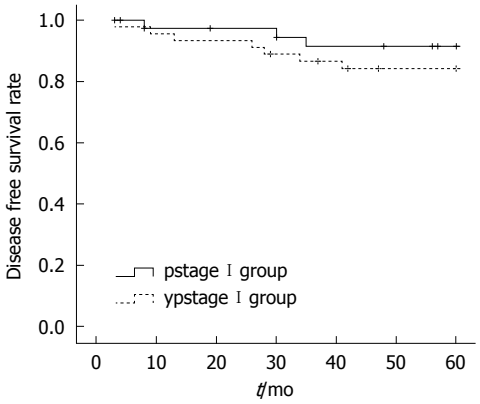

Figure 1 Five-year disease-free survival rate for patients in two groups.

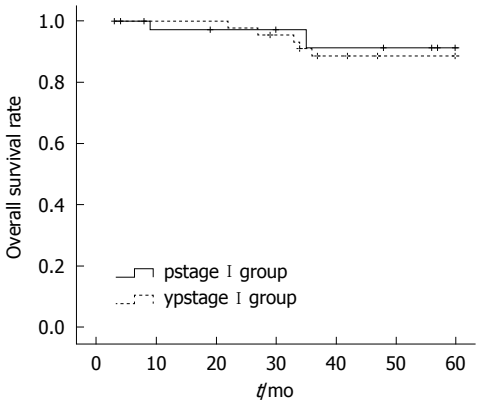

Figure 2 Five-year overall survival rate for patients in two groups.

Figure 1, Table 2) while the 5-year OS rate was 88.9% and 92.3%, respectively ($P = 0.692$, Figure 2, Table 2) for the two groups.

Effectiveness of chemotherapy for ypstage I patients

Of the 45 patients in ypstage I group, 19 received adjuvant chemotherapy and 26 rejected chemotherapy. The 5-year disease progression rate was 10.5% and 19.2%, respectively, for the patients who accepted chemotherapy and those who rejected chemotherapy (OR = 0.09-2.87, $P = 0.681$, Table 4).

Prognostic factors affecting DFS rate

Multivariate analysis demonstrated that the pretreatment serum CEA level was the major factor affecting the 5-year DFS rate for early stage rectal cancer patients (Table 5). Gender, age, neoadjuvant radiotherapy, DAV, histological differentiation, pathologic T stage, lymph nodes, LVI, and adjuvant chemotherapy were not the independent factors for the long-term DFS rate.

DISCUSSION

Currently, multidisciplinary management of advanced rectal cancer has gained wide acceptance[3-5]. Neoadjuvant radiotherapy is an effective treatment modality for locally advanced rectal cancer with respect to resectability, local control, and survival benefit[19-21]. The tumor stage decreases in approximately 40%-60% of rectal cancer patients obtain after neoadjuvant radiotherapy, which is related to a long-term favorable oncologic outcome[11,13,22]. One fifth of patients with ypstage I rectal cancer can directly benefit from neoadjuvant therapy[23,24]. Although it is widely acknowledged that ypTNM stage and primary TNM stage are different in terms of clinical meaning[9,25,26], few studies have specially compared the difference in early stage rectal cancer between post-irradiated patients and those undergoing direct surgery. In this study, patients with early stage rectal cancer were selected to undergo radical surgery instead of transanal local resection as a control in order to enhance the comparability of the two arms.

In the present study, the pstage I rectal cancer patients undergoing radical surgery had a favorable outcome, with a 5-year disease progression rate of < 10% and an OS rate of > 90%, which is consistent with the reported data[27,28]. Compared the patients in pstage I group, those in ypstage I group had several potential risk factors for poor oncologic outcomes, such as higher CEA level, more advanced T stage, and poorer histological differentiation, which is consistent with the reported data[29,30]. However, the patients in ypstage I group did not exhibit a higher disease progression rate or cancer-related death than those in pstage I group. Multivariate analysis also revealed that only serum CEA level was the major factor affecting DFS rate for early stage rectal cancer patients. Reerink *et al*[25] also showed that the prognosis of of patients with initially unresectable rectal tumor down-staged to pT2 and those

Table 4 Correlation between adjuvant chemotherapy and disease progression in ypstage I group

Adjuvant chemotherapy	Disease progression (*n*)		OR (95% CI)	*P* value
	Yes	No		
Yes	2	17	0.09-2.87	0.681[1]
No	5	21		

[1]Fisher's exact test. OR: Odds ratio.

Table 5 Multivariate analysis of disease-free survival rate by COX model (forward method)

Variable	Hazard ratio	95% CI	*P* value
Pretreatment serum CEA	5.535	1.574-19.468	0.008
Distance from anal verge	0.715	0.453-1.130	0.064
Gender	0.483	0.174-1.337	0.154
Age	1.057	0.999-1.119	0.054
Neoadjuvant radiotherapy	0.490	0.176-1.362	0.244
Histological differentiation	1.161	0.452-2.980	0.929
Pathologic T stage	0.827	0.232-0.953	0.271
Lymphovascular invasion	1.643	0.164-16.480	0.829
NELN	0.946	0.880-1.017	0.244
Adjuvant chemotherapy	1.381	0.512-3.725	0.670

NELN: Number of examined lymph nodes; CEA: Carcinoembryonic antigen.

with primary resectable cancer with the same T classification is similar. Our study further demonstrated that post-irradiated early stage rectal cancer has no significant heterogeneity in prognosis compared to primary early stage rectal cancer.

Up to date, no general agreement has been reached on the indications of adjuvant chemotherapy for post-irradiated patients. Short-course radiation is predominantly used in European countries, although no consensus has been reached. Most doctors believe that postoperative pathologic stage of rectal cancer is still the decisive factor for adjuvant chemotherapy, since its down-staging rate is less than 1%[31]. The role of adjuvant chemotherapy in down-staged patients after preoperative long-course radiation is controversial. It has been demonstrated that chemotherapy, whether preoperative or postoperative, reduces the overall risk of disease progression in patients undergoing neoadjuvant radiotherapy[16]. However, which patients would benefit from adjuvant chemotherapy needs to further investigated[32]. Fietkau *et al*[33] reported that postoperative chemotherapy is not necessary for patients with ypN0 after neoadjuvant chemo- and radiotherapy because no obvious improvement has been achieved in 3-year DFS rate. Collette *et al*[13] suggested that patients with down-staged ypT0-2, irrespective of ypN status, can benefit from adjuvant chemotherapy. No consensus has been reached on the indications of chemotherapy for post-irradiated patients in China. The results of this study suggest that adjuvant chemotherapy may be beneficial for the patients with ypT1-2N0 after neoadjuvant radiotherapy alone, since its outcome is better in patients after chemotherapy. Although the difference in disease progression rate did not show statistical significance, this two-fold difference is of clinical significance. Moreover, the results were largely influenced by the small sample size of patients in this study, thus further randomized study with a large sample size is needed.

In conclusion, the oncologic outcome of primary and post irradiated early stage rectal cancer after neoadjuvant radiotherapy is similar. Furthermore, patients with ypstage I rectal cancer may slightly benefit from adjuvant chemotherapy.

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COMMENTS

Background

Currently, neoadjuvant radiotherapy has been regarded as an effective treatment modality for locally advanced rectal cancer in terms of improving its local control and increasing the sphincter preservation rate. The pathologic stage of rectal cancer after neoadjuvant therapy (ypstage) is one of the most important factors affecting its oncologic outcome, and the clinical and pathologic meanings of ypstage are different from those of primary pathologic TNM stage (pstage). However, few studies have specially investigated the prognosis of post-irradiated early stage rectal cancer patients. Thus, this study was conducted to compare the long-term outcomes of ypstage I and pstage I rectal cancer patients after radical resection, and the outcomes of ypstage I patients who received postoperative chemotherapy with those who did not receive chemotherapy.

Research frontiers

This study specially addressed the long-term oncologic outcome of post-irradiated early stage rectal cancer by well-designed cohort study.

Innovations and breakthroughs

This study demonstrated that the oncologic outcome of primary and post-irradiated early stage rectal cancer was similar. Furthermore, patients with ypstage I rectal cancer may slightly benefit from adjuvant chemotherapy.

Applications

The post-irradiated early stage rectal cancer has a good oncologic outcome, and the clinical value of postoperative adjuvant chemotherapy should be further studied with a large sample size.

Peer review

This retrospective study comparing the ypstage I and pstage I rectal cancer patients after radical resection demonstrated that down-staging of rectal cancer was related to a long-term favorable oncologic outcome (5-year local recurrence rate, disease-free and overall survival rate). Pretreatment serum Carcinoembryonic antigen level was the major factor affecting the 5-year disease-free survival rate. On the other hand, it showed that patients with ypstage I rectal cancer could benefit from adjuvant chemotherapy.

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BRIEF ARTICLE

Influence of irritable bowel syndrome on treatment outcome in gastroesophageal reflux disease

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Abstract

AIM: To investigate the influence of irritable bowel syndrome (IBS)-like symptoms on treatment outcomes with pantoprazole in gastroesophageal reflux disease (GERD) in a real life setting.

METHODS: For this prospective, open-label, multinational, multicentre study, 1888 patients assessed by the investigators as suffering from GERD were recruited. The patients were additionally classified as with or without IBS-like symptoms at baseline. They were treated with pantoprazole 40 mg once daily and completed the Reflux Questionnaire™ (ReQuest™) short version daily. Response rates and symptom scores were compared after 4 and 8 wk of treatment for subgroups defined by the subclasses of GERD [erosive (ERD) and non-erosive reflux disease (NERD)] and the presence of IBS-like symptoms.

RESULTS: IBS-like symptoms were more prevalent in NERD than in ERD (18.3% *vs* 12.7%, $P = 0.0015$). Response rates after 4 and/or 8 wk of treatment were lower in patients with IBS-like symptoms than in patients without IBS-like symptoms in both ERD (Week 4: $P < 0.0001$, Week 8: $P < 0.0339$) and NERD (Week 8: $P = 0.0088$). At baseline, ReQuest™ "lower abdominal complaints" symptom scores were highest in NERD patients with IBS-like symptoms. Additionally, these patients had the strongest symptom improvement after treatment compared with all other subgroups.

CONCLUSION: IBS-like symptoms influence treatment outcome and symptom burden in GERD and should be considered in management. Proton pump inhibitors can improve IBS-like symptoms, particularly in NERD.

Key words: Pantoprazole; ReQuest™; Clinical practice; Irritable bowel syndrome; Gastroesophageal reflux disease

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INTRODUCTION

Complaints in the lower abdomen associated with altered stool habits were commonly considered the leading char-

acteristic of irritable bowel syndrome (IBS). However, despite the overlap between gastroesophageal reflux disease (GERD) and IBS being recognised for more than 20 years[1-3], there is still much debate about the pathophysiology and clinical management of lower abdominal complaints in patients with GERD[4-7]. Two theoretical frameworks have been proposed to account for the high prevalence (up to 71%[4,8,9]) of IBS symptoms in GERD patients[8,10].

The first defines GERD and IBS as distinct disorders that share common underlying mechanisms [e.g. gastrointestinal (GI) dysmotility[9] or visceral hypersensitivity[7]] in symptom genesis. In contrast, the second theory integrates IBS-like symptoms into the spectrum of GERD complaints, and is supported by evidence that shows that IBS-like symptoms improve after the administration of proton pump inhibitors (PPIs) for the treatment of GERD[11-14].

Interestingly, the prevalence of IBS-like symptoms[15-17], symptom burden[15], and treatment response following PPI administration[18,19] differ among patients with erosive GERD (ERD) and non-erosive GERD (NERD). Furthermore, current data suggest that the presence of IBS-like symptoms unfavourably affects treatment outcome with PPIs in some patients with GERD[8,20].

Given that symptom resolution is the primary goal of treatment for both physicians and patients, identifying and understanding the spectrum of symptoms influencing the treatment outcome in patients with GERD seen in routine clinical practice is essential for providing optimal primary patient care. We therefore designed a study to closely resemble the conditions of ordinary clinical practice to investigate these issues of practical clinical concern in the management of GERD. The aim of the current paper is to evaluate the influence of the presence of IBS-like symptoms on treatment response to the PPI pantoprazole in patients with GERD (including both ERD and NERD) in a real-life setting.

MATERIALS AND METHODS

Study design and patients

This prospective, open-label, phase Ⅲ study (BY1023/M3-341, ClinicalTrials.gov Identifier: NCT00312806) used a pragmatic trial design to assess the effectiveness of treatment with pantoprazole in managing GI symptoms in patients considered by physicians to have both GERD and IBS-like symptoms. A pragmatic trial design (as opposed to an explanatory trial) was chosen for this study to reflect variations among patients and doctors in real clinical settings, thus more accurately reflecting patients within the community in whom the treatment will be applied and optimising the applicability of the trial findings to those clinical settings[21,22]. The current study was conducted worldwide in 167 centres in 21 countries (Argentina, Australia, Austria, Belgium, Brazil, Canada, France, Germany, Hong Kong, India, Italy, Malaysia, Mexico, Poland, Singapore, South Africa, South Korea, Spain, Switzerland, Taiwan, and the United Kingdom) according to ICH-GCP guidelines. Ethics approval was obtained locally by all participating centres.

Patients aged ≥ 18 years (≥ 21 years in Argentina) were included if they were considered by the investigator to have symptoms of GERD and to likely comply with requirements for the completion of the ReQuest™. All patients provided written informed consent prior to participation.

Patients were excluded if they: had a history of Zollinger-Ellison syndrome or other gastric hypersecretory condition; were suffering from acute peptic ulcer and/or ulcer complications, inflammatory bowel disease, or other severe concomitant diseases; or had acid lowering surgery or any other surgery of the esophagus and/or upper gastrointestinal tract (excluding polypectomy and cholecystectomy). Those who had used acid suppressing medication in the last 7-10 d prior to enrolment, or any medication for *Helicobacter pylori* eradication during the last 28 d prior to the study were also excluded, as were those who had received systemic glucocorticoids or non-steroidal anti-inflammatory drugs including COX 2 inhibitors more than 5 d on demand but not more than 3 consecutive days during the last 28 d (with the exception of regular intake of acetylsalicylic acid in dosages up to 163 mg/d). Medications for the relief of acid-related symptoms, and systemic glucocorticoids or non-steroidal anti-inflammatory drugs, were not permitted during the study.

At baseline, all patients underwent upper GI endoscopy to determine whether they had ERD or NERD; the grade of GERD was determined according to the LA classification for all patients[23]. During this visit, the investigator also enquired about the presence of symptoms that would be consistent with IBS and answered "yes", "no", or "I do not know" to the question: "Is it possible that this patient does not only suffer from GERD-related symptoms, but also from symptoms caused by irritable bowel syndrome (IBS)?".

Enrolled patients were treated with the PPI pantoprazole-Na, 40 mg, provided as an oral enteric-coated tablet, to be taken one hour before breakfast once daily for eight weeks. During the eight-week period, three study visits were performed: visit 0 (V0, baseline), visit 1 (V1) after four weeks of treatment (Week 4), and visit 2 (V2) after eight weeks of treatment (Week 8).

Assessments

Patients completed the ReQuest™ at baseline and then daily starting on Day 1 (the day after V0), and continuing until V2 on Day 56. The ReQuest™ is a fully validated and easy-to-handle self-administered symptom assessment tool reliable for the evaluation of treatment effects in ERD and NERD[11,24-27]. It comprises 67 items assigned to six dimensions of GERD: acid complaints (e.g. heartburn, acid regurgitation, and esophageal or upper abdominal burning); upper abdominal/stomach complaints (e.g. upper abdominal pressure, pain or burning, feeling or fullness or incomplete bowel evacuation, and upper or lower back pain); lower abdominal/digestive complaints (e.g. pressure, cramps or pain in the lower abdomen, flatulence, diarrhoea and constipation); nausea; sleep disturbances; and other complaints[28]. For each dimension, symptom intensity is assessed on a 100 mm visual analogue scale (VAS; ranging from "not at all" to "extremely severe") and symptom frequency is measured using a 7-point Lik-

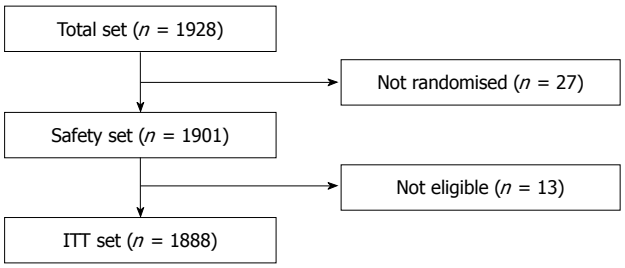

Figure 1 Flowchart of patient disposition. *n*: Number of patients; ITT: Intention-to-treat.

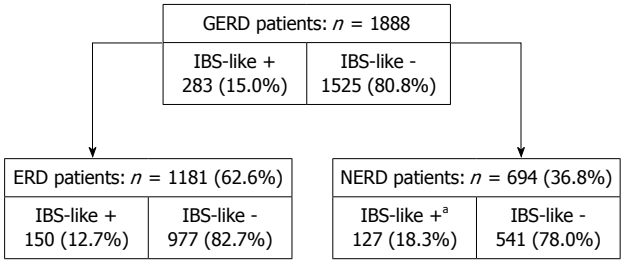

Figure 2 Subgroups of gastroesophageal reflux disease patients according to esophagitis and irritable bowel syndrome-like symptoms. Percentages do not equal 100% due to missing values. [a]*P* = 0.0015, irritable bowel syndrome (IBS)-like +: patients with non-erosive reflux disease (NERD) *vs* patients with erosive (ERD). *n*: Number of patients; GERD: Gastroesophageal reflux disease.

ert scale (ranging from "0" to "more than 10 times per day" or "continuously"). The ReQuest™ also includes a question about general well-being, evaluated using a VAS ranging from "wonderful" to "extremely poor".

The dimensions of the ReQuest™ can be grouped into two subscales: ReQuest™-GI, which includes acid complaints, upper abdominal/stomach complaints, lower abdominal/digestive complaints, and nausea; and ReQuest™-WSO, comprising general well-being, sleep disturbances, and other complaints[28]. Each dimension is weighted, resulting in the following score ranges: (1) ReQuest™ total score: 0 to 46.28; (2) ReQuest™-GI: 0 to 30.77; and (3) ReQuest™-WSO: 0 to 15.51.

The score range of the two weighted individual dimensions of special interest, i.e. "acid complaints" and "lower abdominal complaints", is 0 to 7.692. Patients were considered to have responded to treatment (responders) if their ReQuest™-GI score was below 1.6[28] over three consecutive days prior to the two scheduled visits at Week 4 and Week 8.

Statistical analysis

The sample size was calculated based on response rates seen in previous studies after 8 wk of treatment and envisaged a total of 2000 patients, approximately half with and half without esophagitis. A two-sided 95% confidence interval for a single proportion using the large sample normal approximation was expected to extend 0.019 from the observed proportion for an expected proportion of 0.100 in patients with esophagitis (sample size 1000) and 0.025 from the observed proportion for an expected proportion of 0.200 in patients without esophagitis (sample size 1000). Subsequently, a total of 1928 outpatients were screened, resulting in an intention-to-treat (ITT) population of 1888 patients.

Table 1 Demographic data and baseline characteristics [intention-to-treat population, number of patients (*n*) = 1888]

Age (yr), mean (SD)	47 (14.3)
Height (cm), mean (SD)	167.5 (9.6)
Weight (kg), mean (SD)	74.2 (15.8)
BMI (kg/m^2), mean (SD)	26.4 (4.8)
Gender, *n* (%)	
Female	978 (51.8)
Male	910 (48.2)
Esophagitis, *n* (%)	
Non-erosive	694 (36.8)
Grade A	680 (36.0)
Grade B	381 (20.2)
Grade C	97 (5.1)
Grade D	23 (1.2)
Ethnic origin, *n* (%)	
White	1326 (70.2)
Asian	352 (18.6)
Other	167 (8.8)
Black	43 (2.3)
Smoker, *n* (%)	
Never	1179 (62.4)
Former	360 (19.1)
Current	349 (18.5)

BMI: Body mass index.

Response rates were calculated at Week 4 and Week 8, and baseline scores and the pre-post differences (baseline to Week 4 and Week 8) were determined for the individual dimensions "acid complaints" and "lower abdominal complaints" of the ReQuest™-GI. Subgroup analyses were performed based on the presence or absence of esophagitis (i.e. ERD or NERD), IBS-like symptoms, and the combination thereof. The comparison of the response rates between these subgroups was performed using Fisher's exact test. The baseline scores were compared with the Wilcoxon rank sum test, the pre-post differences with the Wilcoxon signed rank test.

RESULTS

Demographics and baseline characteristics

In total, 1928 outpatients were recruited in 167 centres in 21 countries worldwide; 27 patients were not randomised and 13 were not eligible for inclusion, resulting in an ITT population of 1888 patients with GERD (Figure 1). Table 1 presents patient demographic and baseline characteristics; percentages do not equal 100% due to missing values. In total, 694 patients (36.8%) were diagnosed with NERD and 1181 (62.6%) with ERD. Of the ERD patients, 680 (57.6%) had esophagitis grade A, 381 (32.3%) grade B, and 97 (8.2%) grade C. Esophagitis grade D was diagnosed in 23 ERD patients (1.9%). Of the 1888 GERD patients, 283 presented with IBS-like symptoms (15.0%) and 1525 presented without those symptoms (80.8%). IBS-like symptoms were significantly more frequent in NERD (18.3%) than in ERD patients (12.7%, *P* = 0.0015; Figure 2).

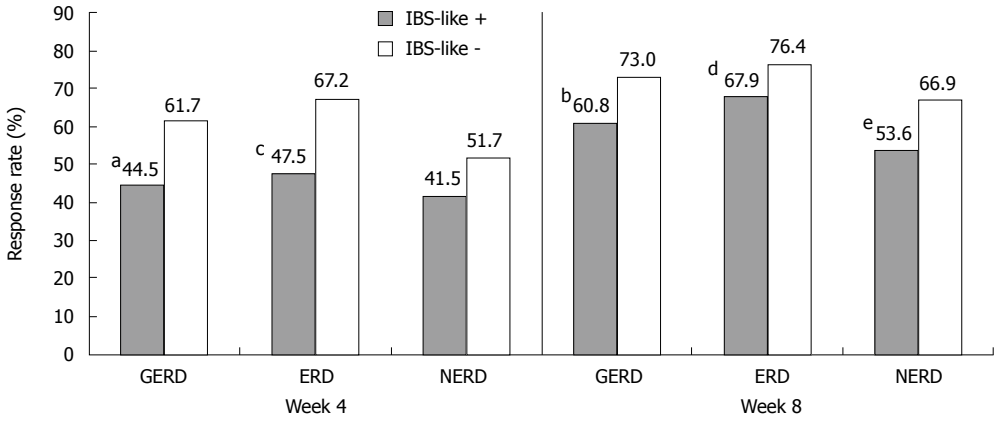

Figure 3 Response rates for gastroesophageal reflux disease/erosive/non-erosive reflux disease patients with or without irritable bowel syndrome-like symptoms after 4 and 8 wk of treatment with pantoprazole. [a]*P* < 0.0001 irritable bowel syndrome (IBS)-like + *vs* IBS-like - in patients with gastroesophageal reflux disease (GERD) after 4 wk; [b]*P* < 0.0001 IBS-like + *vs* IBS-like - in patients with GERD after 8 wk, [c]*P* < 0.0001 IBS-like + *vs* IBS-like - in patients with erosive reflux disease (ERD) after 4 wk, [d]*P* = 0.0339 IBS-like + *vs* IBS-like - in patients with ERD after 8 wk, [e]*P* = 0.0088 IBS-like + *vs* IBS-like - in patients with non-erosive reflux disease (NERD) after 8 wk.

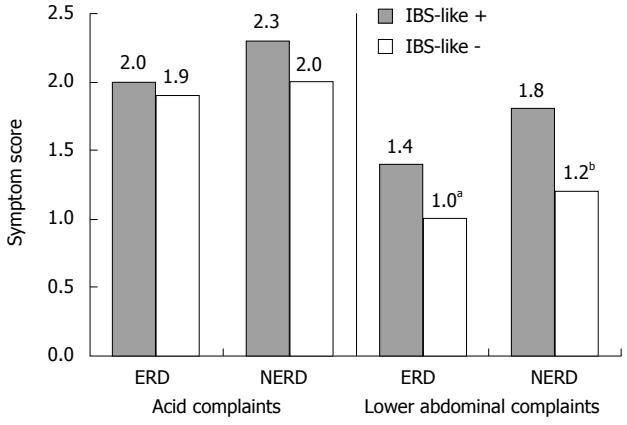

Figure 4 Symptom scores for "acid complaints" and "lower abdominal complaints" in erosive and non-erosive reflux disease patients with and without IBS-like symptoms at baseline. [a]*P* < 0.0001 irritable bowel syndrome (IBS)-like + *vs* IBS-like - in patients with erosive reflux disease (ERD) at baseline, [b]*P* < 0.0001 IBS-like + *vs* IBS-like - in patients with non-erosive reflux disease (NERD) at baseline.

Treatment response rates for ReQuest™-GI

In total, 58.9% of patients with GERD responded to pantoprazole treatment at Week 4; this increased to 71.2% of patients at Week 8. Response rates were significantly higher for ERD patients than for NERD patients at both Week 4 (64.6% *vs* 49.2%, $P < 0.0001$) and Week 8 (75.5% *vs* 64.5%, $P < 0.0001$). Both patients with and without IBS-like symptoms responded to treatment at Weeks 4 and 8 (Figure 3); however, response rates in GERD patients without IBS symptoms were significantly higher than in those with IBS-like symptoms at both time points (both $P < 0.0001$; Figure 3). Similarly, for patients with either ERD (Week 4: $P < 0.0001$; Week 8: $P < 0.0339$) or NERD (Week 8: $P = 0.0088$) response rates were higher if IBS-like symptoms were absent than if they were present (Figure 3).

Acid complaints and lower abdominal complaints

At baseline, the symptom scores for the ReQuest™ dimension "acid complaints" were 2.0 for both ERD and NERD ($P = 0.6269$). Scores remained similar irrespective of the presence of esophagitis or IBS-like symptoms (Figure 4). In contrast, baseline symptom scores for "lower abdominal complaints" were statistically significantly different in patients with ERD compared with those with NERD (1.1 *vs* 1.3, $P = 0.0012$). Furthermore, in both the ERD and NERD subgroups, patients with IBS-like symptoms had a significantly higher baseline score than those without IBS-like symptoms (both $P < 0.0001$, Figure 4).

There were no statistically significant differences in changes from baseline for the dimension "acid complaints" at Week 4 or Week 8 in ERD or NERD patients with IBS-like symptoms compared with those without IBS-like symptoms (Table 2). Similarly, for patients with ERD, pre-post differences in the dimension "lower abdominal complaints" did not statistically differ in those with IBS-like symptoms compared with those without IBS-like symptoms at either timepoint. In contrast, NERD patients with IBS-like symptoms showed significantly higher pre-post differences in the dimension "lower abdominal complaints" at Week 4 ($P = 0.049$)

Table 2 Pre-post differences of "acid complaints" and "lower abdominal complaints" in erosive and non-erosive reflux disease patients with and without irritable bowel syndrome-like symptoms after 4 and 8 wk

	ERD		NERD	
	IBS-like +	IBS-like -	IBS-like +	IBS-like -
Acid complaints				
4 wk	1.6	1.6	1.6	1.4
8 wk	1.6	1.7	1.7	1.6
Lower abdominal complaints				
4 wk	0.7	0.7	1.0[a]	0.7
8 wk	1.0	0.8	1.2[b]	0.8

[a]*P* = 0.049 irritable bowel syndrome (IBS)-like + *vs* IBS-like -; [b]*P* = 0.0092 IBS-like + *vs* IBS-like -. ERD/NERD: Erosive/non-erosive reflux disease.

and Week 8 (P = 0.0092) than did NERD patients without IBS-like symptoms (Table 2). Furthermore, for both ERD and NERD patients a higher increase of the pre-post differences could be shown between Week 4 and Week 8 for patients with IBS-like symptoms.

DISCUSSION

Using a pragmatic trial design, we investigated the influence of IBS-like symptoms on treatment response to pantoprazole and symptom burden in patients with GERD in a real-life setting. To represent the situation in real life clinical practice, patients were identified as suffering from GERD solely by their symptomatology, i.e. without knowledge of presence/absence of esophageal lesions. All patients then underwent upper GI endoscopy at baseline to determine whether they had ERD or NERD and to grade GERD (LA classification). Consistent with a pragmatic design, the presence of IBS-like symptoms was determined by the investigator by answering "yes", "no", or "I do not know" to the question: "Is it possible that this patient does not only suffer from GERD-related symptoms, but also from symptoms caused by Irritable Bowel Syndrome (IBS)?". This is reflective of real life clinical practice in many countries where general practitioners may not be familiar with the Rome Ⅲ criteria and IBS is largely diagnosed based on patient history[29,30]. Similarly, so as to reflect ordinary practice, pragmatic trials do not usually monitor patient compliance with trial medication in the way that is necessary in most explanatory trials[31]. Consequently, we did not do so. Results demonstrated a significantly higher occurrence of IBS-like symptoms in patients with NERD than in those with ERD. The presence of IBS-like symptoms reduced response to PPI treatment in patients with GERD, irrespective of endoscopic diagnosis of ERD or NERD. However, IBS-like symptoms may also show substantial improvement with placebo, making the lack of a placebo arm a limitation of this study. Nonetheless, results are reflective of what may be expected in clinical practice.

The significantly higher occurrence of IBS-like symptoms in patients with NERD (18.3%) than in those with ERD (12.7%) observed in the current study has also been shown in the literature (ERD range: 12.0%-48.0%; NERD range: 21.2%-63.6%)[15,17], although inconsistently[32,33]. However, the prevalence of IBS-like symptoms reported in the literature was generally higher than that in our study, possibly because of differences in symptom reporting and patient groups. For example, prevalence in the current study was based on the investigator's assessment of symptoms as opposed to prevalence derived from patient-reported symptoms in the literature. Furthermore, the current investigation was restricted to patients with GERD seeking help in a real life setting in contrast with population based studies presented in the literature[34].

We demonstrated a statistically significantly lower treatment response to pantoprazole therapy in GERD patients with IBS-like symptoms than in GERD patients without these symptoms. This difference occurred in both ERD and NERD, although in the latter group statistical significance was only found after 8 wk of treatment. The response rates observed in the real life setting of this study were similar to those reported in the literature and were consistent with lowered treatment responses in patients with NERD than in those with ERD[19]. They were also consistent with data from the literature showing that the presence of IBS-like symptoms unfavourably affects the treatment response to PPIs, although data remain limited, especially in subpopulations of patients with ERD and NERD[8,15]. In the current study, the calculated response rates were based on the symptom burden assessed using the ReQuest™, whereas previous studies defined response solely based on a reduction of symptom burden[20,32,35], thus lacking a valid definition of a response threshold. Nonetheless, findings based on these two approaches are likely to be comparable.

In accordance with a previous analysis of baseline symptom burden in GERD patients[33], baseline "acid complaints" symptom scores were similar in ERD and NERD patients and in patients with and without IBS-like symptoms in the current study. Furthermore, the observed similar pre-post differences in "acid complaints" in the two GERD subgroups were expected based on previous studies using the ReQuest™, which have demonstrated similar improvements of acid-related symptoms during PPI therapy in both ERD and NERD patients[11,24,25]. This effect was independent of IBS status, which is in line with existing data[32].

In this real life setting, "lower abdominal complaints" scores at baseline were significantly higher in NERD than in ERD patients, supporting previous results from Neumann *et al* (2008), which showed that NERD patients suffered more frequently from IBS symptoms than did patients with ERD or Barrett's esophagus in a small patient population[15]. Both studies contrast with earlier evaluations of the ReQuest™ database, which revealed no baseline score differences between ERD and NERD[33]. The ReQuest™ database contains data from 23 clinical studies with symptom assessment based on the ReQuest™, thus representing a selected patient population, which may account for the differences in results compared with the current study conducted under real life conditions, in which patients were included based only on their GERD symptomatology.

We also found that patients with IBS-like symptoms had significantly higher baseline scores for "lower abdominal complaints" than patients without IBS-like symptoms in both GERD subclasses, a finding concordant with previous studies[20,32]. Baseline symptom score differences between IBS+ and IBS- patients were even more pronounced than those between ERD and NERD. These results show that the investigator assessment of the presence of IBS-like symptoms was congruent with patient symptom assessment *via* the ReQuest™.

Lower abdominal symptoms improved with pantoprazole treatment in both ERD and NERD patients. This finding corresponds with earlier findings in therapeutic

studies using PPIs as GERD treatment[28]. Interestingly, of the four subgroups, patients with NERD and IBS-like symptoms had the highest "lower abdominal complaints" symptom score at baseline and the largest clinical improvement following PPI therapy after 4 and 8 wk.

Although typical GERD symptoms, i.e. acid complaints, improved with pantoprazole therapy at 4 wk in this study, treatment response rates continued to increase from Week 4 to Week 8, suggesting that continued elevation of response rates may result from a reduction of other symptoms. These results support the concept that GERD is more than heartburn and IBS-like symptoms can be a part of the GERD symptom spectrum.

The proportion of patients with ERD in the current study was higher than what might be expected based on the literature. However, because our patients were a mix of primary and secondary care cases and came from many different countries, there is no good basis for estimating what the ERD:NERD ratio should be in this study. We can therefore only speculate on the possible explanations for the observed ratio. Symptom-based diagnosis of GERD is most reliable when heartburn and/or regurgitation are the patient's dominant or only symptoms. When abdominal symptoms such as dyspepsia or IBS, which are more prevalent in NERD than in ERD, are also present, physicians may be less confident about making a diagnosis of GERD. Enrolment into the trial was based on "patients considered to have symptoms of GERD"; thus, recruitment may have been inadvertently biased against enrolling patients with NERD, thereby affecting the ERD:NERD ratio. Nonetheless, this possibility should not affect the validity of the conclusions as the results relate to patients in whom physicians are prepared to make the GERD diagnosis on the basis of symptoms.

In conclusion, IBS-like symptoms have a distinct influence on the response of GERD patients to treatment with the PPI pantoprazole. The presence of IBS-like symptoms reduces response rates and appears to be higher in patients with NERD than in those with ERD. The exact underlying mechanisms for this remain to be elucidated. These findings are of particular interest for the medical community as the symptom based identification of GERD is reflective of the real life situation in daily practice. The results suggest the need to enhance the awareness of physicians for the possible co-morbid occurrence of IBS symptoms in GERD to improve diagnosis and treatment of individuals bothered by this intricate complex of symptoms.

COMMENTS

Background

The overlap of symptomatology between gastroesophageal reflux disease (GERD) and irritable bowel syndrome (IBS) has been increasingly recognised, and one theory integrates IBS-like symptoms into the spectrum of GERD complaints. The presence of IBS-like symptoms may unfavourably affect treatment outcome in some patients with GERD, thus highlighting the importance of identifying and understanding the spectrum of symptoms influencing the treatment outcome in GERD patients in ordinary clinical practice.

Research frontiers

Research suggests that IBS is common in patients with GERD (both ERD and NERD), although these data are from a small study of outpatients (n = 71) from only one clinic. In the current study, the authors further evaluate the interrelationship between IBS-like symptoms and GERD in a large (n = 1888), prospective, multicentre study of outpatients in a real-life setting, to help understand the spectrum of symptoms influencing the treatment outcomes for improved patient management in practice.

Innovations and breakthroughs

In a recent publication by Neumann *et al* (2008), IBS symptoms were common in GERD and found to be slightly more prevalent in NERD than in ERD; however, differences were not statistically significant, which may have resulted from the small sample size. The study is the largest to date to evaluate the presence of IBS-like symptoms in both ERD and NERD patients, and to establish the impact these symptoms may have on treatment response in GERD patients.

Applications

By understanding the impact of IBS-like symptoms on response to proton pump inhibitor (PPI) treatment in patients with GERD, physicians can better predict treatment response in their patients.

Terminology

ReQuest™: a fully validated, reliable, self-administered symptom assessment tool for the evaluation of treatment effects in ERD and NERD. It comprises six dimensions of GERD: acid complaints, upper abdominal/stomach complaints, lower abdominal/digestive complaints, nausea, sleep disturbances, and other complaints which are assessed for symptom intensity and symptom frequency. The ReQuest™ also includes a question about general well-being.

Peer review

The authors investigated the influence of IBS-like symptoms on treatment outcomes with pantoprazole in GERD in a real life setting. The authors concluded that the presence of IBS-like symptoms influences treatment outcome and symptom burden in GERD. They also supported the theory incorporating IBS-like symptoms into the spectrum of GERD by demonstrating good PPI response. Overall, this topic would raise quite an interest with readers. In view of the rarity of studies of IBS in GERD with a pragmatic attitude, this manuscript could possibly get frequent citations by other researchers.

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BRIEF ARTICLE

Symptom-based tendencies of *Helicobacter pylori* eradication in patients with functional dyspepsia

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Abstract

AIM: To investigate whether there were symptom-based tendencies in the *Helicobacter pylori* (*H. pylori*) eradication in functional dyspepsia (FD) patients.

METHODS: A randomized, single-blind, placebo-controlled study of *H. pylori* eradication for FD was conducted. A total of 195 FD patients with *H. pylori* infection were divided into two groups: 98 patients in the treatment group were treated with rabeprazole 10 mg twice daily for 2 wk, amoxicillin 1.0 g and clarithromycin 0.5 g twice daily for 1 wk; 97 patients in the placebo group were given placebos as control. Symptoms of FD, such as postprandial fullness, early satiety, nausea, belching, epigastric pain and epigastric burning, were assessed 3 mo after *H. pylori* eradication.

RESULTS: By per-protocol analysis in patients with successful *H. pylori* eradication, higher effective rates of 77.2% and 82% were achieved in the patients with epigastric pain and epigastric burning than those in the placebo group ($P < 0.05$). The effective rates for postprandial fullness, early satiety, nausea and belching were 46%, 36%, 52.5% and 33.3%, respectively, and there was no significant difference from the placebo group (39.3%, 27.1%, 39.1% and 31.4%) ($P > 0.05$). In 84 patients who received *H. pylori* eradication therapy, the effective rates for epigastric pain (73.8%) and epigastric burning (80.7%) were higher than those in the placebo group ($P < 0.05$). The effective rates for postprandial fullness, early satiety, nausea and belching were 41.4%, 33.3%, 50% and 31.4%, respectively, and did not differ from those in the placebo group ($P > 0.05$). By intention-to-treat analysis, patients with epigastric pain and epigastric burning in the treatment group achieved higher effective rates of 60.8% and 65.7% than the placebo group (33.3% and 31.8%) ($P < 0.05$). The effective rates for postprandial fullness, early satiety, nausea and belching were 34.8%, 27.9%, 41.1% and 26.7% respectively in the treatment group, with no significant difference from those in the placebo group (34.8%, 23.9%, 35.3% and 27.1%) ($P > 0.05$).

CONCLUSION: The efficacy of *H. pylori* eradication has symptom-based tendencies in FD patients. It may be effective in the subgroup of FD patients with epigastric pain syndrome.

Key words: *Helicobacter pylori*; Functional dyspepsia; Eradication; Symptom

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INTRODUCTION

Functional dyspepsia (FD) is considered to possess a wide spectrum of nonspecific upper gastrointestinal symptoms without any organic alteration[1], accounting for 60% of patient referrals to gastroenterology clinics[2]. Despite being barely life-threatening, this disease can lead to a poor quality of life and a high economic burden in the patients[3].

However, without a clear understanding of its pathogenesis, the optional clinical strategy for FD remains a controversial issue. *Helicobacter pylori* (*H. pylori*) is considered to play a role in the pathogenesis of FD, and therefore, *H. pylori* detection and eradication are performed for FD treatment. But it is still dubious that patients with FD can benefit from *H. pylori* eradication. In our opinion, one of the factors that could contribute to the various outcomes of trials regarding the efficacy of *H. pylori* eradication for FD may be the diversity and inconsistency in FD symptoms, suggesting that this strategy might be effective only for certain dyspeptic symptoms in FD patients.

We conducted a prospective randomized, single-blind and placebo-controlled study and analyzed the efficacy of *H. pylori* eradication therapy for FD patients with *H. pylori* infection to find symptom-based predictors that can guide the clinical application of *H. pylori* detection and eradication.

MATERIALS AND METHODS

Patients

Patients with FD were admitted to the digestive outpatient clinics of three medical centers.

Patients who met the following criteria were enrolled into this study: (1) aged 18-75 years; (2) a definitive diagnosis of FD defined by Rome Ⅲ criteria (2006)[1]; (3) absence of organic diseases, such as ulcer, bleeding, erosion, atrophy, tumor, and esophagitis in gastroscopic examination; (4) laboratory tests, ultrasonography, and X-ray showing no structural diseases in other organs, such as liver, gall bladder, pancreas, kidney, intestine, and colon; (5) both rapid urease test (RUT) and ^{13}C-urea breath test (^{13}C-UBT) confirmed *H. pylori* infection; and (6) not receiving antacids, antibiotics, prokinetic drugs, or nonsteroidal anti-inflammatory drugs within the previous 4 wk.

We excluded the patients who (1) had a drug hypersensitivity history; (2) were previously treated with *H. pylori* eradication therapy; (3) were complicated with irritable bowel syndrome (defined by Rome Ⅲ criteria); (4) were pregnant or nursing; (5) could not describe subjective complaint accurately; (6) were complicated with diabetes, connective tissue disease, neuromuscular disease, or any other severe systematic diseases; (7) had a history of abdominal surgeries; and (8) drank alcohol more than 40 g a day.

This study was approved by the Institutional Review Board and the Ethics Committee of each medical center and was conducted in accordance with the Helsinki Declaration. Written informed consent was obtained from each subject before enrollment into this study.

Intervention

Gastroscopy and ^{13}C-UBT were performed, and samples of the gastric mucosa were collected during gastroscopy and used for further RUT testing. Patients who were positive in both the RUT and ^{13}C-UBT tests were considered suitable candidates for the proposed *H. pylori* eradication therapy. Patients were blinded to this study and randomly assigned by an independent investigator using a computer-generated random number table into one of the two groups, the treatment group and placebo group. The allocation ratio was 1:1. Patients in the treatment group were administered with rabeprazole 10 mg *bd* for 2 wk, and amoxycillin 1 g *bd* and clarithromycin 500 mg *bd* for 1 wk; patients in the placebo group were given placebos as control. No other medication was used during the proposed treatment.

Data collection and follow-up

The symptoms of suitable candidates were recorded on admission, and the proposed 2-wk eradication therapy was administered. Patients were returned for a ^{13}C-UBT test 4 wk after completing the therapy, wherein negative results were considered as definitive evidence of successful eradication. All patients were followed up for 3 mo.

Assessment of clinical efficacy

Six common symptoms of FD, including postprandial fullness, early satiety, nausea, belching, epigastric pain and epigastric burning, were assessed before and 3 mo after the eradication therapy. Symptom scores were graded according to severity as follows: (0) absent; (1) being aware of symptoms but no interference with daily activities; (2) having persistent discomfort with some interference with daily activities; (3) having severe discomfort and being unable to conduct daily work; and (4) suffering from worsening symptoms and having an extreme influence on daily life.

Clinical efficacy was calculated using the reduction rate of the total score of the six symptoms with the following formula: Reduction rate = (total score before treatment - total score after treatment)/ total score before treatment × 100%.

If the reduction rate was ⩾ 75%, the patient was considered to experience clinical recovery; a reduction rate ⩾ 50% referred to a significant improvement, a reduction

rate ⩾ 25% meant an improvement, and a rate < 25% meant invalidation. The total effective rate was calculated based on the clinical recovery rate and the significant improvement rate.

Safety assessment

Before treatment and during the follow-up, the basic vital signs and laboratory results, including routine blood, urine and stool tests, were monitored and documented. Any suspected adverse events observed during treatment were also recorded for further analysis.

Statistical analysis

Sample size was calculated based on the assumption of a 40% response in the drug arm *vs* 20% in placebo arm using the χ statistic to compare dichotomous variables with $\alpha = 0.05$ (two-tailed) and $\beta = 0.20$. The estimated sample size was 81 patients per arm. All data were processed using SPSS 11.0. The effective rates of symptoms were analyzed according to per-protocol (PP) and intent-to-treat (ITT) methods. The χ^2 test with 2 × 2 tables was used to analyze the efficacy. $P < 0.05$ was considered significant difference.

RESULTS

Patient population

From September 2008 to May 2010, 195 FD patients with *H. pylori* infection were enrolled into this study. Number, mean age, gender distribution and total scores of symptoms before treatment were similar in the two groups (Table 1).

H. pylori eradication rate and safety analysis

Totally, 195 FD patients with *H. pylori* infection were included. Twenty-two were excluded for discontinuation of medication due to severe diarrhea (3 in the treatment group) or for being lost to follow-up (11 in the treatment group and 8 in the placebo group). The others (84 in the treatment group and 89 in the placebo group) received complete treatment and follow-up examinations on schedule. In the treatment group, the successful *H. pylori* eradication rate was 85.7% (72/84).

Adverse reactions documented during treatment included: mild diarrhea in 7 cases, severe diarrhea in 3 cases, stomach discomfort in 4 cases, and simultaneous diarrhea and stomach discomfort in 1 case. Except for one patient who withdrew due to severe, unbearable diarrhea, the adverse events in the patients disappeared spontaneously after the eradication treatment. The basic vital signs and routine blood, urine and stool tests were normal in all the participants.

Total clinical efficacy

By PP analysis, in 72 patients with successful *H. pylori* eradication, the total effective rate was 44.4% 3 mo after treatment. In 84 patients who received *H. pylori* eradication therapy, the total effective rate was 42.9%, which was higher than that in the 89 patients of the placebo group (21.4%) ($P < 0.05$) (Table 2).

By ITT analysis, the total effective rate was 36.7% in the treatment group, and 19.6% in the placebo group. There was significant difference between the two groups ($P < 0.05$) (Table 2).

Table 1 Baseline demographic and clinical characteristics of patient population

	Treatment group	Placebo group
No. of patients	98	97
Age (yr, mean ± SD)	49.2 ± 14.1	45.5 ± 14.9
Gender (male/female)	47/51	42/55
Total scores of symptoms before treatment (mean ± SD)	9.0 ± 2.8	8.4 ± 2.6

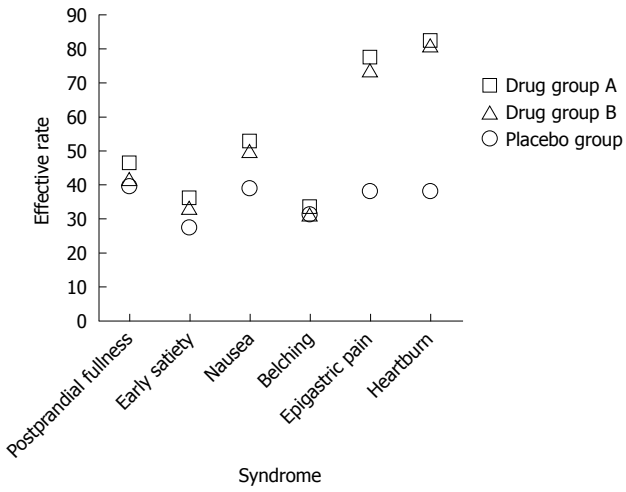

Figure 1 Respective effective rates of six common symptoms in functional dyspepsia patients by per-protocol analysis.

Efficacy for symptom improvement

By PP analysis 3 mo after treatment, among the 72 patients with successful *H. pylori* eradication, those with epigastric pain and epigastric burning achieved higher effective rates of 77.2% and 82% than the patients in the placebo group (38.2% and 38.2%) ($P < 0.05$). The effective rates for postprandial fullness, early satiety, nausea and belching were 46%, 36%, 52.5% and 33.3%, and 39.3%, 27.1%, 39.1% and 31.4%, respectively in the placebo group ($P > 0.05$) (Table 3 and Figure 1).

Correspondingly, in 84 patients who received *H. pylori* eradication therapy, the effective rates for epigastric pain (73.8%) and epigastric burning (80.7%) were higher than in the placebo group ($P < 0.05$). The effective rates for postprandial fullness, early satiety, nausea and belching were 41.4%, 33.3%, 50% and 31.4%, respectively, and did not differ from those in the placebo group ($P > 0.05$) (Table 3 and Figure 1).

Similar results were obtained by ITT analysis. In the treatment group, patients with epigastric pain and epigastric burning also achieved higher effective rates of 60.8%

Table 2 Total efficacy of *Helicobacter pylori* eradication therapy in functional dyspepsia patients with *Helicobacter pylori* infection *n* (%)

	Group	*n*	Clinical recovery	Significant improvement	Improvement	Invalidation	Total effective rates
PP	Treatment group						
	Patients with successful *H. pylori* eradication	72	11 (15.3)	21 (29.1)	30 (41.7)	10 (13.9)	32 (44.4)[a]
	Patients receiving *H. pylori* eradication therapy	84	12 (14.3)	24 (28.6)	33 (39.3)	15 (17.8)	36 (42.9)[a]
	Placebo group	89	3 (3.4)	16 (18.0)	34 (38.2)	36 (40.4)	19 (21.4)
ITT	Treatment group	98	12 (12.2)	24 (24.5)	33 (33.7)	29 (29.6)	36 (36.7)[a]
	Placebo group	97	3 (3.1)	16 (16.5)	34 (35.1)	44 (45.4)	19 (19.6)

[a]$P < 0.05$ *vs* the placebo group. *H. pylori*: *Helicobacter pylori*; PP: Per-protocol; ITT: Intent-to-treat.

Table 3 Respective efficacy for six common symptoms in functional dyspepsia patients *n* (%)

	Group	*n*	Effective rates					
			Postprandial distress syndrome				Epigastric pain syndrome	
			Postprandial fullness	Early satiety	Nausea	Belching	Epigastric pain	Epigastric burning
PP	Treatment group							
	Patients with successful *H. pylori* eradication	72	23 (46.0)	18 (36.0)	21 (52.5)	15 (33.3)	44 (77.2)[a]	41 (82.0)[a]
	Patients receiving *H. pylori* eradication therapy	84	24 (41.4)	19 (33.3)	23 (50.0)	16 (31.4)	48 (73.8)[a]	46 (80.7)[a]
	Placebo group	89	24 (39.3)	16 (27.1)	18 (39.1)	16 (31.4)	26 (38.2)	21 (38.2)
ITT	Treatment group	98	24 (34.8)	19 (27.9)	23 (41.1)	16 (26.7)	48 (60.8)[a]	46 (65.7)[a]
	Placebo group	97	24 (34.8)	16 (23.9)	18 (35.3)	16 (27.1)	26 (33.3)	21 (31.8)

[a]$P < 0.05$ *vs* the placebo group. *H. pylori*: *Helicobacter pylori*; PP: Per-protocol; ITT: Intent-to-treat.

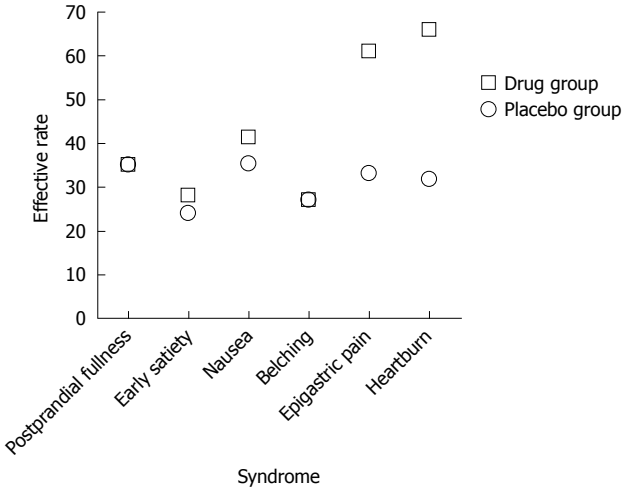

Figure 2 Respective effective rates of six common symptoms in functional dyspepsia patients by intent-to-treat analysis.

and 65.7% than those in the the placebo group (33.3% and 31.8%) ($P < 0.05$). The effective rates for postprandial fullness, early satiety, nausea and belching were 34.8%, 27.9%, 41.1% and 26.7% in the treatment group, and 34.8%, 23.9%, 35.3% and 27.1% in the placebo group ($P > 0.05$) (Table 3 and Figure 2).

DISCUSSION

FD is a common disease, accounting for a majority of upper gastrointestinal symptoms. The pathogenesis of FD is unknown. Various pathophysiological mechanisms, such as gastroduodenal dyskinesia[4], visceral paraesthesia[5], vagal dysfunction[6], *H. pylori* infection[7], and psychosocial factors[8], have been proposed to promote the development of FD. Without a clear understanding of its pathogenesis, it is not surprising that standard and effective strategies for treatment of this disease are still unavailable. Consequently, patients with FD now receive symptomatic treatment with prokinetics, antacids, and digestive enzymes empirically, which are prone to unfavorable responses and an extremely high risk of relapse.

H. pylori infection is the sole cause of FD that can be successfully eliminated by well-established medical interventions. Furthermore, 40%-60% of FD patients suffer from a detectable *H. pylori* infection[9,10], which can induce chronic inflammation and in turn the development of symptoms[11]. Evidence suggests that *H. pylori*-associated dyspepsia is caused by effects of the bacteria, such as increased secretion of gastric acid, elevated fasting and postprandial levels of serum gastrin, and declines in somatostatin in gastric mucosa. All these abnormalities can be corrected after *H. pylori* eradication[12]. If symptomatic benefits can be achieved after *H. pylori* eradication, the potential implications for this treatment will be enormous.

Unfortunately, whether *H. pylori* eradication is beneficial in FD remains controversial. Several studies have demonstrated that *H. pylori* eradication is efficacious[13-17]. A double-blind study of Asian populations suggested that patients with FD benefit from the *H. pylori* treat-

ment, who gained as much as a 13-fold greater chance for symptom improvement[18]. Another systematic review of FD patients with *H. pylori* infection showed that improvement of symptoms could be achieved by up to 3.5 folds with *H. pylori* eradication therapy[19].

Other clinical trials, however, have noted that *H. pylori* eradication therapy has a moderate but statistically significant effect on FD patients with *H. pylori* infection[13,20]. A long-term study attributed the relief of symptoms to natural fluctuations in dyspeptic symptoms[21]. Evenly, low relief rates[22] and the persistence of symptoms[23] were observed in some reports, indicating no benefit of *H. pylori* eradication. Some researchers also found that dyspeptic symptoms had a negative correlation with the severity of *H. pylori*-associated inflammation and oxidative damage in FD patients[11].

Nevertheless, in most trials, the follow-up period was less than 1 year, creating a dearth of information about the long-term effects of *H. pylori* eradication. Recently, several long-term prospective researches have shown promising benefits[24,25]. Maconi *et al*[24] reported that at a 7-year follow-up, 33.9% of patients with successful *H. pylori* eradication were more likely to be asymptomatic. This percentage was slightly higher than in symptom-free patients with persisted bacteria infection. Yet, there were some confounding factors in the study. Some patients continued to use antisecretory agents, and 40% of patients still used or had been using anti-dyspeptic medications, which might bias the results and render them disputable[24]. Therefore, the evidence that FD patients with *H. pylori* infection benefit from *H. pylori* eradication therapy is inconclusive[26].

The consensus on *H. pylori* treatment in China, reached in 2003[27], and its new version published in 2007[28], recommended but did not stipulate *H. pylori* eradication therapy in "some patients with FD" or those who had "dyspeptic symptoms that were accompanied by chronic gastritis." As such, questions on whether routine *H. pylori* detection should be applied in patients with FD, whether *H. pylori* eradication treatment must be conducted in these patients, and whether the relief of symptoms can be expected following successful eradication have become critical issues. These issues can result in negative consequences, including over-examination and over-treatment, vague evaluations of prognoses, panic psychology in patients with *H. pylori* infection, and decreased treatment compliance among irresponsive patients.

To our knowledge, no study has yet focused on the symptom-based tendencies of *H. pylori* eradication. Based on our prospective study, patients with epigastric pain and epigastric burning could experience greater improvement 3 mo after treatment than those with postprandial fullness, early satiety, nausea, and belching. The Rome Ⅲ consensus classifies FD into two subgroups: postprandial distress syndrome, which presents with postprandial fullness, early satiety, postprandial nausea and over-belching; and epigastric pain syndrome, which features epigastric pain or epigastric burning[1]. This indicated that *H. pylori* eradication might be more effective in the subgroup of FD patients with epigastric pain syndrome.

But the mechanism of this symptom selectivity and tendency of *H. pylori* eradication therapy, however, is unknown. Gastric emptying in patients with FD is usually delayed or accelerated. The former can lead to abdominal distention, early satiety and nausea, and the latter can result in epigastric pain[15]. *H. pylori* infection is associated with faster gastric emptying and aggravates abdominal pain[15]. This result supports our findings that *H. pylori* eradication significantly improves the symptoms of epigastric pain. With regard to the relief of epigastric burning in our patients, the intake of antacids might be a half-convincing explanation.

In the further study, we will classify FD patients into two subgroups by the Rome Ⅲ consensus to observe the symptom response to *H. pylori* eradication therapy, in order to investigate whether the efficacy of *H. pylori* eradication can be predicted by assessing the initial dyspeptic symptoms.

There are, however, regional differences in the prevalence of various dyspeptic symptoms in patients with FD. For instance, compared with Asian populations, the symptoms of epigastric pain and epigastric burning are more common in Western FD patients[29]; there is a higher prevalence of *H. pylori* infection in Asian populations than in Western populations[30]. Hence, global multicenter studies regarding the symptom-based tendencies of *H. pylori* eradication treatment are needed for further evaluation of this regimen.

COMMENTS

Background

Helicobacter pylori (*H. pylori*) eradication is performed commonly as a treatment in functional dyspepsia (FD) patients with *H. pylori* infection. But whether *H. pylori* eradication is beneficial for the improvement of symptoms in FD is still controversial.

Research frontiers

Characteristics of FD patients with *H. pylori* infection who benefit from *H. pylori* eradication are being investigated.

Innovations and breakthroughs

Up to now, no study has yet focused on the symptom-based tendencies of *H. pylori* eradication in FD patients. The authors conducted a prospective randomized, single-blind and placebo-controlled study and analyzed the efficacy of *H. pylori* eradication therapy for FD patients with *H. pylori* infection to find symptom-based predictors that can guide the clinical application of *H. pylori* detection and eradication.

Applications

This article indicated that *H. pylori* eradication might be more effective in the subgroup of FD patients with epigastric pain syndrome.

Peer review

The authors hypothesized that *H. pylori*-infected FD patients should have significant improvement of symptoms after *H. pylori* eradication, and the study was designed to test it. This study (hypothesis and methodology) is simple and clearly presented. The manuscript is well organized. However, a little bit more details are expected in the methodology section.

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BRIEF ARTICLE

GST polymorphisms are associated with hepatocellular carcinoma risk in Chinese population

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Abstract

AIM: To investigate the association between *GSTM1* and *GSTT1* polymorphisms and the risk of hepatocellular carcinoma (HCC) in Chinese population.

METHODS: Literature databases including PubMed, ISI web of science and other databases were searched. Pooled odds ratio (OR) and 95% CI were calculated using random- or fixed- effects model. Subgroup analysis and sensitivity analysis were also performed.

RESULTS: Nineteen studies of *GSTM1* (2660 cases and 4017 controls) and 16 studies of *GSTT1* (2410 cases and 3669 controls) were included. The *GSTM1*/*GSTT1* null genotypes were associated with increased risk of HCC in Chinese population (for *GSTM1*, OR = 1.487, 95% CI: 1.159 to 1.908, *P* = 0.002; for *GSTT1*, OR = 1.510, 95% CI: 1.236 to 1.845, *P* = 0.000). No publication bias was detected. In subgroup analysis, glutathione S-transferases polymorphisms were significantly associated with HCC risk among the subjects living in high-incidence areas, but not among the subjects living in low-incidence areas.

CONCLUSION: The present meta-analysis suggests that *GSTM1*/*GSTT1* null genotypes are associated with increased risk of HCC in Chinese population.

Key words: *GSTM1*; *GSTT1*; Polymorphism; Hepatocellular carcinoma; Liver cancer

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INTRODUCTION

Liver cancer is one of the most common types of cancer and one of the most common causes of cancer-related

death[1]. The death rates have increased for both men and women with liver cancer over the past two decades[2]. The incidence and mortality rates of liver cancer vary considerably among racial and ethnic groups[3]. Asians, particularly Chinese, have a high risk of developing liver cancer[4]. About 80%-90% of all cases of primary liver cancer are hepatocellular carcinoma (HCC).

The pathogenesis of HCC may have a genetic and environmental basis[5,6]. Epidemiological studies have shown that HCC is associated with many environmental factors, including alcoholism, chronic infection with hepatitis B virus (HBV), hepatitis C virus (HCV), and dietary exposure to aflatoxin B1 (AFB1)[7]. These hepatocarcinogens result in increased generation of reactive oxygen species and free radicals that cause liver damage and repair. Thus the accumulated multistage genetic mutations may lead to liver carcinogenesis[8-11].

During liver carcinogenesis, cellular defense mechanisms can alleviate the effects of oxidative stress and exogenous toxins. One of the essential antioxidant is the reducing compound glutathione. Reduced glutathione can be conjugated to various xenobiotics and endobiotics by glutathione S-transferases (GST), a superfamily of cytosolic soluble detoxification enzymes. In humans, cytosolic soluble GSTs are encoded by seven distinct genes: Alpha, Mu, Omega, Pi, Sigma, Theta and Zeta[12-14]. GSTs play an important role in cellular protection against oxidative stress and exogenous toxins. Homozygous deletion of GST genes (null genotype) results in decreased enzyme activity, which will impede detoxification and may ultimately increase the risk of many diseases, including HCC[13-16]. *GSTM1* and *GSTT1* have been the most extensively studied GST genes. While many studies have investigated the relationship between *GSTM1* and *GSTT1* polymorphisms and HCC risk, so far the results have been inconsistent.

Recently, a meta-analysis result did not suggest a statistically significant increased risk of HCC with the *GST* null genotypes[17]. However, a large number of studies were not reported in that meta-analysis (which included only 9 studies of *GSTM1* and 8 studies of *GSTT1* in Chinese population)[17]. Since the ethnic background and the environmental exposures may vary greatly across populations in different geographic regions, the conclusion could not be drawn[17]. During the preparation of this paper, another meta-analysis was published showing that the null genotypes of *GSTM1* and *GSTT1* are both associated with an increased HCC risk[18]. However, these investigators omitted some important data and introduced some incorrect information[18]. For example, Indians are mainly of Indo-European and Dravidian ancestries, which should be distinguished from East Asian population[18]. Many studies, including our previous studies[16,19-22], have reported on the effects of ethnic differences on genetic predisposition to human diseases. In addition, it is important to note that the allele frequencies and the genotype distributions for GST genes differed significantly across ethnic groups[23]. For example, the frequency of *GSTM1* null genotype is about 0.53 in Caucasians/Asians and about 0.28 in Africans; and the frequency of *GSTT1* null genotype is about 0.20 in Caucasians and about 0.52 in Asians[23]. Therefore, heterogeneity was introduced in that meta-analysis, which may not accurately assess the effects of *GSTM1* and *GSTT1* null genotypes on the risk of HCC[18]. In this study, we reinvestigated the relationship between *GSTM1*/*GSTT1* polymorphisms and the risk of HCC. We focused on the association between GST polymorphisms and the risk of HCC in Chinese population, because Chinese are at a much greater risk of developing HCC compared to other ethnic groups. A total of 19 studies of *GSTM1* (2660 cases and 4017 controls) and 16 studies of *GSTT1* (2410 cases and 3669 controls) were included. This meta-analysis has a much greater number of subjects and thus a much greater statistical power; therefore, it may define the effects of GST gene polymorphisms on the risk of HCC more precisely.

MATERIALS AND METHODS

Literature and search strategy

We searched the literature databases including PubMed (1950 to 2010), ISI web of science (1975 to 2010), Embase (1966 to 2010), Chinese Biomedical Database (1978 to 2010), China National Knowledge Infrastructure (1979 to 2010, in Chinese) and Wanfang Data (1982 to 2010, in Chinese).

The search strategy to identify all possible studies involved use of combinations of the following key words: ("glutathione S-transferase" or "GST" or "GSTM1" or "GSTT1") and ("hepatocellular carcinoma" or "liver cancer" or "HCC") and ("China" or "Chinese"). The reference lists of reviews and retrieved articles were also searched. Supplementary data were searched for missing data points. If more than one article were published using the same case series, only the study with largest sample size was selected. The literature search was updated on Oct. 20th, 2010.

Inclusion criteria and data extraction

The studies included in the meta-analysis must meet all the following inclusion criteria: (1) evaluating the association between *GSTM1* or *GSTT1* null genotypes and HCC risk; (2) case-control design; (3) in Chinese population; and (4) sufficient data for calculation of odds ratio (OR) with CI. The following information was extracted from each study: (1) name of the first author; (2) year of publication; (3) language of publication; (4) residence area of the subjects; (5) source of control subjects; (6) numbers of cases and controls; (7) numbers of null genotypes for *GSTM1* and *GSTT1* in cases and controls; and (8) OR and 95% CI. The authors independently assessed the articles for compliance with the inclusion/exclusion criteria, resolved disagreements and reached a consistent decision.

Statistical analysis

The association between *GSTM1*/*GSTT1* polymorphisms and the risk of HCC was estimated by calculating pooled

Table 1 Characteristics of studies included in the meta-analysis of *GSTM1*

Ref.	Language of publication	Residence area of subjects	Case			Control			Source of control	OR (95% CI)	Earlier or smaller reports
			Null	Positive	Total	Null	Positive	Total			
Dong *et al*[28], 1997	Chinese	Jiangsu, Guangxi and Hebei	62	48	110	50	62	112	Population	1.602 (0.943-2.721)	[29,30]
Yu *et al*[31], 1999	English	Taiwan	42	42	84	216	159	375	Hospital	0.736 (0.458-1.183)	[32-37]
Wu *et al*[38], 2000	Chinese	Hu'nan	38	16	54	62	74	136	Population	2.835 (1.444-5.565)	NA
Sun *et al*[39], 2001	English	Taiwan	26	43	69	77	51	128	Population	0.400 (0.219-0.731)	NA
Zhu *et al*[40], 2001	Chinese	Guangdong	34	18	52	41	59	100	Hospital	2.718 (1.354-5.455)	NA
Chen *et al*[41], 2002	English	Taiwan	60	41	101	19	16	35	Hospital	1.232 (0.568-2.674)	NA
Liu *et al*[42], 2002	Chinese	Jiangsu	56	28	84	69	75	144	Population	2.174 (1.243-3.803)	[43-46]
McGlynn *et al*[47], 2003	English	Jiangsu	NA	NA	231	NA	NA	256	Population	0.830 (0.570-1.210)	[48-50]
Li *et al*[51], 2004	Chinese	Jiangsu	122	85	207	118	89	207	Population	1.083 (0.733-1.600)	NA
Chen *et al*[52], 2005	English	Taiwan	322	255	577	231	158	389	Population	0.864 (0.666-1.121)	[53]
Deng *et al*[54], 2005	English	Guangxi	117	64	181	172	188	360	Hospital	1.998 (1.383-2.888)	[55-58]
Guo *et al*[59], 2005	Chinese	Henan	67	28	95	52	51	103	Population	2.347 (1.306-4.218)	NA
He *et al*[60], 2005	Chinese	Guangxi	68	37	105	77	74	151	Hospital	1.766 (1.059-2.947)	[61, 62]
Long *et al*[63], 2005	Chinese	Guangxi	92	48	140	254	282	536	Hospital	2.128 (1.444-3.137)	[64]
Ma *et al*[65], 2005	Chinese	Guangxi	37	25	62	29	44	73	Population	2.246 (1.125-4.481)	NA
Zhang *et al*[66], 2005	Chinese	Hubei	37	23	60	28	45	73	Hospital	2.585 (1.281-5.219)	NA
Zhu *et al*[67], 2005	Chinese	Zhejiang	56	35	91	61	69	130	Hospital	1.810 (1.049-3.121)	NA
Long *et al*[68], 2006	English	Guangxi	179	78	257	312	337	649	Hospital	2.479 (1.823-3.370)	NA
Yang *et al*[69], 2009	Chinese	Guangxi	59	41	100	41	19	60	Hospital	0.667 (0.340-1.309)	NA

NA: Not available; OR: Odds ratio.

OR and 95% CI. The significance of the pooled OR was determined by Z test with $P < 0.05$ considered statistically significant. Q test was performed to evaluate whether the variation was due to heterogeneity or by chance. A random- (DerSimonian-Laird method[24]) or fixed- (Mantel-Haenszel method[25]) effects model was used to calculate pooled effect estimates in the presence ($P \leqslant 0.10$) or absence ($P > 0.10$) of heterogeneity, respectively. Begg's funnel plot, a scatter plot of effect against a measure of study size, was generated as a visual aid to detecting bias or systematic heterogeneity[26]. An asymmetric funnel plot indicates a relationship between effect and study size, which suggests the possibility of either publication bias or a systematic difference between smaller and larger studies ("small study effects"). Publication bias was assessed by Begg's test and Egger's test[27] with $P < 0.05$ considered statistically significant. Subgroup analyses were performed to examine the effect of heterogeneity on meta-analysis results. The following subgroup comparisons were analyzed: residence area of the subjects (high-incidence area *vs* low-incidence area), number of cases (< 100 *vs* ⩾ 100), and source of controls (population-based *vs* hospital-based). To evaluate the stability of results, sensitivity analysis was performed by removing one study at a time and calculating the overall homogeneity and effect size. Data analysis was performed using STATA version 10 (StataCorp LP, College Station, Texas, USA).

RESULTS

Characteristics of the studies

The literature search identified a total of 137 potential relevant papers. The full text articles were retrieved and carefully reviewed to assess the eligibility according to the inclusion criteria. Forty-three papers met the inclusion criteria[28-70]. However, 23 papers were excluded because they were earlier or smaller reports from the same groups[29,30,32-37,43-46,48-50,53,55-58,61,62,64]. Nineteen studies of *GSTM1* (2660 cases and 4017 controls) and 16 studies of *GSTT1* (2410 cases and 3669 controls) were included in the meta-analysis, respectively. Most of the cases and controls included in this meta-analysis were HBV carriers. The characteristics of the included studies are listed in Tables 1 and 2.

Meta-analysis results of the association between GSTM1 polymorphisms and HCC

Nineteen studies of *GSTM1*, including 2660 cases and 4017 controls, were included in the meta-analysis. The relative frequency of *GSTM1* null genotype among control groups ranged from 0.384 to 0.683 in Chinese population. Using a random-effects model, the overall meta-analysis result showed that there was a statistically significant association between *GSTM1* null genotype and HCC risk in Chinese population (OR = 1.487, 95% CI: 1.159 to 1.908, $P = 0.002$). The forest plot is shown in Figure 1.

Meta-analysis results of the association between GSTT1 polymorphisms and HCC

Sixteen studies of *GSTT1*, including 2410 cases and 3669 controls, were included in the meta-analysis. The relative frequency of *GSTT1* null genotype among control groups ranged from 0.183 to 0.602 in Chinese population. Using a random-effects model, the overall meta-analysis result showed that there was a statistically significant association between *GSTT1* null genotype and HCC risk in Chinese

Table 2 Characteristics of studies included in the meta-analysis of *GSTT1*

Ref.	Language of publication	Residence area of subjects	Case			Control			Source of control	OR (95% CI)	Earlier or smaller reports
			Null	Positive	Total	Null	Positive	Total			
Dong *et al*[28], 1997	Chinese	Jiangsu, Guangxi and Hebei	63	47	110	42	70	112	Population	2.234 (1.305-3.825)	[29,30]
Yu *et al*[31], 1999	English	Taiwan	41	42	83	181	194	375	Hospital	1.046 (0.650-1.683)	[32-37]
Sun *et al*[39], 2001	English	Taiwan	30	37	67	77	51	128	Population	0.537 (0.295-0.976)	NA
Liu *et al*[42], 2002	Chinese	Jiangsu	34	50	84	36	108	144	Population	2.040 (1.146-3.630)	[43-46]
McGlynn *et al*[47], 2003	English	Jiangsu	NA	NA	231	NA	NA	256	Population	0.880 (0.590-1.310)	[48-50]
Liu *et al*[70], 2003	Chinese	Guangxi	28	23	51	18	35	53	Population	2.367 (1.072-5.227)	NA
Li *et al*[51], 2004	Chinese	Jiangsu	108	99	207	97	110	207	Population	1.237 (0.841-1.820)	NA
Chen *et al*[52], 2005	English	Taiwan	298	279	577	199	190	389	Population	1.020 (0.788-1.319)	[53]
Deng *et al*[54], 2005	English	Guangxi	108	73	181	154	206	360	Hospital	1.979 (1.377-2.845)	[55-58]
Guo *et al*[59], 2005	Chinese	Henan	58	37	95	45	58	103	Population	2.020 (1.146-3.562)	NA
He *et al*[60], 2005	Chinese	Guangxi	43	62	105	50	101	151	Hospital	1.401 (0.836-2.347)	[61,62]
Long *et al*[63], 2005	Chinese	Guangxi	82	58	140	234	302	536	Hospital	1.825 (1.251-2.660)	[64]
Ma *et al*[65], 2005	Chinese	Guangxi	35	27	62	21	52	73	Population	3.210 (1.573-6.551)	NA
Zhang *et al*[66], 2005	Chinese	Hubei	38	22	60	34	39	73	Hospital	1.981 (0.986-3.982)	NA
Long *et al*[68], 2006	English	Guangxi	146	111	257	297	352	649	Hospital	1.559 (1.165-2.086)	NA
Yang *et al*[69], 2009	Chinese	Guangxi	33	67	100	11	49	60	Hospital	2.194 (1.010-4.765)	NA

NA: Not available; OR: Odds ratio.

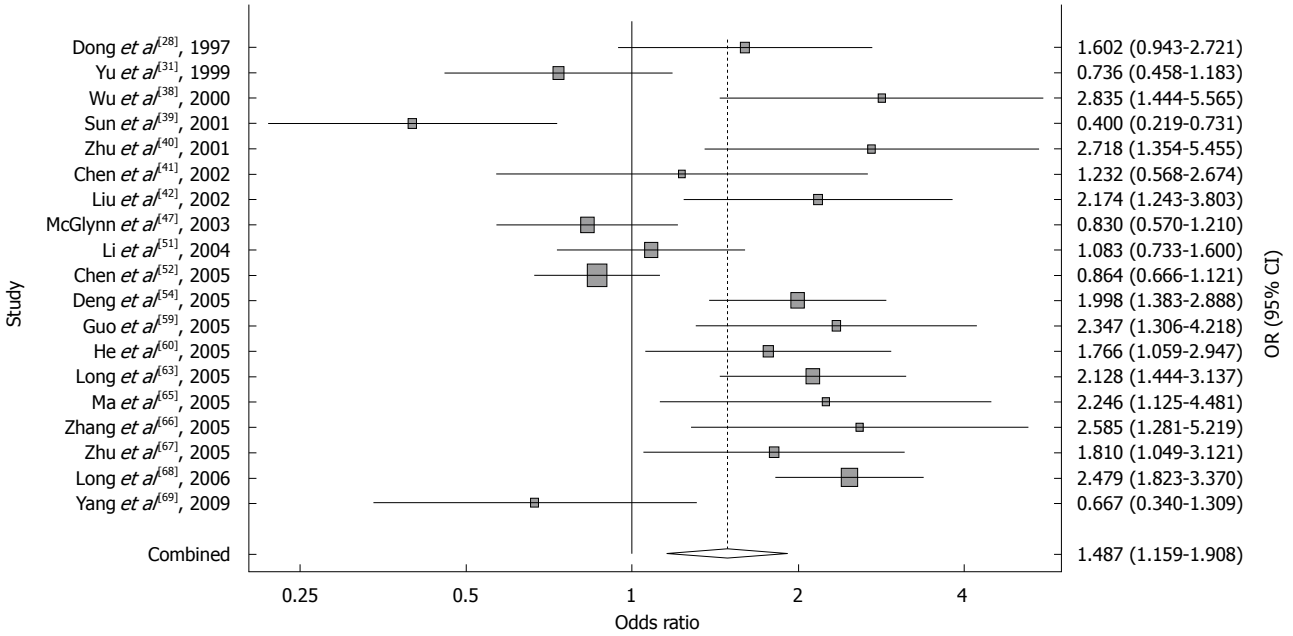

Figure 1 Forest plot of the meta-analysis of the association between *GSTM1* polymorphism and hepatocellular carcinoma risk.

population (OR = 1.510, 95% CI: 1.236 to 1.845, P = 0.000). The forest plot is shown in Figure 2.

Subgroup analysis

To examine the effect of heterogeneity between studies on meta-analysis results, we conducted subgroup analyses stratified by the following: residence area of the subjects (high-incidence area vs low-incidence area), number of cases (< 100 *vs* ≥ 100), and source of controls (population-based vs hospital-based). GST polymorphisms were significantly associated with HCC risk among the subjects living in high-incidence areas (Jiangsu, Zhejiang, Guangxi and Guangdong provinces), but not among the studies living in low-incidence areas. The result of subgroup analysis is shown in Tables 3 and 4.

Sensitivity analysis

Sensitivity analysis was performed by excluding each study at a time. The analysis confirmed the stability of the association between *GSTM1* and *GSTT1* polymorphisms and HCC risk (data not shown).

Potential publication bias

Begg's funnel plots were generated to assess potential publication bias (Figure 3 for *GSTM1* and Figure 4 for *GSTT1*). No publication bias was detected (Egger's test,

Table 3 Subgroup analysis of the association between *GSTM1* polymorphism and hepatocellular carcinoma risk

Group	No. of studies (cases/controls)	Statistical method	OR (95% CI)	*P*
All studies	19 (2660/4017)	Random	1.487 (1.159-1.908)	0.002
Residence area of the subjects				
High-incidence area	11 (1510/2666)	Random	1.659 (1.264-2.177)	0.000
Low-incidence area	7 (1040/1239)	Random	1.235 (0.753-2.026)	0.402
Mixed areas	1 (110/112)	-	1.602 (0.943-2.721)	0.081
No. of cases				
< 100	9 (651/1262)	Random	1.676 (1.061-2.649)	0.027
⩾ 100	10 (2009/2755)	Random	1.365 (1.005-1.853)	0.046
Source of controls				
Population-based	9 (1489/1548)	Random	1.316 (0.915-1.892)	0.139
Hospital-based	10 (1171/2469)	Random	1.675 (1.251-2.243)	0.001

OR: Odds ratio.

Table 4 Subgroup analysis of the association between *GSTT1* polymorphism and hepatocellular carcinoma risk

Group	No. of studies (cases/controls)	Statistical method	OR (95% CI)	*P*
All studies	16 (2410/3669)	Random	1.510 (1.236-1.845)	0.000
Residence area of the subjects				
High-incidence area	10 (1418/2489)	Random	1.641 (1.328-2.027)	0.000
Low-incidence area	5 (882/1068)	Random	1.152 (0.777-1.707)	0.483
Mixed areas	1 (110/112)	-	2.234 (1.305-3.825)	0.003
Number of cases				
< 100	7 (502/949)	Random	1.617 (1.035-2.528)	0.035
⩾ 100	9 (1908/2720)	Random	1.457 (1.173-1.810)	0.001
Source of controls				
Population-based	9 (1484/1465)	Random	1.441 (1.039-1.997)	0.028
Hospital-based	7 (926/2204)	Fixed	1.635 (1.391-1.921)	0.000

OR: Odds ratio.

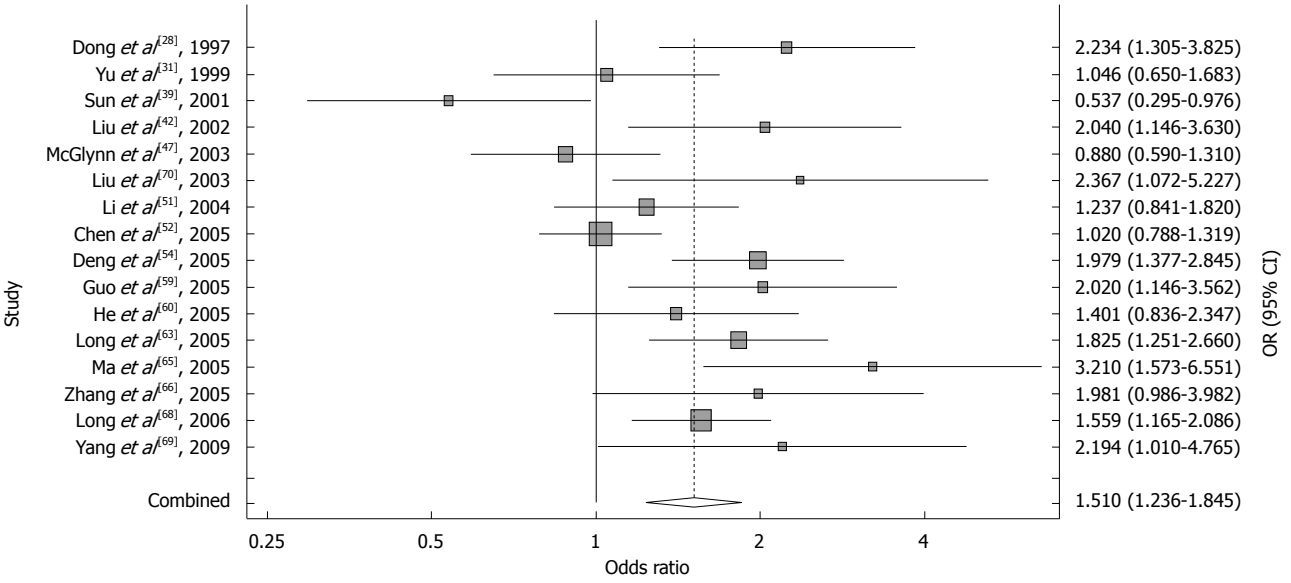

Figure 2 Forest plot of the meta-analysis of the association between *GSTT1* polymorphism and hepatocellular carcinoma risk.

P = 0.542 for *GSTM1* and *P* = 0.136 for *GSTT1*; Begg's test, *P* = 0.677 for *GSTM1* and *P* = 0.299 for *GSTT1*).

DISCUSSION

GST polymorphisms are implicated in the development of HCC. In the present study, we investigated the relationship between *GSTM1* and *GSTT1* polymorphisms and the risk of HCC in Chinese population. To minimize language bias, all available studies published in both English and Chinese languages were assessed, including a total of 19 studies of *GSTM1* (2660 cases and 4017

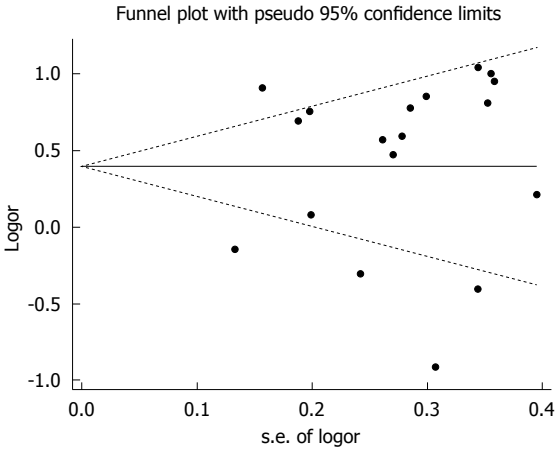

Figure 3 Funnel plot of the meta-analysis of the association between *GSTM1* polymorphism and hepatocellular carcinoma risk.

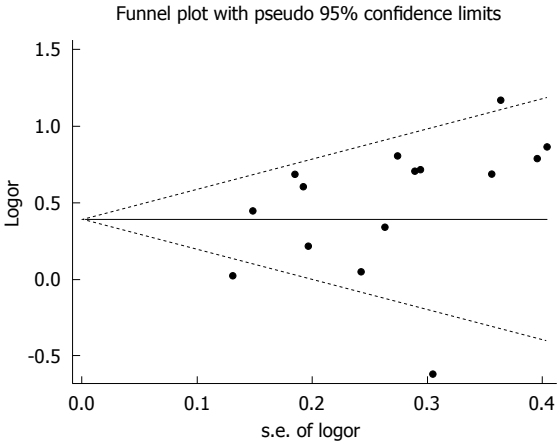

Figure 4 Funnel plot of the meta-analysis of the association between *GSTT1* polymorphism and hepatocellular carcinoma risk.

controls) and 16 studies of *GSTT1* (2410 cases and 3669 controls). The present meta-analysis has a much greater number of subjects and thus a much greater statistical power; therefore, it can define the effect of GST genes polymorphisms on HCC risk more precisely. The pooled analysis of two genes produced similar risk estimates (for *GSTM1*, OR = 1.487, 95% CI: 1.159 to 1.908, P = 0.002; for *GSTT1*, OR = 1.510, 95% CI: 1.236 to 1.845, P = 0.000), suggesting that both *GSTM1* and *GSTT1* null genotypes are associated with an increased risk of HCC in Chinese population.

The difference between our meta-analysis and the previous metaanalysis may be due to language bias introduced in the previous metaanalysis, which was based primarily on reports in English[17]. Therefore, all available studies published in both English and Chinese languages were assessed in the current meta-analysis. Another recent meta-analysis suggested that null genotypes of *GSTM1* and *GSTT1* were both associated with increased risk of HCC[18]. However, these investigators omitted some data and included some studies with incorrect information[52,53,59,65,69]. Therefore, the effects of *GSTM1* and *GSTT1* null genotypes on HCC was not assessed accurately[18]. Considering that Chinese people are at a much greater risk of developing HCC, we focused on the relationship between GST polymorphisms and HCC risk in Chinese population, thereby minimizing ethnic/racial differences. In the subgroup analysis, GST polymorphisms were significantly associated with HCC risk among the subjects living in high-incidence areas, but not among the subjects living in low-incidence areas. This result suggests that the effect of GST polymorphisms on HCC risk may be enhanced by environmental risk factors, such as dietary exposure to AFB1. Since sample size could influence the results, we also performed subgroup analysis stratified by sample size of cases. The results showed that the studies with either large (number of cases $\geqslant$ 100) or small sample size (number of cases $<$ 100) had similar risk estimates, indicating that small study effects may not exist in this meta-analysis. In addition, we generated Begg's funnel plots and found no publication bias among the studies included in this meta-analysis (Begg's test and Egger's test, $P > 0.1$).

The current meta-analysis has vital advantages compared to other studies; however, it does have some limitations. First, the present meta-analysis was based on unadjusted effect estimates and confidence intervals due to insufficient data available for most of the studies. Although the cases and controls were matched on age, sex and residence in all studies, these confounding factors might slightly modify the effect estimates. Second, the effect of gene-environment interactions was not studied in this meta-analysis. Alcoholism, HBV/HCV infections, and dietary exposure to AFB1 may be environmental risk factors that modify the effect estimates. Third, although most primary liver cancer cases are HCC, some of the included studies did not state whether the primary liver cancer patients were histologically confirmed to be HCC. Fourth, the heterogeneity between studies was not well addressed by subgroup analysis, suggesting there were other potential confounding factors in the included studies. Fifth, the results of subgroup analysis should be interpreted with caution because of limited statistical power. We anticipate these issues will be addressed in future studies.

In summary, our research suggests that *GSTM1*/*GSTT1* null genotypes are associated with increased risk of HCC in Chinese population. Considering the increasing prevalence of HCC in China and other countries worldwide, our finding may have important clinical and public health implications. More epidemiological and mechanistic studies are needed to further elucidate the role of GST polymorphisms in HCC and other liver cancers.

COMMENTS

Background

Asians, particularly Chinese, have a high risk of developing liver cancer. About 80%-90% of all cases of primary liver cancer diagnosed are hepatocellular carcinoma (HCC). Previous studies suggest that glutathione S-transferase (GST)

polymorphisms (*GSTM1* and *GSTT1*) may be risk factors for HCC. However, recent findings have been inconsistent.

Research frontiers

Meta-analysis was performed to assess the association between *GSTM1* and *GSTT1* polymorphisms and HCC risk in Chinese population.

Innovations and breakthroughs

The meta-analysis provided new evidence for the association between *GSTM1* and *GSTT1* polymorphisms and HCC risk in Chinese population. The results of this meta-analysis show that the *GSTM1* and *GSTT1* null genotypes are both associated with increased risk of HCC in Chinese population, suggesting that *GSTM1*/*GSTT1* null genotype carriers have 1.5 fold higher risk of developing HCC.

Applications

Since *GSTM1*/*GSTT1* polymorphisms are implicated in the pathogenesis of HCC, population-based genetic screening in future may help to identify the individuals at high risk of developing liver cancers.

Terminology

Meta-analysis, which combines the results of several studies that address a set of related research hypotheses, is an important component of a systematic review procedure.

Peer review

This meta-analysis provided new insights into liver cancer research.

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BRIEF ARTICLE

Survival trends in gastric cancer patients of Northeast China

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Abstract

AIM: To describe survival trends in patients in Northeast China diagnosed as gastric cancer.

METHODS: A review of all inpatient and outpatient records of gastric cancer patients was conducted in the First Affiliated Hospital of China Medical University. All the gastric cancer patients who satisfied the inclusion criteria from January 1, 1980 through December 31, 2003 were included in the study. The main outcomes were based on median survival and 3-year and 5-year survival rates, by decade of diagnosis.

RESULTS: From 1980 through 2003, the median survival for patients with gastric cancer (n = 1604) increased from 33 mo to 49 mo. The decade of diagnosis was not significantly associated with patient survival for gastric cancer (P = 0.084 for overall survival, and P = 0.150 for 5-year survival); however, the survival rate of the 2000s was remarkably higher than that of the 1980s (P = 0.019 for overall survival, and P = 0.027 for 5-year survival).

CONCLUSION: There was no significant difference of survival among each period; however, the survival rate of the 2000s was remarkably higher than that of the 1980s.

Key words: Survival trends; Gastric cancer; Northeast China

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INTRODUCTION

Although the prognosis of gastric cancer have improved due to early diagnosis, radical operation, and the development of adjuvant therapy, patients with gastric cancer still have a poor prognosis[1,2]. Since the 1980s, there have been substantial changes in the incidence of gastric cancer[3,4] and the causes for this change remain highly debated. Possible causes include the obesity epidemic, decreasing *Helicobacter pylori* (*H. pylori*) prevalence, and dietary changes[5,6].

Currently, various surgical approaches are being practiced, including conventional surgery, function preserving surgery, minimally invasive surgery and less extensive lymph node dissection. Previously, surgical interventions had been associated with significant perioperative risk; recently, however, this risk appears to be decreasing[7,8]. The role of chemotherapy, both preoperatively and postoperatively, has been extensively studied[9-11]. Despite these changes in treatment, it is unclear whether the survival of patients with gastric cancer has significantly improved since the 1980s.

The purpose of the current study was to use a sample population over a 24-year period to describe changes in the survival of gastric cancer patients. We compared patient survival in the 1980s with the 1990s and the 2000s, since patient survival might have improved as a result of advances in the quality of surgical techniques and other medical management.

MATERIALS AND METHODS

Patients

We enrolled 1604 histologically confirmed gastric cancer patients who underwent an operation at the First Affiliated Hospital of the China Medical University between 1980 and 2003. Of these patients, 496 were allocated to 1980s, 673 to 1990s and 435 to 2000s. The inclusion criteria were as follows: (1) gastric cancer was histologically confirmed; (2) an operation was performed; and (3) a complete medical record was available.

Follow-up for all patients was conducted by mailing letters or telephone interviews. The follow-up was completed in December 2008, with a total follow-up rate of 89%. Clinical findings, surgical findings, pathological findings and every follow-up were collected and recorded in the database. The study protocol was approved by the Ethics Committee of China Medical University.

Statistical analysis

Kaplan-Meier survival curves were used to estimate patient survival. Cox proportional hazards regression models were used to assess associations of risk factors with survival. For univariate analyses, we put the prognostic factor of interest and the diagnosis period as covariates in the Cox regression model. In multivariate analyses, the prognostic factor detected in univariate analysis and the diagnosis period were the covariates included in the Cox regression model.

Two-sided *P* values were calculated for all tests and are reported here. *P* values less than 0.05 were considered statistically significant. Analyses were performed using SPSS software, version 16.0.

RESULTS

In total, 1604 patients diagnosed with gastric cancer were enrolled in the 24-year study period. The median survival for gastric cancer patients increased during the 2 decades studied from 33 mo in the 1980s, and 39 mo in the 1990s to 49 mo in the 2000s. The 3-year survival for patients in the 1980s, 1990s, and 2000s was 47.6% (95% CI: 43.3%-51.9%), 51.4% (95% CI: 47.7%-55.1%), and 55% (95% CI: 49.5%-60.5%), respectively. Five-year survival estimates were 39.1% (95% CI: 34.8%-43.4%), 39.8% (95% CI: 36.1%-43.5%), and 45% (95% CI: 37.9%-52.1%) in the 1980s, 1990s, and 2000s, respectively. There was no significant difference in survival among the three periods (P = 0.084 for overall survival, and P = 0.150 for 5-year survival); however, the survival rate in the 2000s was remarkably higher than that of the 1980s

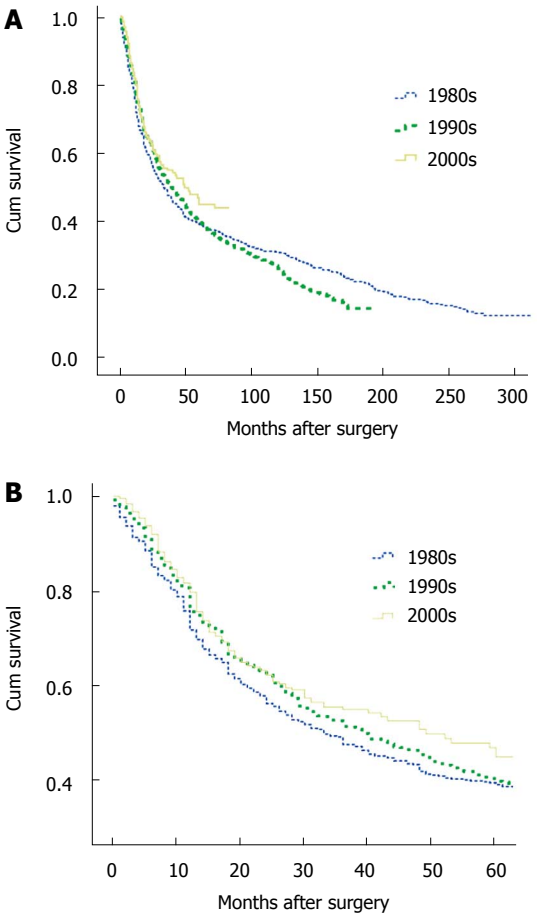

Figure 1 Kaplan-Meier survival curves for patients with gastric cancer by decade. A: Overall survival for gastric cancer patients; B: Five-year survival for gastric cancer patients.

(P = 0.019 for overall survival, and P = 0.027 for 5-year survival). Kaplan-Meier survival curves for patients with gastric cancer, by decade, are shown in Figure 1.

For those patients who had undergone resection with curative intent, the median survival of patients was 85 mo in the 1980s, 58 mo in the 1990s, and 72 mo in the 2000s, respectively The 3-year survival for patients in the 1980s, 1990s, and 2000s were 65.3% (95% CI: 60.0%-70.6%), 60.8% (95% CI: 56.5%-65.1%), and 61.0% (95% CI: 55.3%-66.7%), respectively. Five-year survival estimates were 55.6% (95% CI: 50.1%-61.1%), 48.9% (95% CI: 44.6%-53.2%), and 50.4% (95% CI: 42.8%-58.0%) in the 1980s, 1990s, and 2000s, respectively. There was no significant difference in survival among the three time periods (P = 0.169 for 5-year survival).

The distributions of patient characteristics by decade are shown in Table 1. Significant changes were detected in all areas except in the distribution of number of involved lymph nodes, hepatic metastasis and type of gastrectomy during the 24 years studied (P = 0.072, 0.244 and 0.073, respectively). The median age was 56 in the 1st period, 60 in the 2nd period, and 58 in the 3rd period. There was also a change in male to female ratio from 4:1 to 7:3. Among tumor factors, whole stomach tumors decreased from 12% to 5%, while T1 stage tumor and node negative

Table 1 Characteristics of population from the three periods (*n* = 1604)

Characteristics	1980s (*n* = 496)	1990s (*n* = 673)	2000s (*n* = 435)	*P* value
Age (yr)				0.000
Median	56	60	58	
Sex (%)				0.000
Male	397 (80)	469 (70)	306 (70)	
Female	99 (20)	204 (30)	129 (30)	
Number of lymph nodes removed				0.000
Mean	12	12	18	
Number of involved lymph nodes				0.072
Mean	2	2	3	
Tumor size (cm)				0.000
Median	6	5	5	
Site of tumor (%)				0.000
Whole stomach	60 (12)	49 (7)	23 (5)	
Upper stomach	55 (11)	80 (12)	44 (10)	
Middle stomach	39 (8)	69 (10)	49 (11)	
Lower stomach	213 (43)	298 (44)	254 (58)	
> 2/3 stomach	129 (26)	177 (26)	65 (15)	
Pathological tumor stage (%)				0.003
T1	99 (20)	148 (22)	104 (24)	
T2	179 (36)	215 (32)	121 (28)	
T3	154 (31)	242 (36)	178 (41)	
T4	64 (13)	68 (10)	32 (7)	
Pathological nodal stage (%)				0.006
N0	159 (32)	195 (29)	152 (35)	
N1	193 (39)	269 (40)	161 (37)	
N2	84 (17)	155 (23)	94 (22)	
N3	60 (12)	54 (8)	28 (6)	
TNM stage (%)				0.000
Ⅰ A	47 (10)	58 (9)	36 (8)	
Ⅰ B	62 (13)	102 (15)	98 (23)	
Ⅱ	81 (16)	168 (25)	100 (23)	
Ⅲ A	108 (22)	149 (22)	86 (20)	
Ⅲ B	73 (15)	71 (11)	42 (10)	
Ⅳ	125 (25)	125 (19)	73 (17)	
Gross type (%)				0.000
Borrmann Ⅰ	15 (3)	5 (1)	2 (1)	
Borrmann Ⅱ	158 (35)	97 (16)	44 (11)	
Borrmann Ⅲ	227 (50)	439 (71)	311 (77)	
Borrmann Ⅳ	50 (11)	79 (13)	47 (12)	
Surgery (%)				0.000
Absolutely curative	277 (56)	357 (53)	172 (40)	
Relatively curative	47 (10)	163 (24)	225 (52)	
Palliative	172 (35)	153 (23)	38 (9)	
Lymph node dissection (%)				0.000
D1	51 (10)	52 (8)	46 (11)	
D2	188 (38)	399 (59)	347 (80)	
D3	90 (18)	73 (11)	16 (4)	
Palliative resection	167 (34)	149 (22)	26 (6)	
Complication (%)				0.001
Intestinal obstruction	16 (3)	7 (1)	11 (3)	
Anastomotic leakage	10 (2)	12 (2)	0 (0)	
Pneumonia	3 (1)	3 (0)	1 (0)	
Abdominal abscess	8 (2)	11 (2)	7 (2)	
Anemia	3 (1)	1 (0)	4 (1)	
Other	7 (1)	12 (2)	22 (5)	
Hepatic metastasis (%)	21 (4)	21 (3)	10 (2)	0.244
Peritoneum metastasis (%)	67 (14)	50 (7)	25 (6)	0.000
Adjunctive therapy (%)	0 (0)	41 (6)	153 (35)	0.000
Type of gastrectomy (%)				0.073
Total	94 (19)	95 (14)	76 (17)	
Subtotal	402 (81)	578 (86)	359 (83)	
Combined organ resection				0.000
Pancreas or spleen	53 (11)	56 (8)	18 (4)	
Liver or gall	11 (2)	12 (2)	19 (4)	
Transverse colon	20 (4)	51 (8)	52 (12)	
Other	15 (3)	20 (3)	12 (3)	

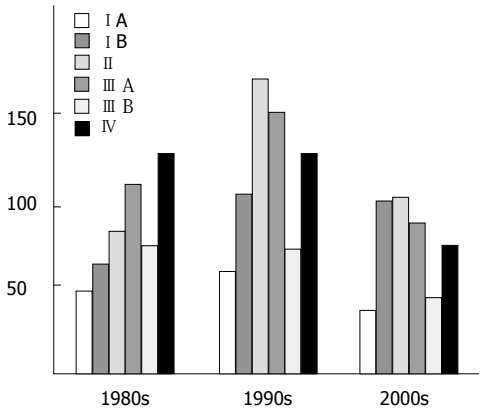

Figure 2 The shift in the distribution of TNM stage of disease at diagnosis.

cancers were found more frequently in the later periods, though this may not be significant, as early gastric cancers increased only from 20% to 24%. Curative resection rates were markedly increased during the 24-year period. Most recently, the curative resection rate was 92% (Table 1). Most cases were diagnosed at advanced stages (T2-T4, N1-N3) throughout the 2-decade period. Figure 2 shows the shift in the distribution of TNM stage of disease at diagnosis.

The operative mortality rate in the 1st period was 2% and 1.3% in the 2nd period, whereas it was less than 1% in the 3rd period. The multivariate Cox proportional hazards models for gastric cancer are shown in Table 2. In the Cox model for gastric cancer, adjusting for sixteen variables, there was no significant association between the decade of diagnosis and patient survival ($P = 0.385$). For the decade of the 1990s relative to the 1980s, the hazard ratio for gastric cancer cases was 1.025 (95% CI: 0.807-1.301), and for the decade of the 2000s relative to the 1980s, the hazard ratio was 0.914 (95% CI: 0.674-1.241). Patient survival was significantly associated with surgical extent ($P = 0.000$). Cases involving curative surgery were associated with prolonged survival. Figure 3 illustrate the Kaplan-Meier survival curves of patients with gastric cancer, by surgical intervention (absolutely curative, relatively curative, and palliative). Stage-by-stage comparison was performed among the 3 periods; for the Ⅱ stage patients the survival rate of 1990s was significantly worse than that of the 1980s.

DISCUSSION

In this study of gastric cancer diagnosed in Northeast China, we found that the median survival of patients with gastric cancer actually appeared to increase between 1980 and 2000. There was a significant change in patient survival in the 2000s compared to that in the 1980s, and decade of diagnosis was not significantly associated with

Table 2 HR for death in population (n = 1604) - univariable and multivariable analyses

	Univariable analyses		Multivariable analyses	
	HR (95% CI)	*P*	HR (95% CI)	*P*
Age (yr)		0		0.005
≤ 55	1 (Ref)		1 (Ref)	
> 55	1.383 (1.222-1.565)	0	1.240 (1.036-1.484)	0.019
Sex		0.734		0.582
Male	1 (Ref)		1 (Ref)	
Female	0.977 (0.855-1.117)	0.734	1.030 (0.854-1.241)	0.760
Tumor size		0		0.024
≤ 5 cm	1 (Ref)		1 (Ref)	
5-7 cm	1.782 (1.534-2.071)	0	1.250 (1.006-1.553)	0.044
> 7 cm	2.587 (2.244-2.982)	0	1.211 (0.956-1.533)	0.113
Tumor site		0		0.071
Whole stomach	1 (Ref)		1 (Ref)	
Upper stomach	0.625 (0.489-0.798)	0	1.247 (0.825-1.886)	0.295
Middle stomach	0.358 (0.273-0.471)	0	0.844 (0.541-1.315)	0.453
Lower stomach	0.401 (0.327-0.490)	0	0.846 (0.574-1.247)	0.399
> 2/3 stomach	0.588 (0.475-0.727)	0	0.845 (0.590-1.210)	0.358
Gross appearance		0		0.002
Borrmann types Ⅰ	1 (Ref)		1 (Ref)	
Borrmann types Ⅱ	0.541 (0.338-0.867)	0.011	0.405 (0.179-0.915)	0.030
Borrmann types Ⅲ	0.807 (0.510-1.278)	0.361	0.626 (0.284-1.381)	0.246
Borrmann types Ⅳ	1.357 (0.841-2.191)	0.211	1.328 (0.317-1.672)	0.454
Tumor stage		0		0.408
T1	1 (Ref)		1 (Ref)	
T2	3.336 (2.542-4.377)	0	1.458 (0.797-2.667)	0.221
T3	4.275 (3.218-5.678)	0	1.780 (0.510-2.287)	0.841
T4	7.873 (5.689-10.894)	0	1.991 (0.459-2.590)	0.844
Lymph-node stage		0		0.874
N0	1 (Ref)		1 (Ref)	
N1	2.980 (2.411-3.682)	0	1.176 (0.913-2.073)	0.127
N2	3.430 (2.754-4.271)	0	1.443 (0.595-1.826)	0.883
N3	5.174 (3.934-6.804)	0	1.756 (0.324-2.766)	0.518
TNM stage		0		0.019
ⅠA	1 (Ref)		1 (Ref)	
ⅠB	1.920 (1.381-2.671)	0	0.642 (0.288-1.431)	0.279
Ⅱ	2.645 (1.938-3.608)	0	0.779 (0.293-2.068)	0.616
ⅢA	4.306 (3.171-5.847)	0	1.303 (0.429-3.961)	0.641
ⅢB	4.847 (3.510-6.693)	0	1.459 (0.409-5.204)	0.561
Ⅳ	8.982 (6.613-12.199)	0	3.286 (0.779-13.857)	0.105
Surgery		0		0
Absolutely curative	1 (Ref)		1 (Ref)	
Relatively curative	1.907 (1.631-2.230)	0	1.372 (1.114-1.690)	0.003
Palliative	4.368 (3.782-5.044)	0	3.361 (1.752-6.448)	0
Lymph node dissection		0		0.867
D1	1 (Ref)		1 (Ref)	
D2	0.931 (0.750-1.155)	0.515	1.169 (0.821-1.664)	0.387
D3	0.807 (0.616-1.058)	0.121	1.146 (0.738-1.780)	0.543
Palliative resection	3.236 (2.573-4.070)	0	1.359 (0.457-1.612)	0.636
Joint organ removal		0		0.007
None	1 (Ref)		1 (Ref)	
Pancreas or spleen	1.997 (1.640-2.433)	0	1.086 (0.709-1.372)	0.933
Liver or gall	1.514 (1.029-2.227)	0.035	1.108 (0.649-1.891)	0.707
Transverse colon	2.093 (1.699-2.579)	0	1.466 (1.107-1.942)	0.008
Other	2.453 (1.797-3.350)	0	1.008 (0.586-1.731)	0.978
Gastrectomy		0		0.512
Total	1 (Ref)		1 (Ref)	
Subtotal	0.573 (0.494-0.664)	0	0.912 (0.693-1.200)	0.511
Hepatic metastasis		0		0.796
No	1 (Ref)		1 (Ref)	
Yes	4.002 (2.991-5.354)	0	1.285 (0.403-1.529)	0.476
Peritoneum metastasis		0		0.947
No	1 (Ref)		1 (Ref)	
Yes	2.835 (2.359-3.406)	0	1.127 (0.382-1.381)	0.329
Complication		0.41		0.157
None	1 (Ref)		1 (Ref)	
Intestinal obstruction	0.796 (0.522-1.216)	0.292	1.311 (0.670-2.565)	0.428
Anastomotic leakage	1.474 (0.946-2.294)	0.086	1.893 (0.904-3.965)	0.091
Pneumonia	1.069 (0.400-2.856)	0.894	1.207 (0.323-4.525)	0.929
Abdominal abscess	1.212 (0.795-1.850)	0.372	1.295 (0.700-2.397)	0.410
Anaemia	0.487 (0.157-1.514)	0.214	0.479 (0.116-1.980)	0.309
Other	0.702 (0.421-1.172)	0.176	0.488 (0.240-0.992)	0.047
Adjunctive therapy		0.022		0.364
No	1 (Ref)		1 (Ref)	
Yes	0.744 (0.577-0.959)	0.022	0.850 (0.643-1.124)	0.254
Diagnosis period		0.395		0.385
1980s	1 (Ref)		1 (Ref)	
1990s	1.072 (0.937-1.226)	0.311	1.025 (0.807-1.301)	0.840
2000s	0.884 (0.735-1.063)	0.191	0.914 (0.674-1.241)	0.565

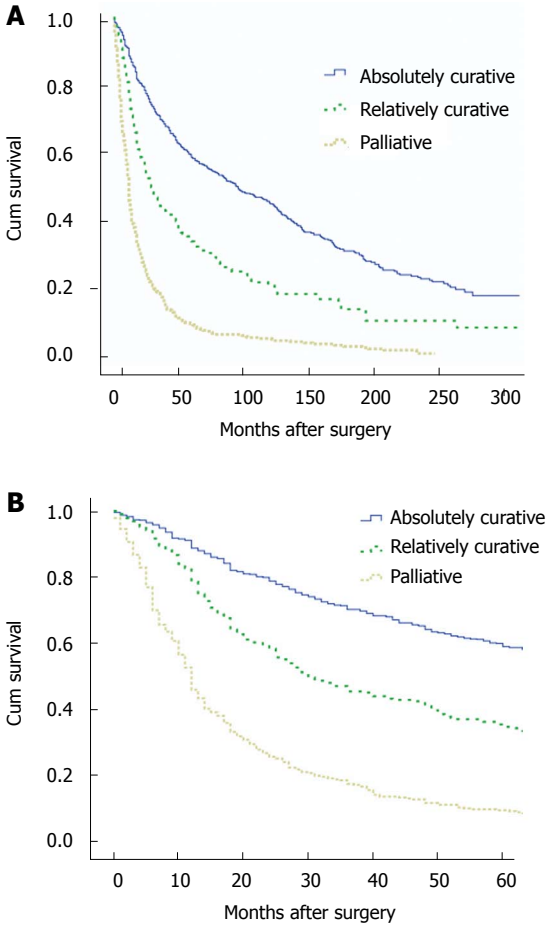

Figure 3 Kaplan-Meier survival curves of patients with gastric cancer, by surgical intervention (absolutely curative, relatively curative, palliative). A: Overall survival for gastric cancer patients; B: Five-year survival for gastric cancer patients.

patient survival for gastric cancer during the 24 years. The improving prognosis of gastric cancer in recent years has been reported[12-18]. For example, the 5-year relative survival rate for gastric cancer increased from 13% to 18% in Sweden from 1960-1964 to 1985-1986[13]. However, for those patients who had a curative intent resection, there was no significant difference in survival between 1980 and 2000. As the patients operated on for palliative purpose decreased with percentages over time, it is possible that the inclusion of these patients could explain

some of the trend of increasing survival between 1980 and 2000.

In this study, we found that the proportion of patients with early gastric cancer increased to 24% in the 3rd period. With the increasing incidence of early gastric cancer, node negative cancers were also increased; however, as the increasing proportions were too little and may not be significant, there were still too many patients diagnosed at an advanced stage. We also found that the incidence among women increased in the later period from 20% to 30%. We speculate that this may be due to the changes in lifestyle of Chinese women, such as more smoking and greater alcohol intake, suggesting that we should strengthen the primary prevention of gastric cancer. Currently, a surgical cure remains the only intervention that may significantly improve a patient's chance of survival; moreover, surgery is the only treatment modality offering hope for a cure. Nevertheless, most patients die from locoregional recurrence or distant metastasis even after curative surgery for advanced stage cancers[19]. As metastasis to lymph nodes is linked to the outcome, extensive lymph node dissection is a statistically favorable prognostic factor[20]. Without surgical intervention, 2-year survival for patients with gastric cancer remained essentially zero[21]. Baba *et al*[22] reported that the rate of recurrence was higher in patients treated with dissection of group 1 lymph nodes than for those with dissection of group 2 or 3 nodes. In our study, 84% of the patients were treated with D2 or D3 lymph node dissection in the later period, which was much more than in the previous two periods. Besides the increase of early gastric cancer patients, advances in treatment factors mostly contributed to the improved survival.

Early diagnosis has markedly improved the survival of patients with gastric cancer, and mass screening has a definite role in diagnosing gastric cancer in its early stages[23]. In Japan, where the incidence of gastric cancer is high, survival of patients with gastric cancer does seem to be improving. This improvement appears to be the result, at least in part, of the frequent diagnosis of early-stage gastric cancer in a mass population screening program in Japan[24]. The high curative resectability rate in the screened group is related to a smaller tumor size and to a lower incidence of lymph node metastasis, liver metastasis and peritoneal dissemination than in the non-screened group. Depth of tumor invasion, lymph node involvement and distant metastasis are important prognostic factors according to the UICC/AJCC staging system of gastric cancer[25]; therefore, every attempt should be made to increase early diagnosis. Currently, gastric cancer is one of the most prevalent cancers in China, making endoscopic or radiologic examinations more common. Awareness among Chinese has also increased, similar to colorectal or prostate cancer in Western countries[26,27]. However, because of the huge rural population whose diseases are often diagnosed at a more advanced stage, current efforts at cancer prevention and early screening of high-risk populations for premalignant lesions have not resulted in a significant change in the stage of presentation of disease. Following the mass population screening being carried out widely in China, especially these past ten years, the proportion of early gastric cancer has increased very slowly.

Another important change observed in this study was the decreased prevalence of operative mortality. Although the surgical extent of recent years was more extensive than before, postoperative mortality in the 3rd period was decreased to less than 1%. The large decrease in operative mortality is due to improved surgical techniques and also to improvements in anesthesia, metabolic care and intravenous nutrition[28]. Besides these factors, the accumulation of treatment experience with gastric cancer and becoming a large volume hospital also have an impact on the improved treatment outcome of gastric cancer patients[29-34]. The specialization in gastric cancer treatment might also influence the lower mortality rate, especially in addition to technical advances.

Therefore, currently in China, we need increased efforts at refining prevention and early diagnosis of gastric cancer as only resection offers the best hope for a cure[21]. We should put emphasis on rural gastric cancer screening, improve the rural level of diagnosis and treatment, and progress the health education of gastric cancer-related knowledge.

COMMENTS

Background

Although the results of gastric cancer have improved due to early diagnosis, radical operation, and the development of adjuvant therapy, patients with gastric cancer still have poor prognosis. To describe survival trends in patients in Northeast China diagnosed as gastric cancer, the authors conducted a review of records of gastric cancer patients from 1980 to 2003 in the First Affiliated Hospital of China Medical University.

Research frontiers

Currently, gastric cancer in China is one of the most prevalent cancers, making endoscopic or radiologic examinations more common. However, because of the huge rural population whose diseases are often diagnosed at a later stage, current efforts at cancer prevention and early screening of high-risk populations for premalignant lesions have not resulted in a significant change in the stage of presentation of disease, and following the mass population screening being carried out widely in China, especially these ten years, the proportion of early gastric cancer has increased very slowly.

Innovations and breakthroughs

In this article, the authors found that there was no significant difference of survival among the three periods. Besides that, they also found that the proportion of patients with early gastric cancer increased in the later period, as well as the proportion of node negative cancers, and the incidence among women increased from 20% to 30%.

Applications

All the findings indicate that, nowadays in China, the authors need increased efforts at refining primary prevention and early diagnosis of gastric cancer as only resection offers the best hope for cure, and they should put emphasis on rural gastric cancer screening, improve the rural level of diagnosis and treatment, and improve health education with regard to gastric cancer-related knowledge.

Peer review

Dr. Zhang *et al*. described survival trends of gastric cancer among Chinese between 1980 and 2003. Although there is no significant change in survival between these periods, the authors observed a significantly increased survival rate in 2000s as compared to that of 1980s. Results of this study were supported by many previous studies. Strengths of this study are (1) a large sample size; and (2) long follow-up data.

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CASE REPORT

Clinical experience of Pseudo-Meigs' Syndrome due to colon cancer

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Abstract

We report a rare case of Pseudo-Meigs' Syndrome caused by ovarian metastasis from sigmoid colon cancer, which was accompanied by peritoneal dissemination. A 58-year-old female patient presented with massive right pleural effusion, ascites and a huge pelvic mass. Under the diagnosis of an advanced ovarian tumor, bilateral oophorectomy was performed and sigmoidectomy was also carried out after intraoperative diagnosis of peritoneal dissemination involving the sigmoid colon. However, immunohistochemical staining revealed that the ovarian lesions were metastasis from the primary advanced colon cancer. Postoperatively, ascites and pleural effusion subsided, and the diagnosis of Pseudo-Meigs' Syndrome due to a metastatic ovarian tumor from colon cancer was determined. The patient is now undergoing a regimen of chemotherapy for colon cancer without recurrence of ascites or hydrothorax 10 mo after the surgery. Pseudo-Meigs' Syndrome due to a metastatic ovarian tumor from colon cancer is rare but clinically important because long-term alleviation of symptoms can be achieved by surgical resection. This case report suggests that selected patients, even with peritoneal dissemination, may obtain palliation from surgical resection of metastatic ovarian tumors.

Key words: Pseudo-Meigs' Syndrome; Colon cancer; Ovarian tumor; Metastasis; Ascites

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INTRODUCTION

Meigs' Syndrome is characterized by pleural effusion and ascites associated with ovarian fibroma or fibroma-like tumors, which are relieved by resection of the responsible lesions. When associated with other types of ovarian tumor, the condition is termed Pseudo-Meigs' Syndrome[1,2]. Malignancy from the gastrointestinal tract, including colon cancer, is a rare etiology for this syndrome and only a few cases have been reported[3-7]. This case report documents our clinical experience, reviews previously reported cases and discusses

the diagnosis and treatment of Pseudo-Meigs' Syndrome caused by ovarian metastasis from colorectal cancer.

CASE REPORT

A 58-year-old female presented to the hospital with a 1-mo history of general fatigue, dyspnea, abdominal distention, loss of appetite and decreased urine volume, which had worsened progressively over the previous few days. Her past medical history was unremarkable. Physical examination found a large mass in her lower abdomen. The peripheral blood test showed elevated levels of carcinoembryonic antigen (CEA): 63.8 ng/mL (normal, < 5 ng/mL), and carbohydrate antigen (CA) 125: 921 U/mL (normal, < 45 U/mL) and a normal level of CA19-9: 2 U/mL (normal, < 37 U/mL). Enhanced computed tomography (CT) demonstrated fluid retention and a round mass with a maximum diameter of 15 cm in the pelvic cavity (Figure 1A). Peritoneal dissemination was highly suspected (Figure 1B), and further examination of the ascites was not performed. The massive right pleural effusion occupied the pleural cavity causing atelectasis and compression of the mediastinum to the left side (Figure 1C). Intermittent drainage of pleural effusion was performed to alleviate severe dyspnea, yielding 4000 mL of serous fluid without malignant cells on cytological examination over 3 d. The patient's poor general condition and refusal prevented us from further examination including routine gastrointestinal endoscopy, and advanced ovarian cancer was tentatively diagnosed. Subsequent palliative surgery disclosed an enlarged right ovarian tumor and sparsely distributed peritoneal dissemination with mid-sigmoid colon involvement. In addition to bilateral oophorectomy, sigmoidectomy and primary anastomosis were carried out in case of colonic obstruction.

Histopathological examination of resected specimens showed that both the ovarian and colonic lesions were composed of well-differentiated adenocarcinoma (Figure 2A and B). The dissected paracolic nodes showed positive malignant cells. However, immunohistochemical staining of cytokeratin 7, mucin-5AC and CA125 was negative and that of cytokeratin 20, CEA and CDX-2 was positive (Figure 2C and D), confirming the ovarian tumors were metastases from primary colon cancer[8]. The postoperative course was uneventful and ascites and pleural effusion subsided. 5-fluorouracil (5-FU), leucovorin and oxaliplatin (FOLFOX) were administered every 2 wk for 5 mo and then changed to 5-FU, leucovorin and irinotecan (FOLFILI) due to neuropathy. Ten months after the surgery, tumor markers were within the normal range and abdominal CT showed no sign of tumor growth or recurrent fluid retention either in the pleural or abdominal cavity. She did not experience a marked change in bowel habit after the surgery and during chemotherapy. The diagnosis of Pseudo-Meigs' Syndrome due to metastatic ovarian tumor from colon cancer was determined.

DISCUSSION

Pseudo-Meigs' Syndrome caused by ovarian metastasis from colon cancer is a rare condition. In 2000, Nagakura *et al*[3] reviewed 5 cases including 3 Japanese cases. Thereafter only 4 subsequent reports of Pseudo-Meigs' Syndrome with the same etiology were reported (Table 1). The age of the patients was relatively low considering that the mean age of occurrence of colorectal cancer is approximately 64-71 years[9,10]. Pleural effusion is more prevalent in the right side, and the ovarian tumor is usually large (mean diameter 16 cm) and predominantly bilateral.

The diagnosis of Pseudo-Meigs' Syndrome is difficult because of its rarity and the similarity of the symptoms and signs to those of terminal malignant disease. Negative cytological results of ascites and pleural effusion might be informative for differentiating between Pseudo-Meigs' Syndrome and advanced cancer. Ascites due to peritoneal dissemination or peritonitis carcinomatosa is generally refractory to surgical or medical treatment, rendering sur-

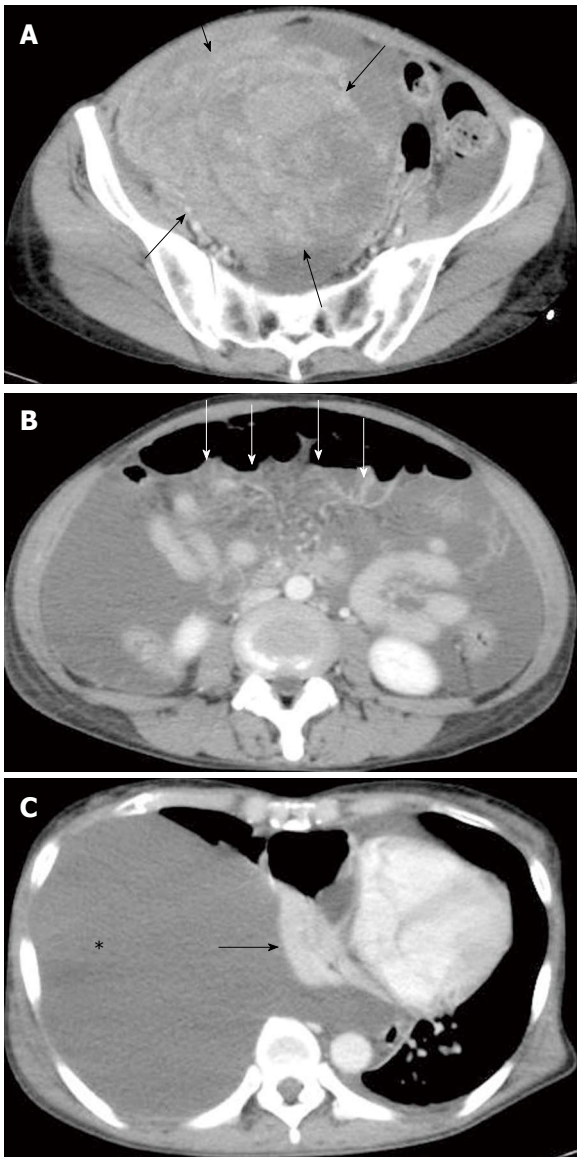

Figure 1 Computed tomography. A: A huge round mass in the pelvis suggesting a bilateral ovarian tumor (black arrows); B: Massive ascites and thickness of omentum (white allows) suggesting peritoneal dissemination; C: Right pleural effusion with atelectasis (black allow) and left-side compression of the mediastinum.

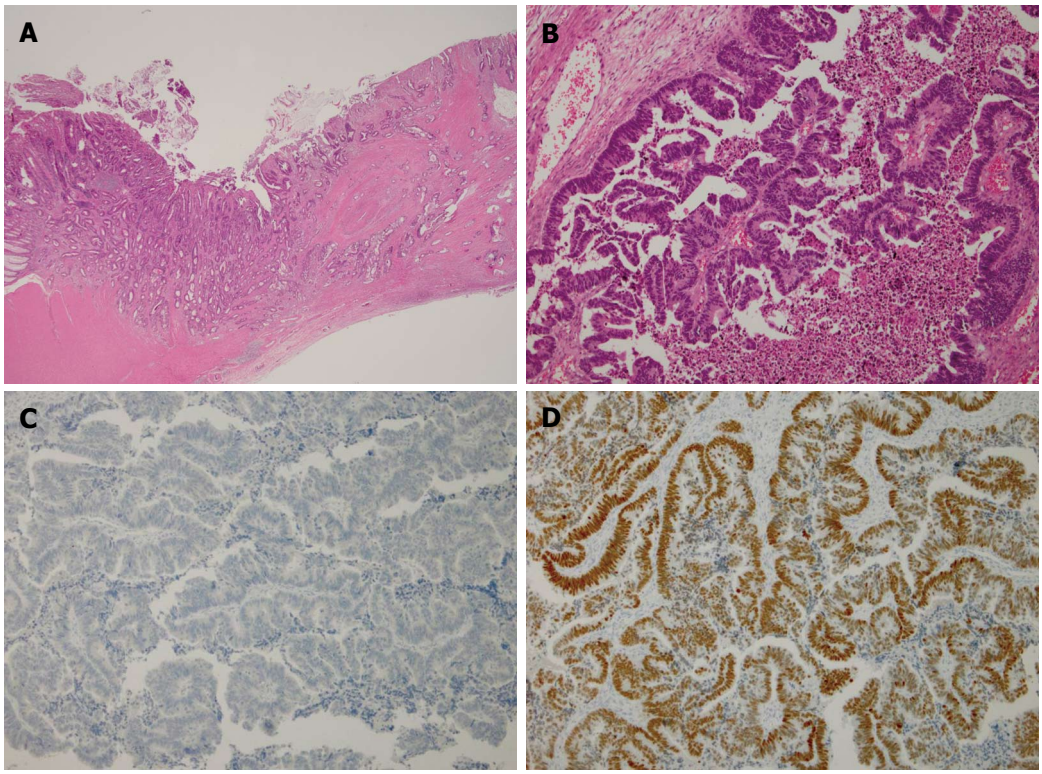

Figure 2 Microscopic features. A: Well-differentiated adenocarcinoma compatible with primary colon cancer; hematoxylin and eosin staining; B: The ovarian tumor is composed of tumor cells compatible with metastasis of colon cancer; C: Cytokeratin 7 showing negative result of staining in the ovarian tumor; D: CDX-2 showing staining in tumor cells in the ovary.

Table 1 Summary of reported cases of Pseudo-Meigs' Syndrome resulting from ovarian metastasis of colorectal cancer

Reference	Age (yr)	Onset of syndrome	Site of pleural effusion	Synchronous metastasis	Diameter of ovarian tumor (cm)	Site of ovarian tumor	Long-term outcome
Nagakura *et al*[3]	35	Metachronous	Right	None	15	Unilateral	108 mo, alive
Nagakura *et al*[3]	40	Synchronous	Right	None	Not given	Bilateral	1.5 mo, alive
Nagakura *et al*[3]	39	Synchronous	Bilateral	None	24	Unilateral	12 mo, alive
Nagakura *et al*[3]	75	Synchronous	Right	Peritoneum	21	Unilateral	Not given
Nagakura *et al*[3]	53	Synchronous	Right	None	18	Bilateral	52 mo, alive
Feldman *et al*[4]	49	Metachronous	Left	None	13	Right	6 mo, alive
Ohsawa *et al*[5]	41	Synchronous	Bilateral	Peritoneum	16	Bilateral	10 mo, died
Rubinstein *et al*[6]	61	Synchronous	Bilateral	None	13	Bilateral	Not given
Okuchi *et al*[7]	42	Synchronous	Right	Liver, lung	11.5	Left	12 mo, died
Present case	58	Synchronous	Right	Peritoneum	15	Bilateral	10 mo, alive

gical intervention for patients with this condition mostly harmful. Nevertheless, a negative result of cytology or peritoneal dissemination is not necessarily a requirement for the diagnosis of Pseudo-Meigs' Syndrome. Three out of 10 reported cases of Pseudo-Meigs' Syndrome due to metastatic colorectal cancer had peritoneal dissemination (Table 1).

Recently, an elevated level of vascular endothelial growth factor (VEGF) in blood and ascites were reported in a patient with Pseudo-Meigs' Syndrome due to colon cancer[7]. In that case, the hypersecretion of VEGF from oviducts was considered to play a key role in the pathogenesis of ascites and pleural effusion[7]. VEGF levels in blood and ascites might be useful for determining which patients would recover from massive fluid retention by surgical resection of metastatic ovarian tumors, even with a positive cytological result of ascites.

In the cases reviewed, the postoperative course was uneventful, symptoms were rapidly relieved, and palliation of the symptoms was long lasting[3-7]. A study from a single institute in Japan reported that the 3-year survival-rate of 5 cases of pseudo-Meigs' Syndrome was 37.5%[11]. However, surgeons should be prudent in the use of surgery. In general, colon cancer with ovarian metastasis is hard to cure[12]. Colon cancer with ascites is associated with frequent postoperative complications[13]. In addition, publication bias must be considered when interpreting rare conditions. Without obvious evidence, a regional lymphadenectomy should be reserved for selected patients, and oophorectomy with colorectal resection with minimal invasion should be the first choice surgical strategy.

In conclusion, ovarian metastasis from sigmoid colon cancer is a potential cause of Pseudo-Meigs' Syndrome. Surgical resection can be the treatment of choice for a selected patient even with the apparent peritoneal dissemination, because it can provide long-term palliation.

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CASE REPORT

Duodenal pseudolymphoma: A case report and review of literature

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Abstract

We report a rare case of duodenal pseudolymphoma without any symptoms. The lesion located in front of the head of the pancreas was found accidentally during a medical examination. The findings of computed tomography and positron emission tomography-computed tomography suggested a stromal tumor or malignant lymphoma. Surgical resection was performed. The lesions were pathologically diagnosed as duodenal pseudolymphoma.

Key words: Pseudolymphoma; Duodenum

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INTRODUCTION

Pseudolymphoma is rare, and is thought to be a benign nodule lesion characterized by marked proliferation of polyclonal and non-neoplastic lymphoid cells forming follicles and active germinal centers. It is difficult to differentiate pseudolymphoma from a malignant tumor, and the accurate diagnosis of pseudolymphoma depends on histopathological examinations. Pseudolymphoma may be found in various organs, including the skin, orbit, thyroid,breast, lung, stomach, liver, small intestine, spleen, pancreas, kidney, uterus and testis[1,2]. However, to our knowledge, duodenal pseudolymphoma has never been reported before. We report a case of duodenal pseudolymphoma and review the literature below.

CASE REPORT

A 23-year-old woman underwent a routine medical examination, during which a space-occupying lesion was found in front of the head of the pancreas by ultrasonography (USG) on April 28, 2010. Computed tomography (CT) performed on May 4th revealed several isodense masses in front of the pancreatic head. The largest one was 30 mm in diameter and well-defined, and the surrounding pancreas, pylorus and duodenal bulb appeared compressed. There was no significant invasion, and it was strongly enhanced after injection of contrast medium. Pancreas was of uniform density without a significant space-occupying lesion. A para-aortic lymph node was seen, approximately 5 mm × 8 mm in size, well-defined, moderately enhanced in arterial phase. The CT scan diagnosis was metastatic stromal tumor (Figure 1A and B); white blood cell count 7.5×10^9/L and hemoglobin 92 g/L, and platelets 419×10^9/L; carcinoembryonic an-

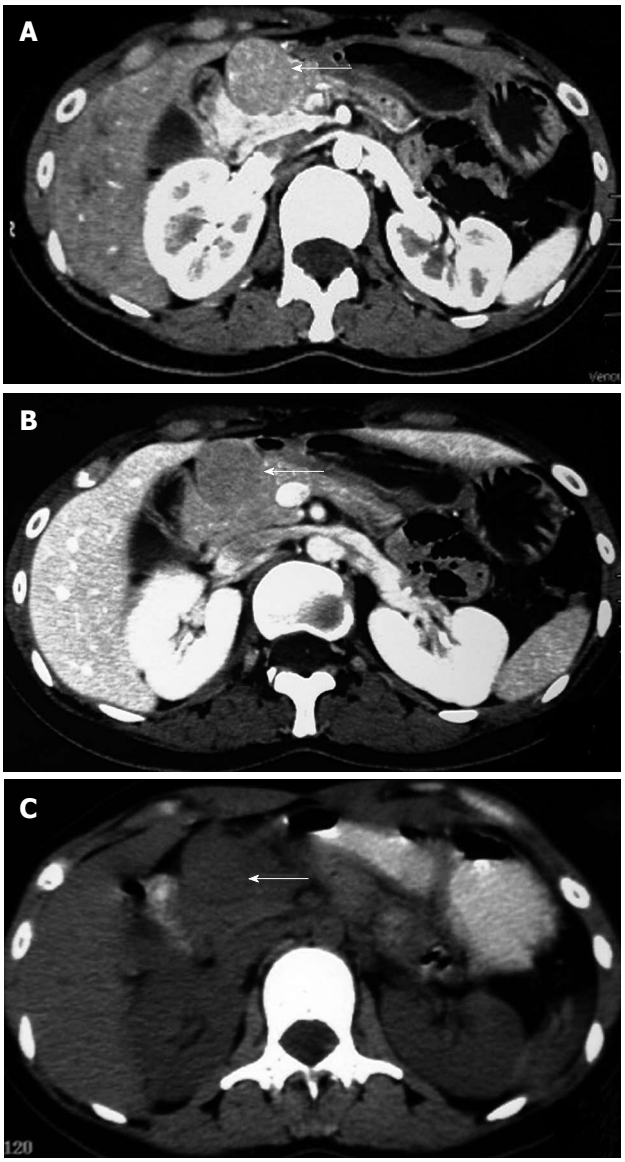

Figure 1 The abdominal imaging examination. A, B: Abdominal computed tomography (CT) showing a lesion in front of the pancreatic head; C: Positron emission tomography-CT showing a lesion in front of the pancreatic head.

tigen 0.58 ng/mL (normal, 0.00-5.00) and carbohydrate antigen 8.64 U/mL (normal, 0.00-5.30). The patient was asymptomatic and physical examination revealed no abnormalities. She was admitted to our hospital for further treatment on May 17th, and laboratory data on admission including liver function, renal function and electrolyte tests were all unremarkable. PET-CT performed on May 25th demonstrated multiple low-density nodules behind the gastric antrum and in front of the pancreas head. PET regions showed a non-uniform abnormal focus of uptake. Standard uptake value (SUV) was about 2.9-5.8. The pancreas, skeleton, muscle and tissue showed no obvious abnormalities. There was no retroperitoneal and pelvic lymph node enlargement. PET-CT diagnosis was stromal tumor or malignant lymphoma (Figure 1C).

Laparotomy was performed on May 26th. The tumor was found located in the duodenal bulb with extended

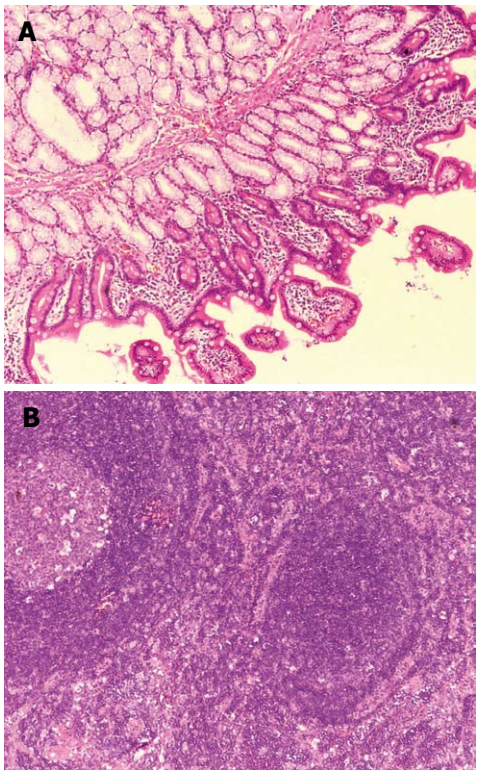

Figure 2 The histological examination. A: Duodenal pseudolymphoma was confirmed by histopathologic examination, HE × 100; B: Lymph nodes showing reactive lymphoid hyperplasia, HE × 100.

growth from the back to the bottom of the intestinal wall, and firmly attached to the head of the pancreas and the root of the mesocolon. There were several hard enlarged lymph nodes located near the root of middle colic vessel and behind the head of pancreas about 10-30 mm in diameter. Lymph nodes near the transverse colon were resected for frozen biopsy, which showed that lymph nodes had chronic inflammation and lymphoid hyperplasia. Due to the limitation of the frozen biopsy and the general form of the lymph nodes and the tumor growth pattern, we could not completely rule out the possibility of malignancy. Thus the complete resection of the tumor and enlarged lymph nodes was performed. The postoperative pathological examination report suggested lymph nodes with chronic inflammation and lymphoid hyperplasia. Duodenal pseudolymphoma was confirmed (Figure 2A and B). Immunohistochemical staining for CD79a, L-26, CD3 and UCHL-1 was positive, and for TDT it was negative (Figure 3A-E). The patient was discharged 10 d after surgery and showed no sign of recurrence in the follow-up of five months.

DISCUSSION

Pseudolymphoma, also named reactive lymphoid hyperplasia, was first described in 1963 by Saltzsein[3], and since then cases of pseudolymphoma of different organs have been successively reported although it is a rare disease.

At present, the exact pathogenesis of pseudolymphoma remains unknown. It has been reported that drugs (Phenyt-

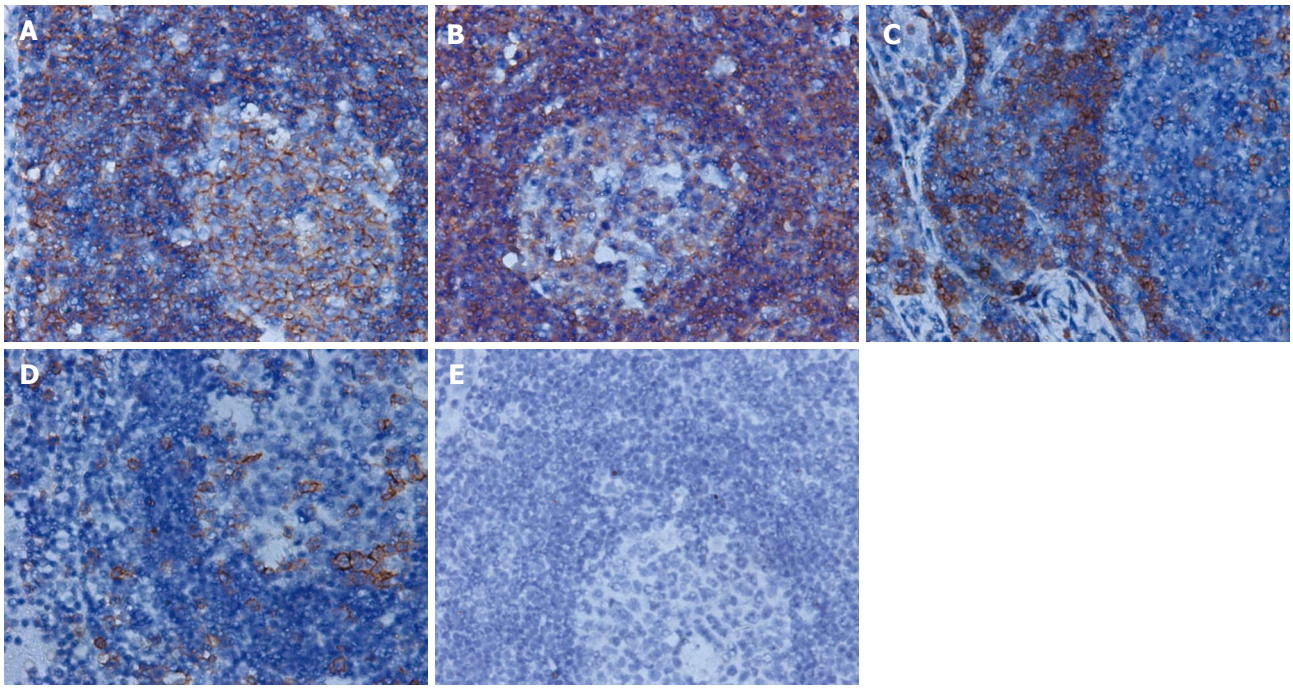

Figure 3 The immunohistochemical finding of the lesion. A, B: Immunohistochemical study revealed follicles mainly consisting of L-26 and CD79a positive B cells, × 400; C, D: Interfollicular distributed CD3 and ubiquitin carboxyl-terminal hydrolase L1 positive T cells, × 400; E: Negative staining for terminal deoxynucleotidyl transferase, × 400.

oin, Carbamazepine, Felodipine), foreign antigens (trauma, tattoo dyes and immunization), infection and sunlight allergy can cause cutaneous pseudolymphoma[4]. Autoimmune diseases (Sjogren's syndrome and thyroiditis) are thought to be associated with pulmonary and hepatic pseudolymphoma[5]. *Helicobacter pylori* infection plays an important role in the etiology of gastric pseudolymphoma[6].Our patient had no obvious predisposing factors. She was asymptomatic and physical examination result was not remarkable. It was accidentally discovered during a routine USG. Therefore, more data are needed for further studies on the risk factors and onset mechanism of pseudolymphoma.

The preoperative diagnosis of pseudolymphoma is difficult. It should be differentiated from malignant lymphoma and other malignant tumors. Gastric pseudolymphoma is easily misdiagnosed as gastric cancer on barium meal and gastroscopy examinations. Radiologically, hepatic pseudolymphoma has been often misdiagnosed as hepatocellular carcinoma. The diagnosis of our case on imaging studies could not be discriminated from stromal tumor and malignant lymphoma and a final diagnosis relied on the pathological examinations. Microscopically, pseudolymphoma is characterized by the presence of hyperplastic lymphoid follicles with germinal centers, consisting of polymorphic and polyclonal cell populations and aggregations of mature lymphocytes, mature plasma cells, macrophages and other types of inflammatory cells. Immunohistochemical tests showed negative results for bcl-2. Immunoglobulin heavy chains (IgH) and T cell receptor (TCR) gene rearrangement analysis revealed polyclonal proliferation or no gene rearrangement[7]. In malignant lymphoma, lymphoid follicles and germinal centers were not found, cellular atypia was significant, caryocinesia was easily observed and the infiltrating lymphocytes were monoclonal. Muria *et al*[8] considered that it is not possible to rule out the diagnosis of pseudolymphoma with the absence of germinal centers and that the pseudolymphoma has mainly mature lymphocytes while lymphoma has non-mature lymphocytes, so they were able to be distinguished according to the differentiation degree of the infiltrating lymphocytes. Takeshita *et al*[9]reported that the Southern blot analysis showed immunoglobulin heavy chain JH and light chain J-κ clone rearrangement, which helped distinguish pseudolymphoma from malignant lymphoma. Therefore, in addition to pathological examination, immune phenotype and gene rearrangements can also be used to further confirm the diagnosis of pseudolymphoma. On the histopathological examination, various sized and shaped lymphoid follicles with reactive germinal center formation were seen in our patient. Infiltrating polymorphic lymphocytes and polyclonal cell populations were composed of lymphoblastoid cells, plasma cells and macrophages without atypical and obvious caryocinesia.

Immunohistochemical studies revealed polyclonality, lymphoid cells positive for L-26 and CD 79a (B cell marker), mainly aggregated in the germinal centers, while those positive for CD3 and UCHL-1 (T cell marker) were present around the germinal centers. These findings were compatible with reactive lymphoid hyperplasia. Positive TDT has been often seen in lymphoblastic lymphoma. In our case, immunohistochemical staining for TDT was negative, which further confirmed the diagnosis of pseudolymphoma.

Although pseudolymphoma is generally considered to be a benign tumor, there is a risk of malignant transformation into lymphoma. It has been reported that some cases such as cutaneous, pulmonary, gastric and hepatic pseudo lymphomas initially diagnosed as pseudolymphoma had

transformed into lymphoma several years later[10,11]. So surgical resection remains the best option for treatment of pseudolymphoma at present**.** Intraoperative frozen biopsy can be used to diagnose pseudolymphoma and if cell variation within frozen section makes the feature of the lesion unclear, it can be treated according to the therapeutic principle of malignant tumors. Whether it is necessary to perform postoperative adjuvant chemotherapy remains inconclusive, as pseudolymphoma is mainly composed of mature lymphocytes, and less sensitive to chemotherapy. The lesions are not easily dissipated, and it has been reported that pseudolymphoma after chemotherapy still transformed into malignant lymphoma. Therefore, more cases should be accumulated to determine whether chemotherapy is necessary. Duodenal pseudolymphoma has never been reported before and its prognosis is not so clear. Although our patient had an early diagnosis, complete tumor resection, enlarged lymph nodes dissection and uneventful postoperative course, she must be followed up carefully because of the possible later development of malignant lymphoma.

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Acknowledgments

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In press

3 **Tian D**, Araki H, Stahl E, Bergelson J, Kreitman M. Signature of balancing selection in Arabidopsis. *Proc Natl Acad Sci USA* 2006; In press

Organization as author

4 **Diabetes Prevention Program Research Group**. Hypertension, insulin, and proinsulin in participants with impaired glucose tolerance. *Hypertension* 2002; **40**: 679-686 [PMID: 12411462 PMCID:2516377 DOI:10.1161/01.HYP.0000035706.28494.09]

Both personal authors and an organization as author

5 **Vallancien G**, Emberton M, Harving N, van Moorselaar RJ; Alf-One Study Group. Sexual dysfunction in 1, 274 European men suffering from lower urinary tract symptoms. *J Urol* 2003; **169**: 2257-2261 [PMID: 12771764 DOI:10.1097/01.ju.0000067940.76090.73]

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6 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303 DOI:10.1136/bmj.325.7357.184]

Volume with supplement

7 **Geraud G**, Spierings EL, Keywood C. Tolerability and safety of frovatriptan with short- and long-term use for treatment of migraine and in comparison with sumatriptan. *Headache* 2002; **42** Suppl 2: S93-99 [PMID: 12028325 DOI:10.1046/j.1526-4610.42.s2.7.x]

Issue with no volume

8 **Banit DM**, Kaufer H, Hartford JM. Intraoperative frozen section analysis in revision total joint arthroplasty. *Clin Orthop Relat Res* 2002; (**401**): 230-238 [PMID: 12151900 DOI:10.1097/00003086-200208000-00026]

No volume or issue

9 Outreach: Bringing HIV-positive individuals into care. *HRSA Careaction* 2002; 1-6 [PMID: 12154804]

Books

Personal author(s)

10 **Sherlock S**, Dooley J. Diseases of the liver and billiary system. 9th ed. Oxford: Blackwell Sci Pub, 1993: 258-296

Chapter in a book (list all authors)

11 **Lam SK**. Academic investigator's perspectives of medical treatment for peptic ulcer. In: Swabb EA, Azabo S. Ulcer disease: investigation and basis for therapy. New York: Marcel Dekker, 1991: 431-450

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12 **Breedlove GK**, Schorfheide AM. Adolescent pregnancy. 2nd ed. Wieczorek RR, editor. White Plains (NY): March of Dimes Education Services, 2001: 20-34

Conference proceedings

13 **Harnden P**, Joffe JK, Jones WG, editors. Germ cell tumours V. Proceedings of the 5th Germ cell tumours Conference; 2001 Sep 13-15; Leeds, UK. New York: Springer, 2002: 30-56

Conference paper

14 **Christensen S**, Oppacher F. An analysis of Koza's computational effort statistic for genetic programming. In: Foster JA, Lutton E, Miller J, Ryan C, Tettamanzi AG, editors. Genetic programming. EuroGP 2002: Proceedings of the 5th European Conference on Genetic Programming; 2002 Apr 3-5; Kinsdale, Ireland. Berlin: Springer, 2002: 182-191

Electronic journal (list all authors)

15 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/index.htm

Patent (list all authors)

16 **Pagedas AC**, inventor; Ancel Surgical R&D Inc., assignee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 20020103498. 2002 Aug 1

Statistical data

Write as mean ± SD or mean ± SE.

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Express *t* test as *t* (in italics), *F* test as *F* (in italics), chi square test as χ^2 (in Greek), related coefficient as *r* (in italics), degree of freedom as υ (in Greek), sample number as *n* (in italics), and probability as *P* (in italics).

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AGA Clinical Congress of Gastroenterology and Hepatology: Best Practices in 2011 Miami, FL 33101, United States

January 20-22, 2011
Gastrointestinal Cancers Symposium 2011, San Francisco, CA 94143, United States

January 27-28, 2011
Falk Workshop, Liver and Immunology, Medical University, Franz-Josef-Strauss-Allee 11, 93053 Regensburg, Germany

January 28-29, 2011
9. Gastro Forum München, Munich, Germany

February 4-5, 2011
13th Duesseldorf International Endoscopy Symposium, Duesseldorf, Germany

February 13-27, 2011
Gastroenterology: New Zealand CME Cruise Conference, Sydney, NSW, Australia

February 17-20, 2011
APASL 2011-The 21st Conference of the Asian Pacific Association for the Study of the Liver
Bangkok, Thailand

February 22, 2011-March 04, 2011
Canadian Digestive Diseases Week 2011, Vancouver, BC, Canada

February 24-26, 2011
Inflammatory Bowel Diseases 2011-6th Congress of the European Crohn's and Colitis Organisation, Dublin, Ireland

February 24-26, 2011
2nd International Congress on Abdominal Obesity, Buenos Aires, Brazil

February 24-26, 2011
International Colorectal Disease Symposium 2011, Hong Kong, China

February 26-March 1, 2011
Canadian Digestive Diseases Week, Westin Bayshore, Vancouver, British Columbia, Canada

February 28-March 1, 2011
Childhood & Adolescent Obesity: A whole-system strategic approach, Abu Dhabi, United Arab Emirates

March 3-5, 2011
42nd Annual Topics in Internal Medicine, Gainesville, FL 32614, United States

March 7-11, 2011
Infectious Diseases: Adult Issues in the Outpatient and Inpatient Settings, Sarasota, FL 34234, United States

March 14-17, 2011
British Society of Gastroenterology Annual Meeting 2011, Birmingham, England, United Kingdom

March 17-19, 2011
41. Kongress der Deutschen Gesellschaft für Endoskopie und Bildgebende Verfahren e.V., Munich, Germany

March 17-20, 2011
Mayo Clinic Gastroenterology & Hepatology 2011, Jacksonville, FL 34234, United States

March 18, 2011
UC Davis Health Informatics: Change Management and Health Informatics, The Keys to Health Reform, Sacramento, CA 94143, United States

March 25-27, 2011
MedicReS IC 2011 Good Medical Research, Istanbul, Turkey

March 26-27, 2011
26th Annual New Treatments in Chronic Liver Disease, San Diego, CA 94143, United States

April 6-7, 2011
IBS-A Global Perspective, Pfister Hotel, 424 East Wisconsin Avenue, Milwaukee, WI 53202, United States

April 7-9, 2011
International and Interdisciplinary Conference Excellence in Female Surgery, Florence, Italy

April 15-16, 2011
Falk Symposium 177, Endoscopy Live Berlin 2011 Intestinal Disease Meeting, Stauffenbergstr. 26, 10785 Berlin, Germany

April 18-22, 2011
Pediatric Emergency Medicine: Detection, Diagnosis and Developing Treatment Plans, Sarasota, FL 34234, United States

April 20-23, 2011
9th International Gastric Cancer Congress, COEX, World Trade Center, Samseong-dong, Gangnam-gu, Seoul 135-731, South Korea

April 25-27, 2011
The Second International Conference of the Saudi Society of Pediatric Gastroenterology, Hepatology & Nutrition, Riyadh, Saudi Arabia

April 25-29, 2011
Neurology Updates for Primary Care, Sarasota, FL 34230-6947, United States

April 28-30, 2011
4th Central European Congress of Surgery, Budapest, Hungary

May 7-10, 2011
Digestive Disease Week, Chicago, IL 60446, United States

May 12-13, 2011
2nd National Conference Clinical Advances in Cystic Fibrosis, London, England, United Kingdom

May 19-22, 2011
1st World Congress on Controversies in the Management of Viral Hepatitis (C-Hep), Palau de Congressos de Catalunya, Av. Diagonal, 661-671 Barcelona 08028, Spain

May 21-24, 2011
22nd European Society of Gastrointestinal and Abdominal Radiology Annual Meeting and Postgraduate Course, Venise, Italy

May 25-28, 2011
4th Congress of the Gastroenterology Association of Bosnia and Herzegovina with international participation, Hotel Holiday Inn, Sarajevo, Bosnia and Herzegovina

June 11-12, 2011
The International Digestive Disease Forum 2011, Hong Kong, China

June 13-16, 2011
Surgery and Disillusion XXIV SPIGC, II ESYS, Napoli, Italy

June 14-16, 2011
International Scientific Conference on Probiotics and Prebiotics-IPC2011, Kosice, Slovakia

June 22-25, 2011
ESMO Conference: 13th World Congress on Gastrointestinal Cancer, Barcelona, Spain

June 29-2, 2011
XI Congreso Interamericano de Pediatria "Monterrey 2011", Monterrey, Mexico

September 2-3, 2011 Falk Symposium 178, Diverticular Disease, A Fresh Approach to a Neglected Disease, Gürzenich Cologne, Martinstr. 29-37, 50667 Cologne, Germany

September 10-11, 2011
New Advances in Inflammatory Bowel Disease, La Jolla, CA 92093, United States

September 10-14, 2011
ICE 2011-International Congress of Endoscopy, Los Angeles Convention Center, 1201 South Figueroa Street Los Angeles, CA 90015, United States

September 30-October 1, 2011
Falk Symposium 179, Revisiting IBD Management: Dogmas to be Challenged, Sheraton Brussels Hotel, Place Rogier 3, 1210 Brussels, Belgium

October 19-29, 2011
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October 22-26, 2011
19th United European Gastroenterology Week, Stockholm, Sweden

October 28-November 2, 2011
ACG Annual Scientific Meeting & Postgraduate Course, Washington, DC 20001, United States

November 11-12, 2011
Falk Symposium 180, IBD 2011: Progress and Future for Lifelong Management, ANA Interconti Hotel, 1-12-33 Akasaka, Minato-ku, Tokyo 107-0052, Japan

December 1-4, 2011
2011 Advances in Inflammatory Bowel Diseases/Crohn's & Colitis Foundation's Clinical & Research Conference, Hollywood, FL 34234, United States

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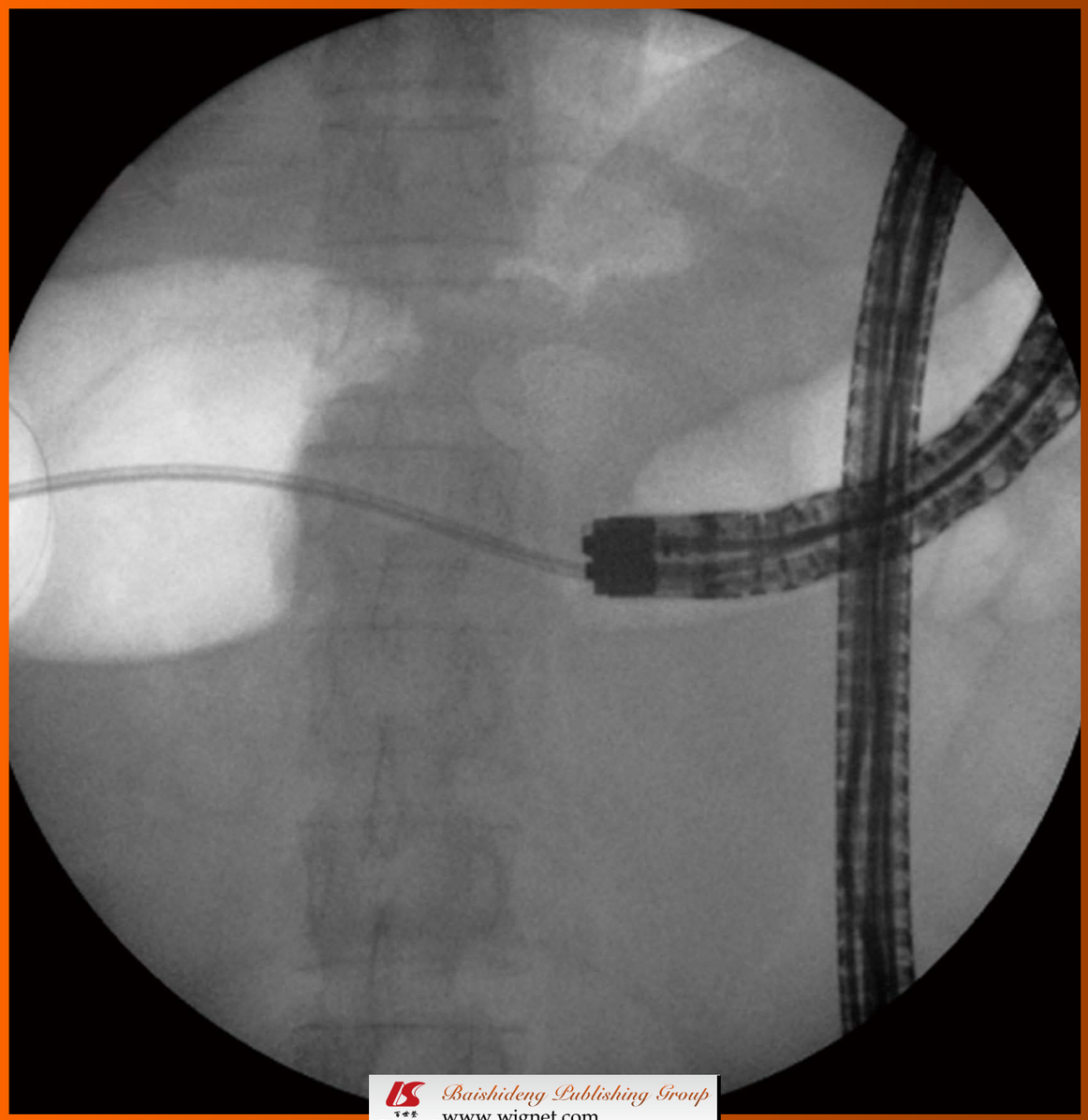

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EDITORIAL

Management of fistula-in-ano: An introduction

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Abstract

Peri-anal fistulae are a worldwide health problem that can affect any person anywhere. Surgical management of these fistulae is not free from risks. Recurrence and fecal incontinence are the most common complications after surgery. The cumulative personal surgical experience in managing cases with anal fistulae is significantly considered as necessary for obtaining better results with minimal adverse effects after surgery. The purpose for conducting this survey is to facilitate better outcome after surgical interventions in idiopathic anal fistulae' cases.

Key words: Peri-anal fistulae; Surgery; Fecal incontinence; Recurrence; Complications

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A fistula-in-ano is a granulating track between the anorectum and the perineum. A fistula may consist of primary and secondary tracks. Many fistulas are low lying, consisting of a single straight track from the skin to the anal canal, just passing through the lower fibers of the internal sphincter The majority of such fistulas can, therefore, be managed by simply lying opening the track (fistulotomy), which produces a good prospect of cure and with no impairment of continence. However, the same can not be said for fistulae which pass through the external sphincter. Some of these fistulas are complex, with secondary pararectal or supralevator tracks. Opening such fistulas may be risky and there is growing evidence that even division of a part of the external sphincter leaving the puborectalis undisturbed is associated with considerable impairment of anorectal function[1]. Unless all the secondary tracks are also treated, there is a risk of recurrent sepsis and fistulation.

This highlights the quandary for the surgeons. Possibly in no other field of anorectal surgery is appropriate treatment so critical. The potential for incurring life-long morbidity may be greater than in any other area of large bowel surgery, for example, even partial sphincter division is complicated by perirectal fibrosis and the resulting gutter deformity can rarely be resolved by later reconstructive surgery[1]. Thus fistulas-in-ano have an unenviable reputation for recurrence ad compromised continence. There are few procedures in surgery where the outcome is so greatly influenced by the experience and judgment of the surgeon. For improving our awareness and for better outcome after surgical management of cases with non-specific fistulae-in-ano, I was invited, on behalf of the *World Journal of Gastroenterology* (*WJG*) to ask experienced senior colorectal surgeons to report their considerate and views.

The benefits of face to face meetings as a source for exchange of information can not be overlooked but they are limited. Yet, *WJG* offers 24/7 service on its website and this channel could be used efficiently for exchanging this valuable data. I hope that the readers of the journal will enjoy reading the following manuscripts and that this trial will be repeated.

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TOPIC HIGHLIGHT

AM El-Tawil, MSc, MRCS, PhD, *Series Editor*

Anal fistula: Intraoperative difficulties and unexpected findings

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Abstract

Anal fistula surgery is a commonly performed procedure. The diverse anatomy of anal fistulae and their proximity to anal sphincters make accurate preoperative diagnosis essential to avoid recurrence and fecal incontinence. Despite the fact that proper preoperative diagnosis can be reached in the majority of patients by simple clinical examination, endoanal ultrasound or magnetic resonance imaging, on many occasions, unexpected findings can be encountered during surgery that can make the operation difficult and correct decision-making crucial. In this article we discuss the difficulties and unexpected findings that can be encountered during anal fistula surgery and how to overcome them.

Key words: Anal; Cryptogenic; Fistula; Surgery

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INTRODUCTION

Anal fistula is a common disease that has long challenged surgeons' skills. It is no wonder that Frederick Salmon, more than 170 years ago, was frustrated by the high recurrence rate after surgery for perianal fistula that he launched "The Infirmary for the Relief of the Poor afflicted with Fistula and other Diseases of the Rectum". This later became St Marks Hospital, the famous British hospital for treating colorectal and small intestinal disorders.

Perianal fistula, if not treated properly will result in one of two terrible complications, recurrence or incontinence. Despite many preoperative investigations that can help to identify the correct anatomy of the fistula, one might face difficult or unexpected intraoperative findings that require wise decisions. Appropriate decisions in such circumstances have a significant impact on the outcome of surgery and the patient' quality of life.

Difficulties in anal fistula surgery can be related to the anatomy of the fistula or the integrity of the anal canal. In this review, we will attempt to draw a roadmap to be followed if a difficult situation is encountered during anal fistula surgery.

INTERNAL OPENING COULD NOT BE IDENTIFIED

Proper identification of the internal opening is an integral part of fistula surgery if an unacceptable high recurrence rate is to be avoided[1,2]. In our opinion, recurrence is inevitable if the correct internal opening is not identified and dealt with. This is simply because in such cases,

the original source of sepsis will not be eliminated.

Every effort should be made to localize the correct internal opening preoperatively. Besides clinical examination, many investigations can help in preoperative localization of the internal opening[3-7]. Unfortunately, none of these investigations are particularly accurate. Thus, it is not uncommon to operate on patients with unidentified or inaccurately identified internal openings. In such cases, accurate intraoperative localization is essential. This can be a simple task if external probing is easy and successful. Otherwise, we are dealing with a difficult internal opening which requires careful maneuvers for correct localization.

First, one should know where the internal opening is. The famous Goodsall's rule relates the radial location of the internal opening of the fistula to the position of the external opening. However, fallacies in Goodsall's rule have been reported and its overall accuracy is limited[8,9]. Thus, it is unsafe to confidently rely on Goodsall's rule for identification of the internal opening. In our practice, we still use Goodsall's rule as a preliminary guide for localization of the internal opening. With regard to the cranio-caudal disposition of the internal opening along the anal canal, the vast majority of internal openings are located at the dentate line[10].

Palpation and internal probing should then be attempted starting at the expected site of the internal opening. Probing should be carried out gently in order to avoid the creation of a false internal opening. Sometimes, the internal opening can be palpated as a dimple, an elevation, a fibrous pit or soft granulation tissue pimple. In addition, a palpable trans-sphincteric track can guide to the site of the internal opening. Posterior horseshoe fistulae almost exclusively have their internal openings at the level of the dentate line at the 6 o'clock position.

Another method which has been used for localization of the internal opening is the injection of hydrogen peroxide[11] or methylene blue[12] into the external opening and observing the flow of air bubbles in the former or colored material in the latter from the internal opening. We personally prefer injection of air and have found this method useful on many occasions. This is because we believe that the injection of colored material will cause undesirable staining of tissues which will obscure vision of the opened track and its granulation tissue, both of which are important for confident laying open of the track to its very end into the anal mucosa, an essential step to avoid recurrence. We also believe that the effervescence of hydrogen peroxide is too much and unnecessary for localization of the internal opening.

Finally, if all methods fail to identify the internal opening, we then perform a preliminary fistulectomy. This is not a complete fistulectomy, but it is a limited fistulectomy aimed at proper localization of the internal opening as a prerequisite for a successful lay open procedure. In a preliminary fistulectomy, the track is dissected short of the external sphincter, at which point, the track is gently pulled while the anal mucosa is carefully inspected. The track will pull on the offending pit, and the anal mucosa at this site will be indrawn. At this stage, the probe can be inserted confidently into the correct track, and the internal opening and fistula can be confidently laid open.

If after using all means, the internal opening is not identified, we prefer to abandon the procedure and to give the patient another chance of accurate assessment and investigations. Although we are confident that, at this stage, we are not curing the patient, we believe that this is better than harming the sphincters without major benefit.

THE FISTULOUS TRACK COULD NOT BE PROBED

Probing of the fistulous track is essential to perform the lay open operation. A fistulous track can be difficult to probe if it is narrow, obstructed, kinked or branched. Forcible probing in such cases will result in the creation of a false track or a false internal opening or both. This will very likely result in recurrence.

If external probing fails, it is advisable to try gentle internal probing starting at the site of a palpable or expected internal opening. If internal probing fails, one can lay open the track piecemeal until the whole track is eventually opened. We do not prefer this technique because inadequate hemostasis can obscure the track making further progression with the lay open procedure difficult. This is especially true when the track is deep, narrow or recurrent. Our preferred technique is preliminary fistulectomy, as mentioned in the previous section. In this situation, preliminary fistulectomy has many advantages. First, if failure of probing was due to the presence of kinks in the track, all such kinks are straightened and this, by itself, can make probing possible. Second, probing of the track is done under vision so that all stenotic segments in the track, if any, are probed with confidence and without fear of creating a false passage. Third, the site of the internal opening can be identified confidently by pulling on the mobilized track and observing dimpling in the anal mucosa as previously mentioned. Finally, during fistulectomy if dissection is performed in the wrong plane and the track is opened, granulation tissue will immediately be seen raising a red flag to warn the surgeon to return to the proper plane.

UNEXPECTED ANATOMY

We believe that the correct anatomy in the majority of perianal fistulae can be disclosed preoperatively[13-15]. However, occasionally, undiagnosed difficult anatomy is encountered during surgery. Special types of difficult fistulae which can be missed clinically only to be discovered during surgery are as follows: classic posterior horseshoe fistula with or without a blind supralevator track, intersphincteric fistula with high component, high arching trans-sphincteric fistula and anterior track with special proximity to the male urethra. These fistulae do not re-

quire specific treatment, however, they need expert experienced management. Anterior fistulae near the base of the scrotum can be very close to the bulbous urethra risking its injury during the procedure. We advise inserting a urethral catheter in such cases for better identification of the urethra. For high arching fistulae, one of the sphincter-saving procedures[16-20] can be performed or otherwise a seton can be placed[21,22] and the situation is reassessed with the patient awake. Placement of a seton will also allow the different treatment options to be discussed with the patient. Inters-phincteric fistulae can reach high into the rectum entailing the use of a self-retaining anal retractor, special instruments that suit endoanal surgery, good suction and light.

FISTULAE WITHOUT AN EXTERNAL OPENING

The vast majority of anal fistulae are cryptogenic in origin[23]. This means that the fistula starts as an infection in one of the perianal glands that extends to drain itself in the perianal skin. A fistula without an external opening simply presents before external drainage occurs. This problem is uncommon and is not specifically discussed in the literature. In our experience, the majority of fistulae without an external opening are inter-sphincteric fistulae, however, as the pathogenesis implies, any type of cryptogenic fistula can present without an external opening.

Absence of an external opening is unlikely to be first discovered intraoperatively, However, because the location of the external opening is significantly related to the complexity of the fistula[13], such fistulae are frequently underestimated and the presence of a fistula of significant magnitude might be discovered at operation. Thus, the main track may not be completely palpable, probing can be difficult or impossible and side tracks might be missed. Our own plan of management is as follows: First, we try to disclose the anatomy and complexity of the fistula by palpating the fistulous tracks to assess their number, extent and depth. Then we try to identify the internal opening and carry out internal probing. If this is successful, we proceed to lay open the track keeping the risk of incontinence and recurrence in mind, in view of the anatomy of the fistula and the status of the anal canal. If internal probing fails, we then perform a limited fistulectomy in which we mobilize a limited segment of the track that will allow proper handling and manipulation. We start the procedure by making a curvilinear incision incorporating the blind end of the fistulous track (the presumed site of external opening). This point is located by meticulous palpation. In inter-sphincteric fistulae, this point might be high in the rectum entailing the use of special instruments as previously mentioned. When a good segment of the track is dissected, it is opened transversely, probed and laid open as in the classic lay open operation. If probing fails, we proceed with complete fistulectomy until the whole track is excised (in inter-sphincteric fistula) or until we reach the external sphincter (in trans-sphincteric fistula) and then we proceed as mentioned previously in preliminary fistulectomy.

Absence of an external opening is particularly difficult in cases of classic posterior horseshoe fistulae. Internal probing and lay open of this track is not enough because it will only drain the trans-sphincteric component of the fistula and not the horseshoe component. Thus, in this type of fistula, we start by mobilizing the anal canal from the sacrum and coccyx, then we lay open the horseshoe track, possibly guided by a probe placed from the internal opening for correct identification of the horseshoe track and then eventually we lay open the trans-sphincteric track.

SYNCHRONOUS FISTULA

A synchronous fistula is a fistula which has a separate track and a separate internal opening and which exists synchronously with the original fistula. This is to be differentiated from a branching fistula in which all branches eventually open into a single internal opening. A synchronous fistula can have an external opening or it can be without an external opening.

Synchronous fistulae are generally uncommon. With personal experience of over 1300 fistulae, we encountered synchronous fistulae in nine patients (unpublished data). In two patients there were three synchronous fistulae.

Synchronous fistulae should be detected preoperatively. However, this is not always the case, especially if they are minute or when they lack external openings. They can even be missed at operation or may be considered to be a branch of the original fistula. In this latter case, the synchronous fistula track might be laid open or excised together with the original fistula, missing its internal opening which will result in recurrence. This dictates careful anal and perianal examination at the beginning of surgery.

Dealing with synchronous fistulae is not difficult and they do not require a special surgical technique. The problem is deciding whether to lay them open or not. If the anal canal is adequate and the fistulae are superficial, one can lay open all the synchronous fistulae without major risk of incontinence. However, if there is any doubt about the integrity of the anal canal or the anatomy of the fistulae, it is advised that only the primary or offending fistula be laid open keeping the other tracks either for staged procedures or other sphincter-saving operations.

COMPROMISED ANAL CANAL

Anal fistula surgery carries an inherent risk of fecal incontinence because part of the anal sphincter is essentially divided in the procedure. The incidence of fecal incontinence after surgery can reach up to 64%[24-26]. Postoperative incontinence is a function of the anatomy and complexity of the fistula on the one hand, and the adequacy of anal canal function on the other hand. Thus, laying open a high fistula with adequate anal canal function can result in a reduced degree of incontinence than

laying open a low fistula with a compromised anal canal. This implies that assessment of the anal canal integrity is very important in anal fistula surgery.

Assessment of the anal canal integrity is basically performed preoperatively, clinically in the majority of situations and by anorectal physiology testing in certain selected patients[26]. However, due to the difficulty of anal examination while the patient is awake, it is not uncommon to encounter unexpected intraoperative findings of compromised anal canal. Patients at risk are female patients, patients with previous anal surgery, patients who had previous proctectomy or extensive colectomy, patients with chronic diarrheal states, anterior fistulae, high arching trans-sphincteric fistulae, supra-sphincteric fistulae and fistulae with high internal openings.

Generally speaking, one should never divide the sphincter if there is any doubt about the integrity of the anal canal or the anatomy of the fistula. In this situation, it is better to abandon the procedure and refer the patient to an expert center where a sphincter-saving procedure can be performed or placement of a seton. We usually place a draining seton rather than a cutting seton as the latter has been shown to result in incontinence and recurrence[27]. The seton allows for better assessment of the anal canal while the patient is awake. The patient then has one of three options depending on the relative risk of incontinence: If there is a high risk of incontinence, the patient can live with the seton in place permanently to drain the fistula track, or he can have one of the sphincter-saving procedures risking higher recurrence. If the risk of incontinence is low, then the patient can have the classic lay open operation that has a high chance of curing the fistula. If the operation results in a degree of incontinence, the patient still has the option of a sphincter repair at a later date.

Many sphincter-saving procedures are available for fistula patients, however, the results in terms of fistula recurrence are always inferior to the classic lay open operation. Moreover, the risk of incontinence is not completely eliminated. Injection of a mixture of fibrinogen and thrombin into the fistulous track was first described by Hjortrup in the early 1990s aimed at the formation of a fibrin plug that would obliterate the track and cure the fistula[28]. The author reported healing in half of the patients. More recent studies have reported poorer results[29-31]. We believe that the high recurrence rate after fibrin glue injection is because the glue will never fill a complex track, a kinked track, a narrow track or a long track. It can also be extruded from the track after successful injection. The Surgisis fistula plug has been introduced to avoid these drawbacks. A healing rate as low as 13.9% has been reported[32] with the majority of studies reporting a healing rate around 40%[33,34]. We believe Surgisis is only suitable for short wide fistula tracks. A mucosal advancement flap depends on obliterating the internal opening by advancing a mucosal flap from the anorectum. Recurrence has been reported in up to two thirds of patients, with slightly better results when a musculo-mucosal flap is used rather than a pure mucosal flap[18,35,36]. Our personal experience with mucosal advancement is disappointing. Recurrence is probably caused by infection in one or more of the stitches holding the flap. This is very likely in view of the septic medium in which the flap resides.

FISTULA IN INFLAMMATORY BOWEL DISEASE

The last point we are going to discuss is fistula in inflammatory bowel disease (IBD). Basically, this should be a preoperative diagnosis. However, fistula in a patient with IBD may only be discovered intraoperatively when perianal disease is the first presentation in the patient. This is especially true in places where IBD is uncommon. Fistulae commonly occur in patients with Crohn's disease but can also be seen in ulcerative colitis. Classic and aggressive surgery in these patients will not cure the fistula but will very likely result in significant incontinence. The procedure in this situation is to take a frozen section biopsy if this is available or a biopsy for paraffin section examination. If IBD is confirmed, minimum action should be taken such as drainage of an abscess or placement of a seton to drain the fistulous track. Attention should then be directed to treatment of the original disease.

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TOPIC HIGHLIGHT

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Idiopathic fistula-in-ano

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Abstract

Fistula-in-ano is the most common form of perineal sepsis. Typically, a fistula includes an internal opening, a track, and an external opening. The external opening might acutely appear following infection and/or an abscess, or more insiduously in a chronic manner. Management includes control of infection, assessment of the fistulous track in relation to the anal sphincter muscle, and finally, definitive treatment of the fistula. Fistulotomy was the most commonly used mode of management, but concerns about post-fistulotomy incontinence prompted the use of sphincter preserving techniques such as advancement flaps, fibrin glue, collagen fistula plug, ligation of the intersphincteric fistula track, and stem cells. Many descriptive and comparative studies have evaluated these different techniques with variable outcomes. The lack of consistent results, level I evidence, or long-term follow-up, as well as the heterogeneity of fistula pathology has prevented a definitive treatment algorithm. This article will review the most commonly available modalities and techniques for managing idiopathic fistula-in-ano.

Key words: Anal fistula; Seton; Fistulotomy; Advancement flap; Fibrin glue; Fistula plug

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INTRODUCTION

Fistula-in-ano is a devastating problem which has been described since the "Corpus Hippocratum". Although the disease has been well described, no definitive mode of management has been established. The most widespread traditionally accepted treatment has been fistulotomy. Problems with management are due to the heterogeneity of fistulas, and to the potential adverse effects on continence arising from division of the involved anal sphincter. Because of a desire to maintain continence, a variety of therapeutic alternatives to fistulotomy have been described[1]. The etiology includes idiopathic and iatrogenic fistulas, and fistulas secondary to other causes. This article will focus on idiopathic fistula-in-ano.

Idiopathic fistula-in-ano most commonly occurs in healthy subjects, with cryptoglandular infection being the most widely accepted etiologic factor. The anal crypt gland penetrates the anal sphincter to varying degrees. Once obstructed, infection will ensue and suppuration will follow the least resistant path, which accordingly determines the location of the abscess (perianal, ischiorectal, inter-sphincteric) and the type of fistula. Therefore, understanding the anatomy is essential to manage this disease.

Based on the relationship with the anal sphincter muscles, fistulas are classified into 5 main types: (1) submucosal: the fistula track passes superficially beneath the submucosa and does not involve any sphincter muscle; (2) intersphincteric fistula: the track passes through the internal sphincter and continues in the inter-sphincteric plane to the perianal skin, not including the external anal sphincter;

(3) trans-sphincteric fistula: the track cross through the internal and external anal sphincter on its exit towards the perianal area. The amount of involved external anal sphincter further subdivides the type of fistula into low when up to one-third of the distal external anal sphincter or less is involved, and high if a larger area of the external sphincter is included; (4) suprasphincteric fistula: the fistulous tract passes through the internal sphincter but traverses the external sphincter below the puborectalis muscle; and (5) extrasphincteric fistula: the fistulous track may pass outside the sphincter complex through the ischiorectal fossa to the perianal skin. In this case, the origin of the fistula is not from the dentate line but could be from a rectal, pelvic or supralevator origin, usually secondary to an inflammatory or neoplastic process.

A fistula-in-ano can be "simple" or "complex"[2,3]. Fistulas with multiple external openings, those involving more than 30%-50% of the anal sphincter (high transsphincteric fistulas), those lying above the sphincter (suprasphincteric), extrasphincteric, or have high blind extensions, or horseshoe tracts, or are anterior in a female patient are considered complex, with higher risk of continence disturbance posed by surgically managing these fistulas. Alternatively, an anal fistula in patients with a preexisting history of fecal incontinence, Crohn's disease, or local irradiation are also considered complex. Submucosal and low intersphincteric (traversing less than 30% of anal sphincter muscle) as well as low transsphincteric fistulas are considered simple[4].

The management of fistulas-in-ano includes 3 main goals: to cure the fistula, to prevent or minimize recurrence, and to retain continence. Regardless of the type of fistula, there are certain principles that lead to successful management: the infection/inflammatory process should be resolved, the source of pathology should be addressed, and when treating the fistula, the internal opening should be closed with least risk to continence. This goal can be achieved with care taken not to jeopardize the integrity of the anal sphincter complex. In a retrospective study of 404 male patients with fecal incontinence, the most common confounding factor in patients younger than 70 years was a surgical history of fistulectomy or hemorrhoidectomy[5].

PREOPERATIVE ASSESSMENT AND PLANNING

Preoperative evaluation is very important. Medical history and physical examination are most important in the assessment phase. The patient's continence is another important facet that needs to be included, as well as any history of anorectal surgery. Anal ultrasound with hydrogen peroxide is another important adjunct for preoperative evaluation. This procedure is simple, fast, may be done in the same office visit, and provides information about the fistula track, the type and complexity and whether the anal sphincter muscle is normal, scarred, or disrupted, as well as the presence of an abscess cavity[6,7]. Anal ultrasound is operator-dependent; scars and defects may confuse sonographic interpretation and might render delineation of the fistulous track difficult[8].

Magnetic resonance imaging (MRI) has become an integral part of the assessment of fistula as it can distinguish between sepsis and granulation tissue from sphincter muscles[9]. A prospective study compared the preoperative MRI assessment of the anal fistula and intraoperative findings. High concordance rates were reported in terms of recognizing the course of primary tracks (86%), demonstrating secondary tracks (91%), and horseshoe extension (97%), as well as identifying internal opening (80%)[10]. Furthermore, MRI accurately detected previously missed secondary tracks when compared with the clinical examination, and correctly identified the level of the fistula with respect to the anal sphincters[11]. An endocoil greatly increases tissue resolution and provides superior anatomical details[12,13]. However, it has a limited field of view (about 2-3 cm from the coil) in which any fistulous extension in this zone might be missed, and is not widely available and is very uncomfortable for the patient[14,15].

Although anal manometry may provide information about sphincter pressures, obtaining a fecal incontinence score will also yield clinically relevant information[16]. Collectively, this information will help select the best choice for treatment, and allows the surgeon to counsel the patient about expectations and probabilities of success. Pescatori *et al*[17] prospectively studied the impact of preoperative anal manometry for guiding the surgical approach, on functional outcomes of fistula surgery. In this study 96 patients underwent pre- and post-operative anal manometry and were compared with a control group of 36 patients who did not have anal manometry. Internal sphincterotomy was performed for effective drainage of the intersphincteric plane in less than 50% of both groups (45 and 15, respectively). While 24% (11/45 patients) from the anal manometry group had postoperative soiling, 53% (8/15 patients) of the control group did. As for recurrence, 3% and 13%, respectively, experienced recurrence. Another study of 45 patients assessed the clinical and manometric effects of fistulotomy for intersphincteric fistulas on anal sphincter function. Although there was a significant decrease in resting pressures throughout the distal 2 cm of the anal canal after surgery, the maximum squeeze pressures did not change. Continence problems arose in patients who had lower preoperative resting pressures[18].

In the majority of patients with idiopathic fistulas-in-ano, a thorough history and physical examination may suffice for surgical planning. When in doubt or if a complex fistula is suspected, an ultrasound is justified for further evaluation. Complexity and or sepsis might require further magnetic resonance imaging assessment. Anal manometry is especially useful in patients in whom postoperative continence is a concern.

MANAGEMENT OF FISTULA-IN-ANO

Management may be achieved by one of the following methods: (1) keep the track from closing around a remnant septic focus preventing further abscess formation using a non-cutting seton; (2) expose the track and let it

heal secondarily heal following a fistulotomy; (3) excise the whole fistulous tract: fistulectomy; (4) excise the internal opening and cover the defect with healthy mucosa as an advancement flap; (5) obliterate the fistulous track with glue, or a collagen and fistula plug; (6) ligate and disconnect the fistula tract in the inter-sphincteric plane as a LIFT (ligation of the intersphincteric fistula tract) procedure; (7) ablate the tract and induce scarring with radiofrequency waves; and (8) induce regeneration in the tract with biologic agents or stem cells.

OPERATIVE APPROACH AND SURGICAL MANAGEMENT

Seton

One of the simplest modes of treating anal fistulas involves passing a thread through the anal fistula tract. The material used may be a non-absorbable suture, drain, rubber band, or even a vessel loop. Setons are a viable option for high trans-sphincteric fistulas, for those fistula involving more than half of the sphincter, and anterior fistulas in female patients[19,20]. There are basically two types of setons used in treating anal fistulas. A cutting seton is used to slowly cut through the tissue allowing for healing from inside to outside thus minimizing the risk of incontinence. In this case, after identifying the external and internal openings of the fistula tract, the skin and anal mucosa overlying the sphincter are incised, and subsequently the seton is passed through and tightened. In a study conducted among 160 patients with a fistula-in-ano, 10% received seton placement for either high transsphincteric or suprasphincteric fistulas. The authors reported that all of the patients in whom a seton was placed had encountered temporary alterations in continence to gas only, for an unreported length of time[20]. Parks and Stitz treated 80 patients with a seton for high transsphincteric fistulae (n = 23) and suprasphincteric fistula (n = 57), of whom a total of 30 (8 and 22, respectively) patients underwent division of residual external sphincter muscle at the time of seton removal. Sixty eight patients were available for functional assessment. The authors found that 17% of those patient who had a seton alone without muscle division complained of partial loss of continence as opposed to 39% of those who had muscle division, emphasizing the importance of conserving muscle as much as possible even if it necessitates a longer healing time[21]. Thirty-four patients (aged between 20 and 57 years), of whom 31 patients had normal preoperative continence, were offered a 2-stage seton procedure for high anal fistulas (16 extrasphincteric and 18 trans-sphincteric). All trans-sphincteric fistulas healed and there were only 2 recurrences. Among 29 patients with preoperative normal fecal control who were available for follow-up, postoperative continence was normal in 12 patients; while 5 patients had no control over flatus, 11 were incontinent for liquid stool or flatus, and one had continued fecal leakage. The authors did not recommend the 2-stage seton technique for fistulas with high anal or rectal openings[22].

In a review of the literature, including 37 different studies on cutting setons, the average incontinence rate was 12%. The more proximal the location of the internal opening the higher was the incontinence rate. Among the studies which described alterations to continence, incontinence to liquid was most common followed by incontinence to flatus. The authors concluded that, when feasible, other sphincter-preserving techniques should be employed especially with high anal fistulas[23]. The cutting seton requires further follow-up visits for subsequent tightening of the seton to achieve the desired effect. The time for a fistula to heal with this method ranged from 1 mo to more than a year[24,25].

The second type of seton, the non-cutting seton, is used primarily for draining, especially in the acute setting, or where other modalities cannot be implemented or have failed previously, and in certain disease entities such as Crohn's disease, and HIV[26,27]. In the acute setting it provides rapid and safe relief of the infection, with no compromise to the sphincter complex, providing time for the inflammation to resolve, and better assessment and decision-making. Furthermore, keeping the seton in place helps prevent abscess recurrence and can act as a guide to the internal and external opening for following treatment(s). A recent consensus conference on fistulas concluded that setons should be used as an initial method until there is no evidence of acute inflammation[28].

Fistulotomy

Fistulotomy is considered to be the procedure of choice for low, single tract, anal fistulas, especially when submucosal, since the risk for incontinence or recurrence is very low[29]. However, others use this procedure for low intersphincteric as well as for trans-sphincteric fistulas[19,30]. According to the practice parameters for the treatment of perianal abscess and fistula-in-ano presented by Whiteford and colleagues (2005), fistulotomy may be used in the treatment of perianal simple anal fistulas in cryptoglandular disease[30]. In this report, a simple fistula was defined as a single, non recurrent tract that crossed less than 30%-50% of the external anal sphincter, not the anterior sphincter in women, and was present in subjects with perfect continence and no history of Crohn's disease or pelvic radiation. Furthermore, the authors suggested the use of tract debridement and/or fibrin glue as these methods do not impose a risk on continence, and, despite the associated higher recurrence rate, still offer the opportunity for alternative treatment.

While some surgeons use fistulotomy only for submucosal fistulas[29], others apply this technique to more complex fistulas, including recurrent, high trans-sphincteric, or extra-sphincteric fistulas[31], in conjunction with sphincter reconstruction. To perform fistulotomy, the internal and external sphincters are divided, accessory tracts are excised, and eventually overlapping sphincter reconstruction is performed. Thirty five patients underwent fistulotomy with sphincter reconstruction for complex anal fistulas (high

transsphincteric = 30, suprasphincteric = 4, extrasphincteric = 1). Eleven patients reported preoperative fecal incontinence, and fistulas were recurrent in 16 patients (8 with preoperative incontinence). Mean follow-up was 32 mo (range, 24-42). Two patients suffered from recurrence at 3 and 6 mo, respectively. One presented with a recurrent fistula. While 3 (12.5%) of the preoperatively fully continent patients (n = 19) experienced minor alterations in incontinence in terms of control of flatus and soiling, all of the preoperatively incontinent patients demonstrated significant improvement in fecal control after surgery. The authors suggested that fistulotomy with sphincter reconstruction provides an effective resource in the management of complex fistula-in-ano[31]. The same technique was applied to 16 recurrent complex fistulas (4 patients experienced at least 2 recurrences) with a mean follow-up of 40 mo. Seven of the 8 patients who had previous fecal incontinence significantly improved. One patient experienced recurrence after 6 mo and was offered the same procedure after 38 mo without recurrence[32]. The same authors, in a randomized controlled study, compared the advancement flap (n = 27) to fistulotomy with sphincter reconstruction (n = 28) for managing primary complex fistula-in-ano, in terms of recurrence and anal function. Fistulas were classified as high transsphincteric fistula (n = 44) and suprasphincteric fistula (n = 11) which were comparable between groups. After a mean follow-up of 36 mo (range, 24-52), there were 2 recurrences in each group (7.4% and 7.1%, respectively). There was no significant difference between the 2 groups regarding continence either before or after surgery, as 77.8% of the flap group and 82.1% of the fistulotomy and reconstruction group were fully continent prior to surgery whereas 70.4% and 67.9%, respectively, maintained postoperative continence[33].

The incontinence rate associated with fistulotomy varies from 0% to 40%[34,35]. In a prospective randomized study among 148 patients with inter-sphincteric fistulas, age, gender, duration of disease, location of the internal orifice, and previous surgery were not found to be significant factors influencing postoperative incontinence[36]. However, in the same study, low voluntary squeeze pressure and multiple prior drainage procedures were deemed as predisposing factors for postoperative incontinence. The authors recommended anal manometry prior to fistulotomy, and not to use this technique in patients with a past history of multiple drainage[36]. Other authors found similar results[34]. However, Garcia-Aguilar *et al*[37] reported that previous surgery, female gender, high internal orifice, type of surgery performed in high fistulas were all risk factors for developing postoperative incontinence following fistulotomy[37].

Others have concluded that trans-sphincteric fistulas and the extent of external sphincter involvement are significant risk factors for post-fistulotomy incontinence[35]. In this retrospective study, 64% of the population (n = 110) experienced at least occasional incontinence episode(s). Lifestyle restriction was found to be mild in 14% and moderate in 10%; mild and moderate depression were encountered in 9% and 4%, respectively, with 5% of patients having moderate embarrassment[35].

Table 1 Results of endorectal advancement flap

Author	n	Follow-up months (mean)	Success rates (%)	Incontinence rates (%)
Dixon *et al*[39]	29	5.7	69.0	0
Koehler *et al*[40]	42	55	73.8	28.6
Ellis *et al*[41]	58	22	62.9	NA
Gustafsson *et al*[42]	83	12	57.0	NA
Perez *et al*[33]	30	36	92.6	7.4
van der Hagen *et al*[43]	103	72	36.6	9.8
Uribe *et al*[44]	56	43.8	92.9	19.6
Zbar *et al*[45]	11	20	81.8	18.2
Mitalas *et al*[46]	87	15	66.7	3.4
Dubsky *et al*[47]	54	53.2	75.9	28.9
Ortiz *et al*[48]	91	24	82.4	12.1
van Koperen *et al*[49]	80	67	73.8	NA
Abbas *et al*[50]	36	27	76.0	12.0

NA: Not available; n: Number of patients

Endorectal advancement flaps

Advancement flaps were implemented as a sphincter-saving method since there is no division of the sphincter muscles, and are mainly used for complex or high fistula. Basically, an incision is made distal to the internal opening of the fistula, a flap of healthy tissue is elevated, the diseased part is excised, and the internal opening is closed followed by advancement of the flap to cover the closed internal opening, and is finally sutured in place. There are a few crucial points to help ensure an optimal outcome. Dissection is started in the submucosal level. As the dissection proceeds proximally, the thickness of the flap increases without injuring the sphincter. In addition, the wide base of the flap should ensure a tension-free flap with a good blood supply. Alternatively a curvilinear (semicircular) flap could be raised to avoid ischemia at the edges[38]. Regardless of the incision used, it should not be very close to the anoderm to avoid ectropion. The healing rates, shown in Table 1, range from about 57% to more than 90% with an acceptable period of follow-up.

In a retrospective review of 91 patients who underwent flap repair for complex fistulas, the recurrence rate was 19% after a median follow-up of 42 mo (range, 24-65)[48]. The authors noted in their cohort that the median time to relapse was 5 mo with no recurrences after 1 year.

Abbas and colleagues[50] conducted a study to determine the long-term outcome of an endorectal advancement flap for complex anorectal fistulas in 36 patients. The primary success rate was 83%. Transient fecal incontinence was reported by 3 male patients, but this problem spontaneously resolved in all 3 patients in 2 mo. Transient and minor continence-related problems have been encountered in other studies[8,29,43,44,51]. Long-term functional outcome was assessed among 179 patients after surgical treatment of cryptoglandular fistulas, 70 of whom received

advancement flaps. The 3-year recurrence rate was 21% soiling was reported in 40% of the patients[34]. Fifty six patients underwent prospective clinical and manometric evaluation after receiving advancement flaps. Four (7.1%) patents had recurrence and were offered the same procedure with a successful outcome. After surgery, 78.6% of patients maintained their continence, 7 patients (12.5%) reported minor incontinence problems and 5 patients (9%) suffered from major continence disturbances. Three months after surgery there was an overall reduction in maximum resting and squeeze pressures. Age, gender, and previous fistula surgery did not affect outcome in multivariate analysis[44].

Dubsky *et al*[47] retrospectively compared full thickness flaps (n = 20) to partial thickness (mucosal) flaps (n = 34). Although incontinence was found in 5 (11.1%) patients, full transection of the rectal wall for flap creation did not pose a threat to continence as only one of the 5 patients belonged to the full thickness flap group. The overall recurrence rate was 24%, occuring mainly in patients who had undergone multiple prior fistula-related procedures. Similarly, one patient (5%) experienced recurrence from the full thickness flap group as opposed to 12 patients (35.3%) from the partial (mucosal) thickness group.

The endorectal advancement flap provides sphincter preservation, and is a relatively safe alternative for managing fistulas-in-ano, with acceptable outcomes. The associated fecal disturbance is temporary in the majority of cases, yet necessitates awareness during preoperative assessment, patient counseling and the operative procedure.

Fibrin glue

During World War I, fibrin glue was initially implemented in surgery for hemostasis. Later, the material was utilized in different fields of surgery mainly as a sealant[52] till 1992 when Hjortrup and colleagues[53] used it to seal anal fistulas. The fibrinogen, thrombin, and calcium mixture in the fibrin glue seal the fistulous track by virtue of inducing clot formation. The initial soluble clot results from cleavage of fibrinogen to fibrin, which transforms to a stable clot once thrombin and calcium activates factor XIII. This reaction takes about 30-60 s. Subsequently, the glue promotes the migration of fibroblasts and pluripotent cells to start healing the fistula by laying down collagen. Over the following 7-14 d, the initially formed fibrin clot starts to dissolve by the lysis action of the plasmin present in the surrounding tissues while the tract is filled with synthesized collagen fibers[54].

For the procedure to be successful, both internal and external openings of the fistula tract need to be identified. Injecting hydrogen peroxide or methylene blue can help to identify the openings. A loaded double-barrel syringe is introduced into the tract till the tip is seen through the internal opening. The syringe is emptied allowing the components of the glue to mix then exit the syringe to fill the fistula tract while steadily withdrawing the syringe outwards with a simultaneous compression to fill the fistula tract from inside out avoiding any filling deficiencies. The injection is followed by a ten minute wait to allow the reaction to stabilize the clot. While some authors advocate suturing the internal and external openings[55], others found no benefits[56,57]. The success rate varies, ranging from 31% to 85% as shown in Table 2.

Table 2 Results of fibrin glue

Author	n	Follow-up months (mean)	Success rates (%)
Patrlj *et al*[58]	69	28	74
Sentovich *et al*[59]	20	10	85
Lindsey *et al*[60]	42	4	63
Sentovich *et al*[61]	48	22	69
Zmora *et al*[62]	60	6	53
Gisbertz *et al*[55]	27	7	33
Dietz *et al*[63]	39	23	31
Adams *et al*[57]	36	3	61
Witte *et al*[64]	34	7	55
Parades *et al*[56]	30	12	50

n: Number of patients

Various reasons have been suggested to explain success or failure, which includes technical-related and postoperative care issues. Dislodgement of the fibrin plug has been one of the most common proposed reasons[56]. Based upon this theory many surgeons instruct their patients to follow a sedentary lifestyle in the immediate postoperative period, and to avoid heavy lifting or any strenuous activity. Others prescribe stool softeners, or suggest that fistula tract preparation by mechanical curettage is a key to success, such that inadequate removal of granulation tissue could lead to failure of the glue[54].

Postoperative infection and abscess formation are other causes of failure. These postoperative septic sequelae may be due to a technical, improper cleansing of the tract prior to instillation of the glue, or a non-resolved infection[60,61]. The length of the fistula tract has also been related to success or failure. While some surgeons have demonstrated higher success rates when using fibrin glue in long tracts (> 3.5 cm)[58,60], assuming that fibrin glue has greater liability to leak from shorter tracts, others have shown greater success rates in shorter tracts[59,61,65].

As previously mentioned, the length of follow-up is an important facet in evaluating such new techniques. Sentovich *et al*[59], in their initial study showed a success rate of 85% over a follow-up period of 10 mo. This rate became 69% after 22 mo of follow-up[61]. Queralto *et al*[66], offered 34 patients with high cryptoglandular fistula synthetic glue. At 1 mo, the healing rate was 67.6%. This rate remained almost the same during a median follow-up period of 34 mo (range, 21-43), with no continence problems.

Despite the inconsistent success rates, the majority of studies showed that in properly selected patients, fibrin glue can achieve 30%-60% success rates. The technique is simple, less invasive to the anal sphincter complex, and in case of failure, it does not preclude the patient from receiving other methods of treatment.

Fistula plug

Another less invasive, sphincter preserving technique is the fistula plug. This biologic plug (Surgisis® Anal Fistula Plug, Cook Surgical, Belington, IN, USA) is manufactured from porcine small intestinal mucosa. The plug characteristically is resistant to infection, and ideally, does not induce a foreign body reaction. Furthermore, it invites host cells to populate it, promoting multiplication and ultimately filling the fistulous tract[67].

Prior to usage, the plug should be rehydrated in 0.9% normal saline for 3-5 min. During this time the surgeon should have identified both the internal and external openings of the fistula tract in the usual manner. The plug should be inserted in the fistula tract through its internal opening. Once light resistance is encountered, the plug is trimmed if needed, and secured at the internal opening, since dislodgement is a primary cause of plug failure[68]. Similarly, the excess at the external opening is trimmed at the skin level, but the external opening is left opened for drainage in an attempt to reduce the possibility of infection and failure of treatment. Similar to fibrin glue, the results of the anal fistula plug vary greatly as shown in Table 3.

Preoperative bowel preparation, perioperative antibiotics, and prone operative position of patient, are not mandatory but preferred by most surgeons[80]. The prior use of a seton not only ensures the elimination of any inflammation or infection, but has been postulated to prepare the tract, making the wall more fibrotic, with an ultimate increase in success rates[75,77]. Conversely, seton use has not been found to correlate with increased healing rates in other studies but seems to facilitate the procedure by identifying the fistula anatomy[68,70].

Several authors have tried to assess predictors of success and to identify causes of failure. Following the previously mentioned technique, with mechanical bowel preparation, hydrogen peroxide irrigation of the fistula tract before plug insertion, strict postoperative limitation of activity, and topical metronidazole, Johnson *et al*[69] reported an 87% success rate at 14 wk, and 83% at 12 mo[68]. O'Connor *et al*[70] achieved an 80% success rate with the addition of seton placement prior to plug insertion, while Garg[71] reported a 71% success rate without using hydrogen peroxide, which is thought to clear the tract of all debris that might interfere with cell migration induced by the plug and hence prevent healing[72].

An anal fistula plug has also been used for complex anal fistulas. Ellis *et al*[81] retrospectively studied the long-term outcomes of an anal fistula plug in complex fistulas in patients who had at least 1 year of follow-up since their last treatment. Sixty three patients were identified, 51 of whom (81%) had had clinical healing of the fistula. When applying multivariate analysis, the authors noted that tobacco smoking, a history of prior plug failure, and a posterior fistula were all predictive factors for failure. This study concluded that the anal fistula plug is an effective method for long-term closure of complex anal fistulas[81]. Similarly, Lenisa *et al*[82] studied 60 consecutive patients with complex fistulas through a prospectively maintained database. Eleven patients had multiple fistula tracts, 17 were located anteriorly in female patients, and the remaining were trans-sphincteric, while 38 tracts were recurrent in nature. At a mean follow-up of 13 mo, the success rate was 60% in all patients and 70% for the fistula tracts. The mean time for recurrence was 5.7 mo. Replugging successfully managed 2 recurrent patients, and 5 patients received successful post-plug fistulotomy. This resulted in a global healing rate of 72% with no continence impairment. The authors concluded that an anal fistula plug remains a safe option in treating complex anal fistulas and the reasons and risk factors for recurrence remain to be discovered.

Table 3 Results of anal fistula plug

Author	*n*	Follow-up months (mean)	Success rate (%)
Johnson *et al*[69]	25	3	87
Champagne *et al*[68]	46	12	83
O'Connor *et al*[70]	20	10	80
Ellis *et al*[4]	18	10	78
Garg *et al*[71]	21	10	71
Schwandner *et al*[72]	19	9.3	61
Ky *et al*[73]	45	6.5	55
Thekkinkattil *et al*[74]	43	11	44
Christoforidis *et al*[75]	47	6.5	43
van Koperen *et al*[76]	17	7	41
El-Gazzaz *et al*[77]	33	7.4	25
Lawes *et al*[78]	20	7.4	24
Safar *et al*[79]	35	4	14

n: Number of patients.

The higher than expected recurrence rate with the glue and plug, and the associated risk of incontinence with other conventional methods prompted the search for other techniques to find an optimal treatment for fistula-in-ano.

Ligation of the intersphincteric fistula tract

Initially described by Rojanasakul[83], Ligation of the intersphincteric fistula tract (LIFT) involves a small incision in the intersphincteric groove where the fistula tract crosses from the internal sphincter to the external sphincter. Dissection is carried out till the fistula tract is clearly identified, ligated, then divided. The initial report showed healing in 17 out of 18 patients by a mean time of 4 wk.

Bleier *et al*[84], applied the technique in 39 patients, 74% of whom had undergone a median of 2 previous attempted repairs. Follow-up data on 35 patients at a median follow-up of 20 wk revealed a success rate of 57% (20/35) and a duration of failure of 10 wk (range, 2-38), with no subjective decrease in continence. Shanwani *et al*[85], applied the same technique on 45 patients (transsphincteric = 33, complex = 12), with 5 patients presenting with recurrent fistula after prior surgical intervention. After a median follow-up of 9 mo (range, 2-16), the primary healing rate was 82% (37/35), with a median healing time of 7 wk

(range, 4-10). Recurrence was encountered in 8 patients over a period of 3 to 8 mo, with no significant morbidity.

Radiofrequency

Radiofrequency ablation of the fistula was used in an effort to reduce continence-related problems by limiting damage to the surrounding muscle. Gupta[86,87] conducted a study among 100 patients with low anal fistulas comparing radiofrequency to conventional fistulotomy. The radiofrequency group was found to have less gas incontinence; 4% *vs* 12%.

Stem cells

Patients' adipose-derived stem cells have been used to treat complex fistulas, either cryptoglandular or Crohn's-related[88]. In a separate procedure, and under complete aseptic techniques, adipose tissue was obtained from the patients, processed and centrifuged to provide adipose-derived stem cells. These cells were cultured, then in a second procedure, were injected into the fistula tract.

In a comparative study in 49 patients with cryptoglandular or Crohn's-related fistulas, the healing rate was 71% in the stem cell group in addition to fibrin glue *vs* 16% in the fibrin glue group, with no difference in adverse reactions among the groups. At 1 year follow-up, recurrence rate was 17.6 % in the stem cell group, with the earliest recurrence occurring at 7 mo, while no recurrences were observed in the control group. The authors concluded that this method was safe and had the potential of healing fistulas in complex disease[88].

CONCLUSION

An anal fistula is a common disease which is devastating to the patients and imposes challenges to the surgeon. Proper management requires knowledge of the etiology and an understanding of the anatomy. So far, the available treatment methods have not achieved the main goals of preventing recurrence and preservation of continence. The lack of level I evidence, absence of long follow-up periods, inconsistent results, and varying methodology among published studies has resulted in the current lack of consensus. However, the higher than accepted recurrence rates, and fear of incontinence, has prompted a search for newer methods. Nevertheless, the variability in success and incontinence and/or recurrence rates could be related to surgeon expertise and/or technique and/or patient selection.

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TOPIC HIGHLIGHT

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Current management of cryptoglandular fistula-in-ano

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Abstract

Fistula-in-ano is a difficult problem that physicians have struggled with for centuries. Appropriate treatment is based on 3 central tenets: (1) control of sepsis; (2) closure of the fistula; and (3) maintenance of continence. Treatment options continue to evolve - as a result, it is important to review old and new options on a regular basis to ensure that our patients are provided with up to date information and options. This paper will briefly cover some of the traditional approaches that have been used as well as some newer promising procedures.

Key words: Cryptoglandular; Fistula; Anorectal; Sphincter sparing; Ligation of the intersphincteric fistula tract procedure

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INTRODUCTION

Fistula-in-ano is a difficult problem that physicians have struggled with since the time of Hippocrates. The ideal treatment is based on 3 central tenets: (1) Control of sepsis; (2) closure of the fistula; and (3) maintenance of continence. Treatment options have continued to evolve - as a result, it is important to review old and new options on a regular basis to ensure that our patients are provided with up to date information and options. The incidence of fistula-in-ano is 9 per 100 000 (compared to 6 per 100 000 for Crohn's disease and 8 per 100 000 for ulcerative colitis)[1], and is therefore a problem commonly encountered. This paper will briefly cover some of the approaches that have traditionally been used as well as some newer promising procedures.

The reader should be aware that despite the long standing history of fistula-in-ano and the multiple approaches that are utilized, there is a paucity of high quality data to guide decision making. A recent Cochrane review concluded among other things that there was a "crying need for well powered, well conducted randomized controlled trials" and that recurrence and rates of incontinence were the most important factors when considering repair[2]. We agree with this assessment, and base our management on the best available data. However, this illustrates the need for continual review of appropriate management techniques.

One of the most clinically useful classification systems for perianal fistulas (by the American Gastroenterological Association) divides them into simple and complex - this classification facilitates operative decision making[3].

Simple fistulas are low - i.e. they involve a small (or sometimes none) portion of the sphincter complex. These fistulas include superficial, low intersphincteric or low transsphincteric fistulae. In addition, communication between the anal canal and skin is only *via* one tract and is not associated with inflammatory bowel disease, radiation or involving any other organs.

Conversely, complex fistulas are anatomically higher: they involve significant portions of the sphincter musculature, may have multiple tracts, involve other organs (i.e. vagina) and may be associated with radiation or inflammatory bowel disease. Recurrent fistulas are usually included in this category as well.

Superficial fistulae

The management of superficial fistulae is fairly straightforward. Superficial or simple fistulas that, by definition, do not traverse any (or an insignificant portion) of the sphincter musculature should be treated with simple fistulotomy. This is a time-honored, effective approach with a success rate that approaches 100%, with little or no effect on continence[4]. Lay-open fistulotomy is easy, and management of these wounds is straightforward. These procedures involve unroofing of the fistula tract and curettage of the epithelialized lining. Conservative wound care with sitz baths and analgesics postoperatively is usually all that is needed. There is some evidence to suggest that healing is quicker with marsupialization of the tract[5].

Intersphincteric fistulae arise from cryptoglandular infections that remain contained between the internal and external sphincters. Partial division of the internal sphincter alone is standard treatment for other benign anorectal disease such as anal fissure, and is similarly safe. This technique involves cutting a portion (usually to a maximum of 30%) of the internal sphincter only, and yet maintains excellent preservation of continence. From this we extrapolate that division of the internal sphincter along the length of an intersphincteric fistula is similarly safe, and with limited changes in continence, is also an acceptable form of treatment of intersphincteric fistula. If an intersphincteric fistula involves a significant portion of the internal sphincter (over 30%) then thought should be given to a sphincter sparing-type procedure - incontinence can result if too much internal sphincter muscle is divided.

Complex fistulae

Fistulae that traverse a significant portion of both sphincter muscles are termed trans-sphincteric and are part of the group of complex fistulae. Lay-open fistulotomy along these tracts are effective at fistula closure for the same pathophysiologic reasons as simple fistulae but, based on the amount of musculature involved, may result in significant changes in continence, violating one of the basic principles of appropriate management. Thus, this is no longer considered an acceptable approach.

Seton placement

Initial management of complex or trans-sphincteric fistulae begins with control of the septic focus. Initially this may involve drainage of anorectal abscess, and if the fistula tract can be identified, a draining seton should be placed. This is a foreign piece of material inserted through the fistula tract which functions to maintain the fistula tract in an open state, preventing a closed space infection, and allowing for drainage and sepsis resolution. This is a safe, and usually simple, method for control of the basic pathophysiologic insult that creates the fistula. Appropriate complex fistula management almost always indicates initial seton placement. By allowing for resolution of sepsis, and establishment of a well-formed tract, the clinician is offered the luxury of time and the ability to characterize the anatomy of the fistula, which underpins effective subsequent management.

The draining seton is usually a silastic or similarly biologically inert, low profile material that does not incite an inflammatory reaction. We typically use a vessel loop. The fistula tract is identified, and traversed with a fistula probe if possible. A suture is then attached and pulled through. The other end is then tied to the vessel loop and then pulled through. The vessel loop is tied to itself with a permanent suture in a loose fashion. In addition, the external fistula opening is usually widened and debrided of chronic granulation tissue. In this manner, almost any fistula, with its associated local sepsis is temporized, and infection and inflammation can clear. Appropriate anorectal hygiene in the form of sitz baths or showering is used until the infection clears. This creates a stable situation in which a fistula can be maintained indefinitely. Once sepsis and inflammation have cleared, the presence of the seton in an uninfected tract allows for accurate delineation of the fistula anatomy, either *via* careful clinical exam, ultrasound or radiologic study. If there is any question as to the possibility of a significant trans-sphincteric component at the time of initial operation, a seton should be placed, and minimal added morbidity will be incurred.

One option for subsequent management of a transsphincteric fistula is the use of a cutting seton. The principle that underlies this is the hypothesis that slow division of the muscle allows for fibrosis and scarring and that overall integrity of the sphincter complex is maintained. The technique involves sequential tightening of the seton through the fistula tract by way of serial placement of additional suture material on the seton and/or by asking the patient to pull on the seton at a regular frequency (once every few days), or by tightening of the original seton. Many different types of setons can be used for this, including the initially placed silastic seton, or more commonly, the silastic seton is replaced with a silk suture which is narrower, and inflammation-inducing. This allows for faster cutting and induction of scarring. This procedure, however, has several disadvantages, despite a sound theoretical basis: It requires frequent office visits for tightening, sometimes weekly or bi-weekly. It is usually quite uncomfortable or painful for the patient, and usually results in the need for narcotic or non-steroidal analgesia. Most importantly, there is substantial literature which demonstrates that despite the scarring through a

maintained sphincter complex, continence can be negatively affected. This procedure must be approached with caution, especially in view of the sphincter sparing procedures, which are available. While reported success rates have been reported as similar to fistulotomy, changes in continence have been reported in greater than 60% of patients[6].

Advancement flaps

The advancement flap is a technique which is designed to address the pathophysiology of the fistula in a sphincter-sparing approach by closing the internal opening, thus depriving the fistula of it's source of sepsis, and allowing the defunctionalized tract to heal by secondary intention.

The endoanal advancement approach involves advancing a healthy sleeve of rectal wall over the debrided internal opening, and suturing the flap over and distal to the internal opening. This is based on a broad pedicled flap dissected from the healthy proximal rectum. This procedure is technically more difficult and is often plagued by difficult exposure, especially on posteriorly located fistulae within the rectal hollow. Fistulas with higher internal openings are often quite difficult to reach as well. Additionally, this procedure involves the creation of a large defect in previously undamaged rectum, and runs the risk of devascularization and loss of a much larger portion of rectal wall. Failure or ischemia of these flaps may result in the creation of a much larger defect than existed previously. In addition, dissection in a scarred or chronically inflamed plane can place the sphincter at risk. Success rates for this approach vary widely through the literature, and range from 0 to 63%[7,8].

Cutaneously based flaps are advanced from the anodermal skin over the internal opening, and are based on pedicled flap principles as well. These flaps avoid placing otherwise healthy rectum at risk, but also have their own associated morbidities. These similarly require extensive experience with maintaining viability of skin-based flaps, and also run the risk of injuring the sphincter. Additionally, advancement of anodermal skin into the anal canal may result in chronic irritation and seepage, pathophysiologically akin to the ectropion, which may be the result of over-extensive hemorrhoidectomy. Data are sparse regarding the optimal flap approach. Mucosa-only flaps may minimize the risk to unaffected rectum, but a randomized trial comparing partial thickness advancement to mucosa alone demonstrated improved efficacy in fistula closure with thicker flaps. A retrospective study by the Cleveland Clinic, Florida found only a 33% recurrence rate for flaps used in non-inflammatory bowel disease (IBD) patients. Zimmerman[9] reported on his group's success with anodermal advancement flaps and found that when used as initial therapy, this method had a greater than 75% success rate, with maintained continence in over 80%. Importantly Mitalas[10] found that repeat approaches using endoanal flaps still had a significant success rate, with 67% of patients having long-term success.

Fistulectomy

Fistulectomy is a less commonly performed technique for trans-sphincteric fistulae. It is based on the principle that removal of the chronic, epithelialized tract will allow healing by secondary intention of healthier tissue. Typically, the fistula tract is cored out over a probe or seton, leaving healthy peri-rectal fat only. Dissection is typically carried out from the external opening up to the external sphincter. This is a difficult and potentially morbid technique which may leave large tissue defects and may involve injury to the sphincter complex. Success rates are similar to fistulotomy, and subsequent incontinence rates have been shown to be as high as 15%[11]. Malik's meta-analysis found only 2 studies comparing fistulectomy to fistulotomy, and found no significant difference between the two[5].

Fibrin glue

Fibrin glue injection was the first exciting modern development in sphincter-sparing approaches to complex fistulae. The technique is based on the injection of a liquid fibrin matrix through any fistula tract which would facilitate healthy tissue ingrowth and fistula closure. The major advantage is the extremely benign nature of the approach. It requires no dissection or risk to the sphincter musculature regardless of the anatomy or complexity of the fistula tract, and has potential applications in IBD as well. However, despite early enthusiasm, long-term results have been quite disappointing with success rates as low as 16%[12-16]. Few randomized trials document fibrin glue efficacy; only one small trial comparing it to conservative management showed improved outcomes with glue therapy. Draining setons were used, resulting in an unsurprisingly low 13% (since no fistulae were deliberately closed) cure rate, while 43% were cured with fibrin glue. A more recent review reported on a recurrence rate ranging from 10%-78%[17]. Newer approaches have modified the use of fibrin glue with the addition of adipose-derived stem cells, and this approach shows promise: in one study 71% of patients with the enhanced approach healed their fistulae, compared to 16% with fibrin glue alone.

Currently, we do not advocate this approach as a primary therapy given its lack of demonstrated success. Currently, it is most often used as an adjunct measure when combined with other methods such as advancement flap, but has demonstrated little success in the literature[13]. Nevertheless, it remains a very safe technique with minimal downside other than expense and time, and may be considered prior to more invasive options when other methods have failed.

Anal fistula plug

The anal fistula plug (AFP) is a simple repair that does not involve an extensive dissection and therefore is a very attractive approach. Essentially, the plug is pulled through the fistula tract and secured in place at the internal opening (the wider portion of the plug is at the internal open-

ing) and trimmed to the skin at the external opening with the external opening left open to drain.

Initial reports documented a very high success rate, with the initial descriptions by Ellis and Johnson documenting close to 80% success[18]. This was further supported by a study from Case Western documenting an 83% success rate[19], however, with more studies the overall success rate has been found to be lower with some studies reporting a 20% success rate[18,20,21]. Jacob and Keighley's recent meta-analysis found success rates ranging from 35%-85%[2].

Thus far, it does not appear to be associated with any major complications and can be regarded as a sphincter-sparing approach. No impact on continence has been found. Wang compared the use of the fistula plug to endoanal flap advancement; both "sphincter-sparing" approaches, and found that the flap approach enjoyed an improved long-term closure rate[22]. These results were echoed by a study from the University of Minnesota[23]. Currently, we are conducting a randomized prospective trial comparing the plug to the ligation of the intersphincteric fistula tract (LIFT) procedure (see below).

LIFT procedure

In 2007, Rojanasakul described the LIFT procedure, in which the fistula tract is identified between the internal and external sphincters (intersphincteric space) and subsequently divided and ligated. His group initially reported a 94% success rate with no impact on continence (Figure 1)[24]. The procedure is appealing as it appears to be a "sphincter-sparing" technique and is a relatively simple operation to perform. The first step is to identify the intersphincteric groove. Once the skin is incised in this area, a combination of blunt and sharp dissection is used to identify the fistula tract - a task made easier if a draining seton has been left in place for at least 6 wk (Figure 1). Once the tract is identified, it is ligated on both sides and divided (Figure 2).

There have been further reports from North America and Malaysia - these have shown lower success rates of 57% and 77%, respectively[25,26]. However, compared to other procedures this still offers a comparable success rate. Other advantages of the procedure are the low cost and the fact that that even if it does not work, other approaches can still be utilized. The long-term success of this technique remains to be determined, as well as waiting to see if these success rates can be duplicated by other centers. Other studies are underway examining the efficacy of the LIFT and there is also a randomized controlled trial comparing the LIFT to the AFP.

BioLIFT

Recently, a modification of the LIFT procedure has been described. After the fistula tract is identified and divided, a biologic mesh is placed in the intersphincteric space to act as a barrier to re-fistulization. A video presented at the 2010 American Society of Colon and Rectal Surgeons meeting demonstrated this technique with promising results in a single surgeon series. This procedure entails a significant dissection of a large portion of the intersphincteric space, all the way up to the levator musculature, and the placement of a large piece of biologic mesh. Given the complexity and magnitude of this method, it may not be appropriate as first-line therapy.

WITH ALL THESE OPTIONS, WHAT IS THE TAKE HOME MESSAGE?

First, is the fistula simple or complex? This will be elucidated by history, physical and appropriate use of imaging.

If it is a simple fistula, a primary fistulotomy with marsupialization of the wound is likely the best option as the addition of marsupialization may lead to faster wound healing.

In a complex fistula, treatment should be individualized. However, the authors advocate sphincter-sparing approaches first (after a draining seton has been placed for 6-12 wk). Based on evidence and simplicity of approach, the authors generally begin with either a fistula plug or LIFT procedure (and since we had clinical equipoise concerning these two repairs we are involved in a randomized controlled trial comparing the two; there is a PLUG trial also underway comparing the plug to advancement flap). We do not use the advancement flap as our first option because it is a more extensive operation and the "downside" of failure is bigger. An important point regarding the use of LIFT is the fact that it appears to "burn no bridges"; if a LIFT procedure fails, it sometimes results in an intersphincteric fistula, which then may more safely be treated with a primary fistulotomy, and there have been no reported issues in continence after this procedure. Alternatively, the failure may still result in a trans-sphincteric fistula (and when the plug fails it always results in the same type of fistula that was originally treated). Prior to attempting definitive management again, a draining seton is usually replaced, remaining in place for 6-12 wk. We usually try a LIFT or plug again but if this fails, we then move on to other options. Further imaging after failures can also be helpful to ensure that additional tracts have not been missed.

Special cases

Crohn's disease: Thirty percent of Crohn's patients will experience perianal disease, including fistula-in-ano. Fistulae in Crohn's patients usually have complex tracts and are often multiple and arborizing, making the treatment challenging. Collaboration between surgeon and gastroenterologist is critical with this condition as medical treatment with immunomodulators (i.e. Remicade and Humira) have demonstrated significant success in perianal fistula closure.

Like other cryptoglandular fistulae, these fistulas can have an acute and chronic phase - in the acute phase they can be associated with local infection and prior to initiating medical treatment, this focus of sepsis usually needs to be dealt with. An examination under anesthesia with drainage of the septic source, as well as placement of a draining seton (or setons) is appropriately employed.

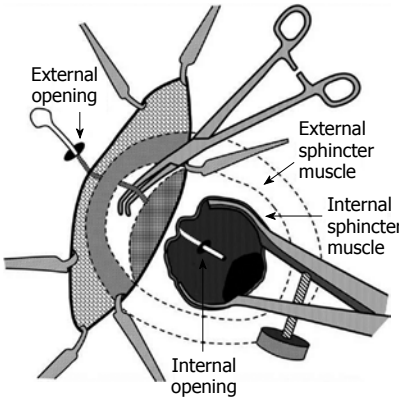

Figure 1 The fistula is identified as it crosses the plane between the internal and external sphincters. Reprinted with permission from the American Society of Colon and Rectal Surgeons.

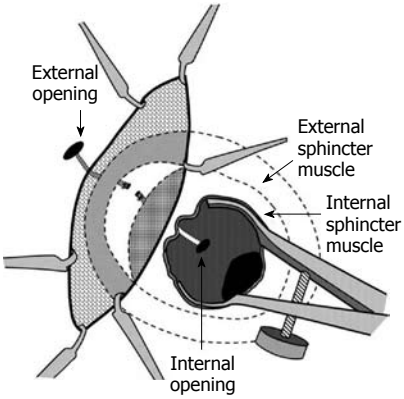

Figure 2 The fistula is dissected free, ligated and divided. Reprinted with permission from the American Society of Colon and Rectal Surgeons.

With the draining seton in place, it is rare to develop a recurrent abscess and medical therapy can be initiated. Chronic inflammation and proctitis usually define the chronic phase of this disease. Communication between surgeon and gastroenterologist should occur to determine optimal initial medical management, and subsequently, to decide if there is a role for removing the seton when the Crohn's disease seems more quiescent. Further options can be considered as discussed below.

If there is a well-established, chronically draining fistula without associated abscess at initial presentation, treatment can be individualized, but in cases where there is still occasional purulent drainage, placement of a draining seton is encouraged to help resolve any residual infection. Primary fistulotomies should be avoided in Crohn's patients as these are usually complex tracts involving significant portions of the sphincter, and impairment of sphincter function, especially in patients who are prone to looser bowel movements, can exacerbate continence issues. In addition, in the presence of multiple fistulas, the cumulative effect of several (even if they are superficial) fistulotomies can cause significant sphincter dysfunction. Treatment of a Crohn's fistula depends on what technique the surgeon is most comfortable with, but conservative, sphincter-sparing approaches are the most appropriate. Options include mucosal advancement flaps, AFPs, the LIFT and possibly, the BioLIFT. Because of the potential for changes in continence that can be seen with a cutting seton, this approach is not one advocated by the authors. It is important to note that most approaches to repair are doomed to failure in the face of active proctitis, and thus all definitive treatment other than sepsis control need to be delayed until effective management of the disease process has been achieved.

Rectovaginal and rectourethral fistula: The vast majority of rectovaginal (RV) fistulas (> 80%) are the result of obstetrical trauma[27]. Other causes include inflammatory bowel disease, infection (from cryptoglandular origin, diverticulitis or Bartholin's gland infection), radiation or neoplasm. RV fistulas are classified as "high" or "low". Anovaginal fistulas are considered low fistulas and involve the sphincter mechanism. High fistulas are those that have their origin above the sphincter complex. The operative approach differs widely based on this anatomic classification. A careful history and physical examination is used to determine the underlying etiology. Further investigations such as colonoscopy, computed tomography scan, ultrasound, and pelvic magnetic resonance imaging can be useful to identify the etiology and anatomy. If obstetrical trauma is the suspected cause, an endoanal ultrasound can be used to assess if there is a sphincter defect along with manometry to document sphincter resting and squeeze pressures. Pudendal nerve latency testing can also be done to give the patient an idea of potential success rate if a sphincteroplasty is performed (if there appears to be nerve damage, a sphincteroplasty will have a lower success rate).

With a RV fistula and concomitant sphincter injury from obstetrical trauma, a sphincteroplasty is the best option. Although the technique may be beyond the scope of this review, this involves a perineal approach and has good short-term success but longer term functional outcome begins to drop off in 3-5 years.

Low RV fistulas that are not associated with a sphincter defect are classified as complex perianal fistulas as discussed in the Introduction. Options that can be used include advancement flaps (both rectal and vaginal), LIFT procedure, BioLIFT, and AFP. In some cases where the fistula is large or after the failure of multiple previous attempts at local closure, the use of pedicled muscle flaps, such as the bulbocavernosus (Martius) or gracilis flap may be required, usually accompanied by temporary gastrointestinal (GI) tract diversion.

High RV fistulas such as those that result from diverticulitis are managed by a transabdominal approach, usually requiring proctectomy or colectomy. Continuity *via* a coloanal anastomosis can be restored depending on the clinical scenario.

Rectourethral (RU) fistula is a rare complication usually seen after intervention in the male genitourinary tract. Appropriate management and maximizing success in treating RU fistulas relies on knowing the etiology and

prior history of the patient. RU fistula most commonly arises as a complication after radical prostatectomy. Iatrogenic injuries to the rectum or local sepsis after anastomotic dehiscence are the most common causes. Rarely, minimally symptomatic patients may be observed, or an initial attempt at local repair with flap techniques may be employed. The use of an indwelling catheter is critical to healing. Although success rates are low, there is little downside to such an attempt in this highly-selected population. In cases where there are significant symptoms, and there is no history of pelvic radiation or IBD, the most appropriate first step in management is GI diversion. Up to one third of RU fistulas may heal with diversion alone[28]. Local flap repairs may then be employed if spontaneous healing does not occur. Success rates of local advancement flaps are improved if the patient is diverted.

If initial attempts at closure fail, the defect is very large, or if the patient has had prior pelvic irradiation, local closure techniques are doomed to failure. Repairs using local pedicle muscle flaps (i.e. gracilis or dartos flaps) are usually required for successful closure. Once closure of the fistula is documented (usually *via* contrast study) the diverting stoma can be closed.

CONCLUSION

Perianal fistulas present a common but challenging problem because of the involvement of the sphincter complex. Complex fistula treatment must always take in to account the need to spare sphincter function. Various treatments exist which indicates that there is no universally successful solution. Sphincter-sparing options continue to evolve and continued review of new techniques is important prior to proceeding with procedures that may impair continence; it is important that clinicians stay abreast of these changes so patients can be given the opportunity to access sphincter-sparing options.

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TOPIC HIGHLIGHT

AM El-Tawil, MSc, MRCS, PhD, *Series Editor*

Why do we have so much trouble treating anal fistula?

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Abstract

Anal fistula is among the most common illnesses affecting man. Medical literature dating back to 400 BC has discussed this problem. Various causative factors have been proposed throughout the centuries, but it appears that the majority of fistulas unrelated to specific causes (e.g. Tuberculosis, Crohn's disease) result from infection (abscess) in anal glands extending from the intersphincteric plane to various anorectal spaces. The tubular structure of an anal fistula easily yields itself to division or unroofing (fistulotomy) or excision (fistulectomy) in most cases. The problem with this single, yet effective, treatment plan is that depending on the thickness of sphincter muscle the fistula transgresses, the patient will have varying degrees of fecal incontinence from minor to total. In an attempt to preserve continence, various procedures have been proposed to deal with the fistulas. These include: (1) simple drainage (Seton); (2) closure of fistula tract using fibrin sealant or anal fistula plug; (3) closure of primary opening using endorectal or dermal flaps, and more recently; and (4) ligation of intersphincteric fistula tract (LIFT). In most complex cases (i.e. Crohn's disease), a proximal fecal diversion offers a measure of symptomatic relief. The fact remains that an "ideal" procedure for anal fistula remains elusive. The failure of each sphincter-preserving procedure (30%-50% recurrence) often results in multiple operations. In essence, the price of preservation of continence at all cost is multiple and often different operations, prolonged disability and disappointment for the patient and the surgeon. Nevertheless, the surgeon treating anal fistulas on an occasional basis should never hesitate in referring the patient to a specialist. Conversely, an expert colorectal surgeon must be familiar with many different operations in order to selectively tailor an operation to the individual patient.

Key words: Fistula; Abscess; Fibrin sealant; Anal fistula plug; Dermal advancement flap; Endorectal flap; Ligation of intersphincteric fistula tract procedure

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INTRODUCTION

The treatment of anal fistula has challenged physicians and healers for millennia. References to fistulous disease and use of both fistulotomy and setons can be found in the writings of Hippocrates, dating from 400 BC[1]. This

disease process has also been mentioned in non-scientific writings through the years. The great English author and playwright William Shakespeare used what many believe to be a historical fact, that the French King, Charles V, battled with fistula-in-ano, as a comedic plot for his play "All's well that ends well"[2]. Two thousand years have passed since Hippocrates' writings and the science of medicine has taken enormous leaps, yet we continue to struggle with fistula-in-ano.

The etymology of the word fistula comes directly from its Latin counterpart which means "pipe". In medical terminology, a fistula translates to an abnormal connection between a set of organs or vessels that do not normally connect e.g. the connection between the distal alimentary tract and the integument. For years, it has been accepted that the abnormal communication of the lower gastrointestinal system with the perianal region is due to a cryptoglandular infection. It is believed that the anal crypts become blocked by inspissated debris or stool. As a result, an infection develops at the anal glands, which extends in a path of least resistance, forming an abscess in the intersphincteric space leading to the development of a fistula in about one third of patients[3]. However, this explanation does not take into account fistulas caused by Crohn's disease, tuberculosis, lymphogranuloma venereum, and actinomycosis, first reported by Swinton in 1964[4]. This duality of cryptoglandular *vs* non-cryptoglandular fistula is also distinguished in differing treatment strategies. For instance, in actinomycosis, effective treatment mandates surgical therapy with the addition of organism-specific antibiotic therapy. Also, with the recent strides seen in Crohn's fistula treatment with immunotherapy, one begins to question whether we truly understand the pathophysiology of what we conveniently group into "cryptoglandular" fistulas in the classical sense[4,5].

Current demographic data for fistula-in-ano in the United States are difficult to ascertain, as the Health Care Utilization Project (HCUP), since 1979, has recorded only inpatient procedures through its discharge data from the National Inpatient Sample. Data from a 1979 Ambulatory Care Survey of the National Center of Health Statistics listed 24 000 individuals with the diagnosis of fistula-in-ano[6]. This corresponds to the incidence of 8.6 per hundred thousand per year reported by Sainio in 1984 in the city of Helskini[7]. Another similarity seen in these studies is the 2:1 ratio of men to women in both the US and Finland[6,7]. A more current analysis of data from Europe has been performed by Zanotti in 2007, where queries of databases in the UK, Spain, Germany and Italy showed an incidence ranging from 1.04 per 10 000 in Spain to 2.32 per 10 000 in Italy[8]. These numbers are considerably higher than those reported from Finland in the 1980s.

A common theme in this disease process in all its forms is the presence of stool within the wound, both before and after any treatment strategies. Surgeons abhor the thought of stool in surgical wounds, yet in fistula-in-ano we have to accept the fact that a fresh surgical wound will be bathed in feces on a daily basis. In Crohn's fistulous disease, it has been shown that proximal diversion helps decrease activity of disease and degree of sepsis. Interestingly, this finding is independent of the severity of Crohn's-related rectal inflammation, and thus is believed to be directly related to the diversion of stool[9]. Nevertheless, proximal diversion for what we classify as routine "cryptoglandular" fistulous disease would be considered excessive to both patients and providers. Surgeons promote cleanliness with vigilant wound care and sitz baths; however, the fact remains that from simple fistulotomy to the most sophisticated repair, we accept that patients' wounds will encounter stool, mucus, and purulence on a daily basis.

The goals of the treatment of fistula-in-ano include resolving the acute-on-chronic inflammatory process, maintaining continence, and preventing future recurrence. In reality, treatment-related incontinence, either to gas, stool or both, is the most important consideration of effective eradication of disease. Continence-related morbidity has plagued physicians through history, a fact that is evidenced even in antiquity, by the use of horse hair setons described by Hippocrates in his writings[1].

For years surgeons have performed a straightforward and effective treatment for fistula-in-ano. Simple fistulotomy, i.e. the complete laying open of the tract between the external secondary opening and the internal primary opening has resulted in success rates in the 95% range[10]. Marsupialization of the tract with a locking absorbable suture (as opposed to allowing healing by secondary intention) has been shown to decrease healing time by reducing the size of the open wound[11]. At first glance, fistulotomy should prove to be the ideal treatment method, especially when compared to the much less desirable success rates seen for treatments of other fistulous diseases such as rectovaginal or enterocutaneous fistulas. However, anorectal fistula may present in a variety of forms, and fistulotomy alone can only be used safely for "simple" disease, comprising of intersphincteric fistulas, which represent about 45% of all fistulas[12]. Similarly, if a fistula is transsphincteric but superficial in nature, and not in the anterior hemisphere in women, one may opt to perform fistulotomy with relative safety. It is important to remember, however, that fistulotomy, even for what is classified as "simple" fistula, will result in some form of incontinence in about 12% of patients[13]. The existence of complex fistulas, or those where a fistulotomy would result in incontinence, comprise approximately 50% of this disease process. These complex fistulas include high transsphincteric, suprasphincteric, extrasphincteric, all anterior transsphincteric fistulas in women and those caused by Crohn's disease and secondary to coloanal anastomosis[14].

The aim of surgical therapy of fistula is cure. If one is too aggressive with fistulotomy, cure may be achieved at a cost of incontinence. On the other hand, being too conservative, while striving to maintain continence, will result in recurrence or persistence of the fistula.

SETONS

The utilization of cutting setons, whose origins can be traced to the writings of Hippocrates, attempts to address the issue of incontinence with the performance of a fistulotomy in "complex" fistulas. In theory, it is believed that sequential tightening of a seton, over the course of weeks, will produce fibrosis and avoidance of a major sphincter defect, thus effectively allowing preservation of external sphincter function. Vial *et al*[15] performed a systematic review of 18 studies, with over 440 patients. They ascertained a recurrence rate of 5.0% for patients where the internal sphincter was not divided at the time of initial surgery, and 3.0% in instances where the internal sphincter was divided. They noted an overall fecal incontinence rate of 5.6% and 25.2%, with the latter number representing the group with intraoperative internal sphincter division. However, others have suggested an overall incontinence rate of up to 67% with the use of this technique[16]. Furthermore, the use of cutting seton for complex disease creates the problem of substantial patient morbidity with severe patient discomfort associated with incremental tightening of the seton.

Others have described attempts in converting high or complex fistulas into processes amenable to fistulotomy. The placement of a draining seton, with the hopes of converting the tract into more superficial processes, or by developing fibrosis within the transsphincteric tract, have been cited as continence-preserving options in "complex" fistulas. The use of draining setons has also been attempted as a bridging therapy in two-stage sphincter-preserving procedures, in order to allow sufficient time for the subsiding of the inflammatory or infectious process, while better defining the epithelial tract of the fistula. This then allows the utilization of one of many relatively recently defined options for sphincter-preserving treatments of fistula-in-ano. Two-stage procedures, with return to the operating room for fistulotomy, have led, however, to subsequent overall incontinence in 66% of patients[16].

FIBRIN SEALANTS AND BIOPROSTHETIC PLUGS

The injection of fibrin sealants and the more recent use of collagen plugs were initially approached with fervor. In theory, the benefits of the avoidance of post-procedure incontinence, due to the lack of sphincterotomy with the use of either modality, were enticing. Furthermore, both procedures are well tolerated by patients due to minimal dissection. It is important to note that salvage treatment is possible and that the use of either technique does not preclude subsequent treatment with other modalities, and thus does not "burn a bridge" to effective treatment of complex fistulas.

FIBRIN SEALANTS

The use of fibrin sealant was initially seen as a promising treatment strategy due to its relative ease of application, and minimal post-procedure discomfort. Its application usually follows the placement of a draining, non-cutting seton. Although authors such as Tyler *et al*[17] have reported up to 62% success rates following application, with 57% resolution at re-application for patients who failed initial treatment, other reports such as those by Loungnarath *et al*[14], with reported 69% overall fistula recurrence, have caused the modality to fall out of favor. Furthermore, Ellis *et al*[18] reported nearly double the percentage of recurrence of fistulas associated with the use of fibrin glue in combination with advancement flap repair of complex fistulas, when compared to flap alone, in a randomized controlled trial.

ANAL FISTULA PLUGS

Most of the studies on bioprosthetic plugs focus on plugs made of treated porcine submucosa; however, newer synthetic plugs have recently come onto the market. A consensus conference for use of the Surgisis plug was held in Chicago in 2007 to address the discrepancy of trials regarding the efficacy of the plug. It was concluded at the meeting that trans-sphincteric fistulas would be ideal candidates for this method of treatment. However, the plug could be used if deemed appropriate in settings of intersphincteric and extrasphincteric fistulas. Absolute contraindications to the use of bioprosthetic plugs included active infectious disease or abscess, simple fistulas, allergy to pork products, and pouch-vaginal and recto-vaginal fistulas, due to the presence of short tracts. The operative procedure entails accurate identification of the external and internal openings and drainage of any active inflammatory disease or abscess with use of a seton. Once all inflammatory disease is resolved in 6 to 8 wk, the plug is placed after debridement of the internal opening. The plug is drawn snug at the internal opening and sutured in place, and then cut flush at the external opening without fixation at this location[19]. Initial studies after the consensus statement showed success rates from 62% to 83%[20,21]. Most recently, however, McGee *et al*[22] showed a 43% success rate at a mean follow-up of 25 mo, and noted a three-fold likelihood of resolution of disease with tracts greater than 4 cm.

FLAP PROCEDURES

The relatively more recent use of endoanal rectal advancement flaps, and subsequently perianal dermal-island anoplasty, has shown some promise. The short term success rate of endoanal advancement flaps in complex fistulas has been seen in some studies to be as high as 82%[23]. Similarly a 77% success rate has been shown with the use of a dermal-island anoplasty in complex transsphincteric fistula[24].

Endoanal advancement flaps were first described in 1902 by Noble *et al* for dealing with rectovaginal fistulas following childbirth[25]. Elting *et al*[26] first reported the use of this technique for use in fistula-in-ano in 1912. However, its usage in complicated anal fistulous disease

became better known in 1985, after Aguilar *et al*[27] published their results in 189 patients, with 3 recurrences and approximately 7% incidence of incontinence to flatus and no reports of incontinence to stool. The surgical technique, as described by Aguilar, includes complete subcutaneous excision of the external opening, along with all other secondary openings, to the external sphincter muscle margin. Subsequently, a flap originating from the intersphincteric groove is raised and includes anoderm, mucosa and submucosa. The flap should extend 3 to 4 cm proximal from the internal opening, and be trapezoidal in nature, with the base wider than the apex to allow adequate blood supply. The internal opening is excised, and the internal sphincter is closed using absorbable sutures. The flap is then sutured, without tension, to the intersphincteric groove[28]. A recent meta-analysis of 35 studies by Soltani *et al*[29], including over 1600 patients, showed a success rate of over 80% for fistulas of cryptoglandular origin, with a 13% incidence of some form of incontinence. Further derivatives of this technique have been described, in combination with the use of bioprosthetic plugs or fibrin glue, with mixed results. The endoanal advancement flap, although not always ideal, has become a promising tool. However, its major limitation is that it frequently results in a mucosal ectropion, which produces mucus and gives patients the false sensation of incontinence due to spontaneous discharge and soiling.

Dermal island-flap anoplasty was first described by Del Pino *et al*[30] in 1996, as an alternative to rectal advancement flaps, in order to reduce risk of mucosal ectropion and anal discharge. The operative technique entails the formation of a tear drop-shaped incision encompassing the perianal skin containing the external opening. The incision is extended just proximal to the internal opening of the trans-sphincteric fistula. Subsequently, the internal opening is excised and debrided, and the internal sphincter at this level is closed using absorbable suture. The flap is mobilized without undermining, and the dermal island is sewn to the rectal mucosa with absorbable sutures. The external opening is neither excised nor debrided. Nelson *et al*[6] found a procedure failure rate of 23% with an associated patient failure rate of 20% with this method, in a mean follow-up period of 28.4 mo. As of recent publications, incontinence data is not available; however, it is safe to assume it to be similar to that of endoanal rectal advancement flaps.

LIGATION OF INTERSPHINCTERIC FISTULA TRACT

Most recently, the introduction of the ligation of intersphincteric fistula tract (LIFT) procedure has sparked interest with good short term results. This procedure, first proposed by Rojansakul in 2007, focuses on the ligation of the intersphincteric tract of the fistula, and can be applicable for both complex and recurrent fistula[31]. In recurrent fistula, previous internal sphincterotomy will impede proper dissection of the tract. This method delineates the trans-sphincteric tract, with careful dissection in the intersphincteric groove, with or without the help of a fistulotomy probe. Once the fistula track is isolated it is ligated with absorbable suture on both proximal and distal sides and divided between the ligatures. The success of LIFT procedure is reported to be 75%-80%[32-34].

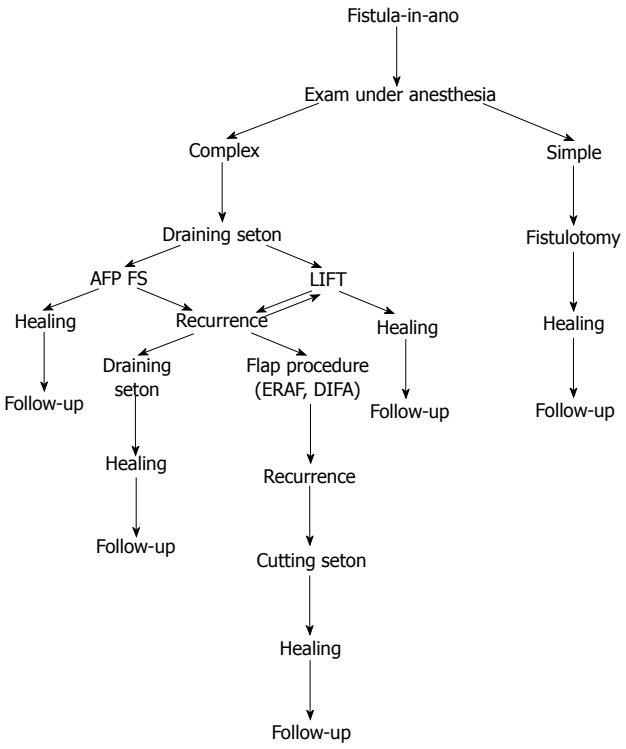

Figure 1 Treatment algorithm. AFP: Anal fistula plug; FS: Fibrin sealant; ERAF: Endoanal advancemnt flap; DIFA: Dermal island flap anoplasty; LIFT: Ligation of intersphincteric fistula tract.

TREATMENT STRATEGY

In our experience, simple fistulotomy is attempted if none or minimal amount of external sphincter is involved in the fistulous tract. In all other cases, a draining seton is placed as a bridging therapy for a minimum of six to eight weeks. After this time, a controlled exam under anesthesia is performed, and if the acute inflammatory process is resolved, then our treatment algorithm follows that of conservative management for continence preservation. If at this point minimal sphincter involvement is identified, then simple fistulotomy with marsupialization is performed. If the tract is deemed "complex" in nature, then a flap, either dermal-island or endoanal advancement, is used, with adequate drainage of the tract through the external opening using a small caliber Malecot drain. If the tract appears to be of sufficient length, then use of bioprosthetic plug is considered. Currently we are studying the utility and efficacy of the LIFT procedure; however, early results seem promising in nature (Figure 1).

Fistula-in-ano continues to prove a formidable chal-

lenge to surgeons. Our understanding of the disease process, although well established, contains gaps in the understanding of complex pathophysiology. While effective treatment has been established for "simple" cases, the concern for iatrogenic sphincter injuries, with resulting incontinence, continues to plague cases of "complex" disease with major sphincter involvement. Recent strides in the development of sophisticated procedures for continence preservation appear to be promising. However, due to location of fistula, contamination of all repairs with feculent soilage challenges the integrity our results. Concern for incontinence has resulted in trading a single-stage curative procedure (fistulotomy) for multiple sphincter-preserving operations, each with varying success rates.

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TOPIC HIGHLIGHT

AM El-Tawil, MSc, MRCS, PhD, *Series Editor*

Why do we have to review our experience in managing cases with idiopathic fistula-in-ano regularly?

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Abstract

"Why do we have to review our experience in managing idiopathic fistula-in-ano regularly?" In order to answer this apparently simple question, we reviewed our clinical and surgical cases and most important relevant literature to find a rational and scientific answer. It would appear that whatever method you adopt in fistula management, there is a price to pay regarding either rate of recurrence (higher with conservative methods) or impairment of continence (higher with traditional surgery). Since, at the moment, reliable data to identify a treatment as a gold standard in the management of anal fistulas are lacking, the correct approach to this condition must consider all the anatomic and clinicopathological aspects of the disease; this knowledge joined to an eclectic attitude of the surgeon, who should be familiar with different types of treatment, is the only guarantee for a satisfactory treatment. As a conclusion, it is worthwhile to remember that adequate initial treatment significantly reduces recurrence, which, when it occurs, is usually due to failure to recognise the tract and primary opening at the initial operation.

"Why do we have to review our experience in managing idiopathic fistula-in-ano regularly?"

The answer to the above question is quite straightforward: surgeons are aware of the poor levels of evidence in anal fistula surgery. Despite the high frequency of suppurative ano-perianal lesions of suspected cryptoglandular origin (idiopathic abscess and fistula-in-ano), the ideal treatment with outcomes of no recurrence, minimal incontinence and good quality of life is still a matter of debate. The traditional surgical treatments which include a division of a continuous part of the sphincteric complex (in particular of the superficial external sphincter in transphincteric fistulas) have been strongly challenged, especially in the last 10-20 years since high rates of impairment of continence have been reported in several experiences[1,2].

In spite of a high successful healing rate varying from 87% to 100%[3], the traditional invasive methods of fistulotomy (with/without draining or slow-cutting seton) and fistulectomy (with closure of internal opening with/without sphincter defect repair) have given way to a number of sphincter-sparing methods: endorectal muscular or mucosal advancement flap[4,5], island flap anoplasty[6], radiof-

requency ablation[7], fistulous tract filling with fibrin or cyanoacrylate glue[8,9], porcine small intestine submucosa-derived anal fistula plug[10], ayurvedic seton[11], ligation of intersphincteric fistula tract (LIFT) procedure[12,13], glue containing adipose-derived stem cells[14], and finally VAAFT (video-assisted anal fistula treatment) carried out with the Storz Meinero fistuloscope®[15].

This continuous research for an ideal conservative method is compelled by concepts recently restated by the Standards of Practice Task Force of the American Society of Colon and Rectal Surgeons[16] and by the Association of Coloproctology of Great Britain and Ireland: division of > 30% of the external sphincter should be undertaken with considerable caution for the relevant risk of impairment of anorectal continence, particularly in females, those with anterior fistulas, advanced aged patients, history of previous anorectal surgery, childbirth, fistula associated with Crohn's disease and obviously in patients with a history of continence impairment not related to the fistula[17].

Indeed, the necessity to identify patients with high risk of incontinence after classic surgical treatment has been stressed over the past few years[18,19]. These patients, representing a limited number of subjects, have been treated with a conservative approach usually represented by the non-cutting draining seton. Thus, considering that the reported rate of impairment of continence after traditional fistula surgery varies from 0% to 82%[20], the doubt arises that the definition of "incontinence" is not the same for all authors and that factors other than the amount of divided sphincter may have a role in continence disturbance. In addition, as already observed by Parks[18], the degree of impairment of anal continence after fistulotomy is not strictly tied to the type of fistula treated and the amount of severed muscle; patients treated for suprasphincteric fistulas (theoretically at higher risk of incontinence) fared better than patients treated for transphincteric fistulas[18]. Nevertheless, it seems obvious that a risk of continence impairment is present when a sphincter is cut or stretched. Also, a trivial lateral internal sphincterotomy for the cure of fissure or a hemorrhoidectomy has a risk of continence impairment[2].

As regards fistula treatment, the question is whether a real advantage is offered by the new proposed methods, especially in the management of the so-called "complex fistulas".

According to several authors[4,18,21], a complex fistula must have one or more of the following features: the tract crosses more than 30% to 50% of the external sphincter; the fistula is anterior in a female; multiple tracts are present; the fistula is recurrent; there is pre-existing incontinence; the perianal area has been irradiated; there is concomitant Crohn's disease.

A recent review of randomized studies in the literature[11] evaluated some proposed conservative methods *vs* traditional surgery (in particular: anal sphincter-preserving seton, conventional seton, ayurvedic seton, conventional fistulotomy with/without seton, radiofrequency, advancement flap with/without fibrin glue, island flap anoplasty, fistulectomy) and concluded that there were no significant differences in recurrence rates or incontinence rates in any of the studied comparisons, except in the case of advancement flaps where the lowest incontinence rates were reported. However, in other experiences of advancement flap procedures, which have been demonstrated as reliable with 77% to 100% healing rates and 21% recurrences, nevertheless 40% of patients had some impairment of continence and 9% presented major disturbance[22,23]. Advancement flap is not a simple procedure and damage of the sphincter is possible. In fact, it has been reported[24] that patients with complex fistulas undergoing fistulectomy with immediate sphincter repair had less recurrences and continence impairment than patients submitted to endoanal advancement flap.

It would appear that whatever method you adopt in fistula management, there is a price to pay regarding either rate of recurrence (higher with conservative methods) or impairment of continence (higher with traditional surgery).

The point is that it is difficult to establish whether, and to what degree, an impairment of continence has a negative effect on the quality of life (QoL) greater than the distress caused by multiple recurrences of a fistulous abscess or fistula.

The assessment of personal impairment in relation to objective medical findings represents a problem in the evaluation of incontinence. The degree of sphincter dysfunction does not always correlate with the patient's subjective awareness of his functional deficit[2]. QoL parameters in fistula surgery are generally based on incontinence scores; however, QoL has a multidimensional aspect that must be taken into account.

The promising results reported by some authors regarding the two least invasive conservative methods, fibrin glue[25] and Surgisis® AFP™ anal fistula plug[10], are interesting (almost none of the patients report impairment of continence); however, their efficacy in healing the fistulas needs to be better evaluated. Healing rates from 31% to 85% have been reported for fibrin glue and from 14% to 87% for the plug[9]. Most of the reported experiences suffer from a small number of patients and short follow-up (often less than 6 mo), with the highest rate of success being for simple uncomplicated fistulas in which traditional treatments have also a high rate of success with low rate of continence impairment[9]. Lack of long-term randomized studies is the other limiting factor for evaluating the efficacy of these procedures. Immediate healing of a fistulous tract does not mean that the infection has disappeared. A fistula can recur after months or years in the same tract or nearby. It must also be considered that when a fistula recurs, patients tend to change surgeon; similarly to what happens in recurring inguinal hernia. Regardless, the adoption of bioprosthetic material as a first-line treatment in complex anal fistulas is recommended by several authors[26,27] ahead of the more prudent suggestions of the consensus conference promoted by the Association of Coloproctology of Great Britain and Ireland[28].

Since, at the moment, reliable data to identify a treatment as a gold standard for the management of anal fistula are lacking, the correct approach to this condition must be to consider all the anatomic and clinicopathological aspects of the disease. This knowledge joined to an eclectic attitude of

the surgeon, who should be familiar with different types of treatment, is the only guarantee for a satisfactory outcome.

As a conclusion, it is worthwhile to remember the following concepts and facts: an adequate initial treatment significantly reduces recurrence, which when it occurs is usually due to failure to recognise the tract and primary opening at the initial operation[19,29-31]; many complex fistulas are iatrogenic in origin[32]; in the acute phase (fistulous abscess) a radical treatment should be attempted only by experienced colorectal surgeons[19]; primary suprasphincteric or extrasphincteric fistulas (according to Parks' classification) of cryptoglandular origin are very rare if not nonexistent[32]; it is essential to have a three dimensional vision of the anorectal region to understand the pathway of diffusion of cryptoglandular infections; a preoperative evaluation of risk factors for incontinence, including frequency of defecation, bowel function and sphincter function, is also mandatory in non-complex fistulas.

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ORIGINAL ARTICLE

Helicobacter pylori arginase mutant colonizes arginase Ⅱ knockout mice

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Abstract

AIM: To investigate the role of host and bacterial arginases in the colonization of mice by *Helicobacter pylori* (*H. pylori*).

METHODS: *H. pylori* produces a very powerful urease that hydrolyzes urea to carbon dioxide and ammonium, which neutralizes acid. Urease is absolutely essential to *H. pylori* pathogenesis; therefore, the urea substrate must be in ample supply for urease to work efficiently. The urea substrate is most likely provided by arginase activity, which hydrolyzes L-arginine to L-ornithine and urea. Previous work has demonstrated that *H. pylori* arginase is surprisingly not required for colonization of wild-type mice. Hence, another *in vivo* source of the critical urea substrate must exist. We hypothesized that the urea source was provided by host arginase Ⅱ, since this enzyme is expressed in the stomach, and *H. pylori* has previously been shown to induce the expression of murine gastric arginase Ⅱ. To test this hypothesis, wild-type and arginase (*rocF*) mutant *H. pylori* strain SS1 were inoculated into arginase Ⅱ knockout mice.

RESULTS: Surprisingly, both the wild-type and *rocF* mutant bacteria still colonized arginase Ⅱ knockout mice. Moreover, feeding arginase Ⅱ knockout mice the host arginase inhibitor S-(2-boronoethyl)-L-cysteine (BEC), while inhibiting > 50% of the host arginase Ⅰ activity in several tissues, did not block the ability of the *rocF* mutant *H. pylori* to colonize. In contrast, BEC poorly inhibited *H. pylori* arginase activity.

CONCLUSION: The *in vivo* source for the essential urea utilized by *H. pylori* urease is neither bacterial arginase nor host arginase Ⅱ; instead, either residual host arginase Ⅰ or agmatinase is probably responsible.

Key words: Arginase; *Helicobacter pylori*; S-(2-boronoethyl)-L-cysteine; Urease; Mice

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INTRODUCTION

Helicobacter pylori (*H. pylori*) causes gastritis[1-3], peptic ulcers[3], and is a significant risk factor for gastric adenocarcinoma[4]. Although the mechanisms behind this spectrum of diseases are not well understood, the very powerful bacterial urease is clearly central to pathogenesis[5-9]. Urease remains a key enzyme for diagnosing *H. pylori* infection[10].

Urease hydrolyzes urea to carbon dioxide (carbonic acid) and ammonium; the latter of which serves to protect the bacterium by neutralizing the acidic microenvironment[11]. Urease is the most abundant protein in *H. pylori*, and accounts for up to 10% of the total cellular protein[5]. It is assumed that urea, the substrate for urease, must be provided abundantly *in vivo* from either the bacterium or the host in order for urease to function efficiently (Figure 1). Indeed, the *in vivo* concentration of urea in the stomach is approximately 5 mmol/L[12,13], which is well above the K_m for urease, and yet the *in vivo* source of the urea for *H. pylori* urease has remained unknown. It originally had been hypothesized that the urea comes from the bacterial arginase, RocF. Arginase catalyzes the hydrolysis of arginine to ornithine and urea (Figure 1). The surprising finding that arginase (*rocF*) mutants of *H. pylori* can still colonize mice suggests that another *in vivo* source of urea must exist[14]. The urea may come from direct release from gastric epithelial cells or other cells in gastric pits lining the stomach. Alternatively, the urea may diffuse into the gastric juice from the bloodstream. In either case, host arginases would be responsible.

We hypothesized that the host arginases are responsible for the urea that is needed by the *H. pylori* urease. There are two known host arginases: arginase Ⅰ and arginase Ⅱ. Arginase Ⅰ is the cytoplasmic enzyme that is expressed heavily in the liver and at lower levels in a few other tissues, whereas arginase Ⅱ is the mitochondrion-associated enzyme that is expressed in many tissues and cells, including the stomach, kidneys and macrophages[15]. Further support for a host-derived stomach source of arginase also comes from the findings that: (1) the arginase Ⅱ gene is expressed in the stomach in mice and humans[16]; and (2) arginase immunohistochemistry staining is markedly elevated in human gastric cancer[17,18]. Both arginase Ⅰ and arginase Ⅱ knockout mice have become available[19,20]. Arginase Ⅰ knockout mice have severe health problems and die between neonatal days 10 and 14[19], which makes it nearly impossible to use this model with *H. pylori*. In contrast, arginase Ⅱ knockout mice have no reported overt health problems[20], although they appear to have reduced fecundity (McGee, unpublished observations).

Arginase Ⅱ is expressed in the human and mouse stomach[16], therefore, we reasoned that the arginase Ⅱ knockout mouse would allow us to determine the relative contributions of host *versus* bacterial arginase to colonization *in vivo*, through the production of urea. The *rocF* arginase mutant of *H. pylori* was included in this study in case the bacterial arginase became an important source of urea in the context of the arginase Ⅱ knockout mice. Also, recent evidence has indicated that *H. pylori* induces murine and human gastric arginase Ⅱ[16], which supports the logical choice of arginase Ⅱ knockout mice as the ideal model for these experiments.

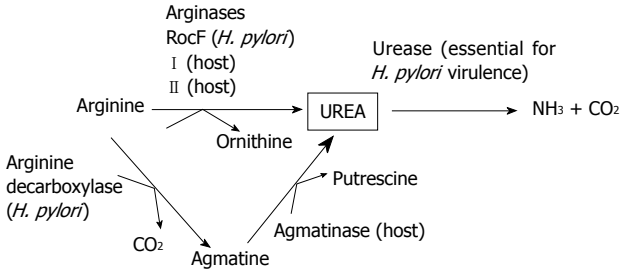

Figure 1 Overview of possible urea sources for *Helicobacter pylori* urease. *Helicobacter pylori* (*H. pylori*) urease is essential for colonization of mice, which indicates that the substrate for the enzyme, urea, is also essential. This study examines the three possible arginase-mediated sources for the urea (underlined): arginase from *H. pylori* (RocF), arginase Ⅰ (host) and arginase Ⅱ (host). Another possible urea source is host agmatinase. *H. pylori* does not have an agmatinase, but does have an enzyme, arginine decarboxylase, that can synthesize agmatine. Arginine is an essential amino acid for *H. pylori* and serves as a substrate for both arginase and arginine decarboxylase.

We infected wild-type or *rocF* mutant *H. pylori* into homozygous arginase Ⅱ knockout mice to decipher whether bacterial or host arginase Ⅱ was important for *H. pylori* colonization. To our surprise, the *rocF* mutant *H. pylori* colonized the arginase Ⅱ knockout mice similar to wild-type mice, which suggests that the *in vivo* source of urea for *H. pylori* urease hydrolysis is from another pathway. Partial inhibition of host arginase Ⅰ by the potent arginase inhibitor S-(2-boronoethyl)-L-cysteine (BEC)[21] still permitted colonization of the *rocF* mutant at or near wild-type levels in the arginase Ⅱ knockout mice.

MATERIALS AND METHODS

Bacterial strains, growth conditions, primers, and plasmids

H. pylori strains SS1[22] and the isogenic *rocF* mutant[14] were routinely grown on *Campylobacter* agar (Becton Dickinson, Sparks, MD, USA) with 10% (v/v) sheep defibrinated blood (CBA; blood from Quad Five, Ryegate, MT, USA) for 2 d, using the CampyPak Plus system (Becton Dickinson), or in a humidified microaerobic atmosphere (5% O_2, 10% CO_2, and 85% N_2). Kanamycin (10-15 μg/mL) was added as needed.

For mouse inoculation, wild-type SS1 and the isogenic *rocF* mutant were passaged an equal number of times from frozen stocks onto CBA. Strains were then grown overnight in a T 25 cm^2 tissue culture flask without aeration in 5 mL Mueller-Hinton broth that contained 1% heat-inactivated fetal bovine serum (FBS). Strains were then diluted 1:40 in 40 mL F-12 plus FBS (2%) in a T 75 cm^2 tissue culture flask and allowed to grow for an additional 16-18 h at 37℃ under microaerobic conditions. All strains grew equally well under these conditions and were inspected by light microscopy for motility and purity. Bacteria were harvested by centrifugation, washed with 1 × PBS (Invitrogen, Carlsbad, CA, USA), resuspended in 1 mL

PBS, and used for inoculation of animals.

Arginase activity by detection of ornithine

Arginase activity to detect ornithine production was determined as described previously[23] using a heat-activation step in the presence of 5 mmol/L cobalt chloride (*H. pylori*) or manganese chloride (mouse/rat) for 30 min at 50-55℃, followed by 1 h incubation at 37℃ in arginase buffer [15 mmol/L MES (pH 6.0) plus 10 mmol/L L-arginine for *H. pylori*; 15 mmol/L Tris (pH 9.0) plus 10 mmol/L L-arginine for mouse or rat]. Absorbance at 515 nm was determined.

Purification of H. pylori arginase

Enzymatically active *H. pylori* RocF was purified as a six-histidine-tagged fusion protein (His_6-RocF) as described previously[23].

Construction of pGEN222-rocF

Plasmid pGEN222, kindly provided by Dr. Jim Galen (University of Maryland, Center for Vaccine Development)[24], was digested with *Sal* I and *Bam*H I and the 1.1 kb *rocF* gene with its own promoter was excised from pBS-*rocF*[14] using the same enzymes. The ligated construct was transformed into *Escherichia coli* (*E. coli*) DH5α and confirmed by digestions and sequencing.

Rat arginase I

A plasmid carrying the rat arginase I gene, pARGr-2[25], kindly provided by Dr. Sid Morris (University of Pittsburgh), was transformed into *E. coli* DH5α, and plasmid extraction and restriction analysis showed that the construct was correct.

Mouse genotyping

Mouse genotyping was similar to that described by Shi *et al*[20], with modifications described below. Chromosomal DNA was isolated from mouse ear (approximately 2 mm diameter) or tail snips using a lysis buffer (10 mmol/L Tris, pH 7.8, 75 mmol/L NaCl, 25 mmol/L EDTA pH 8.0, 1% SDS, 500 μg/mL Proteinase K) followed by phenol-chloroform extraction, ethanol precipitation, and elution in 10 mmol/L Tris/1 mmol/L EDTA, pH 8.0. This template was used in PCR with 200 pmol of each primer, 0.25 mmol/L dNTPs, and *Taq* DNA polymerase under the following conditions: 94℃, 5 min for one cycle; 94℃ for 1 min, 53℃ for 1 min, 72℃ for 1 min for 35 cycles; and 72℃ for 5 min. Primer sequences were as follows: DM52: 5'-TCCTTTCTCCTGTCTAATTC-3'; DM53: 5'-CTAGCATCTAATTGACTGCC-3'; DM54: 5'-CCATGATGGATACTTTCTC-3'. Expected product sizes were: DM 52/53 = 500 bp (wild-type mice); DM 52/54 = 870 bp (arginase knockout mice). Heterozygotes have both products.

Mouse inoculation experiments

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. All animal experiments were approved by the Institutional Animal Care and Use Committee at LSU Health Sciences Center-Shreveport (protocol # 06-020). Euthanasia was done by carbon dioxide asphyxiation. Arginase Ⅱ knockout mice were initially bred from a homozygous knockout female and a heterozygous male generously provided by Dr. W O'Brien (Baylor College of Medicine, Houston, TX, USA). Offspring were genotyped and homozygous knockouts were retained for further breeding. Heterozygotes were euthanized from the colony. Male and female homozygous knockouts were used. The wild-type mice were C57BL/6 (Charles River Laboratories; ⩾ 4 wk old), because homozygous wild-type littermates were euthanized from the breeding colony about 6 mo after the knockout line was established. Following the experiments, the breeding colony and all excess mice were euthanized due to insufficient resources.

Animals were inoculated orally (25-0 μL approximately 10^8-10^9 viable CFU/mL) with bacteria suspended in PBS (pH 7.4). Control animals received PBS. At various time points post-infection, animals were euthanized by cervical dislocation under anesthesia. Stomachs were removed, dissected longitudinally along the greater curvature, and the chyme removed. The rest of the stomach was usually dissected into antrum (distal region), body (middle region) and fundus/cardia (proximal region) portions, weighed, and homogenized (Ultra-Turrax T25, IKA Works, Inc; 10-15 s at a setting of 3) in 1.0 mL sterile PBS. There was insufficient stomach material to measure urea content. Stomach homogenates and dilutions thereof (serial 10-fold in 96-well microtiter dish) in PBS were plated for viable counts in duplicate on CBA plates that contained six antimicrobials to suppress normal flora as described previously[26]. *H. pylori* is resistant to these antimicrobials. Plates were incubated at 37℃ for 5 d in a microaerobic atmosphere. Data are presented as CFU/g stomach or tissue section. Other organs were removed, weighed and homogenized in PBS, and assayed immediately for arginase activity.

BEC experiments

BEC is a potent inhibitor of host arginases and was chemically synthesized according to published procedures[21]. BEC was given to animals (50 mmol/L) in the drinking water *ad libitum*, or directly given orally twice daily to mice (20 μL; approximately 50-100 μmol/L final concentration). BEC was also used *in vitro* for biochemical experiments.

Statistical analysis

Arginase and animal data were analyzed by unpaired, two-tailed *t* test, Welch corrected. $P < 0.05$ was considered significant. InStat software was used (GraphPad Software, San Diego, CA, USA).

RESULTS

Arginase I knockout mice breeding and genotyping

We received a heterozygous male and a homozygous female from the established arginase Ⅱ knockout mouse breeding colony[20], therefore, we internally bred for the ho-

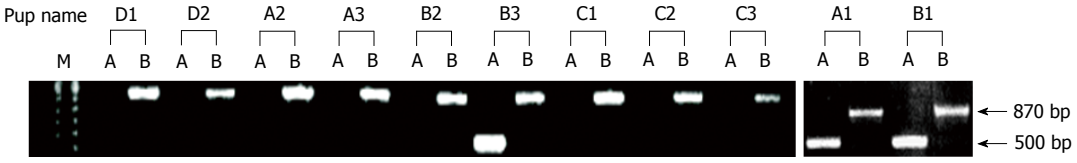

Figure 2 Genotyping of mice to determine whether they are heterozygous or homozygous arginase Ⅱ knockouts. Pups obtained from breeding heterozygous males to arginase Ⅱ knockout females were genotyped by PCR analysis. Wild-type band = 500 bp ("A" lanes); mutant band = 870 bp ("B" lanes). Eight of the 11 pups were homozygous knockouts. Pups A1, B1, and B3 were heterozygous because they contained wild-type and mutant copies of arginase Ⅱ. M: 100 bp marker (Bio-Rad).

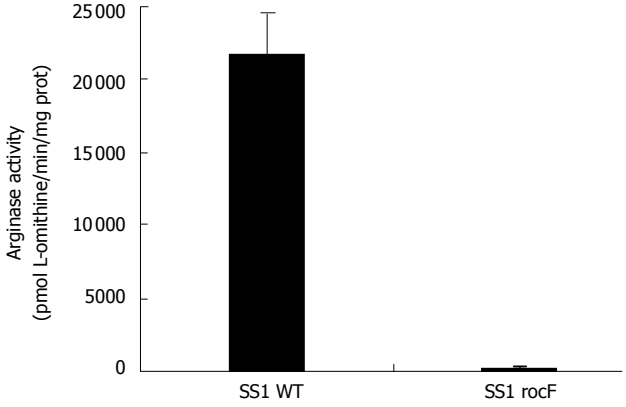

Figure 3 The *rocF* mutant of *Helicobacter pylori* strain SS1 is devoid of arginase activity. Bacteria were harvested in 0.9% NaCl and ice-bath-sonicated (25% intensity, 2 pulses of 30 s each, with 30 s rests on ice between pulses). Following centrifugation (12000 *g*, 2 min, 4℃), supernatants were retained on ice. Equal volumes of extract and 10 mmol/L cobaltous chloride ($CoCl_2$•6 H_2O, final concentration of 5 mmol/L) were preincubated for 30 min at 50-55℃ to activate the enzyme. Arginase buffer (15 mmol/L Tris, pH 7.5, or 15 mmol/L MES, pH 6.0, plus 10 mmol/L L-arginine) was added and incubated at 37℃ for 1 h. The reaction was stopped by addition of 750 μL acetic acid, and the color developed by addition of 250 μL ninhydrin (4 mg/mL) at 95℃ for 1 h and read at 515 nm. The data are presented in units where 1 U is defined as one pmol L-ornithine/min/mg protein + SD. Details of this assay have been reported previously[23].

mozygous arginase Ⅱ knockout mice and genotyped the offspring, similar to that described by Shi *et al*[20]. We successfully obtained homozygous arginase Ⅱ knockout mice (Figure 2). Once the homozygous arginase Ⅱ knockout line was firmly established, we euthanized all heterozygotes from the colony to ensure that no contamination occurred. The homozygous arginase Ⅱ knockout mice were then periodically PCR confirmed for the knockout. Occasionally, we observed reduced fecundity with the arginase Ⅱ knockout colony, although we did not pursue this.

Arginase activity by measurement of ornithine

Arginase activity, determined by ornithine detection in the presence of arginine, was measured from extracts of wild-type SS1 and the isogenic *rocF* mutant grown on CBA plates. No appreciable activity was detected in the mutant, in contrast with the wild-type strain, which had arginase activity (Figure 3). Omission of arginine led to no detectable ornithine, which showed that all of the ornithine that was detected was produced by arginine hydrolysis (data not shown). These results demonstrated that the *rocF* mutant of *H. pylori* had no arginase activity and did not appear to have alternative mechanisms of producing ornithine under these conditions. Using nuclear magnetic resonance, we have previously reported that the *rocF* mutant of SS1 has no arginase activity[14]. There is no evidence for the existence of an alternative urea-generating pathway in *H. pylori*, based on biochemical, genomics and metabolic networking approaches (McGee, unpublished observations)[27], but we cannot completely rule out this possibility.

Arginase Ⅱ knockout mice become infected with arginase mutant H. pylori

We have previously demonstrated that wild-type and *rocF* mutant *H. pylori* strain SS1 colonize wild-type mice[14]. To determine whether arginase Ⅱ knockout mice were colonized by *H. pylori*, these mice were inoculated with wild-type or *rocF* mutant SS1 *H. pylori*. At 2 wk post-infection, there was a trend towards a 1 log_{10} decrease in colonization of the entire stomach by the *rocF* mutant compared with the wild-type strain, but this did not reach statistical significance (Figure 4A). By 10 wk post-infection, there was no difference in colonization between the wild-type and the *rocF* mutant (Figure 4B). We concluded that the *rocF* mutant devoid of arginase activity colonized arginase Ⅱ knockout mice at or near the levels of the wild-type strain of *H. pylori*.

The experiment was repeated, but this time the antrum and body portions were separated, in order to understand whether there might be tropism differences. At 4 wk post-infection, the wild-type and *rocF* mutant both colonized the antrum and body regions of the stomach of arginase Ⅱ knockout mice at similar levels (data not shown).

H. pylori wild-type and *rocF* mutant both induced similar levels of gastritis in the arginase Ⅱ knockout mice (inflammatory score = 2.5 for wild-type; 2.5 for *rocF* mutant, four mice in each group).

BEC decreases in vivo host arginase activity

The *rocF* mutant surprisingly colonized the arginase Ⅱ knockout mice, yet the urea substrate must be available for *H. pylori* urease to function *in vivo*, therefore, we abandoned the hypothesis that arginase Ⅱ generates the essential urea substrate for *H. pylori* urease. Instead, we reasoned that the source of the urea must originate from host arginase Ⅰ. Arginase Ⅰ knockout mice die at neonatal days 10-14, therefore, we could not test the role of arginase Ⅰ directly. Thus, we used the host arginase inhibitor BEC. Preliminary experiments using different concentrations of BEC in mice suggested a concentration of 50 mmol/L would have at least some arginase inhibitory activity in the

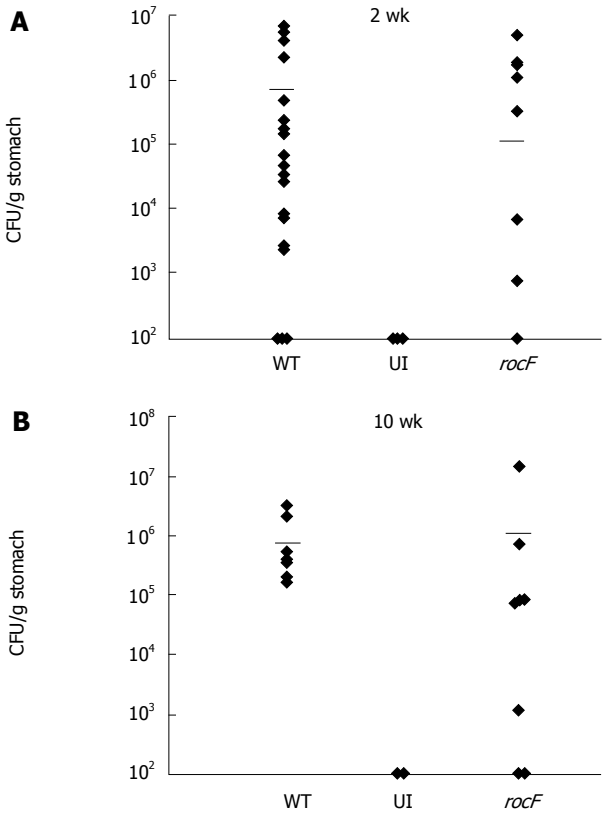

Figure 4 The *rocF* mutant of *Helicobacter pylori* colonizes the stomach of arginase Ⅱ knockout mice. Arginase Ⅱ knockout mice were inoculated with either the wild-type SS1 strain (WT) of *Helicobacter pylori* (*H. pylori*) or the isogenic *rocF* mutant (*rocF*). Number of animals used per group were as follows: 2 wk experiment: 13 for WT, nine for *rocF* mutant; 10 wk experiment: eight for WT, eight for *rocF* mutant. At 2 (A) or 10 (B) wk post-infection, stomachs were removed, completely homogenized, and plated for *H. pylori*. UI, uninfected controls (n = 2 at 2 and 10 wk). Limit of detection: ~100 CFU/g stomach; all animals that lacked *H. pylori* were set to this detection limit. Bar, mean CFU/g stomach. At 2 wk post-infection, P = 0.65 by unpaired two-tailed t test between the mean CFU/g tissue for the wild-type versus the *rocF* mutant. Each symbol represents one mouse. In some cases there are two mice represented by a symbol if the data overlapped.

stomach (data not shown). The expense and concern of toxicity to the animals precluded using higher concentrations. Also, preliminary experiments that compared BEC given *ad libitum* in the drinking water *versus* twice daily by pipetting 20 μL of 50 mmol/L concentration directly into the oral cavity revealed that the latter gave more consistent inhibition of arginase activity (data not shown), and lower quantities of the inhibitor could be used. Thus, oral delivery was used for subsequent experiments.

To demonstrate that BEC inhibited arginase *in vivo*, we fed uninfected arginase Ⅱ knockout mice with BEC by direct oral delivery to inhibit host arginase Ⅰ. The data showed that exceptionally high levels of arginase activity occurred in the liver, as expected for the known location of highest expression of arginase Ⅰ [15]. About 50% of this strong activity was inhibited by BEC (Figure 5A), which indicated that oral BEC delivery had a systemic effect on inhibiting arginase Ⅰ activity. In the kidney, very low levels of arginase Ⅰ activity were detected and this was reduced more than fivefold by BEC (Figure 5B). In the antrum region of the stomach, arginase Ⅰ activity was intermediate to that of the liver or kidney and could be inhibited by more than threefold by BEC (Figure 5C). In contrast, in the body (Figure 5D) and the fundus (data not shown), BEC did not appreciably inhibit the arginase activity, although there was a lot of animal to animal variability. Arginase activity was also much lower in these other gastric regions than in the antrum. We conclude that BEC inhibits at least some of the arginase Ⅰ activity *in vivo*, but residual activity still occurs.

If arginine was omitted from the enzyme buffer, no ornithine was detectable in the stomach (data not shown), which indicated that the tissues did not have appreciable amounts of pre-formed ornithine.

BEC does not inhibit H. pylori arginase activity, but does block arginase Ⅰ activity

BEC is an established, potent eukaryotic arginase inhibitor with a K_i of 400-600 nmol/L[21], therefore, we questioned whether BEC would inhibit *H. pylori* arginase, which shares very limited amino acid homology with human arginases (data not shown). A BEC dose-response curve using extracts from *E. coli* that expressed the *H. pylori rocF* gene (Figure 6A) revealed only partial inhibition of *H. pylori* arginase activity at very high BEC concentrations (≥ 25 μmol/L). Even at 1000 times above the K_i for eukaryotic arginases, 400 μmol/L BEC only inhibited about 50% of the *H. pylori* activity. To show that this lack of inhibition was not due to the complexity of extracts, we purified the *H. pylori* arginase and tested whether BEC would inhibit *H. pylori* arginase activity. BEC used at the K_i for eukaryotic arginases (400 nmol/L) completely failed to inhibit *H. pylori* arginase activity (Figure 6B). To prove that the BEC was working properly under these conditions and also to show directly that BEC can inhibit arginase Ⅰ *in vitro*, we used extracts from *E. coli* that expressed the rat arginase Ⅰ. Rat and mice arginase Ⅰ share 93% amino acid identity and 96% similarity (http://blast.ncbi.nlm.nih.gov/Blast.cgi), therefore, we assume that both enzymes behave in a similar fashion with respect to BEC inhibition. BEC was shown to inhibit 70% of the rat arginase Ⅰ activity at just 80 nmol/L and > 97% at concentrations of ≥ 4 μmol/L (Figure 6C), which was in good agreement with the published K_i values[21]. These results show that BEC strongly inhibits eukaryotic arginase Ⅰ under these enzyme assay conditions, yet BEC is a poor inhibitor of *H. pylori* arginase[21].

rocF mutant of H. pylori colonizes BEC-treated wild-type and arginase Ⅱ knockout mice

Wild-type or arginase Ⅱ knockout mice were pretreated with BEC or water and inoculated with wild-type or *rocF* mutant *H. pylori*. Animals were euthanized at 1 wk post-infection. The *rocF* mutant of *H. pylori* colonized the BEC-treated wild-type mice (Figure 7). Remarkably, the *rocF* mutant also colonized BEC-treated arginase Ⅱ knockout mice (Figure 8) at levels similar to wild-type *H. pylori* (P > 0.05). This similar colonization occurred in all three regions of the stomach (antrum, body and fundus). The experiment was repeated in the arginase Ⅱ knockout mice

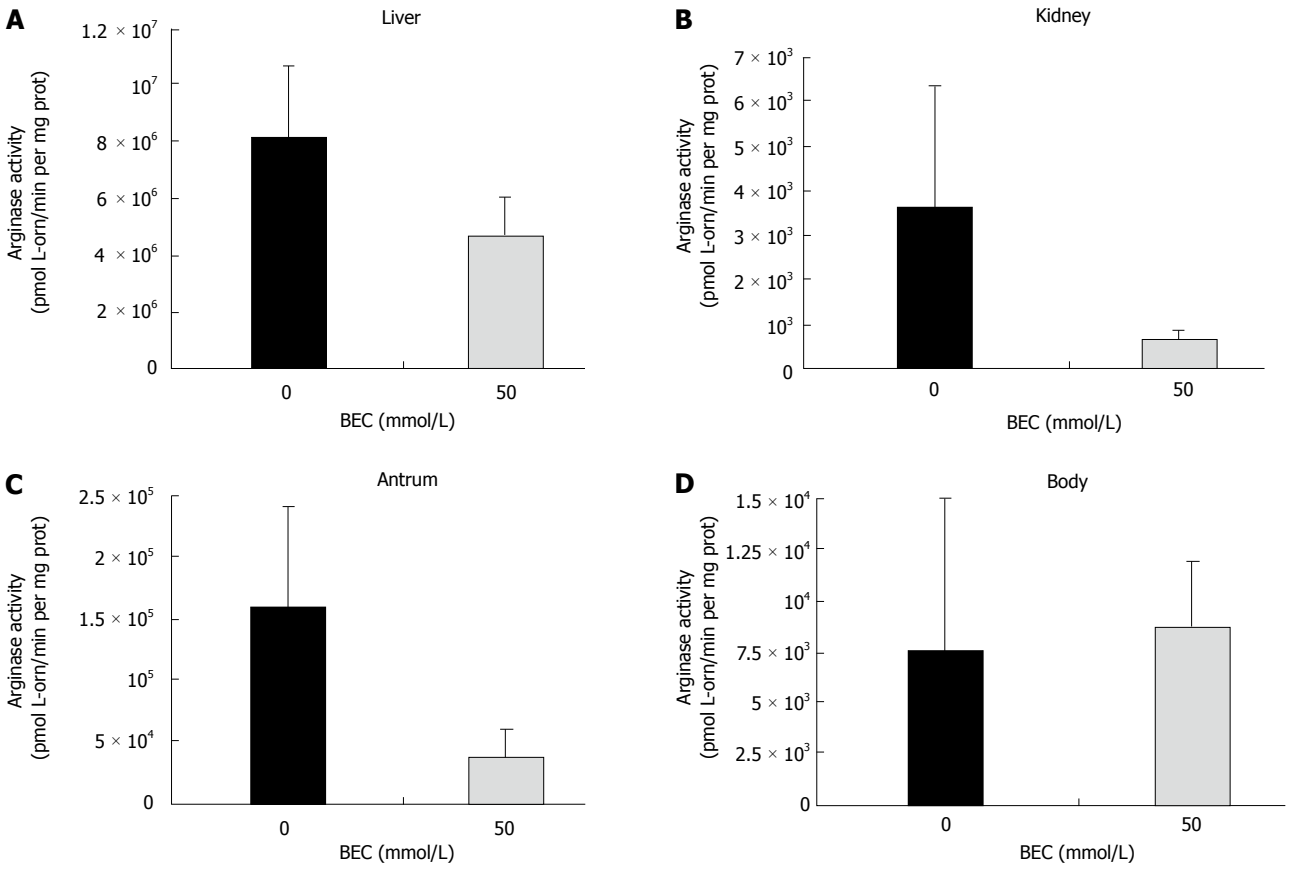

Figure 5 ***Ex vivo*** **arginase activity of organs from arginase Ⅱ knockout mice fed water or the arginase inhibitor S-(2-boronoethyl)-L-cysteine.** Mice were administered 20 μL water or 50 mmol/L S-(2-boronoethyl)-L-cysteine (BEC) once or twice daily for 3 d by direct oral delivery *via* pipetting. Animals were euthanized and organs removed and homogenized. The data represent the average arginase activity of four to five mice ± SD, with each mouse organ measured in duplicate or triplicate using arginine buffer at pH 9.0 in the presence of manganese. A: Liver. Arginase activity was inhibited almost 50% in the liver by BEC. $P = 0.0006$ between the two groups; B: Kidney. Arginase activity was inhibited > 75% in the kidney by BEC. $P = 0.0033$ between the two groups; C: Antrum. Arginase activity was inhibited > 75% in the antrum by BEC. $P = 0.0007$ between the two groups; D: Body. No inhibition was observed in the body ($P > 0.05$).

alone (n = 5 or 6 mice per group), and only the antrum and body were processed. Essentially identical results were obtained (data not shown).

DISCUSSION

We demonstrated that the *H. pylori rocF* mutant, which has no detectable arginase activity, colonized host arginase Ⅱ knockout mice. These data are very surprising, because host arginase Ⅱ is expressed in the stomach, and is even upregulated by *H. pylori* infection[16]. Moreover, inhibiting at least some of the host arginase Ⅰ by BEC treatment failed to block the ability of the *rocF* mutant to colonize arginase Ⅱ knockout mice. The data suggest that *H. pylori* must obtain its urea from either the residual arginase Ⅰ that is not inhibitable by BEC, or an alternative pathway of host urea generation, such as agmatinase. The data also suggest that enzymatically active host arginase Ⅰ occurs in the stomach, as has also been suggested previously[28]. A recent study from Wilson and colleagues also have demonstrated that BEC inhibits murine macrophage arginase activity; this leads to an increase in nitric oxide production *via* increased host nitric oxide synthase protein in an arginine-dependent fashion when stimulated by *H. pylori*[29]. Gastric macrophages from *H. pylori*-infected arginase Ⅱ knockout mice that had been given BEC in the drinking water did not produce more nitric oxide than macrophages from WT mice, which suggests that arginase Ⅱ, but not arginase Ⅰ is important for protection of *H. pylori* from nitric oxide[29]. Although arginase Ⅱ clearly plays a crucial role in protection of *H. pylori* from nitric oxide, it is not essential for *H. pylori* colonization of the gastric mucosa, based on the findings reported here.

One other surprise in this study was a lack of substantial inhibition of *H. pylori* arginase by BEC. Only about 50% of the *H. pylori* arginase could be inhibited by very high concentrations of BEC. This finding may be due to the very distant evolutionary relationship between *H. pylori* and host arginases, as well as the finding that *H. pylori* arginase preferentially uses cobalt for enzyme activity[23] *versus* manganese for the host arginases. BEC is known to bind to eukaryotic arginases where the binuclear manganese center is situated[21], and perhaps the presence of cobalt in the *H. pylori* arginase weakens this inhibitory interaction. The very well conserved DAHAD divalent metal binding motif in eukaryotic arginases is completely conserved in *H. pylori*, which suggests that

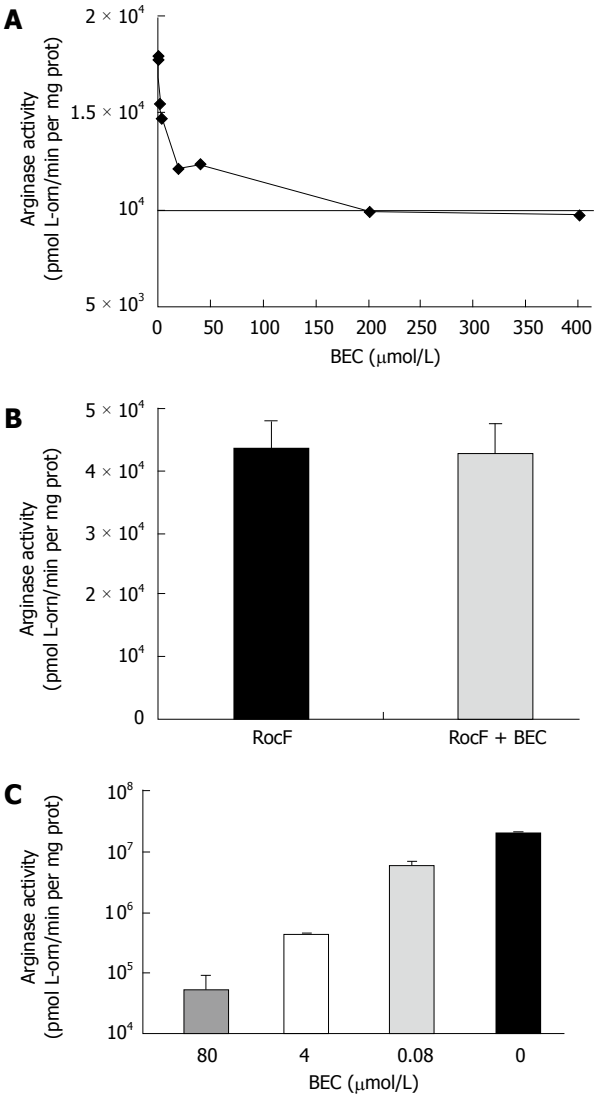

Figure 6 S-(2-boronoethyl)-L-cysteine does not strongly inhibit *Helicobacter pylori* arginase activity *in vitro*. A: Arginase-containing extracts from *Escherichia coli* (*E. coli*) expressing the *Helicobacter pylori rocF* gene on pGEN222. Extracts were incubated in the presence or absence of various concentrations of S-(2-boronoethyl)-L-cysteine (BEC) and measured for arginase activity at pH 6.0 in the presence of cobalt; B: Purified arginase (His6-RocF) was incubated in the presence or absence of BEC (400 nmol/L) and assayed for arginase activity in the presence of cobalt at pH 6.0; C: Extracts from *E. coli* expressing rat arginase Ⅰ were prepared and incubated in the presence of different concentrations of BEC and then assayed for arginase activity in the presence of manganese at pH 9.0. Graph plotted on a logarithmic scale due to magnitude of arginase activity.

other amino acid residues in these proteins are responsible for the differential BEC inhibition observed between eukaryotic and *H. pylori* arginase.

The arginase from *Bacillus anthracis* (*B. anthracis*) has best catalytic activity with nickel[30]. Arginase activity from the purified *B. anthracis* RocF (active site = DAHGD) was not inhibited at all by up to 400 μmol/L BEC (assayed at pH 9.0), and instead, a stimulatory activity was noticed at 40 and 400 μmol/L BEC (Viator and McGee, unpublished observations). Whether the A to G amino acid substitution in the *B. anthracis* DAHGD metal-binding site is responsible for this lack of BEC inhibition is not yet known. Nonetheless, the data suggest that bacterial and eukaryotic arginases have distinct properties with respect to the BEC inhibitor.

The abundant urease must obtain copious amounts of urea for the urease to function efficiently *in vivo*, since urease is absolutely essential for colonization[8,9]. Urea is essential for urease activity, and urease is largely cytosolic in *H. pylori*, therefore, there must be a mechanism to transport urea. Indeed, the *H. pylori* UreI protein, expressed from the urease operon, is an urea transporter[31]. Mutation in *ureI* renders *H. pylori* attenuated in gerbils[32], which illustrates how crucial it is for urea to enter the bacterial cell and allow *H. pylori* to survive at pH < 4.0. Important future experiments are to examine the role of urease in more depth in this arginase Ⅱ knockout mouse background, and to monitor closely intragastric pH and urea concentrations in the different stomach compartments in wild-type and arginase Ⅱ knockout mice.

Although the data showed that BEC could almost completely inhibit arginase Ⅰ *in vitro*, the same was not true *in vivo*. Several possibilities may account for this. First, oral delivery of BEC leads to the dilution of the inhibitor in the gastric juice, which could decrease its effective inhibitory concentration. Second, the acidity of gastric juice could alter inhibitory properties of BEC by protonation of BEC. This may explain why BEC inhibits arginase activity in the antrum, but not the body, where there is much stronger acid production. Third, BEC may not be able to enter the host cell with great efficiency to inhibit arginase Ⅰ, which is found in the host cytosol. In the *in vitro* biochemical experiments, arginase is freed from the cells by a lysis step, which allows BEC direct access to the arginase. Finally, the complexities of the *in vivo* environment may lead to BEC being degraded or sequestered from reaching the arginase Ⅰ. Despite these potential limitations it was demonstrated that oral delivery of BEC could block at least some of the arginase Ⅰ in several tissues examined. However, the residual arginase Ⅰ that remains may have been enough to provide the necessary urea substrate for *H. pylori* urease to utilize. Alternatively, another host urea-generating enzyme, agmatinase[33,34], may be responsible.

Agmatine is prevalent in the stomach[35,36] and 60% of this agmatine goes to the liver[37]. Interestingly, agmatine levels are higher in the gastric juice of *H. pylori*-infected patients versus uninfected patients[36]. Moreover, *H. pylori* has a putative arginine decarboxylase that could theoretically generate the agmatine. There is also the possibility that intestinal normal flora bacteria could provide the urea[38].

Future studies will center on attempting to eliminate host arginase Ⅰ in the stomach. This may pose a challenge, due to the crucial role that arginase Ⅰ plays in mice, which die of hyperammonemia shortly after birth if they are disrupted for arginase Ⅰ[19]. Several possible strategies to inhibit completely host arginase Ⅰ could be inhibitory RNA knockdown experiments, or construction of tissue-specific arginase Ⅰ knockout mice that hopefully would live normally but just fail to produce arginase Ⅰ in the stomach. However, urea could also diffuse to the stomach from arginine hydrolysis in other organs, which might complicate

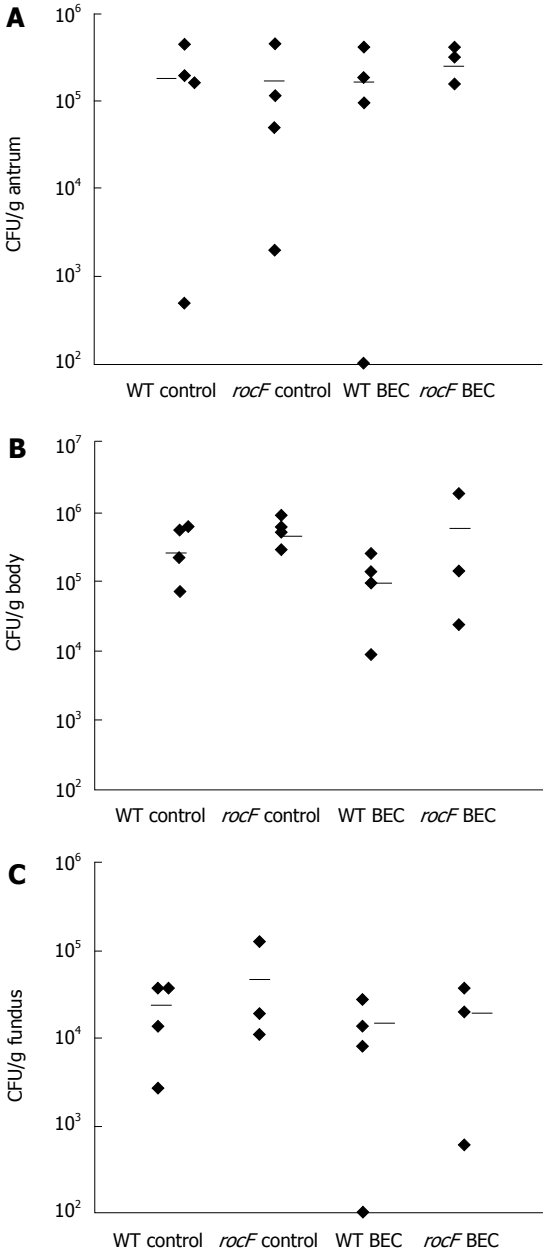

Figure 7 The *rocF* mutant of *Helicobacter pylori* colonizes wild-type mice treated with the arginase inhibitor S-(2-boronoethyl)-L-cysteine. Wild-type (C57 BL/6) mice (n = 3 or 4 per group) were orally fed water or S-(2-boronoethyl)-L-cysteine (BEC) (50 mmol/L) once or twice daily for 3 d before oral inoculation with wild-type or *rocF* mutant *Helicobacter pylori* (*H. pylori*) strain SS1 (~108 CFU). The BEC animal groups continued to receive BEC daily until animals were euthanized. At 1 wk post-infection, animals were euthanized and the stomach dissected into antrum (A), body (B), or fundus (C). Limit of detection was ~100 CFU/g stomach and all animals that lacked *H. pylori* were set to this detection limit. Bar, mean CFU/g tissue. Usually each symbol represents one mouse. In some cases there are two mice represented by a symbol if the data overlapped.

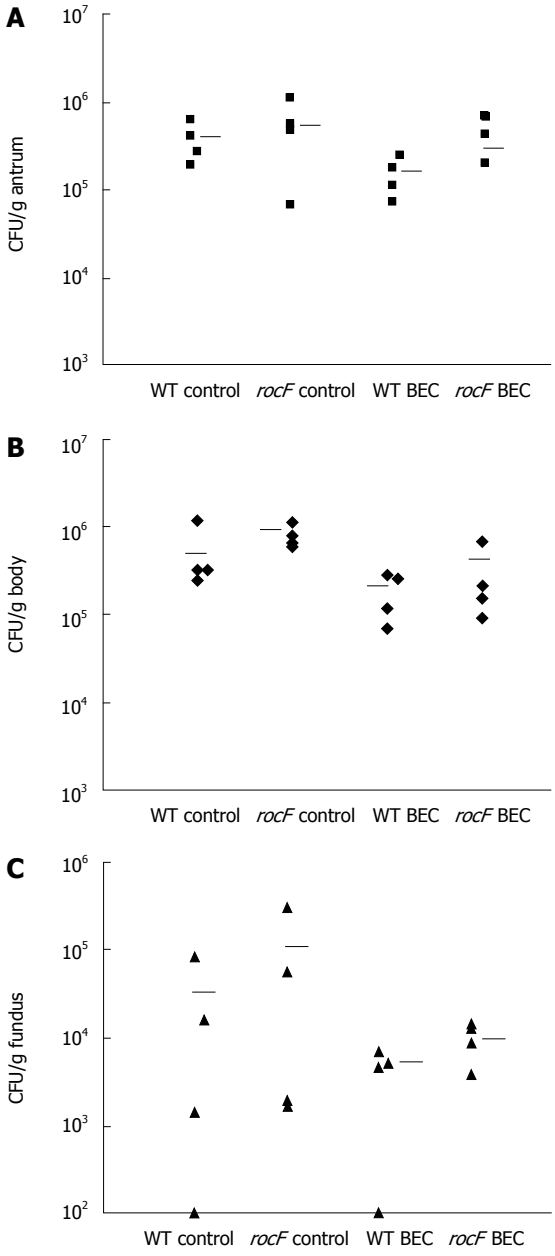

Figure 8 The *rocF* mutant of *Helicobacter pylori* colonizes arginase Ⅱ knockout mice treated with the arginase inhibitor S-(2-boronoethyl)-L-cysteine. Arginase Ⅱ knockout mice (n = 3 or 4 per group) were orally fed water or S-(2-boronoethyl)-L-cysteine (BEC) (50 mmol/L) once or twice daily for 3 d before oral inoculation with wild-type or *rocF* mutant *Helicobacter pylori* strain SS1 (~108 CFU). The BEC animal groups continued to receive BEC daily until animals were euthanized. At 1 wk post-infection, animals were euthanized and the stomach dissected into antrum (A), body (B), or fundus (C). Limit of detection: ~100 CFU/g tissue. Bar, mean CFU/g stomach.

a tissue-specific arginase Ⅰ knockout strategy. The recent study that used an arginase-containing adenovirus delivery system to complement the arginase Ⅰ $^{-/-}$ mouse led to a doubling of survival to 27 d[39]. Further refinements to this exciting model may hold promise for additional exploration of the role of arginase Ⅰ in *H. pylori* pathogenesis.

The *in vivo* source of urea still remains a mystery. However, it is clear that the *in vivo* source of urea for the crucial enzyme urease does not originate from the bacterial arginase or from host arginase Ⅱ. Instead, the data suggest that urea originates from host arginase Ⅰ or an alternative urea-generating pathway.

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COMMENTS

Background

The bacterium *Helicobacter pylori* (*H. pylori*) causes stomach problems, such as ulcers and inflammation and can lead to stomach cancer. One of the important proteins it makes that contributes to disease is called urease, an enzyme that converts urea to ammonia and carbon dioxide. Urease is required for bacteria to cause disease, and thus the substrate urea is also required. In this study, the authors designed experiments to determine from where this urea is coming: bacterial or host sources.

Research frontiers

The most likely enzyme that provides the urea source is arginase and both *H. pylori* and humans have arginases (two in humans, one in *H. pylori*). The authors used a mouse model in which one of the arginases, arginase Ⅱ, was knocked out, along with *H. pylori* wild-type or an arginase knockout bacterium (*rocF*). An arginase inhibitor called S-(2-boronoethyl)-L-cysteine (BEC) was also used to knock down the second mouse arginase (arginase Ⅰ). The authors found that neither the bacterial arginase (*rocF*) nor arginase Ⅱ from the mouse was important to provide the urea, which implies the involvement of either arginase Ⅰ or another pathway (agmatinase).

Innovations and breakthroughs

The study provides the first steps towards the foundation needed to understand where the urea originates for the crucial urease enzyme. Much remains to be done, including completely knocking down arginase Ⅰ in mice and determining whether another enzyme, agmatinase could be involved.

Applications

By understanding where the urea originates, the authors hope to develop novel anti-*Helicobacter* drugs. New therapies are sought because *H. pylori* is becoming resistant to antibiotics.

Terminology

H. pylori causes infection in the stomach, which leads to inflammation, ulcers and cancer. Urease is an enzyme made by *H. pylori* the converts urea to ammonia and carbon dioxide. Arginase is an enzyme that converts arginine to ornithine and urea and is made by humans, mice and *H. pylori*. Knockout refers to a gene that is disrupted and no longer functional.

Peer review

The authors found that the *in vivo* source for the essential urea substrate for the *H. pylori* urease is neither bacterial arginase nor host arginase Ⅱ. Based on these results, they hypothesized that either host urea-generating pathway such as agmatinase or the residual host arginase Ⅰ might be responsible.

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ORIGINAL ARTICLE

Doxycycline blocks gastric ulcer by regulating matrix metalloproteinase-2 activity and oxidative stress

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Abstract

AIM: To examine the effect of doxycycline on the activity of matrix metalloproteinases (MMPs) and oxidative stress in gastric tissues of rats following gastric injury.

METHODS: Gastric ulcers were generated in rats by administration of 70% ethanol, and activity of doxycycline was tested by administration 30 min prior to ethanol. Similarly, the effect of doxycycline was tested in an indomethacin-induced gastric ulcer model. The activities and expression of MMPs were examined by zymography and Western blot analysis.

RESULTS: Gastric injury in rats as judged by elevated ulcer indices following exposure to ulcerogen, either indomethacin or ethanol, was reversed significantly by doxycycline. Indomethacin-induced ulcerated gastric tissues exhibited about 12-fold higher proMMP-9 activity and about 5-fold higher proMMP-3 activity as compared to control tissues. Similarly, ethanol induced about 22-fold and about 6-fold higher proMMP-9 and proMMP-3 activities, respectively, in rat gastric tissues. Both proMMP-9 and MMP-3 activities were markedly decreased by doxycycline in ulcerogen treated rat gastric tissues. In contrast, the reduced MMP-2 activity in ulcerated tissues was increased by doxycycline during ulcer prevention. On the other hand, doxycycline inhibited significantly proMMP-9, -2 and -3 activities *in vitro*. In addition, doxycycline reduced oxidative load in gastric tissues and scavenged H_2O_2 *in vitro*. Our results suggest a novel regulatory role of doxycycline on MMP-2 activity in addition to inhibitory action on MMP-9 and MMP-3 during prevention of gastric ulcers.

CONCLUSION: This is the first demonstration of dual action of doxycycline, that is, regulation of MMP activity and reduction of oxidative stress in arresting gastric injury.

Key words: Doxycycline; Extracellular matrix; Matrix metalloproteinases; Reactive oxygen species; Tissue inhibitor of metalloproteinase

Peer reviewer: Fabrizio Montecucco, MD, Assistant, Division of Cardiology, Department of Internal Medicine, University of Geneva, Avenue de la Roseraie 64, 1211 Geneva, Switzerland

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INTRODUCTION

Matrix metalloproteinases (MMPs) are a class of zinc-de-

pendent endopeptidases that degrade matrix macromolecules and numerous other components such as growth factors, proteinases, and plasma proteins[1,2]. MMPs are best known for their action on extracellular matrix (ECM) remodeling, wound healing and angiogenesis, and have been implicated in the pathogenesis of various diseases including gastric ulcers[3-6]. The endogenous inhibitors of MMPs [tissue inhibitors of metalloproteinases (TIMPs) and α-2 macroglobulin] play a major role in balancing protease-antiprotease action[7,8]. MMPs are synthesized and secreted by gastric epithelial cells, macrophages, and neutrophils[9,10]. Numerous exogenous factors including nonsteroidal anti-inflammatory drugs (NSAIDs), stress, alcohol and *Helicobacter pylori* infection have been implicated in the etiology of gastric ulceration[11-14]. We have previously demonstrated that MMP-9 increases significantly in indomethacin-induced as well as ethanol-induced gastric ulcers[3,4,12]. Overexpression of MMP-9 has been attributed to coronary artery disease, retinopathy, renal disease, chronic obstructive pulmonary disease and atherosclerosis[15-17]. Involvement of several MMPs including gelatinases and stromelysins in various pathological conditions has been documented[5]. However, downregulation of MMP-2 activity in retinal and renal vasculature leads to thickening of the basement membrane due to collagen deposition and contributes to diabetic retinopathy and nephropathy, respectively[18]. Research into the pathophysiology of gastric ulcers suggests a central role of reactive oxygen species (ROS) in the development of the disease. MMP can be activated by ROS *in vitro*, and thus, imbalance of MMP activation and inactivation occurs[19]. An imbalance of MMP activity can lead to release of growth factors, and therefore, inhibition of MMPs can be a potential therapy for various diseases[20]. Ulcer healing is a complicated process that requires interactions between ECM proteins including collagens, proteases, cytokines, and growth factors. Repair of gastric wounds encompasses a series of cell-matrix interactions that involve cellular proliferation, migration, and differentiation[3,4,12,21]. Doxycycline, which belongs to the tetracycline family, is a pluripotent drug that affects many cellular functions including cell proliferation, matrix remodeling and apoptosis, in addition to its use as an antibiotic[22,23].

Doxycycline as well as tetracycline are non-specific potent MMP inhibitors[23-27]. Doxycycline has been used at a dose of 100-300 mg/d for the protection of pancreatitis-associated lung injury and abdominal aneurysm[28,29]. Doxycycline treatment greatly potentiates vasoconstriction, which is attributed to inhibition of MMP-2 and MMP-9 and restoration of elastic fiber integrity during prevention of aortic aneurysm in mice[29]. However, the mechanism of its actions on gastric ulcers, especially at the MMP level, remains unknown and needs to be resolved. Considering a value of 56 for human/rat conversion coefficient, assuming rat and human weights of 200 and 70 kg, respectively, 5-10 mg/kg doxycycline is equal to the 2-3 mg/kg recommended human dose[30,31]. It has been widely available in the market as an antibiotic for over three decades and has become established for long-term use too. However, its use against inhibition of overexpression of MMPs has not been investigated to date in therapeutic trials for gastric ulcers.

This study was conducted to evaluate the ability of the broad-spectrum MMP inhibitor doxycycline to prevent gastric ulcers in rats. In this paper, we report that doxycycline inhibited MMP-3 and MMP-9 activity and expression during prevention of indomethacin- and ethanol-induced gastric ulcers. Remarkably, doxycycline upregulated MMP-2 activity and expression *in vivo*, whereas it downregulated the same activity *in vitro*. Finally, we suggest that doxycycline might exert its regulatory role in addition to its inhibitory role on MMPs *in vivo*, which may be associated with ROS scavenging activity. These results provide an insight into the gastroprotective mechanisms of doxycycline that underlie the dual actions, antioxidant and MMP modulatory role. Hence, establishment of use of doxycycline as an effective therapy will be a boon to gastric ulcer patients.

MATERIALS AND METHODS

Materials

Gelatin from porcine skin, casein, Triton X-100 (TX), 1, 10 phenanthroline, catalase and protease inhibitor cocktail were obtained from Sigma Chemicals (St. Louis, MO, USA). MMP-9 and MMP-2 were purchased from Chemicon (UK). Polyclonal anti-MMP-2, anti-MMP-3, anti-MMP-9, anti-TIMP-1, anti-TIMP-2, and anti-β-tubulin antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Doxycycline was purchased from Dr. Reddy's Laboratory (India), and all other chemicals were purchased from Sisco Research Laboratory (Mumbai, India).

Animals used

Sprague-Dawley rats (180-220 g) bred in-house with free access to food and water were used for all experiments, which were carried out following the guidelines of the animal ethics committee of the institute. Experiments were designed to minimize animal suffering and to use the minimum number associated with valid statistical evaluation. Animals were anesthetized by ketamine (12 mg/kg), followed by cervical dislocation for killing. Animals in control and experimental groups were fasted overnight with free access to water before each experiment.

Gastric ulceration and protection studies

Indomethacin (48 mg/kg) was dissolved in distilled water with a minimum amount of NaOH and was orally fed to rats to induce a maximum level of acute gastric ulcer. The control group received the vehicle only. After 4 h, the animals were sacrificed, gastric lesions in the fundic stomach were scored and expressed as ulcer index as follows: 0 =

no pathology; 1 = a small pinhead ulcer spot; and 2-5 = a band-like lesion of 2-5 mm in length. The sum of the total scores divided by the number of animals was expressed as the mean ulcer index[3]. Again, rats were orally fed with 70% ethanol at 4 mL/kg to induce maximum level of acute gastric ulcer or 2 mL/kg to induce moderate ulcer, while the control group received sterile water only. Animals were sacrificed after 3 h, and stomachs were scored for ulcer index as described by Pradeepkumar Singh *et al*[12]. The sum of the total scores divided by the number of animals indicates the mean ulcer index. Doxycycline at a dose of 50 mg/kg was administered orally 30 min prior to indomethacin or ethanol treatment to test the gastroprotective effects.

Measurement of glutathione level

A homogenate of stomach tissue without the connective tissue layer was made in 0.2 mol/L Tris, pH 8.2, which contained 20 mmol/L EDTA, and centrifuged at 12000 r/min for 15 min at 4℃. An aliquot of 1.0 mL homogenate was precipitated with 10% TCA and centrifuged at 14000 r/min for 3 min at 4℃. The supernatant (1 mL) was added to 2 mL of 0.8 mol/L Tris-HCl, pH 9.0, which contained 20 mmol/L EDTA and mixed with 0.1 mL 10 mmol/L 5,5'-dithiobis-2-nitrobenzoic acid (DTNB). The intense yellow color of nitromercaptobenzoate was read at 412 nm. For calibration, a standard curve was prepared treating varied concentrations of reduced glutathione with DTNB under identical conditions[3].

Measurement of lipid peroxidation

The mitochondrial membrane fraction from gastric tissue homogenate was used for measurement of lipid peroxide content as thiobarbituric acid reactive species (TBARS). Briefly, 1 mL of the membrane fraction was allowed to react with 2 mL of TCA/TBA/HCl (15% TCA, 0.375% TBA, 0.25N HCl) reagent, heated in a boiling water bath for 15 min, cooled and centrifuged at 1000 r/min for 10 min at 4℃. The absorbance of the supernatant was measured at 535 nm and nmol TBARS produced was determined from a standard curve using tetraethoxypropane as standard[3,12].

Measurement of protein carbonyl content

Protein oxidation was measured as carbonyl content in the low speed supernatant of the gastric tissue homogenate[3,12]. The stomach from control, indomethacin-treated and doxycycline (50 mg/kg) pretreated indomethacin-treated rats was homogenized in 50 mmol/L sodium phosphate buffer, pH 7.4, in a Potter-Elvehjem glass homogenizer for 2 min, to obtain 20% homogenate. After centrifugation at 600 *g* for 10 min, the proteins from 1.0 mL of the supernatant were precipitated with 10% TCA and allowed to react with 0.5 mL 10 mmol/L 2,4-dinitrophenylhydrazine for 1 h. After precipitation with 20% TCA, the protein was washed three times with a mixture of ethanol-ethyl acetate (1:1), dissolved in 1.0 mL of a solution that contained 6 mol/L guanidine HCl in 20 mmol/L potassium phosphate, adjusted to pH 2.3 with TCA, centrifuged at 1000 r/min for 5 min at 4℃, and the supernatant was read for carbonyl content at 362 nm (e = 22000/M per centimeter).

Tissue extraction

The whole stomach (including fundic, body and pyloric parts) was washed with saline and used for extraction. Gastric tissues were suspended in 10 mmol/L phosphate buffered saline (PBS) containing protease inhibitors, minced, and incubated for 10 min at 4℃. After centrifugation at 12000 *g* for 15 min, the supernatant was collected as PBS extracts. The pellet was then extracted in lysis buffer (10 mmol/L Tris-HCl, pH 8.0, 150 mmol/L NaCl, 1% TX and protease inhibitors) and centrifuged at 12000 *g* for 15 min to obtain TX extracts. PBS and TX extracts were preserved at -70℃[3,12].

Gelatin and casein zymography

For assay of MMP-2 and MMP-9 activity, PBS and/or TX extracts were electrophoresed in 8% SDS-polyacrylamide gel containing 1 mg/mL gelatin under non-reducing conditions. For assay of MMP-3 activity, tissue extracts were electrophoresed in casein gel instead of gelatin. The gels were washed in 2.5% TX and incubated in calcium assay buffer (40 mmol/L Tris-HCl, pH 7.4, 0.2 mol/L NaCl, 10 mmol/L $CaCl_2$) for 18 h at 37℃, and stained with 0.1% Coomassie Blue followed by destaining[3,12,13]. The zones of gelatinolytic or caseinolytic activity came as negative staining.

MMP inhibition assay

Equal amount of PBS extract from ulcerated tissue were incubated with two different concentrations of doxycycline (100 and 200 μmol/L) in calcium assay buffer (40 mmol/L Tris-HCl, pH 7.4, 0.2 mol/L NaCl, 10 mmol/L $CaCl_2$) for 1.5 h at 37℃. Gelatin and casein zymography were performed from the samples as described previously[3,12,13]. Parallel assays were performed with the addition of phenanthroline (100 and 200 μmol/L in 10% DMSO) to show that inhibition could be attributed to MMP-2, MMP-3 and MMP-9 activity. PBS extract incubated with requisite volume of 10% DMSO was used as a control[32].

Effect of H_2O_2 on MMP-2 activity in vitro

To determine the effect of H_2O_2, equal amounts of PBS extract from control stomach (60 μg) were incubated with 500 μmol/L concentration of H_2O_2 at 37℃ for 1 h. To examine the protective effect of doxycycline on H_2O_2 mediated changes in MMP-2 activity, equal amounts of PBS extract were pretreated with doxycycline (200 μmol/L) prior to incubation with 500 μmol/L H_2O_2. Pretreatment with catalase followed by incubation with H_2O_2 was used as a positive control. Gelatin zymography was performed with the samples as described previously[19].

Western blotting

PBS extracts (100 μg/lane) were resolved by 8% reducing SDS-PAGE and processed for Western blotting[13].

Proteins were transferred to membranes, blocked in 3% BSA solution in 20 mmol/L Tris-HCl, pH 7.4, containing 150 mmol/L NaCl and 0.02% Tween 20 (TBST), and incubated at 4℃ in 1:200 dilutions of the respective primary antibodies in TBST containing 0.2% BSA. The membranes were then washed with TBST, incubated with alkaline-phosphatase-conjugated secondary antibody, and bands were visualized using 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium substrate solution.

Statistical analysis

Ulcer index data were fitted using Sigma plot software. Data were presented as the mean ± SE. Statistical analysis was performed using Student-Newman-Keuls test (ANOVA). $P < 0.05$ was considered statistically significant.

RESULTS

Effect of doxycycline on gastric ulceration induced by indomethacin or ethanol in rats

Indomethacin- and ethanol-induced gastric ulcer models in rat were used to test the protective effect of doxycycline against gastric damage. Doxycycline, a tetracycline analogue, has a number of non-antibiotic properties including inhibition of connective tissue breakdown. The action of doxycycline treatment on gastric damage was evaluated in rats by measuring ulcer index. One single dose of doxycycline (50 mg/kg) was selected for our study. Gastric ulceration was not detected in doxycycline-pretreated, indomethacin-treated rat gastric tissues. Figure 1A illustrates the effect of doxycycline on significant reduction in ulcer indices that occurred due to indomethacin-induced ulceration. In addition, doxycycline caused a significant decrease in ulcer index score from 50 to 15 when administered to high-dose ethanol-treated rats. Similarly, ulcer index score was reduced by less than 5 from 20 in low-dose ethanol-treated rats (Figure 1B). Thus, doxycycline arrested gastric damage by > 70% in rats with severe or moderate ulceration.

Effect of doxycycline on secreted and synthesized proMMP-9 and -2 activities

The gastroprotective action of doxycycline and association with MMP-2 and MMP-9 activities were examined in rat gastric tissues. Figure 2A and C shows that doxycycline inhibited proMMP-9 activity in PBS- and TX-extracted gastric tissues during protection against indomethacin-induced ulcer. PBS and TX extracts represented secreted and synthesized proMMP-9, respectively. ProMMP-9 activity is an inflammatory indicator of gastric damage and it increased with increasing gastric indices. Secreted and synthesized proMMP-9 activity was increased by about 12-fold and about 16-fold in indomethacin-ulcerated gastric tissues as compared to the controls, and doxycycline reduced the activity by 80% and 70%, respectively (Figure 2B and D). In contrast, doxycycline upregulated proMMP-2 activity at the level of secretion and synthesis, while protecting against indomethacin-induced gastric ulcer (Figure 2A and C). Indomethacin treatment decreased proMMP-2 activity by about 50% and docycycline pretreatment reversed the activity almost to control levels for both secretion and synthesis (Figure 2B and D). Doxycycline significantly reduced proMMP-9 activity in gastric tissues both at the level of secretion and synthesis in ethanol-induced ulcerated rats that were doxycycline treated, compared to untreated controls (Figure 3A and C). Doxycycline caused a reduction in secreted proMMP-9 activity to about 5-fold and about 1.5-fold, which was increased about 20-fold and about 7-fold during ulceration by high and low doses of ethanol, respectively (Figure 3B). Similarly, there was a decrease in synthesized proMMP-9 activity by about 18-fold and about 7-fold by doxycycline during protection of ethanol-induced ulceration by low and high doses (Figure 3B). In contrast, doxycycline-pretreated, ethanol-treated rats showed an increase in MMP-2 activity in gastric tissues at the level of secretion and synthesis (Figure 3A and C). Upregulation of MMP-2 activity by doxycycline was more prominent with low-dose compared to high-dose ethanol-induced ulceration (Figure 3D). Thus, MMP-9 activity was downregulated while proMMP-2 activity was

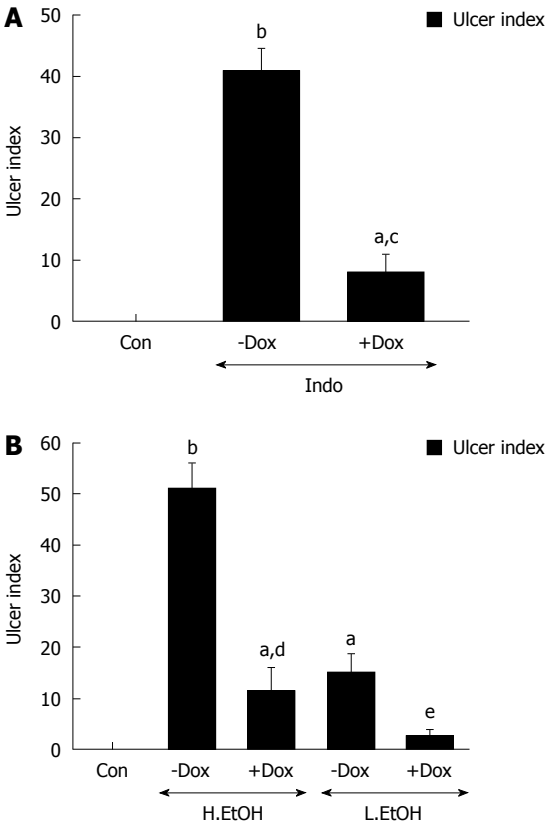

Figure 1 Protective effect of doxycycline on gastric injury. Gastric ulcers were induced in rats by oral administration of 48 mg/kg indomethacin or 70% ethanol (low and high) doses. Doxycycline (50 mg/kg) was administered orally prior to ulcerogen treatment to assess the protective effect. Control rats received sterile water only. After 3 h, rats were sacrificed and ulcer indices were scored. Mean ulcer index for indomethacin-induced (A) and ethanol-induced (B) ulceration in each group of rats was plotted. Dox: Doxycycline; Con: Control; Indo: Indomethacin; L.EtOH: Low dose ethanol; H.EtOH: High dose ethanol. Data expressed as mean ± SE. $^{a}P < 0.05$, $^{b}P < 0.001$ *vs* control; $^{c}P < 0.001$ *vs* indomethacin; $^{d}P < 0.001$ *vs* high dose ethanol; $^{e}P < 0.01$ *vs* low dose ethanol.

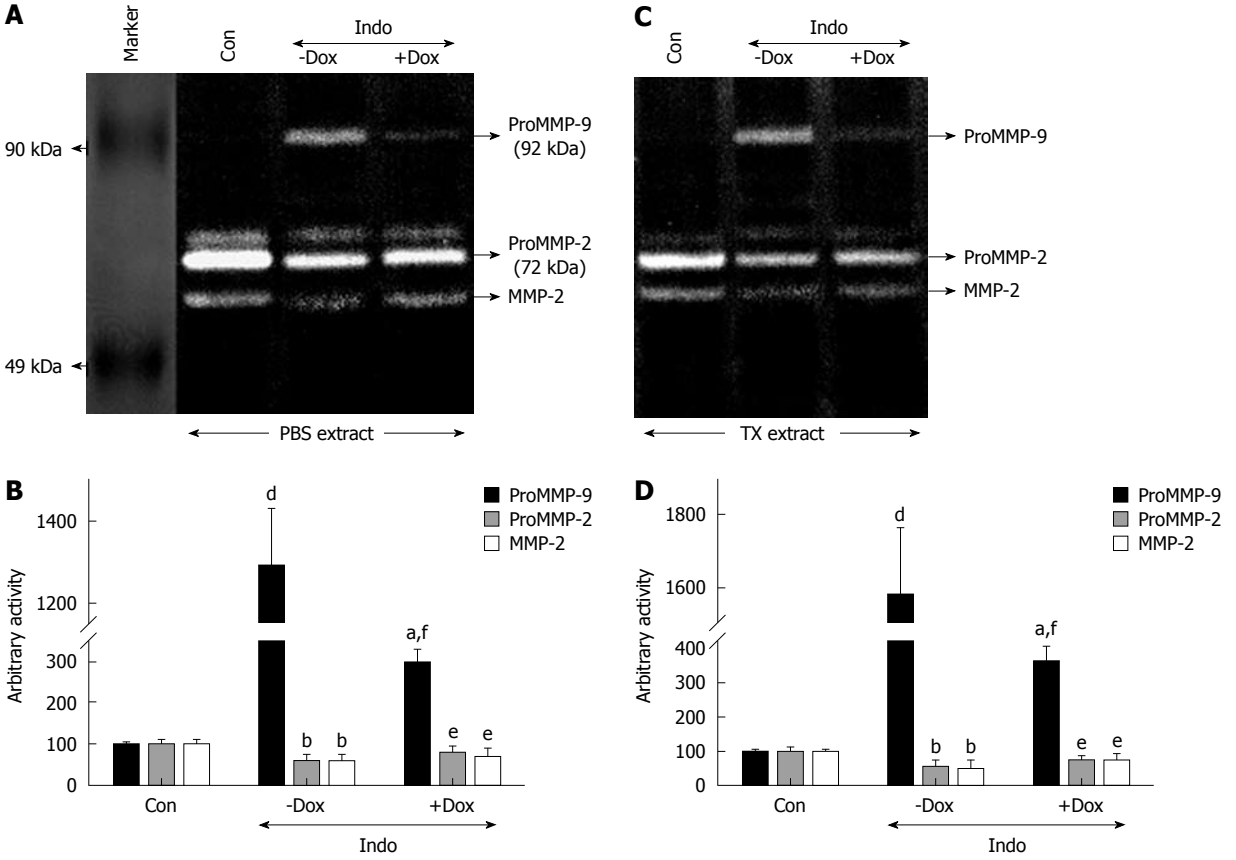

Figure 2 Effect of doxycycline on secreted and synthesized matrix metalloproteinase-2 and -9 activities during protection against indomethacin-induced ulcers. Gastric ulcer was induced by intragastric administration of 48 mg/kg indomethacin in rats, and doxycycline was given 30 min prior to indomethacin for protection studies. Gelatin zymography was performed using phosphate buffered saline (PBS) (A) and Triton X-100 (TX) (C) extracts of gastric tissue from different groups of rats. Histographic representation of arbitrary activity of pro-matrix metalloproteinase (MMP)-9 (B) and proMMP-2 (D) in PBS and TX extracts of gastric tissues, as measured by Lab Image software using the above zymogram and two other representative zymograms from independent experiments. Data expressed as mean ± SE. $^{a}P < 0.05$, $^{b}P < 0.01$, $^{d}P < 0.001$ *vs* control; $^{e}P < 0.05$, $^{f}P < 0.01$ *vs* indomethacin.

upregulated in rat gastric tissues during gastroprotection by doxycycline. Expression of MMP-2 was also examined by Western blot analysis (Figure 3E) in doxycycline-pretreated, ethanol-treated gastric tissues. Figure 3F reveals that upregulation of proMMP-2 reached control levels in doxycycline-pretreated, ethanol-treated (low dose) rat gastric tissues. Similarly, upregulated expression of MMP-2 was also reflected in doxycycline-pretreated, ethanol-treated (high dose) rat gastric tissues. β-tubulin was run in parallel to confirm equal protein loading.

Effect of doxycycline on expression MMPs and TIMPs during gastroprotection

To investigate the ability of doxycycline to regulate MMP-3 during prevention of ulcers, indomethacin- and ethanol-induced ulceration were both examined. Figure 4 suggests that indomethacin increased proMMP-3 activity significantly in ulcerated gastric tissues. Figure 4A and C shows that doxycycline inhibited proMMP-3 activity very significantly in PBS- and TX-extracted gastric tissues during protection against indomethacin-induced ulcer. ProMMP-3 inhibitory activity of doxycycline was at a magnitude of about 75% and about 66% for secreted and synthesized protein, respectively, during protection of indomethacin-induced gastric ulcer (Figure 4B and D). Doxycycline inhibited the activity of secreted proMMP-3 while preventing both severe and moderate gastric ulceration induced by ethanol (Figure 5A). Doxycycline downregulated the activity by about 2-fold while protecting ethanol-treated gastric tissues, and the efficacy was greater for low-dose ethanol-treated tissues (Figure 5B). In other words, doxycycline exhibited an inhibitory effect on proMMP-3 activity by about 50% and about 70% for high-dose and low-dose ethanol-treated gastric tissues, respectively, as depicted in Figure 5B. The effect of doxycycline on MMP-9 and MMP-3 expression in rats was analyzed by Western blotting (Figure 5C). Both high and low doses of ethanol increased the expression of MMP-9 by about 21-fold and about 7-fold, respectively, in rat gastric tissues compared to controls, while treatment with doxycycline inhibited MMP-9 expression by 95% and 85%, respectively. Similarly, MMP-3 expression increased by about 6.5-fold and about 3.5-fold for high- and low-dose ethanol treatment, respectively, and doxycycline reduced their expression by 60% and 80%, respectively (Figure 5C). Moreover, the effect of doxycycline was more pronounced when ulcer was moderate rather than severe. To ascertain the regulatory role of TIMPs in ethanol-induced gastric ulcer,

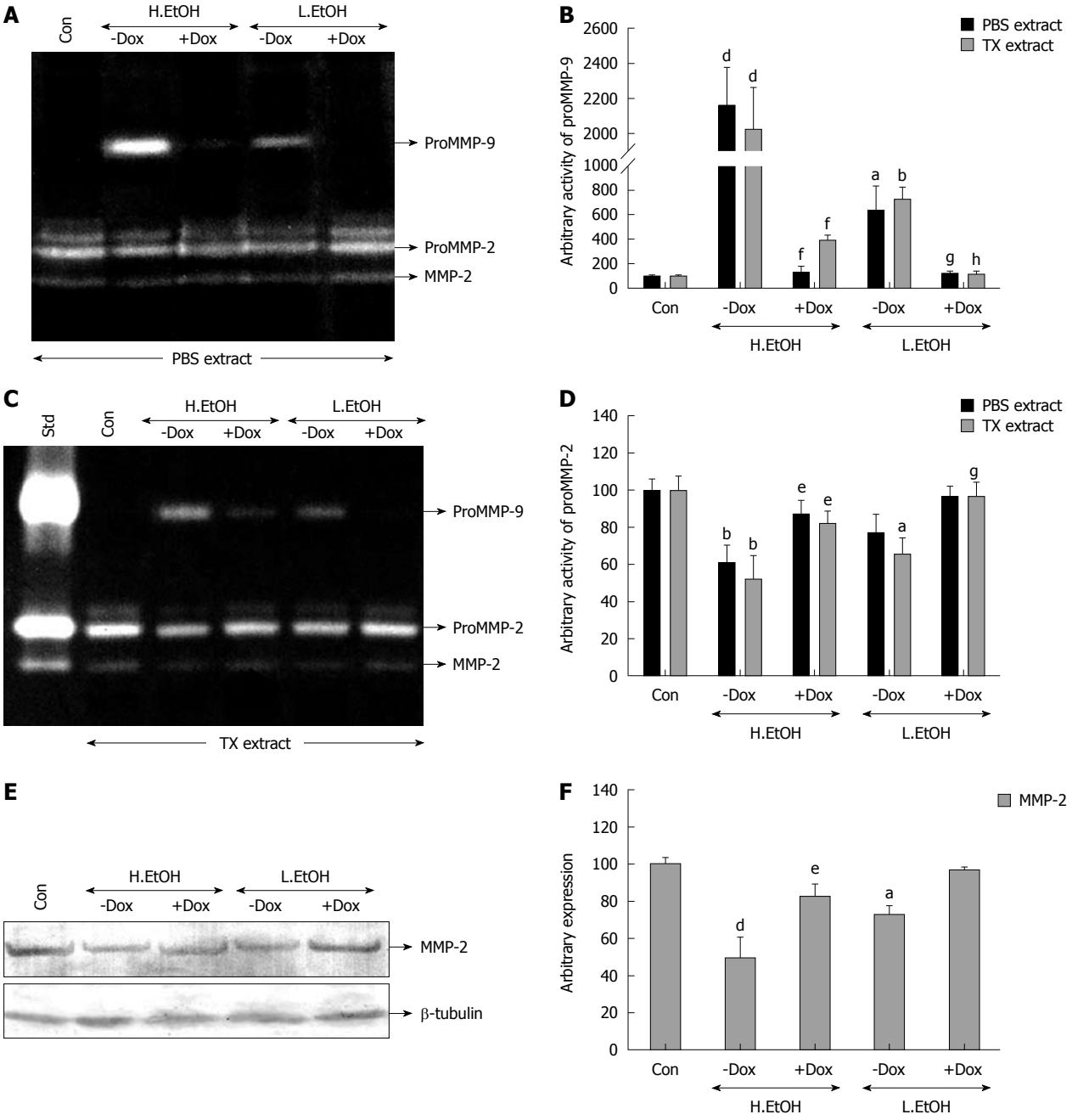

Figure 3 Effect of doxycycline on secreted and synthesized matrix metalloproteinase-2 and -9 activities during protection of ethanol-treated gastric ulcer. Low (0.4 mL/rat) and high (0.8 mL/rat) doses of 70% ethanol were administered intragastrically to induce moderate and maximum gastric ulcers, and doxycycline was administered 30 min prior to ethanol. Gelatin zymography was performed using phosphate buffered saline (PBS) (A) and Triton X-100 (TX) (C) extracts of gastric tissues from different group of rats. Histographic representation of arbitrary activity of pro-matrix metalloproteinase (MMP)-9 (B) and proMMP-2 and MMP-2 (D) in PBS and TX extracts of gastric tissues as measured by Lab Image software using the above zymogram and two other representative zymograms from independent experiments. PBS extracts were subjected to Western blotting (E) and probed with polyclonal anti-MMP-2 and β-tubulin antibody. Fold changes of MMP-2 (F) are represented in a histogram as measured by Lab Image software using the above western blot and two other representative blots from independent experiments. Data expressed as mean ± SE. $^{a}P < 0.05$, $^{b}P < 0.01$, $^{d}P < 0.001$, *vs* control; $^{e}P < 0.05$, $^{f}P < 0.001$ *vs* high-dose ethanol; $^{g}P < 0.05$, $^{h}P < 0.01$ *vs* low-dose ethanol of respective PBS and TX extracts. L.EtOH: Low dose ethanol; H.EtOH: High dose ethanol.

TIMP-1 and TIMP-2 expression was checked by Western blot analysis. TIMP-1 expression was reduced by 40% and 60% for high- and lose-dose ethanol, respectively, and doxycyline reversed TIMP-1 expression almost to control values. On the other hand, TIMP-2 expression followed an opposite trend. It increased in rat gastric tissue after ethanol treatment. TIMP-2 expression induced by 1.6-fold and 1.3-fold, respectively, and doxycyline reduced TIMP-2 expression near to control levels. β-tubulin was run in parallel to confirm equal protein loading.

Inhibitory effect of doxycycline on MMP-9, -2 and -3 activities in vitro

The effect of doxycycline on MMP activity *in vitro* was

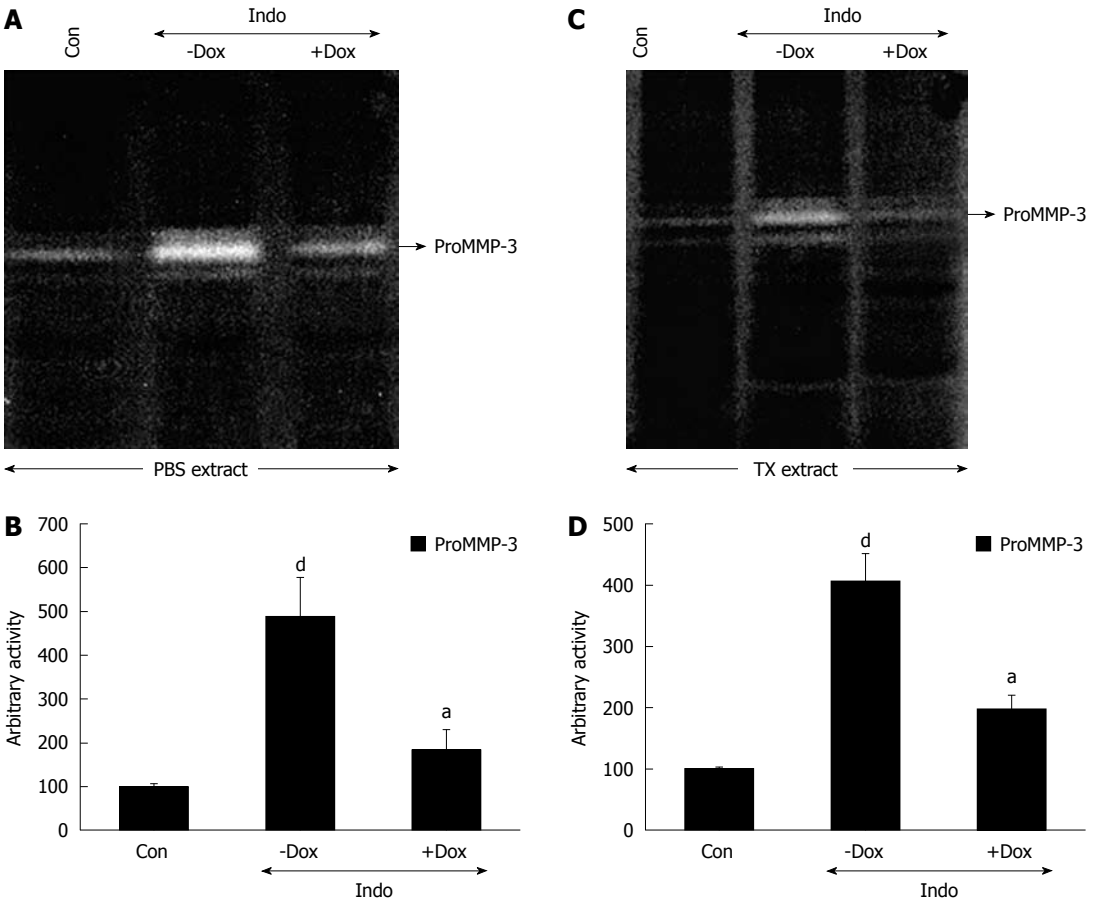

Figure 4 Effect of doxycycline on matrix metalloproteinase-3 activity during protection against indomethacin-induced gastric ulcer. Gastric ulcer was induced by intragastric administration of 48 mg/kg indomethacin in rats, and doxycycline was given 30 min prior to indomethacin for protection studies. Casein zymography was performed using phosphate buffered saline (PBS) (A) and Triton X-100 (TX) (C) extracts of gastric tissue from different groups of rats. Histographic representation of arbitrary activity of pro-matrix metalloproteinase (MMP)-3 in PBS (B) and TX (D) extracts of gastric tissues as measured by Lab Image software designed using the above zymogram and two other representative zymograms from independent experiments. Data expressed as mean ± SE. $^{a}P < 0.05$, *vs* indomethacin; $^{d}P < 0.001$ *vs* control.

evaluated by casein as well as gelatin zymography. Gastric tissue extracts with detectable MMP-2, MMP-3 and MMP-9 activities were used as controls for the inhibition experiment. Doxycycline inhibited MMP-2, MMP-3 and MMP-9 activities dose dependently, as judged by zymography (Figure 6A). Doxycycline at a dose of 100 μmol/L and 200 μmol/L inhibited MMP-9 activity by 30% and 50%, respectively, while MMP-2 and MMP-3 activities were inhibited by 65% in each case. *In vitro* inhibition studies of MMP activity were also performed in parallel using zinc chelator (1, 10 phenanthroline) to confirm the metalloprotease property. Phenanthroline at a dose of 100 μmol/L inhibited MMP-2, MMP-3 and MMP-9 by about 60%, about 62% and about 55%, respectively. In addition, the activity of MMP-2, MMP-3 and MMP-9 were further decreased by 10-15% more at high doses of phenanthroline (200 μmol/L) in each case (Figure 6B).

Reduction of oxidative stress by doxycycline in vivo and in vitro

Oxidative damage of cellular lipid and protein is directly associated with gastric ulceration. To determine the effect of doxycycline on oxidative damage of gastric tissues due to indomethacin, we measured protein oxidation and lipid peroxidation in control, indomethacin-treated, and indomethacin-treated doxycycline-pretreated gastric tissue homogenates. Indomethacin caused excessive oxidative damage that was associated with a about 2.5-fold and about 2-fold increase in lipid peroxidation and protein oxidation, respectively, while doxycycline treatment reduced them by about 80% and about 93%, respectively (Table 1). Moreover, the antioxidant, glutathione was reduced by about 36% compared to controls, and doxycycline recovered the glutathione content up to 92% during gastroprotection. In order to assess the possible antiulcer mechanism of doxycycline, we examined its protective effect on H_2O_2-mediated suppression of proMMP-2 activity *in vitro*. PBS-extracted gastric tissues from control rats were incubated with 200 μmol/L doxycycline followed by H_2O_2 treatment for 1 h at 37℃, and gelatin zymography was performed. The activity of MMP-2 in the zymogram (Figure 7A) was decreased by about 45% compared to the controls by H_2O_2, and doxycycline rescued H_2O_2-mediated suppression of MMP-2 activity by about 80% (Figure 7B). As expected when H_2O_2 treated samples were incubated with catalase, it failed to suppress MMP-2

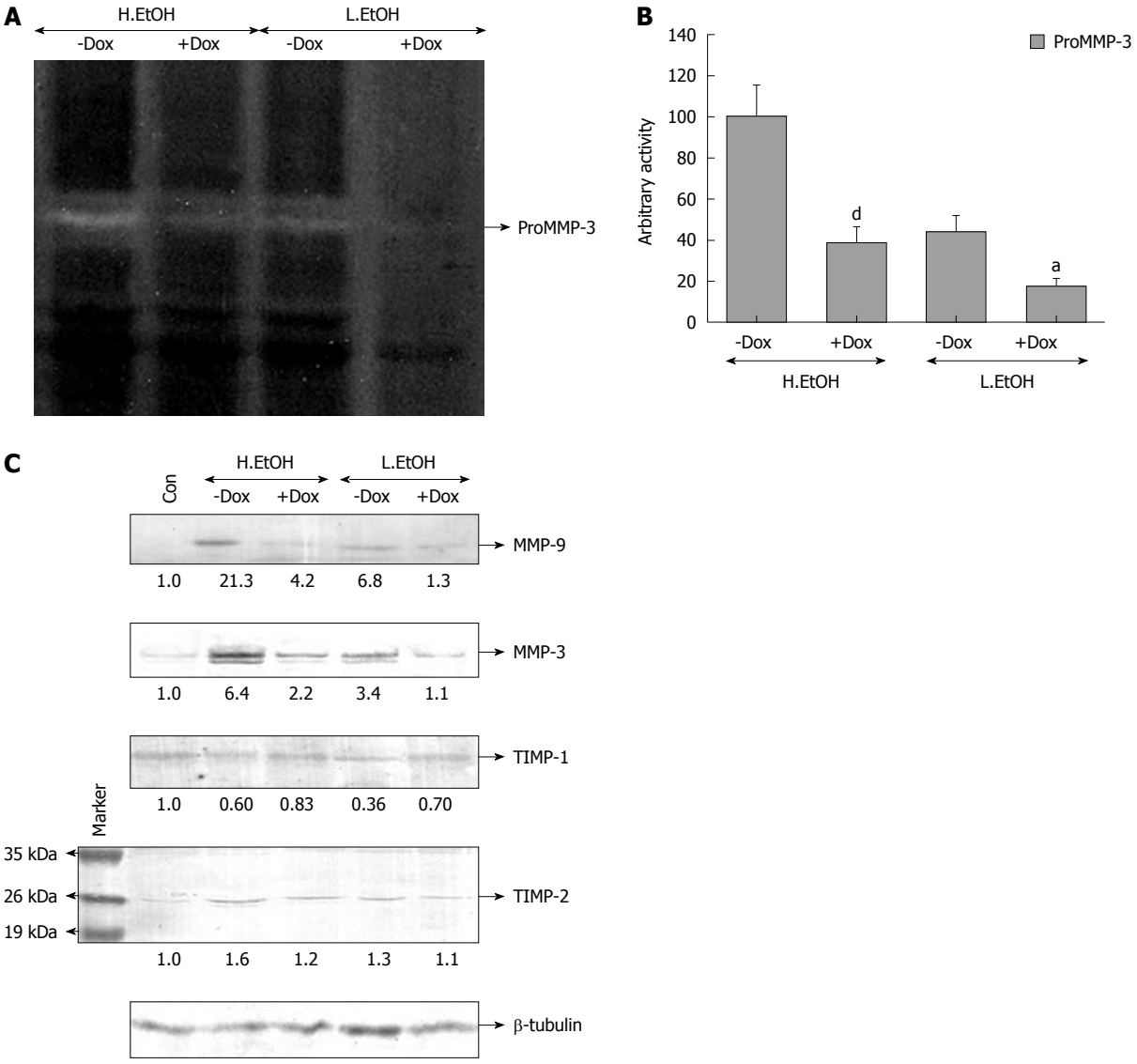

Figure 5 Effect of doxycycline on matrix metalloproteinase and tissue inhibitors of metalloproteinase activity and expression during protection against ethanol-treated gastric ulcer. Ulcers were induced in rat using 70% ethanol at low and high doses, and protection by doxycycline was performed. Casein zymography (A) was performed using phosphate buffered saline (PBS) extract of different gastric tissues. Histographic representation (B) of arbitrary activity of pro-matrix metalloproteinase (MMP)-3 as measured by Lab Image software using the above zymograms and two other representative zymograms from independent experiments. PBS extracts of gastric tissues from different experimental groups were subjected to Western blot blotting (C) and probed with polyclonal anti-MMP-9 and -3, tissue inhibitors of metalloproteinase (TIMP)-1 and -2, and anti-β-tubulin antibodies. Fold changes of proteins as represented below each band were measured by Lab Image software using the above wester blots and two other blots from independent experiments. Data expressed as mean ± SE. $^{a}P < 0.05$ *vs* low-dose ethanol; $^{d}P < 0.01$ *vs* high-dose ethanol. L.EtOH: Low dose ethanol; H.EtOH: High dose ethanol.

activity. Indomethacin-induced ulceration is associated with generation of H_2O_2, which in turn, damages tissues by interacting with protein and lipid. Thus, neutralizing H_2O_2-mediated damage by doxycycline suggests the antioxidant property of doxycycline.

DISCUSSION

NSAIDs, along with other aggressive factors like ethanol, *Helicobacter pylori* infection, stress and steroids, increases the risk of gastric ulcer formation[3,4,11-13,19]. Increased production of ROS and inflammatory cytokines, inhibition of cell proliferation, infiltration of inflammatory cells, reduced production of mucus, growth factors, and prostaglandins are factors involved in the pathogenesis of gastric ulcers[9,11]. Research has been focused recently on the importance of ECM and its turnover in the ulcerated margins of gastric tissue. Increased epithelial cell turnover is associated with the repair process without altering the composition and organization of the gastric ECM. Matrix proteins are secreted by different cell types including fibroblasts and epithelial and endothelial cells and assemble into an ECM network in the spaces surrounding the cells[5,9,10]. Recent studies have described the involvement of MMPs in cleaving and remodeling of ECM. MMPs have been implicated in various processes including tumor growth metastasis, chronic ulceration, fetal tissue development, and bone resorption. MMPs

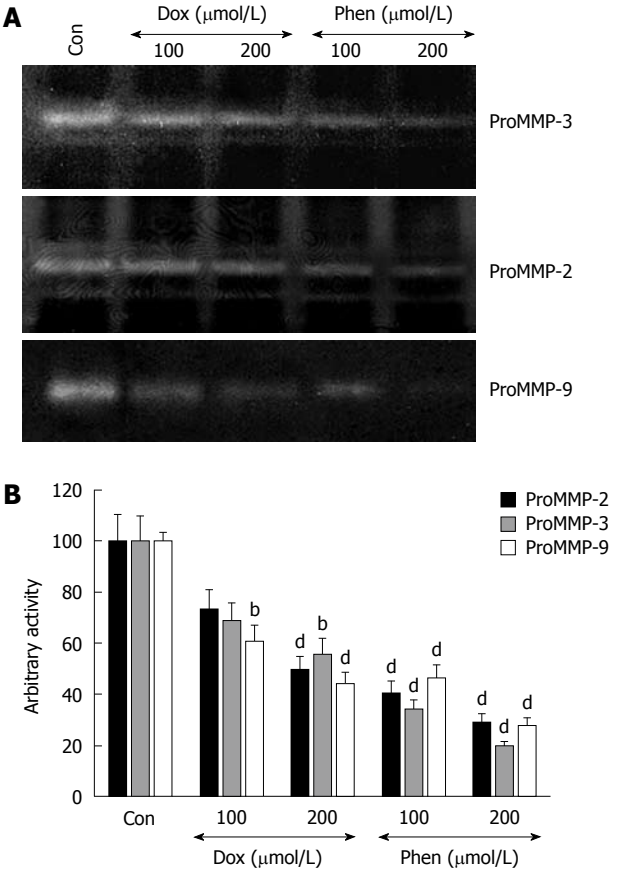

Figure 6 Inhibitory effect of doxycycline on matrix metalloproteinase activity in rat gastric tissue extract *in vitro*. A: Gelatin and casein zymography of gastric tissue extract following treatment with doxycycline or 1, 10-phenanthroline at two different concentrations; B: Histographic representation of pro-matrix metalloproteinase (MMP)-3, proMMP-2 and proMMP-9 activities as measured by Lab Image software using the above zymograms and two other representative zymograms from independent experiments. Data expressed as mean ± SE. $^{b}P < 0.01$, $^{d}P < 0.001$ *vs* control phenanthroline was labeled as Phen in the figure.

Table 1 Effect of doxycycline on glutathione, lipid peroxidation and protein oxidation level in indomethacin-induced gastric ulcer

Samples	Glutathione (nmol/g tissue)	Lipid peroxidation (nmolTBARS/mg protein)	Protein oxidation (nmol/mg protein)
Control	182 ± 6.50	1.13 ± 0.16	2.66 ± 0.12
Indomethacin	116.50 ± 7.72^{b}	2.52 ± 0.07^{b}	5.14 ± 0.32^{b}
Doxycycline + indomethacin	167.25 ± 8^{d}	1.27 ± 0.06^{d}	3.36 ± 0.25^{a,d}

Doxycycline was administered 30 min before indomethacin in Sprague-Dawley rats. Control animals received vehicle orally. Glutathione content (n = 18), lipid peroxidation (n = 18), and protein carbonylation (n = 18) were measured in gastric tissue homogenate. Results are represented as mean ± SE. Data expressed as mean ± SE. $^{a}P < 0.05$, $^{b}P < 0.001$ *vs* control; $^{d}P < 0.01$ *vs* indomethacin.

capable of degrading ECM proteins are believed to play a significant role during the pathogenesis and healing of gastric ulcer[3,4,12]. These enzymes are synthesized as latent proenzymes and their activity is tightly regulated at

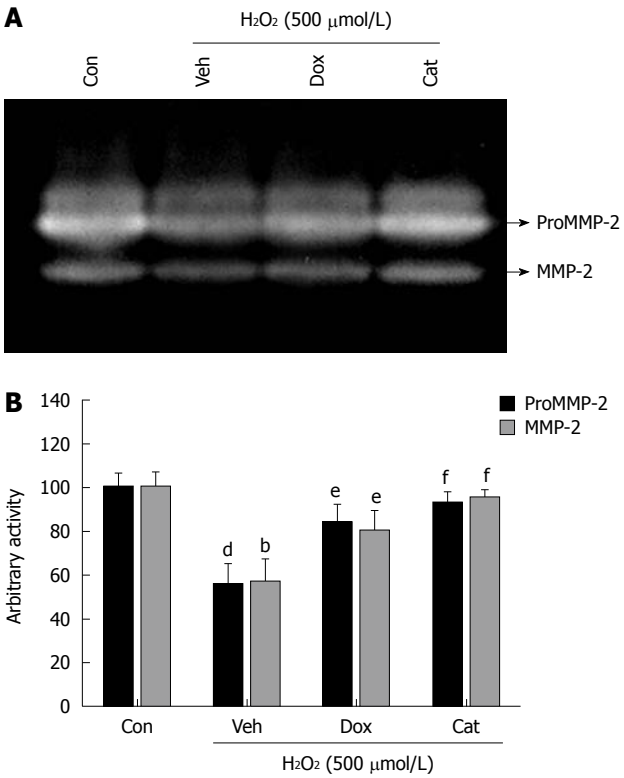

Figure 7 H_2O_2 scavanging activity of doxycycline. A: Gelatin zymography of phosphate buffered saline (PBS) extract from control gastric tissue was incubated with H_2O_2 (500 mmol/L) at 37℃ for 1 h. Equal amounts of PBS extract were pretreated with doxycycline (200 μmol/L) before incubation with H_2O_2. In one lane (Cat, lane 4), the extract was pretreated with catalase before reaction with H_2O_2; B: Histographic representation of pro-matrix metalloproteinase (MMP)-2 and MMP-2 activities as measured by Lab Image software using the above zymograms and two other representative zymograms from independent experiments. Veh: Vehicle; Cat: Catalase. Data expressed as mean ± SE. $^{b}P < 0.01$, $^{d}P < 0.001$ *vs* control; $^{e}P < 0.05$, $^{f}P < 0.01$ *vs* vehicle.

several levels including the post-translational level[33]. The coordinate action of proteases, in particular the MMPs and antiproteases, including the TIMPs, is thought to play an important role in connective tissue degradation[5]. In the present study, the authors intended to assess the relationship between MMP-2, MMP-3 and MMP-9 activity due to induction of gastric injury. Herein, doxycycline has been used to examine its gastroprotective potential.

Doxycycline, a widely prescribed antibiotic, is also a non-specific MMP inhibitor. It also possesses antiangiogenic and anti-inflammatory and neuroprotective properties, and is a convenient drug with low cost, and clinical tolerability without serious side effects[34]. Ryan *et al*[35] have reported divalent chelating activity of doxycycline. The beneficial effects of doxycycline are mainly mediated by its inhibition of MMP-9 and MMP-2. Doxycycline is safe in treating certain diseases because it reduces the level of pathophysiologically excessive MMPs but not to the level below normal physiological conditions. The effective dose of doxycycline is 100-200 mg/d for adult humans[36].

In the present study, we primarily showed that doxycycline potentially inhibited MMP-9 and MMP-3 activity

while arresting gastric damage in rats. It is noteworthy that doxycycline upregulated MMP-2 activity and expression during gastroprotection. Our results suggest that doxycycline acts through mechanisms distinctly different from its inhibitory effect on MMPs. Thus, doxycycline has a different action on MMP-2 activity in gastric damage, and indicates its regulatory role in addition to its inhibitory effect on MMPs. The increased MMP-2 activity may be required for preservation of matrix integrity, because function of the gastric epithelial and mucosal layer could be modulated by ECM components in gastric tissues[3,19]. Herein, an interesting feature of doxycycline is that it reduced MMP-2 activity *in vitro* and increased it *in vivo*. One interpretation is that doxycycline binds to Ca^{2+}/Zn^{2+} at catalytic sites of MMP *in vitro* and inhibits the enzymatic activity of MMPs[22,35]. Another interpretation may be that doxycycline perturbs the signaling pathways related to mitogen-activated protein kinase or Smad[27], and subsequently leads to beneficial systemic changes. *In vivo* upregulation of MMP-2 activity could be explained as an anti-apoptotic role of doxycycline during gastroprotection[34]. An alternative interpretation is that doxycycline inhibits oxidative stress and upregulates expression of MMP-2, or decreases antiprotease expression, thus shifting the protease/antiprotease balance towards high enzymatic activity. The beneficial effect on gastric damage may not be solely attributed to MMP-9 and MMP-3 inhibition. Other proteases and antiproteases may play a critical role, and pleiotropic effects of doxycycline may offer a better approach. This study provided convincing data to suggest a new pharmacological approach for the drug doxycycline for prevention of NSAID- or ethanol-induced gastric ulcers.

While the exact mechanism of gastric ulceration is still not completely understood, various cellular stresses including oxidative stress play a central role in the progression of gastric ulcers. Inflammatory reactions induced by indomethacin are a significant source of ROS, for example, H_2O_2 and the superoxide radical, which play a causative role in gastric injury[19]. ROS generally modulate MMP activity either indirectly through redox-dependent regulation of MMP gene transcription or directly through modification of MMP structure[2]. However, the direct association of ROS with the regulation of MMPs during gastric ulceration is not well studied. Several *in vivo* and *in vitro* cell culture studies have hypothesized activation of gelatinases through ROS or ROS generators when applied time and dose dependently. Herein, incubation of pro-MMP-2 in an *ex vivo* system with H_2O_2 resulted in suppression of enzyme activity, which suggests that ROS play a significant role in modulating MMP-2 activity. We also showed that doxycycline possessed inhibitory activity against H_2O_2-mediated suppression of MMP-2 activity, which indicated the antioxidant action of doxycycline. Doxycycline offered gastroprotection by preventing oxidative damage caused by lipid peroxidation and protein oxidation. Ulcerated samples exhibited reduced thiol content, whereas that was almost completely restored to control levels by doxycycline during gastroprotection. Although doxycycline has been reported as a potent inhibitor of MMPs, the antioxidant activity is worth mentioning in arresting gastric injury.

The major observations of the present study highlight that: (1) gastric injury is associated with upregulation of proMMP-3 and -9 activity, as well as expression in rat gastric tissues; (2) doxycycline is very effective in preventing gastric damage, and the mechanism of gastroprotection is attributable to its inhibitory effect on proMMP-3 and -9, while enhancing MMP-2 *in vivo*; and (3) doxycycline has a modulatory role with TIMP-1 and TIMP-2 during protection against gastric ulcers. The regulatory role of proMMP-2 activity may be dependent on the antioxidant action of doxycyline. We showed that doxycycline provides protection against gastric damage *via* inhibition of protein oxidation, lipid peroxidation and glutathione depletion. Moreover, suppression of MMP-2 activity by oxidative stress is rescued by doxycycline. Overall, this study is believed to be the first to demonstrate effectiveness of doxycycline in prevention of gastric damage *via* regulation of MMPs. Doxycycline therefore, may be considered as a candidate towards a therapeutic strategy for gastric ulcers. Also, proMMP-3 and -9 overexpression in gastric injury may be exploited as therapeutic targets, and doxycycline may be used as target-based therapy.

COMMENTS

Background

A class of zinc-dependent enzyme named matrix metalloproteinases (MMPs) is capable of degrading proteins of cellular and extracellular origin of various tissues. Their function is regulated by endogenous inhibitors such as tissue inhibitor of metalloproteinases and α-2 macroglobulin, which helps to maintain the protease-antiprotease balance. Imbalance in MMPs action is implicated in different diseases including gastric ulcer. Various factors mainly alcohol, non-steroidal anti-inflammatory drugs and infection with *Helicobacter pylori* cause gastric injury. In recent years, there has been a paradigm shift in understanding the mechanism of gastric ulcers, which includes regulation of MMPs and remodeling of the matrix surrounding the cells. Healing of gastric injury is a complicated process that comprises a series of cell-matrix interactions, and cell-cell signaling. Existing drugs against gastric injury have some limitation and side effects. Researchers are looking for novel drugs that have fewer side effects, easy availability, and low cost. A research group in India has examined the effect of doxycycline on gastric injury *via* regulation of MMPs.

Research frontiers

Doxycycline (derivative of tetracycline) is used commonly as an antibiotic. However, the antiulcer mechanism of doxycyline has not yet been explored fully. This study is believed to be the first to investigate the regulation of MMP-2 expression by doxycycline while preventing gastric injury.

Innovations and breakthroughs

The gastroprotective effect of doxycycline was tested against ethanol- or indomethacin-induced gastric mucosal injury in rats. Doxycycline prevented oxidative damage caused by lipid peroxidation and protein oxidation. Ulcerated samples exhibited reduced glutathione content, and it was almost completely restored to control levels by doxycycline during gastroprotection. Injured tissues exhibited significantly higher MMP-3 and MMP-9 activities, while doxycycline treatment markedly decreased both enzymes during gastroprotection. In contrast, the reduced MMP-2 activity in injured tissues was reversed by doxycycline during ulcer prevention. In addition, *in vitro*, doxycycline inhibited significantly all MMPs and proMMP-2, -3 and -9. Swarnakar *et al* have demonstrated a novel role of doxycycline in the upregulation of MMP-2 activity and expression during protection against gastric ulcers.

Applications

Altogether, the work established the use of doxycycline as an antiulcer drug that may lead to effective therapy for gastric ulcer. Combination therapy of doxycycline along with other existing antiulcer drugs such as famotidine, ranitidine or omeprazole may be better for treatment of gastric inflammation.

Peer review

This is believed to be the first demonstration of the dual action of doxycycline, namely, upregulation of MMP-2 activity and reduction of oxidative stress, in arresting gastric injury. Long-term use of doxycycline may provide therapeutic intervention for chronic gastric ulceration that poses a risk of progression to carcinoma.

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ORIGINAL ARTICLE

Immunostaining of PD-1/PD-Ls in liver tissues of patients with hepatitis and hepatocellular carcinoma

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Abstract

AIM: To investigate the expression of programmed death (PD)-1, PD ligand 1 (PD-L1) and PD-L2 in liver tissues in the context of chronic hepatitis and hepatocellular carcinoma (HCC).

METHODS: Liver biopsies and HCC specimens from patients were collected and histologically examined. The expression of PD-1, PD-L1, and PD-L2 in biopsy specimens of chronic hepatitis and HCC specimens was evaluated by immunohistochemical staining. The association between the expression level of PD-1, PD-L1, and PD-L2 and clinical and pathological variables was analyzed statistically.

RESULTS: Expression of PD-1 was found in liver-infiltrating lymphocytes. In contrast, PD-L1 and PD-L2 were expressed in non-parenchyma liver cells and tumor cells. The expression of PD-L1 was significantly correlated with hepatitis B virus infection (1.42 ± 1.165 *vs* 0.50 ± 0.756, $P = 0.047$) and with the stage of HCC (7.50 ± 2.121 *vs* 1.75 ± 1.500 *vs* 3.00 ± 0.001, $P = 0.018$). PD-1 and PD-Ls were significantly up-regulated in HCC specimens (1.40 ± 1.536 *vs* 5.71 ± 4.051, $P = 0.000$; 1.05 ± 1.099 *vs* 4.29 ± 3.885, $P = 0.004$; 1.80 ± 1.473 *vs* 3.81 ± 3.400, $P = 0.020$).

CONCLUSION: PD-L1 may contribute to negative regulation of the immune response in chronic hepatitis B. PD-1 and PD-Ls may play a role in immune evasion of tumors.

Key words: Hepatitis B virus; Programmed death-1; Programmed death ligands; Hepatitis; Hepatocellular carcinoma

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INTRODUCTION

Though active vaccination against hepatitis B virus (HBV) infection is successful, chronic HBV infection remains an important medical issue. There are approximately 400 million individuals chronically infected with HBV worldwide. Chronic HBV infection is one of the major causes of liver cirrhosis and hepatocellular carcinoma (HCC).

It is generally accepted that an appropriate T-cell response to HBV is crucial for viral elimination[1]. It has been suggested that HBV-specific CD8+ T cells have dual functions: the production of antiviral cytokines to down-regulate HBV replication in hepatocytes and the elimination of residual HBV-infected cells by cytotoxic activities[2]. Chronic HBV infection is characterized by weak or absent specific T-cell responses to HBV in peripheral blood. In the liver, low numbers of HBV-specific CD4 and CD8 T cells have been found. However, those T cells show a low expression of interferon (IFN)-γ and low cytotoxic activity[3,4]. Mechanisms leading to decreased cellular immune responses to HBV are not yet defined. Though there are many approaches suitable for priming specific CTL responses, their ability to break immune tolerance mechanisms in chronically infected HBV patients requires further investigation. In this respect, understanding tolerance mechanisms in chronic HBV infection will further help the design of strategies to overcome the unresponsiveness of T cells.

Programmed death (PD)-1 is a member of the CD28 family and is involved in the regulation of T-cell activation[5]. PD-1 is expressed on T cells, B cells, and myeloid cells. Two ligands for PD-1, PD ligand 1 (PD-L1) and PD-L2 (also known as B7-H1 and B7-DC), have been identified and have features as co-stimulatory molecules. A large number of publications have indicated a role for PD-L1 in the negative regulation of T-cell functions and the maintenance of peripheral tolerance[6]. The exact role of PD-L2 requires further definition. In murine liver tissues, PD-L1 was found to be expressed on Kupffer cells (KCs) and liver sinusoidal epithelial cells (LSECs)[7]. Hepatocytes express constitutively low levels of PD-L1 but show enhanced expression of PD-L1 upon stimulation with interferons[8]. It has also been shown in cell culture that PD-L1 expression on hepatoma cells induces apoptosis in T-cells. PD-L1 deficiency leads to hepatic accumulation and impaired apoptosis of T-cells in a murine model[9], and PD-1 deficiency leads to enhanced proliferation of effector cells in the liver during adenoviral infection[10].

Recent data from other chronic viral infections, such as lymphocytic choriomeningitis virus, human immunodeficiency virus, and hepatitis C virus infections, indicate that up-regulation of the PD-1/PD-L1 system may be responsible for the impairment of T-cell function[11-18]. Blocking of PD-L1 led to the rescue of exhausted CD8 T-cells even in the absence of Th functions[15-18]. Kassel *et al*[19] examined the expression of PD-1, PD-L1, and PD-L2 in patient liver samples and found that there was a direct link between the degree of inflammation and the expression of PD-1/PD-L1. Gao *et al*[20] found in patients with HCC that overexpression of PD-L1 significantly associates with tumor aggressiveness and postoperative recurrence.

The expression of PD-Ls was further suggested as a mechanism of immune evasion for tumors[21]. Elevated expression of PD-L1 was found in different tumor entities[22-29]. It could be shown that tumor cells expressing PD-L1 were able to induce apoptosis of T-cells. Thus, it is necessary to investigate the expression of PD-L1/2 and PD-1 in the liver in the context of viral hepatitis and HCC.

MATERIALS AND METHODS

Specimens

Specimens of liver tissues were obtained by biopsy or surgery from 20 hepatitis patients (including 12 with HBV infection and 8 with non-viral hepatitis) and 26 HCC patients (including 21 with HBV infection) at Tongji Hospital of Tongji Medical College, Huazhong University of Science and Technology, China, between 2006 and 2007 (Tables 1-3). Surgically resected or biopsy specimens were fixed in formalin and embedded in paraffin for routine histological diagnosis, and then embedded in OCT compound and snap frozen in liquid nitrogen for immunohistochemical analysis. The histological activity index (HAI) was assessed according to the classification of Ishak *et al*[30], which combines grading and staging scores (Table 2). All patients with hepatitis C virus (HCV) and human immunodeficiency virus (HIV) infection were excluded. No patient underwent antiviral drug treatment prior to biopsy. Tumors were classified as stage I to Ⅲ based on the Chengdu conference[31] and as grade I to Ⅳ based on the Edmondson-Steiner Guidelines (Table 3). The Chengdu conference stage classification was based on tumor dimension and lobar distribution, vascular thrombosis, lymph node metastasis, distant metastasis and Child-Pugh staging. No patient underwent radiation or chemotherapy prior to surgery.

Immunohistochemical staining

Four micron sections of the specimens were air-dried for 10 min and then fixed in acetone for 10 min. Endogenous peroxidase activity was blocked by treatment with 0.3% hydrogen peroxidase in PBS for 30 min at room temperature. Sections were then washed three times in PBS. After blocking nonspecific binding with normal goat serum for 20 min at room temperature, sections

Table 1 Patient information

Patients	Age (mean, yr)	Sex (M/F)	Etiology (viral/non-viral)
Hepatitis	36.6	14/6	12/8
HCC	49.3	21/5	21/5
Total	43.8	35/11	33/13

HCC: Hepatocellular carcinoma.

Table 2 Characteristics of patient samples with hepatitis

No.	Age (yr)	Sex	Disease, etiology	HAI
LB10	35	M	Hepatitis, HBV	2
LB24	18	M	Hepatitis, HBV	2
LB18	43	M	Hepatitis, HBV	3
LB23	33	M	Hepatitis, HBV	3
LB26	36	F	Hepatitis, HBV	3
LB11	72	M	Hepatitis, HBV	4
LB21	40	M	Hepatitis, HBV	4
LB7	26	M	Hepatitis, HBV	4
LB9	40	F	Hepatitis, HBV	5
LB25	25	M	Hepatitis, HBV	6
LB31	28	M	Hepatitis, HBV	6
LB8	22	M	Hepatitis, HBV	13
LB4	36	F	Hepatitis	2
LB19	56	F	Hepatitis	2
LB22	28	F	Hepatitis	2
LB16	43	M	Hepatitis	3
LB20	42	F	Hepatitis	3
LB27	19	M	Hepatitis	3
LB30	55	M	Hepatitis	3
LB17	35	M	Hepatitis	4

HBV: Hepatitis B virus; HAI: Histological activity index.

were incubated with primary antibodies in a humidified chamber at 4℃ overnight. Anti-PD-1 (J116), anti-PD-L1 (MIH1), anti-PD-L2 (MIH18), anti-FoxP3 (236A/E7) and anti-CD3(UCHT1) antibodies were purchased from eBioscience (San Diego, CA) and used as primary antibodies at the final concentrations of 5, 10, 5, 10 and 5 μg/mL, respectively. The bound primary antibodies were detected with a Dako Envision™ Kit according to the manufacturer's instructions, and sections were counterstained with hematoxylin. IgG fractions from naïve mice were used instead of the primary antibody as negative controls.

The expression levels of PD-1, PD-L1 and PD-L2 were defined as the quickscore which was calculated according to the Detre S's method[32]. In brief, the proportion of cells staining positively throughout the section was termed category A and was assigned scores from 1 to 6 (1 = 0%-4%; 2 = 5%-19%; 3 = 20%-39%; 4 = 40%-59%; 5 = 60%-79%; 6 = 80%-100%). The whole section was scanned at low power in order to gauge the general level of intensity throughout. The average intensity, corresponding to the presence of negative, weak, intermediate, and strong staining, was given a score from 0 to 3, respectively, and termed category B. The product (A × B) was recorded as the quickscore.

Table 3 Characteristics of patient samples with hepatocellular carcinoma

No.	Sex	Age (yr)	Disease, etiology	Grade	Stage
c002	M	71	HCC, HBV	Ⅰ	Ⅰ
c005	M	42	HCC, HBV	Ⅰ	Ⅰ
c025	M	34	HCC, HBV	Ⅰ	Ⅰ
c009	M	58	HCC, HBV	Ⅰ	Ⅱb
c021	M	50	HCC, HBV	Ⅰ	Ⅱb
c042	M	48	HCC, HBV	Ⅰ	
c045	M	49	HCC, HBV	Ⅰ	
c014	M	48	HCC, HBV	Ⅱ	Ⅰ
c046	F	53	HCC, HBV	Ⅱ	
c023	F	34	HCC, HBV	Ⅲ	Ⅱa
c027	M	45	HCC, HBV	Ⅲ	Ⅱa
c026	F	18	HCC, HBV	Ⅲ	Ⅱb
c029	M	43	HCC, HBV	Ⅲ	Ⅱb
c043	M	61	HCC, HBV	Ⅲ	
c044	M	56	HCC, HBV	Ⅲ	
c020	M	49	HCC, HBV	Ⅳ	Ⅱb
c040	M	52	HCC, HBV	Ⅳ	
c004	M	64	HCC, HBV		
c011	M	41	HCC, HBV		
c019	F	40	HCC, HBV		
c028	M	50	HCC, HBV		
c015	M	48	HCC	Ⅰ	Ⅰ
c001	M	37	HCC	Ⅰ	Ⅱa
c003	M	62	HCC	Ⅰ	Ⅱa
c007	F	65	HCC	Ⅲ	Ⅱa
c022	M	64	HCC	Ⅳ	Ⅱa

HCC: Hepatocellular carcinoma; HBV: Hepatitis B virus.

Statistical analysis

The association between the expression level of PD-1, PD-L1, and PD-L2 and clinical and pathological variables was analyzed statistically by the Student's t test and Correlation analysis using SPSS 12.0 software. Values of $P < 0.05$ were considered to indicate statistical significance, and all tests were two-tailed.

RESULTS

Immunostaining of PD-1

Typically, lymphocyte infiltration occurred in liver tissues of patients with hepatitis (Figure 1A). In these tissues, lymphocytes were recognized as small mononuclear cells and showed positive staining with anti-PD-1. The pattern of staining was consistent with the fact that PD-1 molecules are associated with cell membranes. The PD-1 positive cells showed a scattered pattern in liver tissues. Liver cells were not stained with anti-PD-1, which is consistent with the tissue specificity of PD-1 expression. The expression of PD-1 in liver tissues from patients with hepatitis had no relation to age, gender, alanine aminotransferase (ALT) and total bilirubin (TB) level, HAI or etiology (Table 4).

In peritumoral regions of HCC and within tumor tissues, PD-1 positive cells accumulated as massive lymphocyte infiltration took place (Figure 1D and G). Therefore, the expression level of PD-1 was determined by lymphocyte infiltration and was not dependent on the parameters

Table 4 Programmed death 1/programmed death ligands expression in liver tissues of patients with hepatitis (mean ± SD)

	n	PD-1	*P*	PD-L1	*P*	PD-L2	*P*
Age (yr)							
< 36.6	12	1.67 ± 1.826	0.297	1.08 ± 1.240	0.873	1.83 ± 1.467	0.905
⩾ 36.6	8	1.00 ± 0.926		1.00 ± 0.926		1.75 ± 1.581	
Gender							
Male	14	1.43 ± 1.604	0.903	1.07 ± 1.207	0.898	1.71 ± 1.437	0.702
Female	6	1.33 ± 1.506		1.00 ± 0.894		2.00 ± 1.673	
ALT level (U/L)							
Normal (⩽ 40)	11	1.45 ± 1.440	0.866	1.27 ± 1.191	0.330	2.00 ± 1.673	0.517
Abnormal (> 40)	9	1.33 ± 1.732		0.78 ± 0.972		1.56 ± 1.236	
TB level (μmol/L)							
Normal (⩽ 19)	13	1.46 ± 1.613	0.814	0.92 ± 0.862	0.496	1.77 ± 1.691	0.903
Abnormal (> 19)	7	1.29 ± 1.496		1.29 ± 1.496		1.86 ± 1.069	
HAI score							
< 4	12	1.00 ± 1.477	0.159	0.67 ± 0.778	0.053	1.50 ± 1.567	0.276
⩾ 4	8	2.00 ± 1.512		1.62 ± 1.302		2.25 ± 1.282	
Etiology							
HBV	12	1.83 ± 1.528	0.125	1.42 ± 1.165	0.047	2.08 ± 1.311	0.304
Unknown	8	0.75 ± 1.389		0.50 ± 0.756		1.38 ± 1.685	

PD: Programmed death; PD-L: Programmed death ligand; HBV: Hepatitis B virus; ALT: Alanine aminotransferase; TB: Total bilirubin; HAI: Histological activity index.

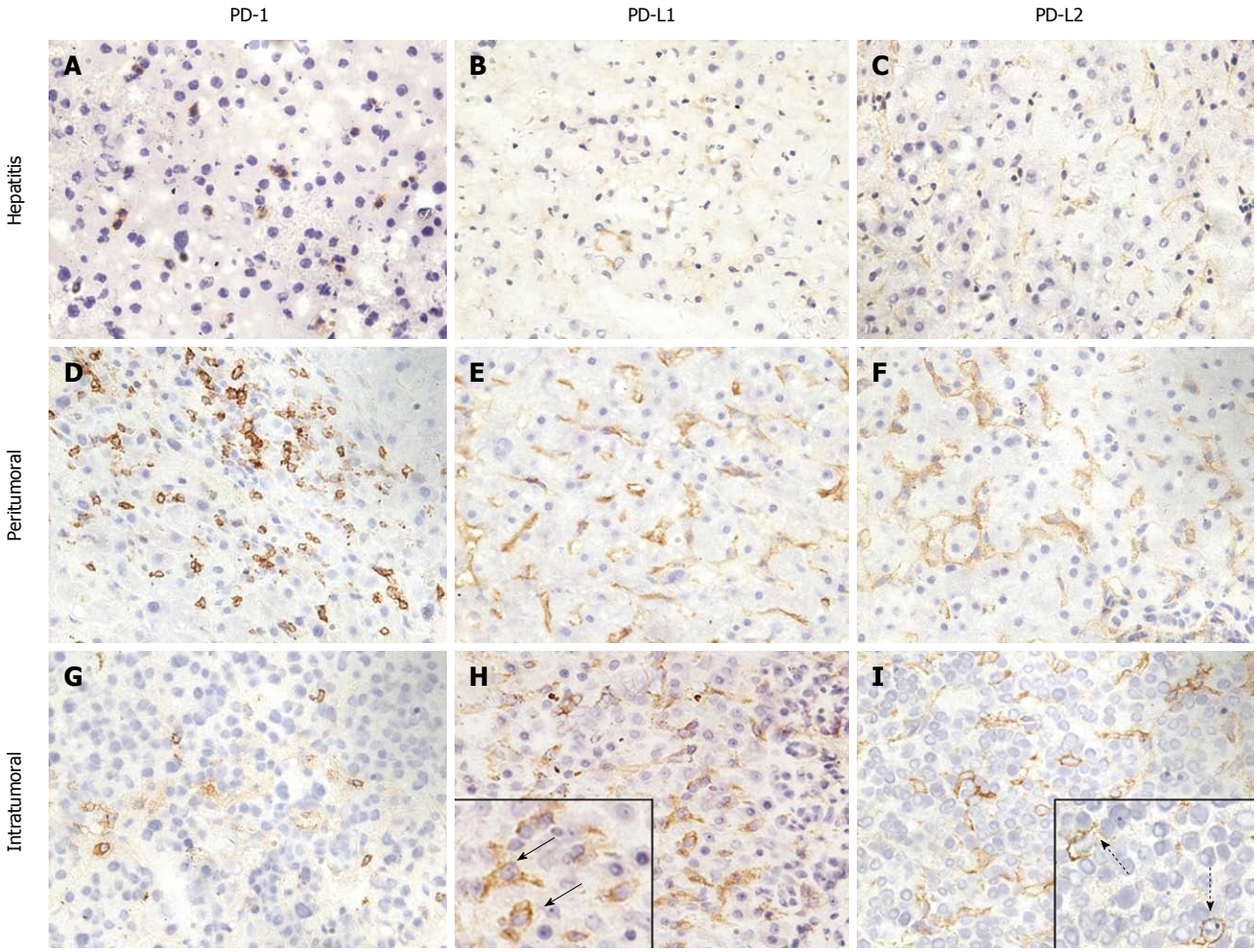

Figure 1 Immunohistochemical staining of liver tissues from patients with hepatitis and hepatocellular carcinoma for programmed death-1, programmed death-L1, and programmed death-L2. A-C: Programmed death (PD)-1 (A), PD ligand 1 (PD-L1) (B), and PD-L2 (C) expression in liver tissues with hepatitis; D-I: PD-1 (D and G), PD-L1 (E and H), and PD-L2 (F and I) expression in liver tissues with hepatocellular carcinoma (HCC); D-F: Peritumoral region; G-I: Intratumoral region. A, D and G: PD-1 was expressed on the membrane of infiltrated lymphocytes in liver tissues with hepatitis and HCC; B, E and H: PD-L1 was expressed on the membrane of hepatic cells and/or tumor cells in liver tissues with hepatitis and HCC; C, F and I: PD-L2 was expressed on the membrane of hepatic cells and/or tumor cells in liver tissues with hepatitis and HCC. Solid arrows indicate PD-L1+ tumor cells, and dashed arrows indicate PD-L2+ tumor cells. Magnification 200 ×.

Table 5 Programmed death 1/programmed death ligands expression in liver tissues of patients with hepatocellular carcinoma (mean ± SD)

	n	PD-1	*P*	PD-L1	*P*	PD-L2	*P*
Age (yr)							
< 49.3	21	6.45 ± 4.510	0.397	4.56 ± 4.304	0.779	4.73 ± 3.524	0.202
≥ 49.3	5	4.90 ± 3.872		4.00 ± 3.625		2.80 ± 3.120	
Gender							
Male	21	5.60 ± 4.154	0.844	4.45 ± 4.390	0.826	3.67 ± 3.619	0.769
Female	5	6.00 ± 4.147		4.00 ± 3.098		4.17 ± 3.061	
Stage							
Ⅰ	5	4.75 ± 2.500	0.252	7.50 ± 2.121	0.018[1]	2.75 ± 1.893	0.624
Ⅱa	6	7.00 ± 5.612		1.75 ± 1.500		2.60 ± 2.302	
Ⅱb	5	1.67 ± 1.528		3.00 ± 0.001		1.33 ± 1.528	
Grade							
Ⅰ, Ⅱ	12	5.78 ± 3.632	0.958	6.80 ± 5.495	0.153	4.00 ± 3.775	0.736
Ⅲ, Ⅳ	10	5.67 ± 4.975		2.44 ± 1.878		3.44 ± 3.046	
Etiology							
HBV	21	5.60 ± 4.154	0.844	4.69 ± 4.231	0.464	3.73 ± 3.615	0.876
Unknown	5	6.00 ± 4.147		3.00 ± 2.449		4.00 ± 3.098	

[1]The significance among different stages is mainly the difference between stage Ⅰ and Stage Ⅱ (including Ⅱa and Ⅱb) according to the SNK analysis. PD: Programmed death; PD-L: Programmed death ligand; HBV: Hepatitis B virus.

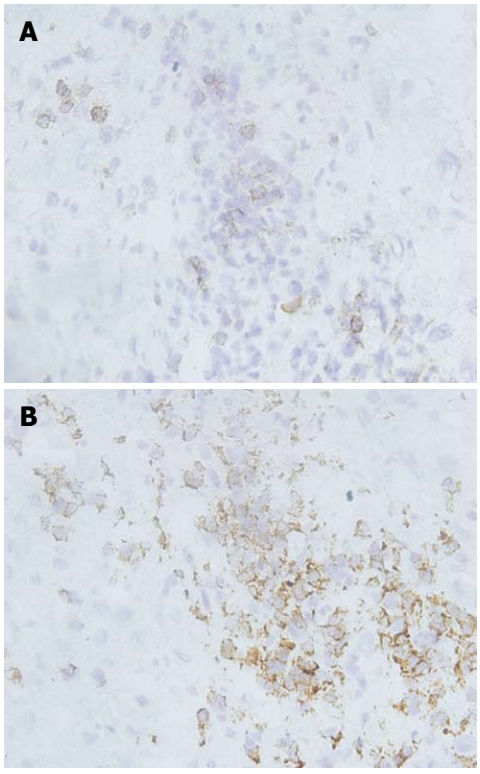

Figure 2 Expression of CD3 and programmed death-1 on regular liver sections from a patient with hepatocellular carcinoma. Serial sections were prepared from the same liver sample of a patient with hepatocellular carcinoma; CD3 and programmed death (PD)-1 were stained on these serial sections. CD3 was expressed on the membrane of liver-infiltrating lymphocytes, and the expression of PD-1 had a similar pattern to that of CD3, indicating PD-1 was also expressed on liver-infiltrating lymphocytes. A: PD-1; B: CD3.

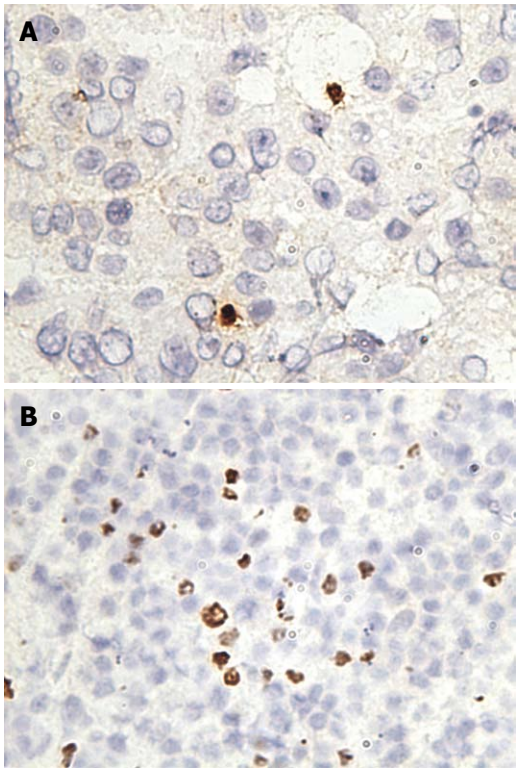

Figure 3 Immunohistochemical staining of liver tissues from patients with hepatocellular carcinoma for FoxP3. FoxP3 was expressed in liver-infiltrating lymphocytes in liver tissues with hepatocellular carcinoma. Liver infiltrating lymphocytes were mainly FoxP3 positive. A: Intratumoral region; B: Peritumoral region.

of age, gender, tumor stage or grade, or HBV infection (Table 5).

PD-1 was expressed on infiltrating lymphocytes (Figure 2A). These lymphocytes were CD3 positive, as both T-cell types were present in liver tissues and HCC tissues (Figure 2B). In addition, a significant portion of infiltrating lymphocytes were FoxP3 positive, indicating that negative regulation by regulatory T cells is operative in HCC (Figure 3).

Immunostaining of PD-L1 and PD-L2 in hepatitis

It has been reported that PD-L1 and PD-L2 are abun-

Table 6 Comparison of programmed death 1/programmed death-L expression in liver tissues of patients with hepatitis or hepatocellular carcinoma (mean ± SD)

Diagnosis	*n*	PD-1	*P*	PD-L1	*P*	PD-L2	*P*
Hepatitis	20	1.40 ± 1.536	0.000	1.05 ± 1.099	0.004	1.80 ± 1.473	0.020
HCC	26	5.71 ± 4.051		4.29 ± 3.885		3.81 ± 3.400	

PD: Programmed death; PD-L: Programmed death ligand; HCC: Hepatocellular carcinoma.

dantly expressed on KCs and LSECs[7]. In liver tissues with hepatitis, the expression of PD-L1 and PD-L2 was weakly detectable (Figure 1B and C), and the percentages of PD-L1 and PD-L2 positive cells were found to be only 0.57% and 1.29% of total cell counts, respectively. Cells with PD-L1 and PD-L2 expression were clearly different from lymphocytes and hepatocytes and represented rather KCs and LSECs.

The PD-L1 expression in liver tissues from patients with hepatitis had no relation to age, gender, or ALT and TB level (Table 4). Enhanced PD-L1 expression seemed to be associated with HBV infection, as compared with non-viral hepatitis (Table 4). In contrast, there was no relationship between PD-L2 expression and age, gender, ALT and TB level, or the etiology of hepatitis (Table 4).

The HAI was assessed to evaluate inflammation in the liver according to the classification of Ishak *et al*[30]. As shown in Table 4, a weak correlation between PD-L1 expression and HAI was found, which is consistent with previous observations that PD-L1 expression can be stimulated by viral infection and further pulse with IFNs[8]. The correlation between HAI and PD-L1 expression might be explained by the production of proinflammatory cytokines by infiltrating lymphocytes in chronic viral hepatitis. In contrast, there was no relationship between PD-L2 expression and HAI (Table 4).

Immunostaining of PD-L1 and PD-L2 in HCC

It has been reported that PD-L1 is abundantly expressed on cancer cells in various types of tumors. Therefore, 26 HCC specimens and peritumoural tissues prepared from surgery were examined for the expression of PD-L1 and PD-L2. The tumors were classified as stage Ⅰ (n = 5), stage Ⅱa (n = 6), or stage Ⅱb (n = 5) based on the Chengdu conference, and as grade Ⅰ (n = 10), grade Ⅱ (n = 2), grade Ⅲ (n = 7), or grade Ⅳ (n = 3) tumors based on the Edmondson-Steiner Guidelines (Table 3).

In peritumoural tissues, the expression of PD-L1 and PD-L2 was significantly elevated. However, the expression of PD-Ls appeared to still be restricted to cell types like KCs and LESCs (Figure 1E and F). Compared with liver tissues from patients with hepatitis, the expression of PD-Ls was recognizably enhanced in intensity for IHC staining with respective antibodies.

PD-L1 and PD-L2 had focal or scattered expression in 24 (92.6%) and 23 (88.9%) of 26 HCC specimens, respectively (Figure 1H and I). Thus, the enhanced expression of PD-Ls was a general phenomenon in HCC. In tumor tissues, tumor cells expressing PD-L1 and PD-L2 were detected. These cells had a different morphological appearance and were characterized by large nuclei surrounded by thick cytoplasm (Figure 1H and I). Tumor cells were heterogeneous in regards to the expression of PD-L1 and PD-L2 molecules, and less than 50% of cells in tumor tissues expressed these molecules. Thus, the expression of PD-L1 and PD-L2 did not appear to be regulated by an exogenous inducer for cancer cells, but rather was an intrinsic characteristic of these cancer cells.

The expression of PD-L1 was more pronounced at an early stage of HCC, while there was a lower level of PD-L1 expression with increasing stage of HCC (Table 5). Thus, the expression of PD-L1 appeared to be an early event during tumor progression. In contrast, there was no significant correlation between tumor grade and PD-L1 expression level. In addition, HBV infection had no significant influence on PD-L1 or PD-L2 expression in HCC (Table 5).

Compared to liver tissues from patients with chronic HBV infection, PD-1, PD-L1, and PD-L2 expression levels in HCC were greatly enhanced (Table 6).

DISCUSSION

It has been demonstrated that the PD-1/PD-L1/PD-L2 system can deliver a negative signal to T cells and lead to T-cell exhaustion or apoptosis[9,10,33,34]. To assess the role of the PD-1/PD-L1/PD-L2 system in chronic HBV infection and HCC, we analyzed the expression of these molecules in liver tissues from patients with these conditions. From the results of this study, we conclude the following: (1) the expression of PD-L1 and PD-L2 is detectable on KCs and LSECs in liver tissues with viral and non-viral hepatitis; (2) PD-L1 expression in liver tissues is enhanced in patients with chronic hepatitis B; and (3) PD-1 and PD-Ls expression are significantly enhanced in peritumoral and tumor tissues. The first two findings are in agreement with previous findings[19,20]. It will be interesting to investigate the role of PD-L1 expression in chronic HBV infection. While KCs and LSECs are the cell types expressing PD-L1 and PD-L2 in peritumoral tissues, a portion of tumor cells in HCC gained the ability to express PD-L1 and PD-L2. PD-L1 and PD-L2 expression in tumor tissues occurred in the early stage and may represent an important contribution to immune evasion during tumor progression. Thus, HCC may evade immune control by different mechanisms including negative regulation of T-cells.

PD-1/PD-L1 expression may play a role in viral hepa-

titis[13,14,17,18]. Upon activation, T-cells express PD-1 and are therefore susceptible to negative signaling by its ligands[33,34]. In our work, it is evident that liver-infiltrating lymphocytes were positive for PD-1. The activated T-cells may enter liver tissues due to attraction by chemokines. Due to the expression of PD-L1 and PD-L2 on KCs and LSECs in liver tissues and on cancer cells in HCC, liver-infiltrating cells would frequently encounter negative signals and become "exhausted" T-cells.

In chronic viral infection, virus-specific T cells are dysfunctional either because of interaction with regulatory T cells[35] or interaction between PD-1 and its ligand PD-L1, which results in the down-regulation of T-cell functions[11-14]. Recently, blockage of PD-L1 emerged as an effective measure to promote the proliferation and functions of T-cells in lymphocytic choriomeningitis virus infection of mice[15]. Barber *et al*[15] demonstrated that virus-specific, exhausted cytotoxic T-lymphocytes regained their ability to proliferate and perform functions such as IFN-γ production and killing of target cells. In HIV- and HCV-positive patients, virus-specific cells also have high expression levels of PD-1, which may explain their dysfunction[12-14,16-18]. Therefore, the blockage of PD-L1 and PD-L2 may restore impaired T-cell functions in individuals with persistent HBV infection and may lead to effective immunological control of viral infection. It is evident that T-cell functions are impaired in patients chronically infected with HBV. In our work, PD-L1 is up-regulated in chronic HBV, suggesting that PD-L1 may play an important role in chronic HBV infection due to the negative control of proliferation and functions of liver-infiltrating T-cells. A recent study demonstrated that a blockage of PD-L1 could restore the functions of peripheral and intrahepatic T-cells from patients with chronic hepatitis B[36].

Our findings have important implications for immunotherapeutic approaches to chronic hepatitis and HCC. Until now, the focus of experimental approaches has been to improve the ability of vaccines to induce a broad, strong T-cell response. However, these efforts could be compromised by the fact that specific T cells may be unable to exert their functions in the liver due to negative regulation, despite effective priming in the peripheral and local lymphoid organs. Thus, future research on immunotherapeutic approaches should consider the blockage of negative T-cell regulation in combination with immunostimulation.

Taken together, PD-L1 and PD-L2 were expressed by KCs and LSECs independent of viral and non-viral hepatitis and were up-regulated by chronic HBV infection and in HCC. The presence of PD-L1 and PD-L2 may lead to the suppression of immune responses and therefore facilitate viral persistence and carcinogenesis. Furthermore, the expression of PD-1, PD-L1, and PD-L2 in HCC was significantly higher than in hepatitis and correlated with the stage of HCC and the number of infiltrating lymphocytes, indicating the importance of the PD-1/PD-Ls system in tumor development.

COMMENTS

Background

Chronic hepatitis B and hepatocellular carcinoma (HCC) remain the big problem in China and around the world. Immune tolerance characterized with weak or absent specific T-cell responses to hepatitis B virus (HBV) or tumor is responsible for the pathogenesis of hepatitis and tumor. In this content, understanding tolerance mechanisms in chronic hepatitis B and HCC will further help the design of strategies to overcome the unresponsiveness of T cells.

Research frontiers

It has been demonstrated that the programmed death (PD)-1/PD ligand 1 (PD-L1)/PD-L2 system can deliver a negative signal to T cells and lead to T-cell exhaustion or apoptosis. Recent data from other chronic viral infections, such as lymphocytic choriomeningitis virus, human immunodeficiency virus, and hepatitis C virus, and other tumors, such as breast cancer, pancreatic cancer, and non-small cell lung cancer, indicated that up-regulation of the PD-1/PD-L1 system may be responsible for the impairment of T-cell function. In this study, the authors investigated the expression of PD-L1/2 and PD-1 in the liver in the context of chronic hepatitis B and HCC.

Innovations and breakthroughs

Recent reports have highlighted the importance of PD-1/PD-Ls system as a negative immune regulator in pathogenesis of chronic viral infection and tumors. In this study, the authors found: (1) the expression of PD-L1 was significantly correlated with HBV infection and with the stage of HCC; (2) PD-1 and PD-Ls were significantly up-regulated in HCC specimens, which indicated for the first time that PD-L1 may contribute to negative regulation of the immune response in chronic hepatitis B; and (3) PD-1 and PD-Ls may play a role in immune evasion of HCC.

Applications

By identifying the fact that expression of PD-1/PD-Ls is highly up-regulated in hepatitis B and HCC, our study indicated the PD-1/PD-Ls system plays a role in immune tolerance of hepatitis B and HCC, and thus may represent a future strategy for therapeutic intervention in the treatment of patients with hepatitis B or HCC.

Terminology

PD-1 is a member of the CD28 family and is involved in the regulation of T-cell activation. PD-1 is expressed on T cells, B cells, and myeloid cells. Two ligands for PD-1, PD-L1 and PD-L2 (also known as B7-H1 and B7-DC), have been identified and have features as co-stimulatory molecules. A large number of publications have indicated a role for PD-L1 in the negative regulation of T-cell functions and the maintenance of peripheral tolerance. The exact role of PD-L2 requires further definition.

Peer review

This manuscript is impressive in that it investigated the expression of PD-1 and PD-Ls in liver tissue from patients with hepatitis and HCC using immunostaining in detail.

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ORIGINAL ARTICLE

Interactions between *CagA* and smoking in gastric cancer

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Abstract

AIM: To examine the interactions between cytotoxin-associated gene (*CagA*) positive *Helicobacter pylori* infection and smoking in non-cardiac gastric cancer.

METHODS: A case-control study (257 cases and 514 frequency-matched controls) was conducted from September 2008 to July 2010 in Xi'an, China. Cases were newly diagnosed, histologically confirmed non-cardiac cancer. Controls were randomly selected from similar communities to the cases and were further matched by sex and age (± 5 years). A face-to-face interview was performed by the investigators for each participant. Data were obtained using a standardized questionnaire that included questions regarding known or suspected lifestyle and environmental risk factors of gastric cancer. A 5 mL sample of fasting venous blood was taken. *CagA* infection was serologically detected by enzyme-linked immunosorbent assays.

RESULTS: Smoking and *CagA* infection were statistically significant risk factors of non-cardiac cancer. *CagA* was categorized in tertiles, and the odds ratio (OR) was 12.4 (95% CI: 6.1-20.3, P = 0.003) for *CagA* after being adjusted for confounding factors when the high-exposure category was compared with the low-exposure category. Smokers had an OR of 5.4 compared with subjects who never smoked (95% CI: 2.3-9.0, P = 0.002). The OR of non-cardiac cancer was 3.5 (95% CI: 1.8-5.3) for non-smokers with *CagA* infection, 3.5 (95% CI: 1.9-5.1) for smokers without *CagA* infection, and 8.7 (95% CI: 5.1-11.9) for smokers with *CagA* infection compared with subjects without these risk factors. After adjusting for confounding factors, the corresponding ORs of non-cardiac cancer were 3.2 (95% CI: 1.5-6.8), 2.7 (95% CI: 1.3-4.9) and 19.5 (95% CI: 10.3-42.2), respectively. There was a multiplicative interaction between smoking and *CagA*, with a synergistic factor of 2.257 (Z = 2.315, P = 0.021).

CONCLUSION: These findings support a meaningful interaction between *CagA* and smoking for the risk of gastric cancer which may have implications for its early detection.

Key words: Non-cardia cancer; Cytotoxin-associated gene; *Helicobacter pylori*; Interaction; Smoking

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INTRODUCTION

Gastric cancer is a multifactorial disease whose pathogenesis is still uncertain. *Helicobacter pylori* (*H. pylori*) infection, a class I human carcinogen, has been identified as a major risk factor of non-cardiac gastric cancer[1,2], particularly cytotoxin-associated gene (*CagA*) positive *H. pylori* infection[3-5]. Recently, smoking has been recognized as an important risk factor associated with the development of gastric cancer[6]. A meta-analysis has suggested that the risk of gastric cancer increased by approximately 50% in smokers compared with non-smokers[7]. In addition, smoking was also shown to increase the risk of gastric intestinal metaplasia (a precancerous lesion) in subjects with *H. pylori* infections[8,9], suggesting that smoking may be involved in altering or modifying the effect of *H. pylori* in gastric carcinogenesis. However, there have been few studies on the potential synergistic association between *H. pylori* infection and smoking for gastric cancer risk[10-13].

The aim of this study was to examine the associations between *H. pylori* infection and smoking and the risk of non-cardiac gastric cancer.

MATERIALS AND METHODS

From September 2008 to May 2010, patients clinically diagnosed with non-cardiac gastric cancer from Grade Ⅲ Level A comprehensive hospitals (ranked among the best hospitals) in Xi'an, China, were identified. They were clinically and pathologically diagnosed with non-cardiac gastric cancer, aged 30-79 years, and living in Xi'an at the time of their diagnosis. These patients or their family members (in some cases) signed informed consent forms to participate in this study. A total of 257 cases and 514 frequency-matched controls were enrolled. For each case, two controls were randomly selected from the same residential community and matched by sex and age (± 5 years). The controls had never been diagnosed with cancer, diabetes, or gastrointestinal disorders. A face-to-face interview was performed by the investigators for each participant using a standardized questionnaire that included questions regarding a wide variety of known or suspected lifestyle and environmental risk factors of gastric cancer.

The questionnaire included the history of socio-demographic characteristics, physical activity, medical history, family history of cancer, alcohol consumption, smoking and lifestyle factors. "Never Smoked" was defined as having smoked less than 100 cigarettes in the participant's lifetime. Quantitative smoking measures included the average number of cigarettes consumed per day and the age when started smoking, and (among former smokers) years since smoking ceased. A sample of 5 mL fasting venous blood was collected from the participants and the sera were isolated and stored at -80℃ until assayed. The antibody to *H. pylori* was tested in batched serum samples using an enzyme-linked immunosorbent assays (San Diego, CA). *CagA*-positive *H. pylori* infection was defined as the presence of *CagA* antibodies in the serum.

A conditional logistic regression model was used to estimate the odds ratios (ORs), 95% CI, and the risk factors using SPSS (version 15.0). *P* values less than 0.05 (two-tailed) were considered statistically significant. To estimate the linear association between *CagA* positive *H. pylori* infection and the risk of non-cardiac gastric cancer, *CagA* was classified into three categories (tertiles), at the nearest tertile based on the distribution in the control group. Smoking status was classified into never and ever smoking. We assessed the joint effects of smoking and *CagA* infection using four categories: *CagA* (-) and never smoked, *CagA* (-) and smoking, *CagA* (+) and never smoked, and *CagA* (+) and smoking. A synergy index (SF) was calculated in terms of the adjusted ORs. The SF is defined as:

$$SF = OR_{AB} \Big/ \left(OR_{\bar{A}B} \times OR_{A\bar{B}}\right)$$

and is the ratio of the observed *OR* for both factors combined, to the predicted *OR* assuming independent effects of each factor.

SF > 1, is defined as a positive interaction between the two risk factors, and SF < 1, means a negative interaction. The opposite applies to protective factors. To obtain the statistical significance of the *SF*, a test of interaction was performed using the *Z* statistic[14].

$$Z = \frac{In\left[OR_{AB} \Big/ \left(OR_{\bar{A}B} \times OR_{A\bar{B}}\right)\right]}{\sqrt{1/n_1 + 1/n_2 + 1/n_3 + 1/n_4 + 1/n_5 + 1/n_6 + 1/n_7 + 1/n_8}}$$

In this equation, n_1, n_2...n_8 are the values of the 8 cells in the 4 × 2 crosstable. Since the null value is 0, the statistic *Z* has asymptotically a standard normal distribution under the null hypothesis of no interaction.

RESULTS

The mean ages of the patients and the controls in this study were 56.4 and 58.2 years, respectively (Table 1). The majority of the participants were male (72.7%) in both cases and controls. Ninety-three percent of the cases and 91.3% of the controls were ethnic Han. Sixty-seven percent of the cases and 72.8% of the controls had a BMI level greater than 25.

Table 2 shows the smoking habits of the cases and controls. The proportion of current smokers and former smokers was significantly higher in the cases than in the controls. Among smokers, however, cases and controls reported a similar smoking intensity (21 and 22 cigarettes per day on average) and the pack-years of consumption were 28 and 25, respectively.

CagA positive *H. pylori* infection was strongly associated with non-cardiac cancer in this study (Table 3). *CagA* was categorized in tertiles, and the OR was 12.4 (95%

Table 1 Basic characteristics of cases and controls

Demographic data	Cases (n = 257)	Control (n = 514)	χ^2	P
Race				
Chinese Han	240	469	1.061	0.303
Others	17	45		
Education level (yr)				
< 6	82	175	2.075	0.557
6-9	87	155		
9-12	47	110		
> 12	41	74		
BMI (kg/m^2)				
≤ 25	172	374	2.824	0.093
> 25	85	140		

BMI: Body nass index.

Table 2 Smoking habits of cases and controls

	Cases (n = 257)	Control (n = 514)
Non-smoker (%)	90 (35)	355 (69)
Current smoker (%)	69 (27)	56 (11)
Former smoker (%)	98 (38)	103 (20)
Smoker (%)	167 (65)	159 (31)
Age of starting smoking	19 (15-20)	18 (15-20.5)
Cigarettes/d	21 (15-27)	22 (10-30)
Pack/yr	28 (16-42)	25 (10-39)

Data represent means, with interquartile ranges in parentheses.

CI: 6.1-20.3, P =0.003) for *CagA* after being adjusted for confounding factors when the high-exposure category was compared with the low-exposure category. Smoking was associated with the risk of non-cardiac gastric cancer. Subjects who smoked had an OR of 5.4 compared with those who never smoked (95% CI: 2.3-9.0, P = 0.002).

Smoking and *CagA* positive *H. pylori* infection had a joint effect in the development of non-cardiac cancer in this study (Table 4). In the absence of *CagA*, smoking was associated with a moderate increase of the risk in non-cardiac cancer (adjusted OR: 2.7). However, the presence of *CagA* and smoking was strongly associated with the risk of non-cardiac cancer (with an adjusted OR = 19.5), suggesting a synergistic interaction between these two factors in the development of non-cardiac cancer. The test for interaction showed that there was a multiplicative interaction between smoking and *CagA* with a synergistic factor of 2.257 (Z = 2.315, P = 0.021).

DISCUSSION

In this case-control study, smoking and *CagA* positive *H. pylori* infection was found to be important risk factors in non-cardiac gastric cancer. When both of these risk factors were present, the risk of non-cardiac gastric cancer was synergistically higher. These results suggest that smoking may somehow influence the carcinogenic processes associated with *CagA* positive *H. pylori* infection. thereby increasing the risk of gastric cancer.

Several previous studies have investigated the association between *H. pylori* infection and smoking in gastric cancer[10,12,13]. Siman and colleagues showed that among *H. pylori* seropositive subjects, smoking was associated with an increased risk of gastric cancer compared with *H. pylori* positive nonsmokers[12]. Similarly, Brenner and coworkers showed that the relative risks of gastric cancer were 2.6 for non-smoking subjects with *CagA* positive *H. pylori* infections and 7.2 for smoking subjects with *CagA* positive infections compared with subjects without smoking and *H. pylori* infection[10]. These findings were statistically significant, and are consistent with those of a case-control study in Russia, which suggested that smoking was only associated with risk of gastric cancer in men with *H. pylori* infection (OR = 2.3, CI = 1.1-4.7)[13].

Overall, it seems that smoking may increase the risk of gastric cancer in individuals with *H. pylori* infection. However, only Zaridze's study in Moscow formally examined the interaction between smoking and *H. pylori* infection and the P value for interaction was not significant. This may be due to the fact that these studies analyzed smoking and *H. pylori* infection in subjects with all types of gastric cancers, thereby potentially diluting the otherwise stronger effects they may have observed among non-cardiac cancers[2].

As mentioned above, two studies explored the association between *H. pylori* infection and smoking in non-cardiac cancer[10,11]. One study found an adjusted OR for non-cardiac cancer of 1.9 (95% CI: 0.4-8.8) for smokers without *H. pylori* infection, 6.4 (95% CI: 2.1-19.7) for never smokers with *H. pylori* infection, and 19.0 (95% CI: 5.4-67.2) for smokers with *H. pylori* infection. However, no significant interaction between smoking and *H. pylori* infection was found, perhaps due to the small number of *H. pylori* negative cases[11]. In the study reported by Brenner and colleagues, the relative risk of non-cardiac cancer was 6.1 (95% CI: 1.2-5.7) in *CagA*-positive smokers compared with nonsmoking subjects without *H. pylori* infection; this relative risk increased to 16.6 (95% CI: 4.3-64.2) in *CagA*-positive smokers[10]. In this study, never and former smokers were combined in the analysis of the joint effects of smoking and *H. pylori* infection. The inclusion of former smokers may have attenuated the estimates of the joint effects due to their potentially increased risk of gastric cancer compared with never smokers. Unlike the previous studies[12,13] that examined the modification of the smoking-gastric cancer association by *H. pylori* status, we separately analyzed those with non-cardiac gastric cancer, which may explain the markedly stronger association we observed in our data. In addition, our controls were randomly selected from the communities, thereby avoiding the potential selection bias in many hospital-based studies, where the appropriateness of the control group is often questionable.

The *CagA* antibody instead of the *H. pylori* antibody was analyzed in the present study. The reasons for using the *CagA* antibody included that over 90% of Chinese *H. pylori* isolates contain the *CagA* gene[15]. Antibodies

Table 3 Odds ratios of *CagA* positive *Helicobacter pylori* infection and smoking in non-cardiac cancer *n* (%)

	Cases (*n* = 257)	Control (*n* = 514)	Crude		Adjusted	
			OR	95% CI	OR	95% CI
CagA						
Tertile 1	45 (17.5)	249 (48.5)	1.0		1.0	
Tertile 2	85 (33.0)	170 (33.0)	2.3	1.2-3.9	3.8	1.4-7.2
Tertile 3	127 (49.5)	95 (18.5)	4.1	2.7-6.3	12.4[1]	6.1-20.3
P value				0		0.003
Smoking status						
Never smoked	90 (35.0)	355 (69.0)	1.0		1.0	
Smoking	167 (65.0)	159 (31.0)	3.6[a]	2.5-5.3	5.4[2,a]	2.3-9.0

[a]$P < 0.05$. [1]Adjusted for education, alcohol consumption, smoking and family history of gastric cancer; [2]Adjusted for education, alcohol consumption, family history of gastric cancer and *Helicobacter pylori* infection. CagA: Cytotoxin-associated gene; OR: Odds ratio.

Table 4 Risk and synergy index[1] of non-cardia gastric cancer according to *CagA* positive *Helicobacter pylori* infection and smoking *n* (%)

	Cases (*n* = 257)	Control (*n* = 514)	Crude		Adjusted[2]		SF	SF (95% CI)	*Z*	*P*
			OR	95% CI	OR	95% CI				
Smoking status							2.257	1.133-4.496	2.315	0.021[a]
CagA (-) and never smoked	30 (11.7)	269 (52.4)	1.0		1.0					
CagA (-) and smoking	45 (17.5)	105 (20.4)	3.5	1.9-5.1	2.7	1.3-4.9				
CagA (+) and never smoked	60 (23.3)	85 (16.5)	3.5	1.8-5.3	3.2	1.5-6.8				
CagA (+) and smoking	122 (47.5)	55 (10.7)	8.7	5.1-11.9	19.5	10.3-42.2				

[a]$P < 0.05$. [1]Synergy index, is the ratio of the observed odds ratio (OR) for both factors combined, to the predicted OR assuming independent effects of each factor; [2]Adjusted for education, alcohol consumption and family history of gastric cancer.

against *CagA* may have persisted longer than the antibodies against other strains of *H. pylori*[16,17]. If so, the *CagA*-Ab would better represent past exposure than the *H. pylori*-Ab. Using the *H. pylori*-Ab as a biomarker of past *H. pylori* infection would underestimate the true association between smoking and *H. pylori* infection in non-cardiac gastric cancer. Nevertheless, one previous study that used *CagA* as an indicator of *H. pylori* infection showed no significant interaction between this factor and smoking[10], possibly because this study used colorectal cancer patients as controls which have been shown to associate positively with cigarette smoking[18-20].

At present, the interaction between smoking and *CagA* positive *H. pylori* is biologically plausible. For example, bile salt reflux and gastric bile salt concentrations are higher in smokers than in nonsmokers[21]. Bile reflux was positively associated with the severity of glandular atrophy, chronic inflammation and lamina propria edema. Bile reflux causes reactive gastritis and modifies the features of *H. pylori* associated with chronic gastritis. Chronic gastritis has been implicated in gastric carcinogenesis[22]. Moreover, subjects with high bile acid concentrations and *H. pylori* infection had an elevated prevalence of intestinal metaplasia, which is also associated with the development of gastric cancer[22]. In addition, the concentration of vitamin C in gastric juices is lower in smokers[23], resulting in reduced scavenging of free radicals that may in fact be enhanced by *H. pylori*, and ultimately inhibit the growth of *H. pylori*[24]. Thus, smokers may lack vitamin C in their gastric juices that likely protect against carcinogens and inhibit *H. pylori* growth[24].

A known limitation of case-control studies is their inherent susceptibility to information and selection bias. In this study, *H. pylori* infection was estimated at the time of sample collection in cases when their gastric cancer was diagnosed. However, the presence of *H. pylori* infection typically initiates much earlier, and the signs of infection might diminish with advancing premalignant lesions[17,25]. Thus, the status of *H. pylori* infection in this study may have been misclassified which could have biased our findings towards the null association. Additionally, our sample size was not optimal for the analysis of the joint effects between smoking and *H. pylori* infection which resulted in wide confidence intervals for some risk factors.

In summary, we reported a significant interaction between smoking and *CagA* positive *H. pylori* infection for the risk of non-cardiac gastric cancer. These findings may have implications for preventive measures aimed at the early detection of gastric cancer.

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COMMENTS

Background

Helicobacter pylori (*H. pylori*) infection has been identified as a major risk factor of non-cardiac gastric cancer, particularly cytotoxin-associated gene (*CagA*) positive infection. Smoking has also been recognized as an important risk fac-

tor associated with the development of gastric cancer. In addition, smoking was shown to increase the risk of gastric intestinal metaplasia (a precancerous lesion) in subjects with *H. pylori* infections, suggesting that smoking may be involved in altering or modifying the effect of *H. pylori* in gastric carcinogenesis.

Research frontiers

Recently, an increasing number of studies have investigated the interactions between risk factors and gastric cancer, because gastric cancer is a multifactorial disease. The authors investigated the interactions between the risk factors and gastric cancer, and the findings support the effect modification by CagA positive *H.pylori* infection and smoking in the risk of non-cardiac gastric cancer.

Innovations and breakthroughs

Few studies have investigated the association between *H. pylori* infection and smoking in gastric cancer, and even fewer have formally examined the interaction between smoking and *H. pylori* infection.

Applications

This paper reported a significant interaction between smoking and *CagA* positive *H.pylori* infection in non-cardiac gastric cancer risk. These findings may have implications for preventive measures aimed at the early detection of gastric cancer.

Terminology

CagA: is the major virulence factor of type I *H. pylori*. *CagA* positive *H. pylori* infection: Infection with *H. pylori*, especially with strains carrying the *CagA*.

Peer review

Increases in numbers and concentration of a particular serotype *CagA*, as well as modeling interaction with smoking, represent improvements in exposure assessment over previous studies that have examined the relationship between *H. pylori* and smoking with respect to stomach cancer risk.

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BRIEF ARTICLE

Correlation of fatty liver and abdominal fat distribution using a simple fat computed tomography protocol

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Abstract

AIM: To evaluate the relationship between hepatic fat infiltration and abdominal fat volume by using computed tomography (CT).

METHODS: Three hundred and six patients who visited our obesity clinic between November 2007 and April 2008 underwent fat protocol CT scans. The age range of the patients was 19 to 79 years and the mean age was 49 years. The male to female ratio was 116:190. Liver and spleen attenuation measurements were taken with three regions of interests (ROIs) from the liver and two ROIs from the spleen. Hepatic attenuation indices (HAIs) were measured as follows: (1) hepatic parenchymal attenuation (CT_{LP}); (2) liver to spleen attenuation ratio (LS ratio); and (3) difference between hepatic and splenic attenuation (LS_{dif}). Abdominal fat volume was measured using a 3 mm slice CT scan starting at the level of the umbilicus and was automatically calculated by a workstation. Abdominal fat was classified into total fat (TF), visceral fat (VF), and subcutaneous fat (SF). We used a bivariate correlation method to assess the relationship between the three HAIs and TF, VF, and SF.

RESULTS: There were significant negative correlations between CT_{LP}, LS ratio, and LS_{dif} with TF, VF, and SF, respectively. The CT_{LP} showed a strong negative correlation with TF and VF (r = -0.415 and -0.434, respectively, $P < 0.001$). The correlation between CT_{LP} and SF was less significant (r = -0.313, $P < 0.001$).

CONCLUSION: Fatty infiltration of the liver was correlated with amount of abdominal fat and VF was more strongly associated with fatty liver than SF.

Key words: Steatosis; Computed tomography; Abdominal fat; Visceral fat; Subcutaneous fat

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INTRODUCTION

Fatty liver is a disease in which excess fat, mainly triglycerides, accumulates to comprise more than 5% of the

weight of the liver[1]. For patients without any history of excessive alcohol ingestion this condition is called non-alcoholic fatty liver disease (NAFLD). NAFLD is pervasive worldwide and its clinicopathologic spectrum ranges from simple steatosis to nonalcoholic steatohepatitis (NASH), which can advance to cirrhosis[1,2]. NAFLD is common in the obese and is correlated with type 2 diabetes mellitus (DM), dyslipidemia, and hypertension. Together, these abnormalities comprise insulin resistance syndrome (metabolic syndrome) and increase the risk of cardiovascular disease[3]. Among similar disease entities, abdominal obesity is more highly correlated with metabolic risk, independent of whole-body obesity. Within the category of abdominal obesity, visceral fat (VF) is more strongly correlated with metabolic risk than is subcutaneous fat (SF)[4].

In routine ultrasonography (US), computed tomography (CT), and magnetic resonance (MR) studies, we often detect fatty liver in patients who have normal body mass indices (BMIs). We also often find normal liver parenchyma in obese patients. As such, it is highly probable that fatty liver might be more strongly correlated with abdominal VF than with total fat (TF).

Histologic confirmation is the gold standard for diagnosing fatty liver[5]. However, biopsies are invasive, induce pain and require six or more hours of bed rest; they also modestly increase the risk of mortality[6]. Given the potential risks, biopsies are not performed in all patients. As a substitute for biopsy, imaging techniques, including US, CT, and MR, are now widely used. Of these, CT had been chosen as the method for this study[6]. CT attenuation values of the liver were strongly correlated with histological evidence of hepatic steatosis[7]. Hepatic attenuation was a reliable indicator of fatty liver if it was considerably lower than splenic attenuation[8]. Therefore, CT can be used as a non-invasive test to confirm the presence of hepatic steatosis.

To the best of our knowledge, no prior studies have explored the relationship between fatty liver and abdominal fat using CT. The purpose of this study was to identify any possible correlations between hepatic fat infiltration expressed as a CT liver attenuation value [in Hounsfield units (HU)] and abdominal fat volume, which was also measured directly from CT.

MATERIALS AND METHODS

Patient selection

This prospective study included a total of 414 patients (160 men and 254 women; mean age, 50.19 years, ranging from 19 to 80 years) who visited our obesity clinic for self-perception of obesity from November 2007 to April 2008. Information including sex, age, height, body weight (WT), BMI, waist-hip ratio (WHR), history of alcohol intake, systolic and diastolic blood pressure, triglyceride level (TG), and low-density lipoprotein (LDL) was collected for each patient. Any patient with a history of significant alcohol consumption, bile duct dilatation, hepatic mass, hepatitis, liver cirrhosis, or history of hepatic surgery was excluded. A fat protocol CT was also conducted on each patient. Finally, the total number of actual participating patients was 306 (116 men and 190 women; mean age 49 years, ranging from 19 to 79 years). According to World Health Organization, a BMI over 25 kg/m^2 is defined as overweight, and a BMI of over 30 kg/m^2 as obese. 186 of the 306 total patients were normal; 95 of the 306 patients were overweight; 25 of the 306 total patients were obese.

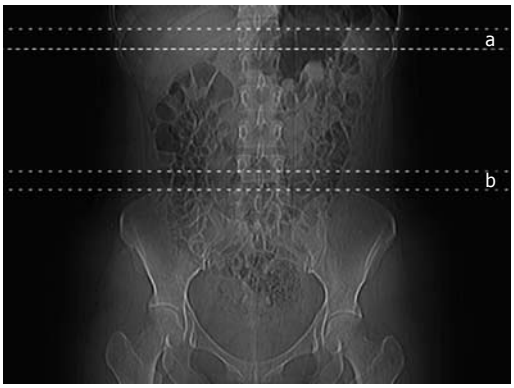

Figure 1 Scanning range of the liver and abdominal fat. Seven serial axial slices with a thickness of 3 mm were scanned for the liver and the spleen. Another seven slices from the iliac crest and above were scanned to assess abdominal fat. a: The range for hepatic fat examination; b: The range for abdominal fat examination.

All patients provided written informed consent and the Korea University Institutional Review Board approved this study protocol in accordance with the Declaration of Helsinki of the World Medical Association.

CT liver attenuation measurement

The attenuation of the liver and the spleen were measured using CT scans taken without intravenous contrast agent administration (Brilliance 64; Philips Medical Systems, Cleveland, OH, USA). The examination was done with a tube voltage of 120 kVp, a tube current of 50 mAs, and a tube rotation time of 750 ms. With the subject in the supine position, seven serial axial slices 3 mm in thickness were taken at approximately the mid-portion of the liver shadow on topogram (Figure 1).

Among the seven serial slices, we chose one image for measurement of the hepatic and splenic attenuation in each patient. Five regions of interests (ROIs) were identified in the liver, avoiding vessels, bile ducts, calcifications, and artifacts; four ROIs were identified in the spleen in the same manner. The highest and lowest values were excluded when calculating the mean attenuation values of the liver and spleen. As such, three liver values and two spleen values were used to calculate the mean values (Figure 2).

We derived hepatic attenuation indices (HAIs) from the calculated mean attenuation values of the liver and spleen. The HAIs included: (1) hepatic parenchymal attenuation (CT_{LP}; mean attenuation of the liver); (2) liver to spleen attenuation ratio (LS ratio; mean attenuation of

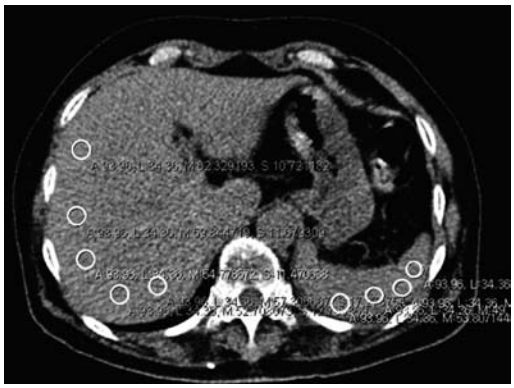

Figure 2 Measurement of attenuation value of the liver and the spleen. Five regions of interests (ROIs) were placed in the liver avoiding vessels, bile ducts, calcifications, and artifacts. In the spleen, four ROIs were placed in the same manner.

the liver/mean attenuation of the spleen); and (3) difference between hepatic and splenic attenuation (LS_{dif}; mean attenuation of liver - mean attenuation of spleen)[7].

The subjects were divided into two groups according to their LS_{dif}. LS_{dif} is among the popular reference values for grading fatty liver with CT[8]. Patients with an LS_{dif} greater than 5 were classified as normal; 249 of 306 (81.4%) patients were included in this group. The remaining 57 patients with an LS_{dif} less than 5 were classified as having fatty liver.

Abdominal fat distribution analysis

Abdominal fat distribution analysis was conducted on all patients. Starting at the level of the iliac crest, 7 slices 3 mm in thickness were scanned in a superior direction (Figure 1) to measure the VF and TF areas. The iliac crest corresponds to the L4/5 level and the level of the umbilicus. The cross-sectional surface areas (cm^2) of different abdominal fat compartments were automatically analyzed in three dimensions using commercially available CT software (Philips EBW2 version 3.0). The adipose tissue area was determined electronically by setting the attenuation values for a region of interest within a range of -175 to -25 HU[9]. The window center was set at 100 and the width was set at 150. The VF area was quantified by determining the size of the intra-abdominal cavity at the internal aspect of the abdominal wall surrounding the cavity. Gas and intestinal contents were excluded (Figure 3).

Abdominal fat is composed of TF, VF, and SF. The SF area was derived by subtracting the VF area from the TF area. The ratio of VF to SF (VS ratio) was also calculated[7]. Furthermore, we assessed the difference of mean ± SD of TF, VF, and SF in men and women among three different groups in hepatic fat infiltration.

Statistical analysis

Statistical analysis was performed with SPSS statistical software (version 12.0 for Windows; SPSS Inc., Chicago, IL, USA). The alpha level was set at $P = 0.05$ for all tests. We used the bivariate correlation method to assess the correlation between HAIs and TF, VF, and SF. Bivariate

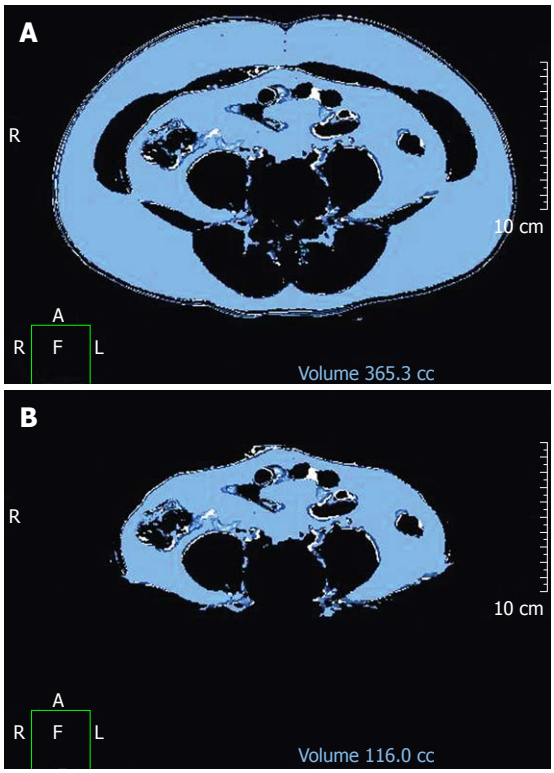

Figure 3 Abdominal fat distribution analysis. At the level of the umbilicus, abdominal fat volume was automatically calculated using a work station (Philips EBW2 version 3.0). Total abdominal fat volume (A) and visceral fat (VF) volume (B). Subcutaneous fat volume was derived by subtracting VF from total abdominal fat.

correlation methods were also used to assess the relationships between the HAIs and BMI, WT, WHR, TG, and LDL and between BMI and TF, VF, and SF. We used Student's *t*-test to compare the means of TF, VF, and SF between the two groups.

RESULTS

The mean ± SD of CT_{LP}, LS ratio, LS_{dif}, and the splenic attenuation value were 63.0 ± 10.7 (ranging from 15.4 to 78.6), 1.2 ± 0.2 (ranging from 0.3 to 1.8), 12.2 ± 10.4 (ranging from -30.4 to 33.4), and 50.8 ± 5.0 (ranging from 34.65 to 65.8), respectively. The mean values ± SD of TF, VF, and SF were 320.3 ± 144.7 (ranging from 68.8 to 869.6), 121.5 ± 68.1 (ranging from 15.7 to 377.2), and 198.8 ± 97.5 (ranging from 12.9 to 628.6), respectively. The mean values ± SD of BMI, WT, WHR, TG, and LDL were 24.5 ± 3.7 (ranging from 14.0 to 38.4), 64.7 ± 12.3 (ranging from 35 to 124), 0.87 ± 0.05 (ranging from 0.69 to 1.01), 137.2 ± 93.5 (ranging from 37 to 762), and 99.5 ± 32.0 (ranging from 12.6 to 331.6), respectively (Table 1).

There were significant negative correlations between CT_{LP}, LS ratio, and LS_{dif} with TF, VF, and SF, respectively. CT_{LP} showed strong negative correlations with TF and VF (r = -0.415 and -0.434, respectively, $P < 0.001$). The correlation between CT_{LP} and SF (r = -0.313, $P < 0.001$) was less significant. Among the three HAIs, CT_{LP} demon-

Table 1 The value of hepatic attenuation indices, splenic attenuation value, body fat volume, body mass index, body weight, waist-hip ratio, triglyceride, and low-density lipoprotein

	mean ± SD	Range
CT_{LP}	63.0 ± 10.7	15.4-78.6
LS ratio	1.2 ± 0.2	0.3-1.8
LS_{dif}	12.2 ± 10.4	-30.4-33.4
CT_S	63.0 ± 10.7	15.4-78.6
TF (cm^2)	320.3 ± 144.7	68.8-869.6
VF (cm^2)	121.5 ± 68.1	15.7-377.2
SF (cm^2)	198.8 ± 97.5	12.9-628.6
BMI (kg/m^2)	24.5 ± 3.7	14.0-38.4
WT(kg)	84.7 ± 12.3	65-124
WHR	0.87 ± 0.05	0.69-1.01
TG (mg/dL)	137.2 ± 93.5	37-762
LDL (mg/dL)	99.5 ± 32.0	12.6-331.6

CT_{LP}: Mean attenuation of the liver; LS ratio: Mean attenuation of the liver/mean attenuation of the spleen; LS_{dif}: Mean attenuation of the liver–mean attenuation of the spleen; CT_S: Splenic attenuation value; TF: Total fat; VF: Visceral fat; SF: Subcutaneous fat; BMI: Body mass index; WT: Weight; WHR: Waist-hip ratio; TG: Triglycerides; LDL: Low density lipoprotein.

Table 2 Correlation between hepatic attenuation indices and total fat, visceral fat, and subcutaneous fat

	TF	VF	SF
CT_{LP}	r = -0.415	r = -0.434	r = -0.313
	P = 0.000	P = 0.000	P = 0.000
LS ratio	r = -0.258	r = -0.298	r = -0.172
	P = 0.000	P = 0.000	P = 0.003
LS_{dif}	r = -0.297	r = -0.330	r = -0.210
	P = 0.000	P = 0.000	P = 0.000

CT_{LP}: Mean attenuation of the liver; LS ratio: Mean attenuation of the liver/mean attenuation of the spleen; LS_{dif}: Mean attenuation of the liver–mean attenuation of the spleen; TF: Total fat; VF: Visceral fat; SF: Subcutaneous fat.

Table 3 Correlation between hepatic attenuation indices and body mass index, body weight, waist-hip ratio, triglyceride, and low-density lipoprotein

	BMI	WT	WHR	TG	LDL
CT_{LP}	r = -0.582	r = -0.593	r = -0.364	r = -0.388	r = -0.060
	P = 0.000	P = 0.000	P = 0.000	P = 0.000	P = 0.300
LS ratio	r = -0.331	r = -0.405	r = -0.219	r = -0.314	r = -0.036
	P = 0.000	P = 0.000	P = 0.000	P = 0.000	P = 0.531
LS_{dif}	r = -0.392	r = -0.454	r = -0.257	r = -0.341	r = -0.036
	P = 0.000	P = 0.000	P = 0.000	P = 0.000	P = 0.531

CT_{LP}: Mean attenuation of the liver; LS ratio: Mean attenuation of the liver/mean attenuation of the spleen; LS_{dif}: Mean attenuation of the liver–mean attenuation of the spleen; BMI: Body mass index; WT: Weight; WHR: Waist-hip ratio; TG: Triglycerides; LDL: Low density lipoprotein.

strated a greater tendency to correlate with the abdominal fat volume than the LS ratio or LS_{dif} (Table 2).

BMI, WT, WHR, and TG were all negatively correlated with the respective HAIs. In contrast, LDL level was not correlated with HAIs (Table 3). BMI was strongly correlated with abdominal fat volume (r = 0.705, 0.601, and 0.624 for TF, VF, and SF, respectively, P < 0.001) (Table 4).

Table 4 Correlation between body mass index and abdominal fat volume

	TF	VF	SF
BMI	r = 0.705	r = 0.601	r = 0.624
	P = 0.000	P = 0.000	P = 0.000

BMI: Body mass index; TF: Total fat; VF: Visceral fat; SF: Subcutaneous fat.

Table 5 mean values of total fat, visceral fat, and subcutaneous fat among three different groups of hepatic fat infiltration

	n	mean ± SD	Minimum	Maximum
TF (cm^2)				
Normal	249	299.8 ± 133.3	68.8	869.6
Fatty liver	57	409.8 ± 159.3	126.6	838.0
VF (cm^2)				
Normal	249	110.1 ± 59.7	15.7	316.6
Fatty liver	57	171.4 ± 79.8	46.5	377.2
SF (cm^2)				
Normal	249	189.7 ± 92.8	12.9	571.3
Fatty liver	57	238.4 ± 108.0	72.4	628.6

The differences in total fat (TF), visceral fat (VF), and subcutaneous fat (SF) between the two groups of normal and fatty liver patients were all statistically significant (P < 0.001). *n*: Number of patients.

Table 6 mean values of total fat, visceral fat, and subcutaneous fat between men and women (mean ± SD)

	Men (*n*)	Women (*n*)	*P* value
TF (cm^2)			
Total	309.5 ± 131.3 (116)	326.9 ± 152.3 (190)	0.005[1]
Normal	292.6 ± 119.0 (89)	303.9 ± 140.8 (160)	0.002[1]
Fatty liver	410.6 ± 160.2 (27)	409.1 ± 161.2 (30)	0.545
VF (cm^2)			
Total	145.9 ± 66.6 (116)	106.7 ± 64.8 (190)	0.914
Normal	133.2 ± 54.3 (89)	97.2 ± 58.8 (160)	0.164
Fatty liver	190.2 ± 74.2 (27)	154.5 ± 82.1 (30)	0.444
SF (cm^2)			
Total	163.6 ± 80.6 (116)	220.3 ± 100.8 (190)	0.000[1]
Normal	159.3 ± 80.0 (89)	206.6 ± 95.3 (160)	0.002[1]
Fatty liver	220.4 ± 116.9 (27)	254.5 ± 98.5 (30)	0.916

TF: Total fat; VF: Visceral fat; SF: Subcutaneous fat; *n*: Number of patients.

The mean ± SD of TF, VF, and SF in normal patients were 299.8 ± 133.3, 110.1 ± 59.7, and 189.7 ± 92.8, respectively. The mean values ± SD of TF, VF, and SF in fatty liver patients were 409.8 ± 159.3, 171.4 ± 79.8, and 238.4 ± 108.0, respectively. The differences in TF, VF, and SF between the two groups of normal and fatty liver patients were all statistically significant (P < 0.001) (Table 5).

In the comparison between men and women, there were statistically significant differences in TF and SF among total or normal patients (Table 6).

The total radiation dose was 25-30 mGy/cm and the effective dose was less than (D_{eff}) 0.37-0.45 mSv.

DISCUSSION

NAFLD is associated with metabolic syndrome and occurs in patients without a history of excessive alcohol ingestion. The presentation of NAFLD varies from asymptomatic elevated liver enzyme levels, through various levels of inflammation and fibrosis, to cirrhosis with complications of hepatic failure and hepatocellular carcinoma[10]. By using accurate non-invasive diagnostic methods that detect NASH (the most severe form of NAFLD and one that can advance to cirrhosis) at an early stage, it is possible to detect patients at risk of developing cirrhosis later in life[5]. Although liver biopsy is the gold standard for diagnosing NAFLD, it has limitations including sampling error and potential risks to the patients[2].

A hepatic attenuation value (CT_{LP}) that is significantly lower than the splenic attenuation value is a reliable indicator for the presence of fatty liver[7]. CT_{LP} and LS ratio both demonstrate strong inverse correlations with degree of histologic steatosis[6]. Therefore, CT can serve as a non-invasive test to confirm the presence of hepatic steatosis[7,8]. The effectiveness of confirming fatty liver using CT has been demonstrated in donor evaluation for liver transplantation[7,8]. A previous study used the difference between mean hepatic attenuation and mean splenic attenuation, which is presented in our study as LS_{dif}, as a parameter for prediction of the degree of macrovesicular steatosis. In that study, an LS_{dif} below -10 HU was correlated with greater than 30% macrovesicular steatosis, a level that is unacceptable for liver transplantation. An LS_{dif} between -10 and 5 HU correctly predicted 6%-30% of steatosis, a relative contraindication for liver transplantation. An LS_{dif} above 5 HU predicted 0%-5% of steatosis[8]. Another study indicated that the highest cutoff values that yielded 100% specificity for the diagnosis of macrovesicular steatosis of 30% or greater (the limit of acceptability for donation) were 0.8 for LS ratio, -9 for LS_{dif}, and 42 for CT_{LP}[11]. Accordingly, CT has functioned as a screening tool to avoid unnecessary liver biopsy in patients with fatty liver who are not suitable for transplantation[11]. Nonetheless, the diagnosis and grading of fatty liver using CT is limited because simple steatosis and NASH that would be demonstrated by biopsy cannot be differentiated using imaging techniques[7].

There are some other tools that can be used to evaluate fatty liver and abdominal obesity. US is relatively cheap and easy to use, but has no reproducibility. CT and MR allow accurate and objective quantification of each body fat components. MR has several disadvantages including long scan time, high cost, and contraindicant to patients with claustrophobia, so that there is a limitation to the use of MR as a screening tool in the obesity clinic. On the other hand, CT is a simple and reproducible tool, and is a popular technique to screen fatty liver and obesity. Though widely used, CT has limitations in the assessment of fatty liver because of its association with significant radiation exposure, especially in serial assessment[6].

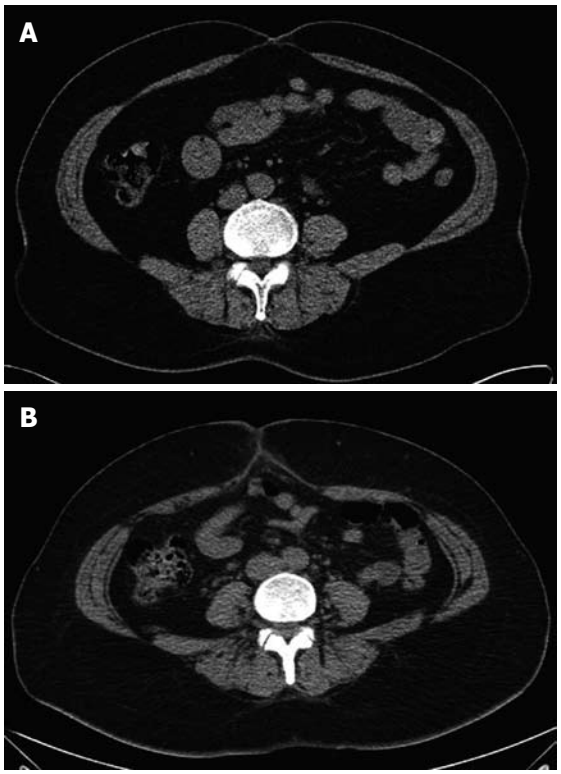

Figure 4 Computed tomography showing cross-sectional abdominal areas at the level of umbilicus in two patients demonstrating variation in fat distribution. A: Visceral type [49-year-old female; body mass index (BMI), 23.1 kg/m^2; visceral fat (VF) area, 146 cm^2; subcutaneous fat (SF) area, 115 cm^2; the ratio of VF to SF (VS ratio), 1.27]; B: Subcutaneous type (40-year-old female; BMI, 24.0 kg/m^2; VF area, 60 cm^2; SF area, 190 cm^2; VS ratio, 0.31).

Obesity is the cause of fatty liver in non-alcoholics. A previous study showed that BMI is directly related to the prevalence of NAFLD[1]. Although BMI is an independent predictor of fatty liver, many studies have indicated that body composition reflects an individual's health status better than body WT or BMI. In a study involving 3 432 Japanese subjects by Omagari *et al*[1], 27.2% of non-alcoholic and non-overweight men and 59.2% of non-alcoholic and non-overweight women were found to have fatty liver. The body fat percentages of these patients as measured using a bipedal bioimpedance instrument were excessive compared to men and women without fatty liver. The present study assumed that a patient with a normal BMI had a central body fat distribution if the body fat percentage was excessive; we also assumed that central body fat distribution is associated with the development of fatty liver. Percentage body fat measurement is useful when determining the cause of fatty liver in non-alcoholic and non-overweight individuals, especially in women[1]. Another study categorized obesity into two types (Figure 4), "android" or "male-type" obesity (central or abdominal depot) showed a stronger correlation than "gyroid" or "female-type" obesity (lower body, gluteofemoral, or peripheral depot) with increased mortality,

DM, hyperlipidemia, hypertension, and atherosclerosis[9]. As such, obesity is not a homogeneous condition and the regional distribution of adipose tissue is important for understanding the link between obesity and disturbances in glucose and lipid metabolism[9]. In contrast, VF and central abdominal fat demonstrated a strong correlation with each other and a strong association with metabolic syndrome. Given that VF in the abdomen is more strongly associated with hyperglycemia, arteriosclerosis, and dyslipidemia than SF, it is useful to distinguish between VF and SF when examining a patient[12]. Anthropometric measurements that evaluate body fat distribution such as skin fold or WHR do not differentiate between VF and SF[9]. Imaging techniques such as abdominal CT and MR are suitable methods for examining abdominal VF because they allow direct measurement of abdominal fat and make an accurate distinction between VF and SF[4,9,13]. We used CT to measure each abdominal fat component and the degree of hepatic fat infiltration; we then analyzed the relationships between these values. To determine visceral intraabdominal and subcutaneous abdominal areas, a simple CT scan was performed at the level of the umbilicus (L4/5). Scanning at the level of the umbilicus was first proposed by Wajchenberg[9], who found that because body fat exists in the highest percentage at the level of the umbilicus, it is easiest to differentiate SF from intraabdominal fat at this level. Kvist *et al*[14] have revealed that VF areas from a single scan at L4/5 region are highly correlated to the total VF volume.

Our study showed that CT_{LP} is more strongly correlated with the amount of abdominal fat than are LS ratio or LS_{dif}. This is most likely because the subjects in this study were quite healthy. The fatty infiltration of the liver was found to be more strongly associated with TF and VF compared to SF. SF is different from VF in that venous drainage from SF is directed towards the systemic circulation while the drainage from VF is directed towards the portal vein. VF also acts as an endocrine organ and affects the risk of developing certain metabolic traits and vasoactive substances[4,15]. VF secretes free fatty acids and adipocytokines and allows fat accumulation in the liver[15]. There is a direct association between the amount of VF and the amount of free fatty acid delivered to the liver. Several circulating cytokines are increased with obesity and may combine with the influence of VF to generate insulin resistance, inflammation, and fibrosis in NAFLD[16].

There are a few limitations in our study. First, pathologic confirmation was not obtained. Diagnosing and grading fatty liver with imaging features alone has limited value without definitive pathologic confirmation[1]. The patients in our study population were primarily outpatients who desired an annual check-up or evaluation of obesity; we were thus ethically prohibited from obtaining liver biopsies. In addition, it is difficult to distinguish between simple steatosis and NASH using imaging alone[6]. Second, we did not consider patients with hemochromatosis or other conditions related to hepatic iron deposition. Increased hepatic iron causes increased attenuation of the liver resulting in misinterpretation of combined fatty liver. Third, CT induces a radiation effect. As part of an effort to minimize radiation exposure, only selected levels of the abdomen were obtained in evaluating the abdominal fat volume. Finally, daily alcohol consumption was not fully assessed[1].

We conclude that fat infiltration of the liver is well correlated with amount of abdominal fat. Fatty liver tends to be more strongly associated with VF compared to SF. In other words, if a non-obese patient exhibits fatty liver, the patient may in fact have visceral obesity. Likewise, not all patients who have a large amount of SF develop fatty liver.

COMMENTS

Background

It has been generally recognized that fatty liver can often be found among obese people. However, we often experienced that the obese patient might always not show the fatty liver on the hepatic ultrasound or computed tomography (CT) examination.

Research frontiers

According to previous studies, fatty liver was found to be more strongly associated with visceral fat (VF) than subcutaneous fat, because of the portal venous drainage and endocrine effect of VF. In this study, the authors proved the result by CT.

Innovations and breakthroughs

The obese patient does not always show fatty liver in the imaging study. Fatty liver tends to be increased in incidence in the patients who have larger amount of VF.

Applications

By using body fat CT, we are able to quantify body fat distribution and degree of fatty liver and evaluate the correlation between the two variables. In the obesity clinic, we can standardize fatty liver and abdominal obesity and use these for the treatment response.

Peer review

This is a good study to correlate the fatty liver with the abdominal fat using CT scan. This as a well designed study with nicely written manuscript. There are few limitations of the study, which are already mentioned by authors.

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BRIEF ARTICLE

Self-expanding metallic stents drainage for acute proximal colon obstruction

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Abstract

AIM: To clarify the usefulness of the self-expanding metallic stents (SEMS) in the management of acute proximal colon obstruction due to colon carcinoma before curative surgery.

METHODS: Eighty-one colon (proximal to spleen flex) carcinoma patients (47 males and 34 females, aged 18-94 years, mean = 66.2 years) treated between September 2004 and June 2010 for acute colon obstruction were enrolled to this study, and their clinical and radiological features were reviewed. After a cleaning enema was administered, urgent colonoscopy was performed. Subsequently, endoscopic decompression using SEMS placement was attempted.

RESULTS: Endoscopic decompression using SEMS placement was technically successful in 78 (96.3%) of 81 patients. Three patients' symptoms could not be relieved after SEMS placement and emergent operation was performed 1 d later. The site of obstruction was transverse colon in 18 patients, the hepatic flex in 42, and the ascending colon in 21. Following adequate cleansing of the colon, patients' abdominal girth was decreased from 88 ± 3 cm before drainage to 72 ± 6 cm 7 d later, and one-stage surgery after 8 ± 1 d (range, 7-10 d) was performed. No anastomotic leakage or postoperative stenosis occurred after operation.

CONCLUSION: SEMS placement is effective and safe in the management of acute proximal colon obstruction due to colon carcinoma, and is considered as a bridged method before curative surgery.

Key words: Endoscope; Proximal colon cancer; Obstruction; Self-expanding metallic stents; Drainage

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INTRODUCTION

At the time of diagnosis, 7%-29% colorectal cancer patients present with an emergent bowel obstruction, which necessitates emergency colectomy with an unprepared bowel (proximal colon cancer) or colostomy (left colon and rectum cancer)[1]. For acute obstruction of colon and rectum cancer, various methods have been reported for a single-stage operation to reduce the cost and to improve patient care, including intraoperative colonic lavage[2-4],

decompression with a metallic stent[5-7], and decompression with a transanal drainage tube[8-10]. But less than 4% of all reported cases shared their experiences in self-expanding metallic stents (SEMS) placement in the colon proximal to the splenic flexure. This is mainly because of the concern about the safety of SEMS in the proximal colon and because acute obstruction of the right colon has traditionally been managed by resection and primary anastomosis[11]. However, recent studies suggest that emergency right-sided colonic resections resulted in a significantly higher morbidity and mortality when compared with elective resections[12].

Endoscopic Center of Zhongshan Hospital is one of the largest centers in the world, which performed more than 15 000 colonoscopies each year. Since 2004, we have used metallic stent placement for 300 cases of acute colorectal obstruction. We retrospectively reviewed the outcomes of the patients with acute proximal colon obstruction treated with SEMS placement as a bridge to curative surgery.

MATERIALS AND METHODS

Patients

Eighty-one colon (proximal to spleen flex) carcinoma patients (47 males and 34 females, aged 18-94 years, mean, 66.2 years) treated between September 2004 and June 2010 for acute colon obstruction were enrolled to this study. The symptoms in these patients were abdominal pain, abdominal fullness, vomiting and constipation. Physical examination showed a distended and tympanic abdomen. Plain abdominal X-ray revealed a distended large bowel and an air-fluid level displaying an acute lower bowel obstruction. After the cleaning enema was administered, urgent colonoscopy was performed for the diagnosis and SEMS placement. Informed consent was obtained from each patient.

Procedure

SEMS used in the present study was 20 mm in diameter and 60 mm, 80 mm and 100 mm in length, depending on the length and caliber of the stricture. These stents have a unique one-step-through-the-scope delivery system (7.3 mm in outer diameter and 190 cm in length) that enables the stent to be passed through the 3.7 mm working channel of the colonoscope before deployment (Micro-Tech Co., Nanjing, China).

A colonoscope (CF260I; Olympus, Tokyo, Japan) was inserted and advanced to the site of the tumor. Combined with fluoroscopy, the site and etiology of acute bowel obstruction can be revealed. Under fluoroscopic and endoscopic guidance, a hydrophilic biliary guidewire (Jagwire, Boston Scientific, Natick, MA, USA) preloaded through a standard biliary catheter was then introduced through the tumor beyond the point of obstruction. After recognizing fluoroscopically the anatomically correct position of the guidewire passing into an air-filled, dilated proximal bowel, water-soluble contrast was injected proximally to the stricture to evaluate the length of the stricture, the degree and the anatomy of the obstruction, and whether a synchronous lesion existed. After the guidewire was positioned, suitable stents were inserted and placed under fluoroscopy. The immediate escape of air and liquid feces through the stents indicated successful decompression.

After stents placement

The patients were asked to take 150 mL paraffine orally to help colonic cleaning. A series of examinations, including chest X-ray, abdominal ultrasound or abdominal computed tomography scan, and blood tests for carcinoembryonic antigen, were performed. After the colon obstruction was relieved 7-10 d later, mechanical bowel preparation using polyethylene glycol or sodium phosphate and one-stage surgery was performed.

RESULTS

Endoscopic decompression by means of SEMS placement was technically successful in 78 (96.3%) of our 81 patients. Three patients' symptoms could not be relieved after SEMS placement and emergent operation was performed 1 d later.

Emergency colonoscopy for initial diagnosis was very useful in differentiating acute colorectal obstruction from obstruction of the small intestine as well as for evaluating the etiology of the obstruction. The obstruction occurred in the transverse colon of 18 patients, in the hepatic flex of 42, and the ascending colon of 21 patients.

All 78 successful patients showed marked improvement in abdominal symptoms shortly after the SEMS placement, and repeated abdominal X-ray showed a reduction of the colonic distention. Following adequate cleansing of the colon, patients' abdominal girth was decreased from 88 ± 3 cm before drainage to 72 ± 6 cm 7 d later (Table 1).

Following appropriate staging and adequate cleansing of the colon, 72 patients received one-stage surgery after 8 ± 1 d (range, 7-10 d), including 5 patients receiving synchronous liver metastasis resection (Figure 1) and 3 receiving synchronous partial duodenal resection. Six patients, who had lung and liver metastasis, avoided major surgeries and accepted SEMS placement as palliative treatment.

The morbidity was 3.8% (3/78), including one case of wound dehiscence and two cases of cardiac complications. No anastomotic leakage and stricture were found in these patients.

DISCUSSION

Acute large-bowel obstruction in primary colorectal carcinoma is an emergent onset and has a poor prognosis, which necessitates immediate surgical treatment[13,14]. The mortality rate of emergent surgery for patients with acute obstruction caused by colorectal carcinoma has

Table 1 Abdominal girth and drainage volume after self-expanding metallic stents placement

	0 d[1]	1 d	2 d	3 d	4 d	5 d	6 d	7 d
Abdominal girth (cm)	88 ± 3	86 ± 4	80 ± 4	78 ± 3	77 ± 5	73 ± 6	73 ± 5	72 ± 6

[1]0 d: The day of ileus tube placement; 1 d: One day after placement.

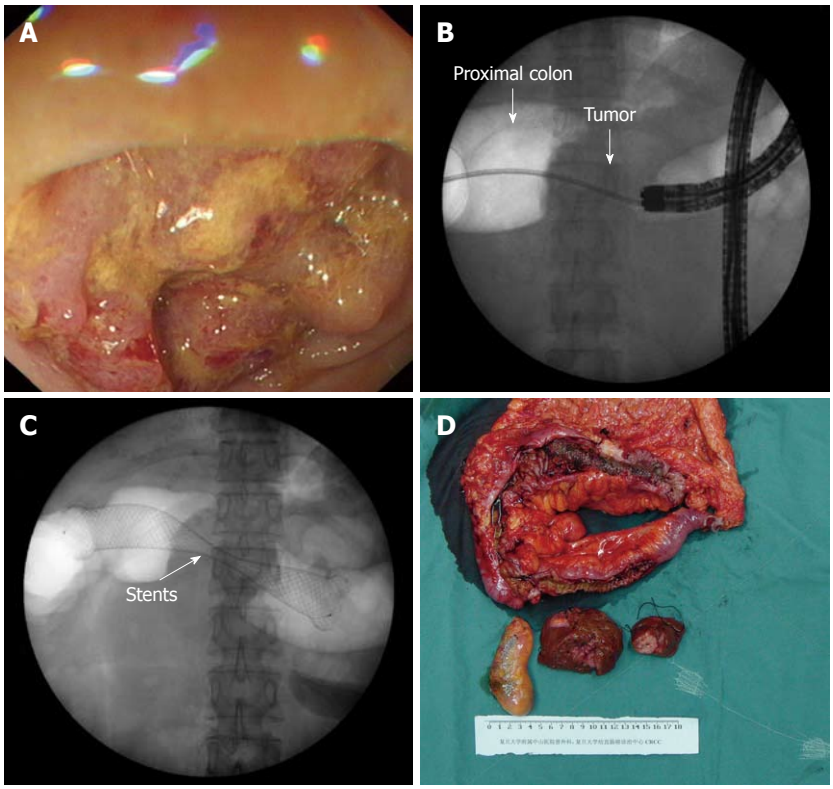

Figure 1 A typical transverse colon cancer patient with resectable liver metastasis. A: Emergent colonoscopy found a tumor in the middle of transverse colon blocked tunnel; B: The 0.052-inch guidewire is introduced through the tumor (arrow) beyond the point of obstruction; C: Self-expanding metallic stents is placed in the obstruction; D: Computed tomography found a liver metastasis in the right liver. After obstruction was relieved, the patients underwent radical transverse colectomy and partial right lobectomy.

been reported to be 17% compared with 7.7% for elective surgery[14]. The use of metallic stent and transanal decompression drainage tube has been reported to avoid emergent surgery, but the majority of reported cases of colonic stenting have involved the distal colon. This is mainly due to the concern about technical safety and different surgical approaches for the right-sided colon obstruction.

Due to the site of proximal colon obstruction, if the patients had a tortuous colon, it will be difficult to deploy the stent to the appropriate site. The literature, largely on distal colon stents, has shown that the technical success rate with colonic SEMS is typically higher than 90%[15-17]. Repici *et al*[11] reported a series of 21 proximal colon obstruction cases treated with SEMS placement. Twenty (95%) cases were technically successful and 85% cases had their symptoms relieved. No early complications (perforation, hemorrhage or deaths) occurred. Dronamraju *et al*[18] reported 16 cases of proximal colon obstruction, including 8 cases with lesions in the ascending colon and 8 with lesions in the transverse colon. The placement of SEMS was technically successful in 15 (94%) patients, which relieved the bowel obstruction (passing stool and flatus) in 14 (87.5%) patients. One patient had post-stent bleeding that was managed conservatively, and there were no perforations or procedure-related deaths, stent dislodgements, or reocclusions. Recently, a multicenter randomized control trial[19] comparing SEMS drainage and emergent surgery for colonic obstruction, ended earlier due to the high incidence of adverse events in SEMS group (6/11 cases, 54.5%). The limited experience of doctors (63 collaborators only finished 11 stents placement in 1 year) may be the reason why the result was different from our data.

Our data suggest that similar outcomes can be seen with proximally placed colon stents. SEMS was deployed successfully in all the patients, regardless of the site of obstruction: transverse colon, hepatic flex or the ascending colon. Three patients still had the obstruction symptoms and had emergent surgery 1 d after SEMS placement. As seen in the operation, the bowl function of the

three patients were destroyed (without any bowl movement) due to the obstruction. So for the patients after SEMS placement, if the symptoms cannot be relieved in 1 d, presence of bowl paralysis should be considered and an emergent surgery should be performed immediately.

In the cases with a tortuous colon, the additional twists and turns in the colonoscope itself sometimes need increased force on the stent delivery system before it actually begins to deploy despite the use of the large-channel colonoscope. In case the colonoscope was looped, manual reduction was sometimes required before the undeployed stent catheter could be fully advanced out of the colonoscope and across the stricture. A relatively straight endoscope was also associated with less resistance during stent deployment.

Surgically, right colonic obstruction is managed differently from the left colonic obstruction. Right sided lesions can be managed with a one-stage operation and ileocolonic anastomosis without the need for formal bowel preparation. However, some patients with right colonic obstruction are elderly persons and have some comorbidities which can increase the postoperative complications. Recent studies suggest that emergency right-sided colonic resections had a significantly higher morbidity and mortality compared with elective resections[12].

On the other hand, right colon cancer with acute obstruction can not be resected in the emergent surgery. For those patients, emergent operation does not benefit patients' survival, while SEMS placement can transfer the emergency situation to a selective state, permitting patients to receive a new adjuvant therapy before surgery. In our study, two patients underwent synchronous liver metastasis resection, two synchronous partial duodenal resection and one patient with lung and liver metastasis received chemotherapy and avoided a major surgery. These patients had a similar postoperative morbidity rate compared with those undergoing elective surgeries.

In conclusion, management of acute proximal colon obstruction due to colon cancer using SEMS placement is safe and effective, which can provide an opportunity for preoperative staging and/or new adjuvant therapy. It is a useful bridge to curative surgery, and should be applicated widely.

COMMENTS

Background

Acute large-bowel obstruction in primary colorectal carcinoma is an emergent onset and has a poor prognosis, which necessitates immediate surgical treatment. The mortality rate of emergent surgery for patients with acute obstruction caused by colorectal carcinoma has been reported to be 17% compared with 7.7% for elective surgery.

Research frontiers

Endoscopic self-expanding metallic stents (SEMS) drainage for proximal colon obstruction has been scarcely reported. The authors retrospectively reviewed the outcomes of the patients with acute proximal colon obstruction treated with SEMS placement as a bridge to curative surgery.

Innovations and breakthroughs

Endoscopic decompression using SEMS placement for proximal colon obstruction had a high successful rate (96.3%). Resectable liver metastasis could be treated after SEMS drainage.

Applications

SEMS placement is effective and safe in the management of acute proximal colon obstruction due to colon carcinoma, and is considered as a bridged method before curative surgery.

Terminology

SEMS drainage: Self-expanded metal stents used to treat acute colorectal obstruction.

Peer review

The authors have done a good job in describing their experiences about SEMS for proximal colon obstruction so that the readers can easily put the findings into the context of their own practice.

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BRIEF ARTICLE

Risk factors for predicting early variceal rebleeding after endoscopic variceal ligation

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Abstract

AIM: To analyze the clinical risk factors for early variceal rebleeding after endoscopic variceal ligation (EVL).

METHODS: 342 cirrhotic patients with esophageal varices who received elective EVL to prevent bleeding or rebleeding at our endoscopy center between January 2005 and July 2010. were included in this study. The early rebleeding cases after EVL were confirmed by clinical signs or endoscopy. A case-control study was performed comparing the patients presenting with early rebleeding with those without this complication.

RESULTS: The incidence of early rebleeding after EVL was 7.60%, and the morbidity of rebleeding was 26.9%. Stepwise multivariate logistic regression analysis showed that four variables were independent risk factors for early rebleeding: moderate to excessive ascites [odds ratio (OR) 62.83, 95% CI: 9.39-420.56, $P < 0.001$], the number of bands placed (OR 17.36, 95% CI: 4.00-75.34, $P < 0.001$), the extent of varices (OR 15.41, 95% CI: 2.84-83.52, $P = 0.002$) and prothrombin time (PT) > 18 s (OR 11.35, 95% CI: 1.93-66.70, $P = 0.007$).

CONCLUSION: The early rebleeding rate after EVL is mainly affected by the volume of ascites, number of rubber bands used to ligate, severity of varices and prolonged PT. Effective measures for prevention and treatment should be adopted before and after EVL.

Key words: Esophageal variceal bleeding; Endoscopic variceal ligation; Loop ligature; Early rebleeding; Risk factor

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INTRODUCTION

Acute esophageal variceal bleeding is a severe and vital complication threatening cirrhotic patients' lives. Variceal bleeding occurs at a yearly rate of 5%-15% in cirrhotic patients. The most important predictor of bleeding is the size of varices, with the highest risk of first bleeding (15% per year) occurring in patients with large varices[1]. Other predictors of bleeding are decompensated cirrhosis (Child-Pugh B/C) and the endoscopic presence of red wale marks[1]. Trials have demonstrated that endoscopic variceal ligation (EVL) is an effective method to prevent variceal bleeding[2]. However, early recurrent bleeding after EVL (rebleeding occurring between 24 h and 14 d after the operation) is also fatal[3], and is mainly due to early spontaneous slippage of rubber bands leaving the

unhealed ulcer[4]. Only a few studies have reported the possible predictive factors for rebleeding after EVL: previous upper variceal bleeding, peptic esophagitis, a high platelet ratio index score, coagulation function, and number of varices[3,4]. Until now, there has been no general consensus on the risk factors and measures to prevent early rebleeding. The aim of this study was to assess the risk factors for early variceal rebleeding after EVL.

MATERIALS AND METHODS

Patients

Enrolled in this study were 342 inpatients who, between January 2005 and July 2010, underwent EVL at our endoscopy center for treatment of variceal bleeding due to cirrhosis. Patients who had accepted injection sclerotherapy prior to the EVL procedure were excluded. There were 242 males and 100 females, and the average age was 52.7 ± 10.9 years (range: 24-79 years). Among these 342 patients, the cause of cirrhosis in 237 cases was viral hepatitis, alcoholism was the cause in 31 cases, and the remaining 48 were due to biliary disease, schistosomiasis infection, autoimmune hepatitis or an unknown pathogenesis.

The 342 patients were divided into rebleeding and non-rebleeding groups. Observations included baseline characteristics (general features, biochemical and ultrasonic data), endoscopic details and medications after EVL (Tables 1-3). The variables were retrospectively collected in a computer database and the medical records of the hospital.

Endoscopic procedure for EVL

The endoscopic procedures followed the guidelines established by the Chinese Endoscopy Institute in 2000[5]. Briefly, selected varices (above the cardia 2-3 cm) were visualized and aspirated into the banding chamber of the ligator. Suction was maintained until the screen became red, and then the band was deployed by rotating the handle clockwise until the band release was felt. The bands were then launched onto varices in ascending order through the esophagus. The devices used were either an Olympus XQ 240 or 260 endoscope, and a 6- or 10-shooter Saeed multi-band ligator (Wilson-Cook Medical).

Follow-up

Following EVL, standard doses of proton pump inhibitors (PPIs) were administered for 2 wk for most patients. Food intake was allowed 24 h after the procedure in cases of prophylactic EVL, and at the discretion of the physician in cases of emergent EVL for acute bleeding. Early rebleeding after EVL was defined as: (1) recurrent hematemesis, and/or melena, and/or bloody fluid drained by nasogastric tube, occurring between 24 h and 14 d after the operation; or (2) a decrease in hemoglobin by at least 20 g/L, or a transfusion of more than 2 units of concentrated RBC needed within 24 h, or hypovolemic shock occurs[3].

In all rebleeding patients, somatostatin (0.25 mg/h) and PPI infusion (omeprazole 40-80 mg/d) were given until active bleeding stopped. Twelve patients received an esophageal balloon tamponade. Endoscopic sclerotherapy injections were performed in 5 patients, and 1 patient had emergency devascularization surgery.

Statistical analysis

Continuous variables were stated as mean ± SD, and Student's *t*-test was used to assess the difference between those variables. Bivariate associations between categorical variables were analyzed by Pearson's χ^2 test or Fisher's exact test. Initially, each risk factor was examined independently, which produced the unadjusted odds ratio (OR) and 95% CI. We then selected the significant candidate variables (hemoglobin, bilirubin, prothrombin time (PT), albumin and portal vein diameter were dichotomized) identified by univariate analysis to undergo binary logistic regression analysis (forward stepwise) to determine the independent risk factors for rebleeding after EVL. Two-sided *P*-values < 0.05 were considered statistically significant. We used SPSS 17 software for all statistical analyses.

All data were managed anonymously. The local ethics committee confirmed that no ethical approval was needed for this study.

RESULTS

Outcome

Among the 342 patients treated with EVL, 26 patients (7.60%) developed early rebleeding. The rebleeding occurred with a mean delay of 8.0 ± 2.3 d (range: 3-13 d). Of these, 21 patients (80.8%) rebled between the 7th and 13th day after EVL, overwhelmingly more than those that rebled within the first 7 d. All of the rebleeding cases were caused by esophageal variceal bleeding, which were confirmed by endoscopy or clinical manifestations. Seven patients (26.9%) died despite positive rescue. We failed to find any benefit in the use of PPIs, somatostatin, β-blockers or sucralfate for the prevention of early rebleeding after EVL.

Baseline characteristics

The characteristics of both the rebleeding and non-rebleeding groups are presented in Table 1. The mean age of the patients who rebled was 57.5 ± 8.3 years, as compared with 52.3 ± 11.0 years for those who did not rebleed ($P = 0.02$). Patients who had had splenectomy or devascularization procedures prior to EVL were more likely to rebleed after EVL ($P < 0.01$). The rebleeding patients had worse Child-Pugh scores (class A, $n = 3.8\%$, class B, $n = 23.1\%$, class C, $n = 73.1\%$) compared with the controls (class A, $n = 39.6\%$, class B, $n = 54.7\%$, class C, $n = 5.7\%$, $P < 0.01$). For the indices scored in the Child-Pugh classification, encephalopathy, ascites, albumin, bilirubin and PT, all were significantly different between the two groups: the *P*-values were < 0.01,

Table 1 Univariate analysis for baseline characteristics

Variable	Non-rebleeding (n = 316)	Rebleeding (n = 26)	P-value	OR	95% CI
Male/female	227/89	15/11	0.13		
Age (yr)	52.3 ± 11.0	57.5 ± 8.3	0.02		
Etiology of cirrhosis					
Virus	237	21	0.60		
Alcohol	31	1			
Others	48	4			
Comorbidities					
Diabetes	35	5	0.21		
Liver cancer	19	2	0.67		
History of surgery					
Splenectomy or devascularization	100	16	< 0.01	3.08	1.38-6.88
Liver cancer surgery	8	0	1		
Liver transplantation	3	0	1		
Child-Pugh score					
A	125	1	< 0.01		
B	173	6			
C	18	19			
Encephalopathy	8	10	< 0.01	24.06	8.36-69.23
Blood loss before EVL (mL)	736 ± 418	1854 ± 657	< 0.01		
Ascites					
None/mild	285	6	< 0.01	26.18	10.25-66.82
Moderate/excessive	31	20			
Portal vein diameter (mm)	12.9 ± 2.1	15.9 ± 2.2	< 0.01		
Portal vein diameter ≥ 14 mm	113	24	< 0.01	21.56	5.00-92.89
Portal vein thrombosis	37	13	< 0.01	7.54	3.25-17.50
Hemoglobin (g/L)	97.0 ± 20.8	71.8 ± 13.2	< 0.01		
Hemoglobin < 90 g/L	112	24	< 0.01	21.86	5.07-94.19
Platelets (10^9/L)	121 ± 77	96 ± 99	0.118		
Albumin (g/L)	35.2 ± 5.6	29.2 ± 4.2	< 0.01		
Albumin < 28 g/L	32	8	< 0.01	3.94	1.59-9.79
ALT (U/L)	32.4 ± 22.2	36.0 ± 18.4	0.42		
AKP (U/L)	108.3 ± 200.1	142.8 ± 96.5	0.39		
Bilirubin (μmol/L)	21.4 ± 13.4	27.7 ± 14.9	0.02		
Bilirubin > 34 μmol/L	34	9	< 0.01	4.39	1.82-10.62
PT (s)	15.7 ± 2.4	20.1 ± 3.5	< 0.01		
PT > 18 s	43	20	< 0.01	19.07	7.21-45.30

EVL: Endoscopic variceal ligation; ALT: Alanine aminotransferase; AKP: Alkaline phosphatase; PT: Prothrombin time; OR: Odds ratio.

Table 2 Univariate analysis for endoscopic data

Variable	Non-rebleeding (n = 316)	Rebleeding (n = 26)	P-value	Odds ratio	95% CI
Esophageal varices grade					
Mild	11	0	< 0.01		
Moderate	179	0			
Severe	126	26			
Number of varies	3.2 ± 0.8	4.5 ± 0.6	< 0.01		
Extent of esophageal varies					
Middle and lower section	281	4	< 0.01	44.16	14.38-135.58
Whole	35	22			
Red sign	267	26	0.04		
Gastric varies	91	25	< 0.01	61.81	8.25-462.97
Portal hypertensive gastropathy	85	23	< 0.01	20.84	6.10-71.18
Number of rubber bands	5.1 ± 0.9	6.5 ± 0.5	< 0.01	22.00	6.46-74.96

< 0.01, < 0.01, 0.02 and < 0.01, respectively. Rebleeding was also associated with more blood loss before EVL (P < 0.01), increased portal vein diameter (P < 0.01), portal vein thrombosis (PVT, P < 0.01) and low hemoglobin (P < 0.01). Gender, etiology of cirrhosis, comorbidities, liver cancer surgery, liver transplantation, platelets, alanine aminotransferase and alkaline phosphatase were not significantly associated with early rebleeding.

Comparison of endoscopic data between the cases and controls

All the patients who rebled had varices classified as "se-

Table 3 Medication after endoscopic variceal ligation

Medication	Non-rebleeding (n = 316)	Rebleeding (n = 26)	P
Proton pump inhibitor	305	26	1
Somatostatin/octreotide	203	26	< 0.01
β-blocker	34	2	1
Sucralfate	176	20	0.04

Table 4 Multivariate analysis

Risk factor	P-value	OR	95% CI
Ascites (moderate to excessive)	< 0.001	62.83	9.39-420.56
Number of rubber bands	< 0.001	17.36	4.00-75.34
Extent of esophageal varies	0.002	15.41	2.84-83.52
Prothrombin time > 18 s	0.007	11.35	1.93-66.70

OR: Odds ratio.

vere", while only 40% of the controls did ($P < 0.01$). The percentage of patients with varices throughout the whole extent of the esophagus in the rebleeding group was 85%, which was nearly 8 times more than that of the controls ($P < 0.01$). The number of rubber bands placed in the rebleeding patients (6.5 ± 0.5) was greater than that of the controls (5.1 ± 0.9, $P < 0.01$). Significant differences between the two groups were also seen for number of varices ($P < 0.01$), gastric varices ($P < 0.01$), portal hypertensive gastropathy ($P < 0.01$) and red signs ($P = 0.04$).

Multivariate analysis

The significant candidate variables were selected for forward stepwise logistic regression analysis to find the independent risk factors for early rebleeding after EVL (Table 4). Four variables were identified: moderate to excessive ascites (OR 62.83, 95% CI: 9.39-420.56, $P < 0.001$), the number of bands placed (OR 17.36, 95% CI: 4.00-75.34, $P < 0.001$), the extent of varices (OR 15.41, 95% CI: 2.84-83.52, $P = 0.002$) and PT > 18 s (OR 11.35, 95% CI: 1.93-66.70, $P = 0.007$).

DISCUSSION

EVL is an effective method to prevent variceal bleeding primarily and secondarily. However, early recurrent bleeding as a vital complication after EVL has not been studied fully. There are only a few studies reporting the possible predictors for early rebleeding after EVL, and the sample sizes are usually too small[4,6]. The large sample size of our study enabled us to find the incidence, predilection time and risk factors for early rebleeding after EVL more credible. Furthermore, the emergency EVL is often supposed to be different from the elective one because of the different patient conditions and technical difficulty. We just focused on the rebleeding risk in prophylactic EVL operations rather than in emergency ones, not as the earlier study[4].

A prior study[7] reported that the rate of early rebleeding following EVL was between 9% and 19%, which is close to our result (7.6%). We also found that post-EVL bleeding was most likely to occur between the 7th and 13th day following the procedure. Vanbiervliet *et al*[4] reported that cases of severe bleeding after EVL were all caused by early slippage of the rubber bands, leaving the unhealed ulcus. Usually, the bands slip spontaneously within the second week after EVL, which can explain the timing of post-EVL rebleeding found in this study. On the basis of the above result, recommending a soft diet and avoiding strenuous exercise is helpful in preventing early slippage, an occurrence which can lead to life-threatening rebleeding.

In this study, we collected more expanded indices than former studies to evaluate patients with esophageal varices more comprehensively, which allowed us to draw convincing conclusions. For example, we took account of extent of varices, number of varices, portal vein diameter, PVT, history of related surgery and so on. As the result showed, there were significant differences between the cases and controls for many characteristics, such as age, surgery history, liver function, severity of varices, number of rubber bands, and so forth. But as demonstrated by the multivariate analysis, there were only four independent risk factors among these, namely moderate to excessive ascites, number of rubber bands placed, extent of varices and PT > 18 s. These four risk factors may therefore be more meaningful than the others for predicting the occurrence of early rebleeding following EVL.

Lee *et al*[8] believed that the more rubber bands that were used to ligate, the greater the possibility of rebleeding, because of the increasing ulcera. In our study, we also found that the number of rubber bands was an independent risk factor for bleeding after EVL. Therefore, for varices which were in the mild to moderate class, it may not be reasonable to launch many rubber bands. For severe varices, however, it's usually unavoidable to use more bands.

The prognosis does not only depend on the EVL procedure, but also relates to the severity of liver damage and bleeding. Yang *et al*[9] found that the Child-Pugh score for liver function was an independent risk factor of post-EVL rebleeding. Berreta *et al*[10] proved that Child-Pugh C was an independent risk factor of death from rebleeding. Our study showed that there was a difference in Child-Pugh score between the rebleeding and non-rebleeding groups. Furthermore, we revealed that ascites and PT, two of the indices for Child-Pugh classification, were independent risk factors for rebleeding after EVL, but the other three indices were not.

Ascites as an independent risk factor for early rebleeding after EVL was not reported in the study of Vanbiervliet *et al*[4]. However, they did not quantify the volume of ascites. We demonstrated that a moderate to excessive volume of ascites was the most dangerous factor predicting post-EVL bleeding (OR 62.83, 95% CI: 9.39-420.56). This may be explained by the elevated portal vein pressure that results from a larger volume of ascites. It was reported in

a previous study[11] that variceal bleeding recurred more in patients with higher basal portal vein pressure, and led to higher mortality. High portal vein pressure, therefore, is crucial for the recurrence of variceal bleeding.

Patients with decompensated cirrhosis often have coagulation disorders. One study[12] showed that an international normalized ratio > 2.3 was a predictor of death within the first 6 wk after patients were treated for their first variceal bleeding. The coagulation index as an independent predictive factor for rebleeding after EVL was reported in some previous studies[3,4], but not in another[13]. Our study showed that PT > 18 s was an independent risk factor of post-EVL bleeding (OR 11.35, 95% CI: 1.93-66.70). It is understandable that the ulcera caused by rubber bands can not heal well without normal coagulation. The prolongation of PT suggests a lack of coagulation factors Ⅰ, Ⅱ, Ⅶ or Ⅹ, or fibrinolysis acceleration. Therefore, for patients with quite prolonged PT, supplementing vitamin K1 and coagulation factors are necessary before EVL. Coagulation disorders in cirrhosis often accompany unusual thrombosis as well. There was a difference in PVT between the rebleeding and non-rebleeding groups, as stated in this study. Kayacetin *et al*[14] considered that slow blood flow in the portal vein was associated with liver damage. When liver function was poor, the blood flow through the portal vein slowed down, raising the likelihood of variceal rebleeding. Recent research reported that PVT without liver cirrhosis caused a low variceal bleeding rate[15], while the rate went up significantly once the cirrhosis presented[16]. Those findings suggest that the primary liver disease may be the dominant factor for variceal bleeding and the prognosis of cirrhosis patients with PVT depends on the severity of liver disease.

We found the other independent risk factor was the extent of varices, which also reflects the severity of varices. Varices that extend along the entire esophagus are much more dangerous than varices that are limited to the middle and lower part. On the other hand, a greater extent of varices often means that more rubber bands are needed, increasing the possibility of rebleeding.

When considering the healing of post-EVL ulcera, the use of PPIs has been reported useful in comparison with a placebo, but the effect on preventing bleeding was not conclusive[17]. In our study, almost every patient received a standard dose of PPIs for 2 wk after EVL, but there was no significant difference between the two groups. We also failed to find any benefit in the use of sucralfate for the prevention of bleeding related to post-banding ulcera. Somatostatin is helpful to reduce portal vein pressure, but it was usually only used for 3 d after EVL. We did not find that it had any preventative effect on rebleeding, which usually occurred 7-14 d after EVL. β-blocker is another useful drug to reduce portal vein pressure, and it can be taken for a long time. Disappointingly, we failed to see any benefit from it too. But the number of treated patients was very small and may not accurately reflect the facts.

A limitation of our study was that relatively few rebleeding cases occurred, which might affect the statistical analysis because of the unbalanced sample size ratio of case to control. It is expected that more samples will be collected from multiple centers in the future. Additionally, the rebleeding cases were not all confirmed by endoscopy (although clinical signs were frequently enough to confirm the source of bleeding in these cirrhosis patients), which precluded us from performing a more detailed analysis.

In conclusion, this large sample size case-control study revealed four risk factors for predicting early post-EVL rebleeding. Part of the result was accordance with some former studies, but the other part was not reported before, such as ascites and the extent of varices. So it provided doctors with some new warnings which should be paid attention to. Patients should be assessed thoroughly according to the risk factors (especially the independent ones) before EVL to minimize rebleeding. Patients with poor liver function, especially those with large ascites and coagulation disorders, should be treated positively before EVL. Improving coagulation function by supplementing vitamin K1 and coagulation factors, reducing ascites by diuretics and albumin are all expected to effectively decrease the rebleeding rate after EVL.

COMMENTS

Background

Acute esophageal variceal bleeding is a severe and vital complication threatening cirrhotic patients' lives. Trials have demonstrated that endoscopic variceal ligation (EVL) is an effective method to prevent esophageal variceal bleeding with fewer complications. However, as a rare complication, early recurrent bleeding after EVL is also fatal. There has been no general consensus on the risk factors of early rebleeding after EVL and measures to prevent this complication.

Research frontiers

Early rebleeding following EVL is mainly due to early spontaneous slippage of rubber bands leaving the unhealed ulcer. Only a few small sample studies have reported the possible predictive factors for the rebleeding. It may be related to not only the EVL procedure, but also the severity of varices and liver damage.

Innovations and breakthroughs

The large sample size of our study enabled us to find the incidence, predilection time and risk factors for early rebleeding after prophylactic EVL. We collected more expanded indices than former studies to elevate patients with esophageal varices more comprehensively, which allowed us to draw convincing conclusions. The four independent risk factors found in our study: moderate to excessive ascites, the number of bands placed, the extent of varices and prolonged prothrombin time, some of which were determined for the first time, are helpful for doctors to predict the risk after EVL.

Applications

The result has actual application values for increasing the safety of EVL. Those independent risk factors found in our study may help doctors to assess patients before EVL better and choose a more suitable time to perform the prophylactic procedure. Correcting as many risk factors as possible with effective treatment is expected to prevent the occurrence of early rebleeding after EVL.

Terminology

EVL is a method to treat esophageal varices endoscopically with fewer complications. EVL works by capturing all or part of a varix with a band ligator resulting in occlusion from thrombosis. The tissue then necroses and sloughs off in a few days to weeks, leaving a superficial mucosal ulceration, which rapidly heals.

Peer review

There are good merits of this study, mainly the very large sample size, which allows the researchers to draw strong conclusions.

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BRIEF ARTICLE

Hepatitis B virus and hepatocellular carcinoma at the miRNA level

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Abstract

AIM: To study Hepatitis B virus (HBV) infection and its association with hepatocellular carcinoma (HCC) at the miRNA level.

METHODS: Three cellular models were used to investigate miRNA expression changes during HBV infection: human HepG2 hepatoblastoma cell line as a model without HBV infection; HepG2 cell line transfected with a 1.3-fold full-length HBV genome as an acute infection model; and HepG2.2.15 cell line, which is derived from HepG2 and stably transfected with a complete HBV genome, as a chronic infection model. The miRNA levels were examined using microarray technology. To explore the relationship between HBV infection and HCC genesis at the miRNA level, we downloaded from national center for biotechnology information Gene Expression Omnibus an miRNA expression dataset derived from HCC patients, most of whom are HBV carriers. We compared the miRNA expression alterations during HBV infection with those in HCC patients, by analyzing miRNA expression change profiles statistically.

RESULTS: Seventy-seven and 48 miRNAs were differentially expressed during acute and chronic HBV infection, respectively. Among these miRNAs, 25 were in common, the intersection of which was significant under the hypergeometric test ($P = 1.3 \times 10^{-11}$). Fourteen miRNAs were observed to change coherently in the acute and chronic infections, with one upregulated and 13 downregulated. Eleven showed inverse changes during the two phases of infection; downregulated in the acute infection and upregulated in the chronic infection. The results imply that common and specific mechanisms exist at the miRNA level during acute and chronic HBV infection. Besides, comparative analysis of the miRNA expression changes during HBV infection with those in HCC indicates that, although miRNA expression changes during HBV infection are distinct from those in HCC patients ($P < 2.2 \times 10^{-16}$), they exhibited significant correlations ($P = 0.0229$ for acute infection; $P = 0.0084$ for chronic infection). Perturbation of miRNA expression during chronic HBV infection was closer to that in HCC patients than that during acute HBV infection. This observation implies the contribution of miRNAs to HCC genesis from HBV infection. According to their patterns of differential expression in acute and chronic HBV infection, as well as in HCC, miRNAs of potential research interest could be identified, such as miR-18a/miR-18b, miR-106a, miR-221 and miR-101. For instance, the gradient expression alteration of miR-221 in the above three phases, which is downregulated in acute HBV infection, normally expressed in chronic HBV infection, and upregulated in HCC, indicates that it may be a key effector for progression of the disease.

CONCLUSION: Our analysis provides insights into HBV infection and related HCC in relation to miRNAs, and reveals some candidate miRNAs for future studies.

Key words: Hepatitis B virus; Hepatocellular carcinoma; MiRNA

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INTRODUCTION

Hepatitis B virus (HBV) is a hepatotropic non-cytopathic DNA virus that belongs to the *Hepadnaviridae* family. It is a major cause of acute and chronic infections of the liver and can lead to hepatitis, cirrhosis and hepatocellular carcinoma (HCC)[1]. Many efforts have been made to investigate the liver diseases caused by HBV. However, attempts at treatment of chronic infections have had only limited success[2,3]. Due to the exclusive dependence of HBV on the host cellular machinery for its propagation and survival, investigation of the interactions between HBV and the host cell is crucial for the understanding of viral pathogenesis and development of novel antiviral therapies. Although the gene regulatory mechanisms involving host and viral proteins have been extensively explored, studies on miRNA-mediated regulation in viral infections are just emerging

As a class of small RNA molecules ~22 nucleotides in length, miRNAs have recently gained widespread attention as crucial regulators in complex gene regulatory networks[4-6]. miRNAs regulate gene expression by base-paring with the 3' untranslated region (3' UTR) of target mRNAs, which leads to mRNA degradation or translational silencing. Recent reports on the interactions between the host and the pathogen in viral infection through miRNAs shed light on the role of miRNAs as crucial effectors in the intricate host-pathogen interaction networks. Lecellier *et al*[7]. demonstrated for the first time that a mammalian miRNA, mir-32, restricts accumulation of the retrovirus primate foamy virus type 1 (PFV-1) in human cells Hariharan *et al*[8] have predicted that five human-encoded miRNAs potentially target the entire repertoire of accessory genes in HIV. Jopling *et al*[9] have reported an interesting case in which a liver-specific miRNA, mir-122, causes viral RNA accumulation of hepatitis C virus (HCV), by binding to the 5' noncoding region of the viral genome. Pedersen and colleagues have shown that the antiviral effects of interferon (IFN)-β against HCV can be at least partially explained by miRNA-mediated modulation, and IFN-β treatment leads to a significant reduction in miR-122 expression, and induces miRNAs with sequence-predicted targets within the HCV genomic RNA[10]. To counteract the small-RNA-mediated interference, viruses have evolved to express suppressors that interfere with miRNA and siRNA pathways[7,11]. Several studies have shown that viruses also encode miRNAs, which can modulate cellular processes as well as regulate themselves[12-14]. Computational analysis has indicated the miRNA-encoding potential of HBV[15].

Previous works have pointed to the possibility of cross-talk between HBV and its host at the miRNA level. In the present study, we investigated human miRNAs that may be involved in acute and chronic HBV infection, via microarray profiling. We found that a significant number of differentially expressed miRNAs during acute and chronic infection overlapped, which were either coherently or inversely changed in the two phases of infection. This indicates that the two processes are both associated and different at the miRNA level. In addition, we explored the relationship between HBV infection and HCC genesis, by integrating our HBV infection dataset with a public miRNA expression dataset derived from a group of HCC patients, most of whom were HBV positive[16]. Our analysis demonstrated that perturbations of miRNA expression during HBV infection were significantly correlated with those in HCC, although they seemed distinct. Compared with acute HBV infection, chronic infection showed more consistent miRNA expression alterations with respect to HCC. In spite of this, there is a long way to go from the miRNA expression states during HBV infection to those that occur in HCC. The results implie that interference therapy at the miRNA level may provide a strategy to control the progression of serious liver diseases in HBV carriers.

MATERIALS AND METHODS

Cell models

Three cell models were used in this study: human HepG2 hepatoblastoma cell line as a model without virus infection; HepG2 cell line transfected with a 1.3-fold full-length HBV genome as an acute infection model; and HepG2.2.15 cell line, which is derived from HepG2 and stably transfected with a complete HBV genome, as a chronic infection model.

Microarray experiments

MiRNA expression profiles were determined at CapitalBio Corp (Beijing, China; www.capitalbio.com) by using mammalian miRNA arrays (version 3.0) which were designed based on the miRBase release 10.0 and contained 924 probes from humans, mice and rats. The arrays were scanned using a LuxScan™ laser confocal scanner and the images obtained were analyzed using LuxScan 3.0™ image analysis package. The raw miRNA expression data were quantile-normalized to correct for between-sample variations. A miRNA was determined as differentially expressed if its expression change was more than 1.5-fold, and it was

Table 1 MiRNAs differentially expressed during acute and chronic hepatitis B virus infection

	Acute infection	Chronic infection
Up	MiR-129-3p, miR-133a, miR-196b, miR-223, miR-296-5p, miR-302c, miR-361-3p, miR-365, miR-372, miR-409-3p, miR-518b, miR-562, miR-564, miR-574-3p, miR-612, miR-638, miR-634, miR-659, miR-663, miR-665, miR-940	Let-7a, let-7b, let-7d, let-7g, let-7i, miR-23a, miR-25, miR-27a, miR-27b, miR-29a, miR-29b, miR-103, miR-146a, miR-146b-5p, miR-181a, miR-181b, miR-181c, miR-181d, miR-182, miR-196b, miR-222, miR-331-3p, miR-499-3p, miR-499-5p, miR-501-3p, miR-660, miR-888
Down	Let-7a, let-7g, miR-15a, miR-15b, miR-16, miR-17, miR-18a, miR-19a, miR-19b, miR-20a, miR-20b, miR-23a, miR-25, miR-26a, miR-26b, miR-27a, miR-27b, miR-29b, miR-29c, miR-30a, miR-30b, miR-30c, miR-30e, miR-32, miR-34a, miR-92b, miR-96, miR-101, miR-103, miR-106a, miR-107, miR-122, miR-128a, miR-129-5p, miR-130a, miR-140-5p, miR-141, miR-146a, miR-146b-5p, miR-148a, miR-181a, miR-186, miR-192, miR-199a-5p, miR-200a, miR-200b, miR-215, miR-217, miR-221, miR-224, miR-301a, miR-338-3p, miR-374b, miR-454, miR-611, miR-923	MiR-17, miR-18a, miR-18b, miR-19a, miR-19b, miR-20a, miR-20b, miR-26b, miR-92a, miR-92b, miR-101, miR-106a, miR-130a, miR-143, miR-148a, miR-193b, miR-199b-5p, miR-325, miR-338-3p, miR-378, miR-483-5p

identified as significantly changed using the Significance Analysis of Microarrays method with FDR < 0.05.

miRNA expression profiles of HCC

We downloaded the miRNA expression profiles of 78 HCC patients from Gene Expression Omnibus (GEO, GSE10694)[16]. For each patient, the expression data for the liver cancer tissue and the corresponding non-cancerous tissue were available. The microarray analysis was performed using a customized miRNA array produced by CapitalBio Corp, which was designed based on miRBase release 7.0 and contained 435 probes for human mature miRNAs, including 122 predicted ones. The data were normalized, and differentially expressed miRNAs identified using the same criteria as described above.

Comparison of miRNA expression changes in HBV infection and HCC

We generated miRNA expression change profiles (miECPs) for acute and chronic HBV infection by comparing the mean miRNA expression levels in the corresponding conditions to those in the uninfected model. Similarly, for the HCC patients, miECPs were obtained by dividing the miRNA expression levels in the patients' non-cancerous tissues into those in the matched liver cancer tissues. Hierarchical clustering of the miECPs was performed to give a qualitative evaluation of the relationship between HBV infection and HCC. The significance of the observed deviations of HBV infection from HCC was determined by comparing the distributions of Pearson correlation coefficients (PCCs) between the miECPs of HBV infection and those of HCC, with the distribution of PCCs in HCC using the Kolmogorov-Smirnov test.

To investigate the association of miRNA expression alterations between HBV infection and HCC, we tested the null hypothesis that the correlation between the HBV infection miECPs and those for HCC was as weak as that between the random miECPs and HCC, which meant that the changes in miRNA expression during HBV infection were irrelevant to those observed in HCC. We first generated 10000 random miECPs by sampling from the elements of the real miECPs. We then computed, for each random miECP, the median of its PCCs with the HCC miECPs. By pooling together the PCC medians of all the random miECPs, we constructed the null distribution for statistical analysis. The correlations between HBV infection miECPs and those for HCC, which were represented by their PCC medians, were evaluated by computing a *P* value, determined as the number of times random PCC medians were larger (one-tailed test for a positive PCC median) or smaller (one-tailed test for a negative PCC median) than the observed one.

RESULTS

Differential expression of miRNAs during HBV infection

We determined the global miRNA expression profiles elicited in the uninfected, acute infection and chronic infection models using CapitalBio mammalian miRNA arrays. Among the 570 human mature miRNAs investigated by the arrays, 77 and 48 were differentially expressed during acute and chronic infection, respectively, with respect to the uninfected model (Table 1). The former consisted of 21 upregulated and 56 downregulated miRNAs, and the latter contained 27 upregulated and 21 downregulated miRNAs. Among these differentially expressed miRNAs, 25 were in common between acute and chronic infection (Table 2); the intersection of which was significant by the hypergeometric test ($P = 1.3 \times 10^{-11}$). Fourteen of these miRNAs were observed to change coherently in acute and chronic infection, with one upregulated and 13 downregulated. Eleven were downregulated in acute infection, but inversely changed in chronic infection.

Relationship between HBV infection and HCC at the miRNA level

The close pathological associations between HBV infection and HCC genesis have been demonstrated by previous studies. It is of great interest to investigate the relationship between the two at the miRNA level. Recently, Li *et al*[16] performed an miRNA expression profiling analysis on a group of 78 HCC patients, 62 of whom were HBV carriers. This dataset provided us an opportunity to explore the problem of whether HBV infection exhibits similar miRNA expression changes as those observed in HCC. We identified 217 common miRNAs between our HBV infection dataset and that of Li *et al*. To summarize

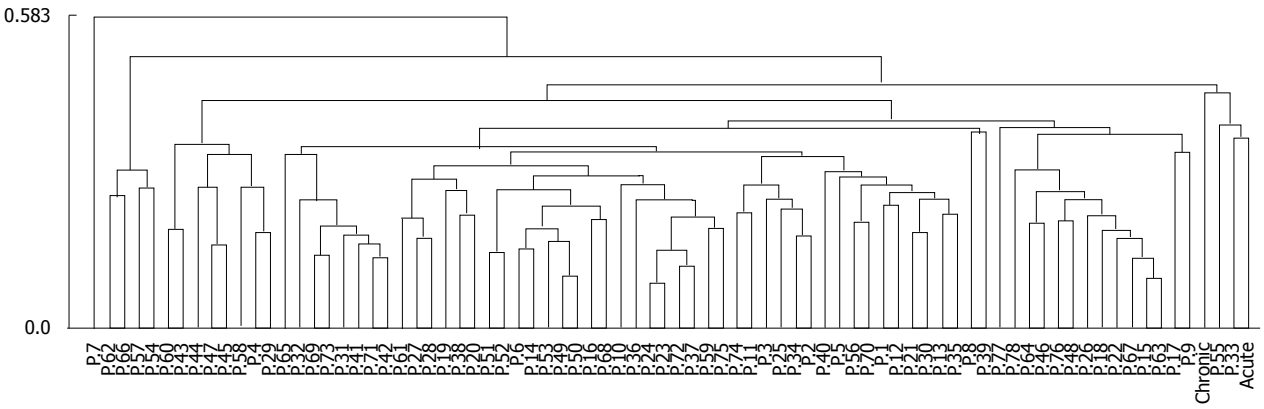

Figure 1 Clustering of miRNA expression change profiles in acute and chronic hepatitis B virus infection, as well as hepatocellular carcinoma (linkage type: Average; similarity measurement: Pearson correlation coefficient). The miRNA expression change profiles (miECPs) of the acute and chronic infections are distant from the majority of the hepatocellular carcinoma (HCC) miECPs, indicating that the alterations of miRNA expression during hepatitis B virus (HBV) infection are different from those of HCC. Despite this, they are statistically correlated (see the main text).

Table 2 Common miRNAs differentially expressed during acute and chronic infection

	Acute	
	Up	**Down**
Chronic		
Up	MiR-196b	Let-7a, let-7g, miR-23a, miR-25, miR-27a, miR-27b, miR-29b, miR-103, miR-146a, miR-146b-5p, miR-181a
Down	-	MiR-17, miR-18a, miR-19a, miR-19b, miR-20a, miR-20b, miR-26b, miR-92b, miR-101, miR-106a, miR-130a, miR-148a, miR-338-3p

the expression changes of these miRNAs in HBV infection, we generated miECPs by taking ratios of the mean miRNA expression levels of acute and chronic infections, with respect to those of the uninfected model. Similarly, miECPs for HCC were generated by comparing the miRNA expression levels in liver cancer tissues against those in matched non-cancerous tissues. Hierarchical clustering of the miECPs indicated that alterations of miRNA expression were different for HBV infection and HCC, and more consistent alterations were observed for HCC (Figures 1). Although the visualization showed the separation of HBV infection from most HCC, however, several patients showed a more extreme distribution. Therefore, we asked whether the observed differences between HBV infection and HCC were significant.

Statistically, it was equivalent to the question of whether the miECPs of HBV infection were from the population represented by the HCC miECPs. To investigate this problem, we used a PCC to quantify the similarity between a pair of miECPs, and tested whether the distribution of PCCs between the miECPs of acute/chronic HBV infection and those from HCC were different from that between the HCC miECPs themselves. It was found that these two kinds of PCC distributions had a significant difference (Kolmogorov-Smirnov test, $P < 2.2 \times 10^{-16}$), and the latter had a significantly high median PCC of 0.26 compared with -0.07 (acute infection) and 0.09 (chronic infection) of the former. This demonstrates that the miRNA expression changes during HBV infection are distinct from those in HCC.

A PCC of 0 indicates linear independence, therefore, we were curious as to whether the observed small PCCs suggested that the miRNA expression alterations between HBV infection and HCC were unrelated. We reasoned that if the extent of correlation between the HBV infection miECPs and those of HCC were indistinguishable from that between the HCC miECPs and random ones, which are obviously irrelevant, they should be deemed as unrelated. The analysis demonstrated that the miECPs of acute HBV infection exhibited a significant negative correlation with those of HCC, with respect to the irrelevant random background ($P = 0.0229$); whereas the miECPs of chronic HBV infection showed a significant positive correlation ($P = 0.0084$). These results indicate that, although subtle, the miRNA expression changes during HBV infection were correlated with those observed in HCC. Compared to acute HBV infection, chronic infection was closer to HCC at the miRNA level

Using the same criteria as for the analysis of the HBV infection dataset, we identified from the HCC dataset 46 differentially expressed miRNAs in liver cancer tissues, with respect to the corresponding non-cancerous tissues. The intersection of differentially expressed miRNAs between HBV infection and HCC is summarized in Table 3. Eight of the 10 differentially expressed miRNAs common to the acute HBV infection and HCC datasets were inversely changed, whereas only three of the eight differentially expressed miRNAs common to the chronic HBV infection and HCC datasets exhibited opposite alterations. The observation also indicates that chronic HBV infection has closer relationship with HCC than acute infection does. According to their patterns of differential expression in acute and chronic HBV infection, as well as in HCC, miRNAs with potential research interest could be identified.

DISCUSSION

Our microarray profiling analysis of miRNAs during HBV

Table 3 Common miRNAs differentially expressed in hepatitis B virus infection and hepatocellular carcinoma

	Acute		Chronic	
	Up	Down	Up	Down
HCC				
	-	MiR-15b	MiR-25	MiR-18a
		MiR-18a	MiR-103	MiR-18b
		MiR-25	MiR-182	MiR-106a
Up		MiR-103	MiR-222	
		MiR-106a		
		MiR-107		
		MiR-221		
		MiR-224		
Down	-	MiR-101 MiR-29c	-	MiR-101

HCC: Hepatocellular carcinoma.

infection showed that a significant number of miRNAs differentially expressed during acute and chronic infection were overlapped, which were either coherently or inversely changed in the two phases of infection. These observations indicate that common and phase-specific mechanisms for acute and chronic infection may exist at the miRNA level. To examine the reliability of our expression dataset, we compared our results with a recent study performed by Liu[17] *et al*, who investigated the miRNA expression alterations in the HepG2.2.15 cell line with respect to HepG2. They used an early version of CapitalBio mammalian miRNA arrays, which contains probes for 435 human mature miRNAs, a subset of the miRNAs present on our arrays. Similar to our analysis, Liu et al used SAM and fold changes to determine differentially expressed miRNAs. However, they required a larger extent of expression alteration of at least three fold, compared with our 1.5. Under their criteria, eleven and seven miRNAs present on the arrays were identified as upregulated and downregulated, respectively. Between our chronic infection data and theirs, seven upregulated (miR-23a, miR-146a, miR-181a, miR-181b, miR-181c, miR-181d and miR-196b) and three downregulated (miR-17, miR-338-3p and miR-378) miRNAs were in common. The intersection was significant by the hypergeometric test ($P = 4.45 \times 10^{-7}$ for the upregulated case; $P = 3 \times 10^{-3}$ for the downregulated case), which demonstrated the reproducibility of our miRNA profiling experiments.

By integrating a miRNA expression dataset of HCC patients[16], most of whom were HBV positive, we were able to investigate the relationship between HBV infection and HCC at the miRNA level, and also had an opportunity to explore miRNA expression changes with the progression of disease, namely from acute HBV infection to chronic infection and at last to HCC status. The statistical analysis of miRNA expression change profiles suggested that the perturbation of miRNA expression during HBV infection is different from that in HCC, however, they are correlated. The pattern of miRNA expression during different phases of disease offers a means to discover miRNAs that might be of great importance for further research. It could be conjectured that miRNAs that exhibit opposite alterations between HBV infection and HCC may be of importance for the entry into a serious disease state, for instance, miR-18a/miR-18b and miR-106a[18], which have already been reported to be involved in HCC. Those showing a gradient alteration may be key effectors for the progression of disease. For example, miR-221, which is downregulated in acute HBV infection, and normally expressed in chronic HBV infection and upregulated in HCC, has been shown recently to contribute to liver tumorigenesis[19]. Also, miRNAs showing coherent alteration patterns among the disease states may be related to the establishment and maintenance of these disease states. For instance, aberrant expression of miR-101, which remains downregulated in acute and chronic HBV infection, as well as in HCC, is closely associated with HCC development[20,21].

In summary, the correlation between HBV infection and HCC can be identified at the miRNA level. Compared with acute HBV infection, miRNA expression changes during chronic HBV infection are closer to those in HCC. However, there is a long way to go from miRNA expression states of HBV infection to those of HCC, which may provide us with opportunities to control the progression of serious liver diseases via interference therapy at the miRNA level.

COMMENTS

Background

Hepatitis B virus (HBV) is a major cause of liver infection and severe liver diseases. The survival and propagation of HBV exclusively depends on the host cellular machinery, therefore, investigation of HBV-host interactions is crucial for understanding viral pathogenesis and development of antiviral therapies.

Research frontiers

Although the gene regulatory mechanisms involving host and viral proteins have been extensively explored, studies on miRNA-mediated regulation in viral infections are just emerging. Recent works on the pathogen-host interactions through miRNAs point to the possibility of crosstalk between HBV and the host at the miRNA level.

Innovations and breakthroughs

The present study indicates that acute and chronic HBV infections are both associated and different at the miRNA level. In addition, the perturbations of miRNA expression during HBV infection are significantly correlated with those in hepatocellular carcinoma (HCC), although they seem distinct. In spite of this, there is a long way to go from miRNA expression states during HBV infection to those that occur in HCC.

Applications

The study implies that interference therapy at the miRNA level may provide a strategy to control the progression of serious liver diseases in HBV carriers and reveals some candidate miRNAs for future studies.

Terminology

miRNAs are a class of small RNA molecules, ~22 nucleotides in length, which regulate gene expression by base-paring with the 3' untranslated region of target mRNAs, leading to mRNA degradation or translational silencing. miRNAs have recently gained widespread attention as crucial regulators in complex gene regulatory networks.

Peer review

This study illustrated that the miRNA level is correlated in HBV infection and HCC. miRNA expression levels during HBV acute and chronic infections were investigated in cell lines, and the clustering between their profiles and those in HCC showed some insights into associations between HBV infection and HCC.

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BRIEF ARTICLE

Laparoscopic fenestration *vs* open fenestration in patients with congenital hepatic cysts: A meta-analysis

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Abstract

AIM: To determine whether the outcomes of laparoscopic fenestration (LF) were superior to open fenestration (OF) for congenital liver cysts.

METHODS: Comparative studies published between January 1991 and May 2010 on Medline (Ovid), Emsco, PubMed, Science Direct; Cochrane Reviews; CNKI; Chinese Biomedical Database, VIP and other electronic databases were searched. Randomized controlled trials (RCTs) and retrospective case-control studies on the management of congenital hepatic cysts were collected according to the pre-determined eligibility criteria to establish a literature database. Retrieval was ended in May 2010. Meta-analysis was performed using RevMan 5.0 software (Cochrane library).

RESULTS: Nine retrospective case-control studies involving 657 patients, comparing LF with OF were included for the final pooled analysis. The meta-analysis results showed less operative time [mean difference (MD): -28.76, 95% CI: -31.03 to 26.49, $P < 0.00001$]; shorter hospital stay (MD: -3.35, 95% CI: -4.46 to -2.24, $P < 0.00001$); less intraoperative blood loss (MD: -40.18, 95% CI: -52.54 to -27.82, $P < 0.00001$); earlier return to regular diet (MD: -29.19, 95% CI: -30.65 to -27.72, $P < 0.00001$) and activities after operation (MD: -21.85, 95% CI: -31.18 to -12.51, $P < 0.0001$) in LF group; there was no significant difference between the two groups in postoperative complications (odds ratio: 0.99, 95% CI: 0.41 to 2.38, $P = 0.98$) and cysts recurrence rates.

CONCLUSION: The short-term outcomes of LF for patients with congenital hepatic cysts were superior to open approach, but its long-term outcomes should be verified by further RCTs and extended follow-up.

Key words: Congenital hepatic cysts; Laparoscopic fenestration; Open fenestration; Systematic review; Meta-analysis

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INTRODUCTION

Hepatic cysts can be divided into two general categories: congenital and acquired. The congenital hepatic cyst has an identifiable epithelium on histological examination, while acquired liver cysts generally arise from post -trau-

matic hematoma and spontaneous intrahepatic infarction or infection. In the past, hepatic cysts were often discovered at laparotomy, but at present it can be recognized due to increased use of imaging modalities and radiographic studies such as ultrasonography, computer tomography and magnetic resonance imaging.

Hepatic cysts were a more common benign liver disease, many are asymptomatic without any particular intervention, only about 16% of cysts are symptomatic[1]. The symptoms caused by a hepatic cyst are related to the size and location of the cyst. Abdominal pain and abdominal distension are the most frequent complaints and are present in more than 50% of the patients[2]. Other less common complaints include nausea, vomiting, fatigue, and jaundice. Only those with symptoms or infection require surgical treatment.

Before the advent of laparoscopy, open fenestration (OF) was regarded as the most popular method for symptomatic liver cysts. However, with the development of minimally invasive surgery and increasingly wide acceptance for laparoscopy approach in abdominal diseases, the laparoscopic fenestration (LF) has become one of the main management for hepatic cyst[3-5]. Although the use of laparoscopic approach produced some uncertain factors, such as the influence on the cardiorespiratory function caused by the increased abdominal pressure, which increased the incidence of hypertension, arrhythmia, cardiac arrest, pulmonary edema, mediastinal emphysema, pneumothorax and so on[6,7]. Cabon diaoxide gas used for this procedure is hazardous for pulmonary-compromised patients. However, successful practice and satisfactory outcome have been reported about the advantages of laparoscopy[8-10], although these reports were about the experiences from a single center. Therefore, we conducted a systematic review and comprehensive analysis of the relevant literatures about the treatment of hepatic cysts to evaluate the outcomes of patients undergoing LF *vs* OF.

MATERIALS AND METHODS

Type of studies

A search of randomized controlled trials (RCTs) or retrospective case-control studies that compared laparoscopic with OF for patients with congenital hepatic cysts was performed, no matter with or without blinding and concealment of allocation. Retrospective case-control studies and case-reports were included besides RCTs. The number of patients included in all the studies was $\geqslant$ 15. All articles should be published in English or Chinese.

Eligibility criteria

Eligibility criteria for all included studies were: (1) explicitly reporting the indications for LF and OF; (2) reporting at least one of the measured outcomes mentioned as follows; (3) comparing the outcomes of LF and OF in patients with congenital hepatic cysts; (4) when two studies were published by the same institution or authors, either one of the higher quality or the most recent article was included; and (5) patients with parasitic liver cysts, liver cancer and intrahepatic bile duct dilation cysts and other cystic diseases were excluded.

Exclusion criteria

Studies were excluded from the analysis if (1) it was impossible to extract the appropriate data from the published articles; (2) there was considerable overlap between authors, institutes, or patients in the published literatures; (3) the measured outcomes were not clearly presented in the literatures; and (4) the laparoscopic and open surgery were performed for other diseases which were companied with liver cysts.

Outcome evaluation

The following outcomes were used to compare LF and OF: (1) operative time; (2) hospital stay; (3) intraoperative blood loss; (4) the time to return to normal diet; (5) the time to return to activities; (6) the incidence of postoperative complications; (7) hospitalization cost; (8) the recurrence rate of hepatic cysts; (9) the time to return to normal liver functions; and (10) the recurrence rate of symptoms.

Literature search strategies and selection

Both an internet based search and a manual search were used to acquire relevant studies. First, eight electronic databases (Medline; Emsco; PubMed; Science Direct; Cochrane Reviews; Chinese Biomedical Database; CNKI; and VIP) were searched and articles published between January 1991 and May 2010 were collected. The following Mesh search headings were used: "laparoscopy", "open fenestration", "congenital hepatic cysts", "comparative study" and "systematic review", and their combinations or similar headings were also searched such as "laparoscopic fenestration", "minimally invasive surgery", and "Meta-analysis". Second, further articles were identified by a manual search of reference lists from retrieved publications. The databases were used again to retrieve the abstracts, and if favorable, the full-text was downloaded for the final review.

Study eligibility assessment

Two reviewers independently screened the title and abstract of each publication for this study. Citations with suspected compliance with our eligibility criteria underwent a full review. If either of the two reviewers identified a citation to be potentially relevant, we obtained the full-text article for a full review. The two reviewers independently determined the eligibility of all included publications for a full text evaluation in the screening process, and disagreements were resolved through discussions by the two reviewers, and when this did not resolve the differences, a third person made a final decision on the eligibility of the study.

Data extraction

The following descriptive data from all eligible studies

Table 1 General characteristics of the included studies

Included studies	Design types	Patients		Age (yr)		Gender (M/F)		Site (left/right/bilobar)		Cyst size (cm)		Cyst types (single/multiple/polycystic)		Main complaints	Measured outcomes
		OF	LF	OF	LF	OF	LF	OF	LF	OF	LF	OF	LF		
Yi *et al*[11]	N-RCT	117	52	45 ± 12.8	43 ± 9.2	43/74	21/32	21/58/ND	16/22/0	9.5 ± 3.4	8.9 ± 4.6	79/38/37	38/14/11	A, B, C, D	1-4, 6, 9
Felizardo *et al*[12]	N-RCT	34	37	52.6 ± 21.8	51.1 ± 20.3	13/21	17/20	05/17/12	04/16/17	12.8 ± 6.3	114 ± 6.7	20/14/ND	19/18/ND	A, B, C, D	1-3, 4, 8
Chen *et al*[13]	N-RCT	19	17	44.2	41.1	23/13		ND	ND	ND	ND	ND	ND	A, B, C, D	1, 2, 3, 7
Qiu *et al*[14]	N-RCT	29	22	ND	ND	22/29		ND	ND	ND	ND	ND	ND	A, B, C, D	1-5, 7
Li *et al*[15]	N-RCT	40	46	18-83 (45)		ND		ND	ND	ND	ND	ND	ND	A, B, C, D	3, 4, 5
Guo *et al*[16]	N-RCT	27	31	42 ± 11.2	45 ± 9.8	09/18	12/19	08/11/ND	09/13/ND	8.4 ± 4.7	9.9 ± 5.8	19/18/21	09/09/09	A, B, C	1-3, 6, 7
Mazza *et al*[17]	N-RCT	37	66	19-87 (62.5)		ND		ND	ND	ND	ND	24/13/ND	46/20/ND	A, B, C, D	3, 4, 5
Gigot *et al*[18]	N-RCT	5	19	45	57	05/0	18/01	01/04/0	12/05/02	7-17 (10)	8-30 (13)	03/02/0	10/06/03	A, B, C, D	3, 4
Treckmann *et al*[19]	N-RCT	17	42	28-86 (62)		07/52		05/08/04	25/09/08	6-20 (11.2)	6-18 (10.8)	11/03/ND	38/04/ND	A, B, C, D	1, 4, 5

OF: Open fenestration group; LF: Laparoscopic fenestration group; A: Abdominal pain; B: Abdominal distension; C: Nnausea; D: Vomiting; ND: Not mentioned; RCT: Randomized controlled trial; 1: Operative time; 2: Intraoperative blood loss; 3: Hospital stay; 4: Incidence of postoperative complications; 5: The time to return to normal diet; 6: The time to return to normal activities; 7: Hospitalization cost; 8: The recovery time of liver functions; 9: The recurrence rates of symptoms.

were abstracted by two reviewers independently: authors, methodology, study period, interventions used, participant characteristics, and the measured outcomes. And disagreements were resolved using the same consensus process mentioned above. We also contacted the authors of all eligible studies if there were missing data or inaccurate information.

Data analysis and statistical processing

Meta-analysis was performed in line with recommendations from the Cochrane Collaboration. Heterogeneity was assessed at first using a random-effect model, $P < 0.10$ as statistically significant heterogeneity. Statistical analysis of continuous variables was carried out using mean difference (MD) as the summary statistics by the Inverse-Variance method, while dichotomous variables were analyzed using odds ratio (OR) by the Mantel-Haenszel method, and both were reported with 95% CI. The MD and OR were considered to be statistically significant at $P < 0.05$ if the 95% CI did not include the value "1". OR was defined as the odds of an adverse event occurring in the LF group compared with the OF group, while MDs represent the differences between the two groups in the continuous variables.

RESULTS

Literature search results and their general characteristics

According to the literature searching strategies, 9 case-control studies, involving a total of 657 cases that compared the outcomes of LF with OF in patients with congenital liver cyst, were identified for pooled analysis, including six studies[11-16] published in Chinese and three[17-19] in English. The quality of all included studies was moderate to poor. The specific literature screening process is shown in Figure 1. The general characteristics and methodological quality assessments of all included studies are summarized in Tables 1 and 2.

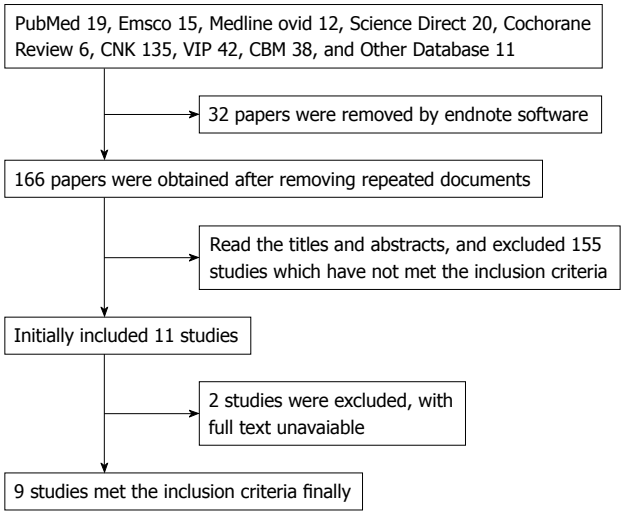

Figure 1 Flow chart for literature screening.

Efficacy evaluation

Operative time: Six studies[11-14,16,19] reported on the operative time, but one study[19] did not provide sufficient information in mean ± SD, so meta-analysis of five studies indicates that the operative time was significantly shorter in LF group than in OF group (MD: -28.76, 95% CI: -31.03 to -26.49, $P < 0.00001$). But this finding was not associated with significant heterogeneity between studies ($P = 0.46$, $I^2 = 0\%$) (Figure 2).

Table 2 Summary of the methodological quality of all included studies

Included studies	Allocation method	Homogeneity analysis	Identification of prognostic factors	Control of bias
Yi *et al*[11]	By doctors and patients	All the included cases come from the same research center, with good homogeneity	Age, sex, cyst location, types, size	No
Felizardo *et al*[12]	By doctors and patients	All the included cases come from the same research center, with good homogeneity	Age cyst location, size, type	No
Chen *et al*[13]	By doctors and patients	All the included cases come from the same research center, with good homogeneity	Age, sex, clinical complaints	No
Qiu *et al*[14]	By doctors and patients	All the included cases come from the same research center, with good homogeneity	Sex, main clinical complaints	No
Li *et al*[15]	By doctors and patients	All the included cases come from the same research center, with good homogeneity	Age, cyst location, type, size	No
Guo *et al*[16]	By doctors and patients	All the included cases come from the same research center, with good homogeneity	Age, sex, cyst location, type, size	No
Mazza *et al*[17]	By doctors and patients	All the included cases come from the same research center, with good homogeneity	Reported the age, cyst type, main complaints	No
Gigot *et al*[18]	By doctors and patients	All the included cases come from the same research center, with good homogeneity	Age, sex, cyst location, type, size	No
Treckmann *et al*[19]	By doctors and patients	All the included cases come from the same research center, with good homogeneity	Age, sex, cyst location , type, size	No

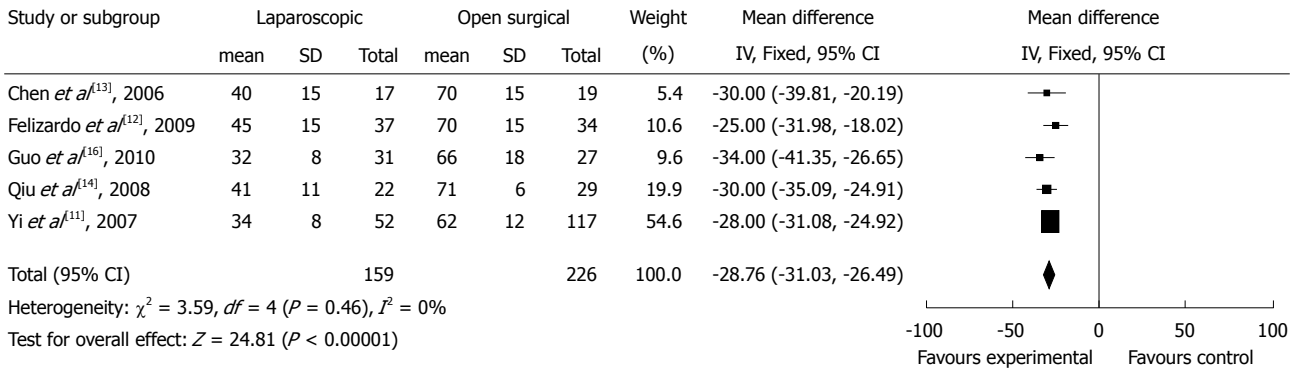

Figure 2 Meta-analysis of all available data in operative time.

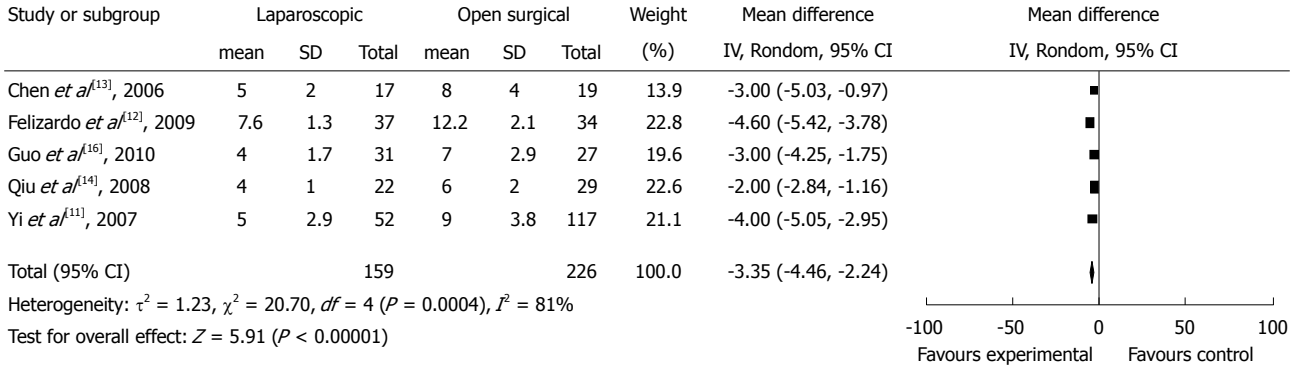

Figure 3 Meta-analysis of all available data in hospital stay.

Hospital stay: Eight studies[11-18] reported on the duration of postoperative hospitalization, but three[16-18] of them did not provide specific time data. Meta-analysis of the remaining five studies indicates that the hospital stay is significantly shorter in the LF group than in the OF group (MD: -3.35, 95% CI: -4.46 to -2.24, $P < 0.0001$) and there is statistically significant heterogeneity between the groups in all available studies for pooled analysis ($P = 0.0004$, $I^2 = 81\%$) (Figure 3).

Intraoperative blood loss: Five studies[11-14,16] reported on intraoperative blood loss. The intraoperative blood loss is significantly lower in the LF than in the OF group (MD: -40.18, 95% CI: -52.54 to -27.82, $P < 0.00001$) and this finding was associated with significant heterogeneity between studies ($P < 0.00001$, $I^2 = 96\%$) (Figure 4).

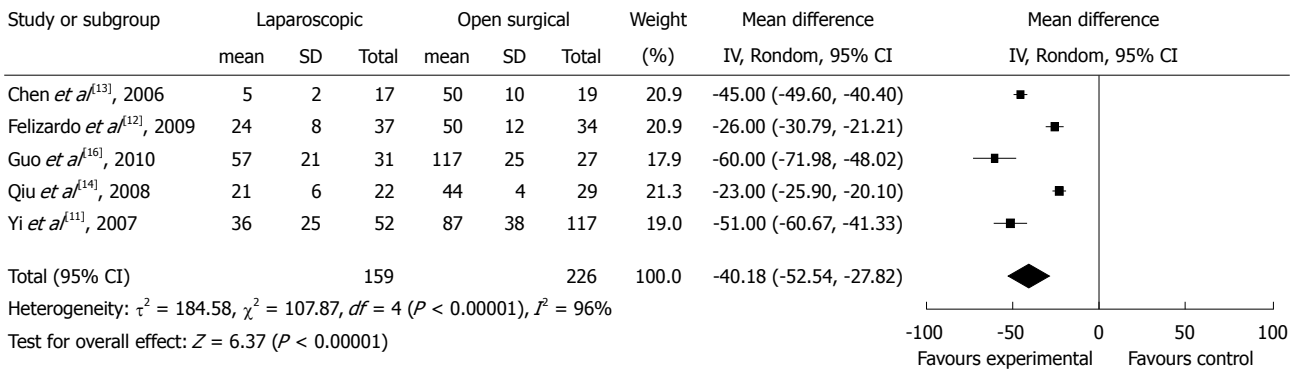

Figure 4 Meta-analysis of all available data in intraoperative blood loss.

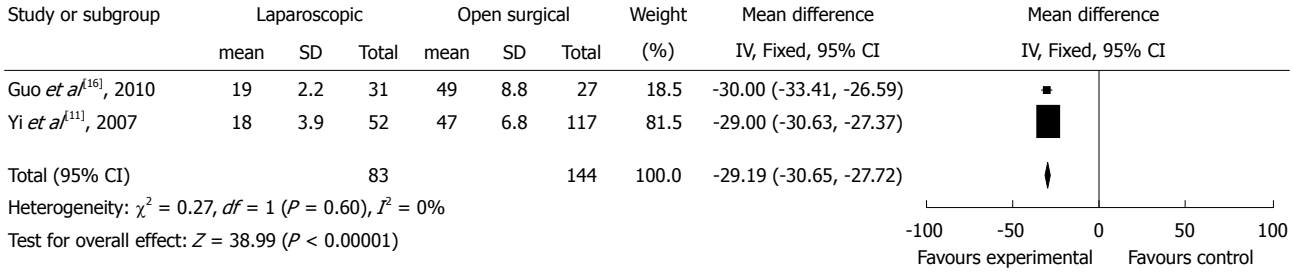

Figure 5 Meta-analysis of all available data in the time to return to normal diet.

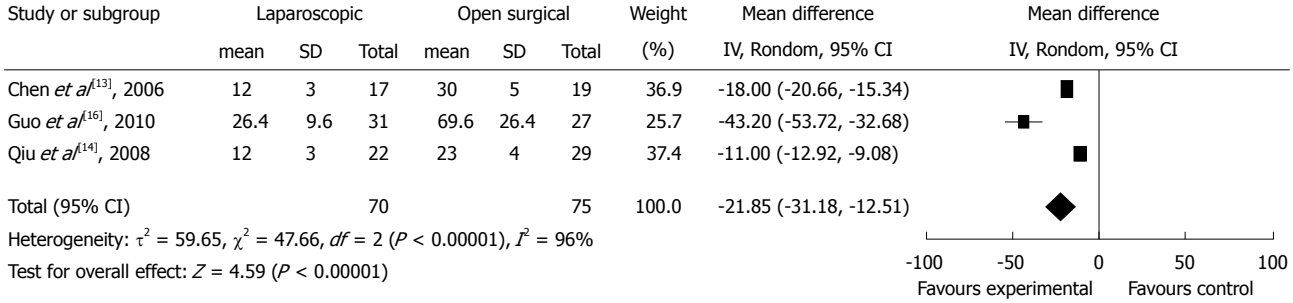

Figure 6 Meta-analysis of all available data in the time to return to normal activities.

Time to return to normal diet: Two studies[11,16] reported on the specific data about the time to return to normal gastrointestinal function. Meta-analysis of the two studies shows that the time to return to normal gastrointestinal function is significantly earlier in the LF group (MD: -29.19, 95% CI: -30.65 to -27.72, $P < 0.00001$), but this finding was not associated with significant heterogeneity between studies ($P = 0.6$, $I^2 = 0\%$) (Figure 5).

Time to return to normal activities: Three studies[13,14,16] reported on the time to return to normal activities in patients after operation. Meta-analysis of the three studies shows that the time to return to normal activities is significantly shorter in LF group than in OF group (MD: -21.85, 95% CI: -31.18 to -12.51, $P < 0.0001$) and this finding was associated with significant heterogeneity between studies for calculated analysis ($P < 0.00001$, $I^2 = 96\%$) (Figure 6).

Incidence of postoperative complications: Seven studies reported on the incidence of postoperative complications, but three[17-19] of them did not provide detailed information, we, therefore, extracted the data from four studies[11,12,14,15] and the meta-analysis shows that there is no significant difference in the incidence of postoperative complications between the two groups (OR: 0.99, 95% CI: 0.41 to 2.38, $P = 0.98$) and this finding was not associated with significant statistical heterogeneity between all available studies ($P = 0.85$, $I^2 = 0\%$) (Figure 7).

Recurrence rates of cysts: Four studies[14,15,17,19] reported on the recurrence rates of cysts through a follow-up from 3 mo to 1 year. However, three[15,17,19] of these studies did not provide sufficient information, but all the four studies showed no significant difference in the recurrence rate of cyst after operation between the two groups.

Postoperative recurrence rates of symptoms: Two studies[14,19] reported on the cyst recurrence rates after op-

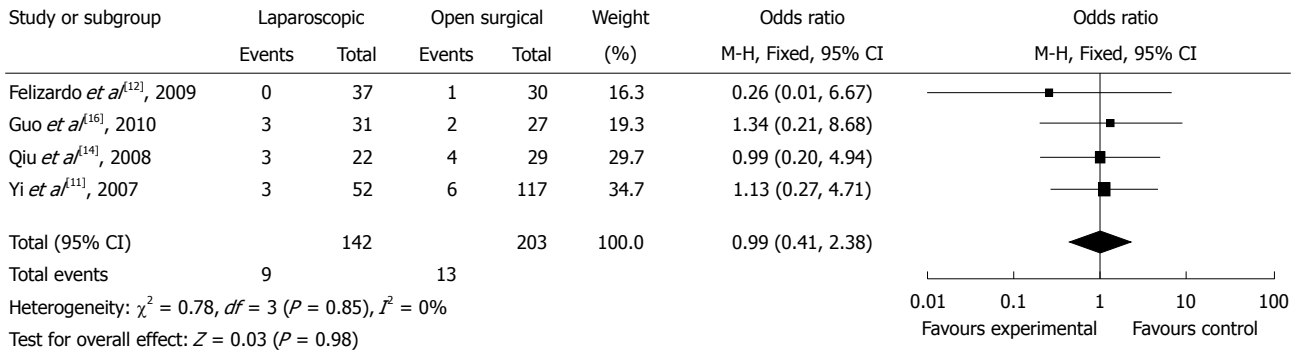

Figure 7 Meta-analysis of all available data in the incidence of complications.

eration, but only one study[14] provided a detailed number of relapse. None of the two studies showed significant difference in the recurrence rates of symptoms after operation between LF and OF group.

Time to return to normal liver functions: Only one study[12] compared the time to return to normal liver function after operation and suggested that the liver function was normalized earlier in the LF than in the OF group ($P < 0.01$).

Hospitalization cost: Only one study[12] reported on the hospitalization costs and suggested that there was no significant difference between the two groups ($P = 0.91$).

Heterogeneity analysis

A significant heterogeneity between the two groups was observed in the operative time, intraoperative blood loss, time to return to diet, time to return to activities and duration of hospital stay.

DISCUSSION

In this systematic review and meta-analysis, we attempted to collect the best evidence-based proofs with respect to LF and OF in patients with congenital hepatic cysts. A total of nine retrospective case-control studies that compared the outcomes of the two approaches for congenital hepatic cysts were identified for final pooled analysis. The methodological quality of all the nine studies was moderate to poor, and some publications have certain methodological deficiencies, such as not mentioning the allocation methods, smaller sample size as well as not adopting methods to reduce the bias in the statistical analysis. All these factors will affect the final reliability of the conclusions from this meta-analysis.

Clinical significance of the results

The conventional therapeutic options for congenital hepatic cyst were either percutaneous aspiration or open surgery, while percutaneous aspiration was accompanied by a high recurrence rate[20]. It has been reported the recurrence rate of hepatic cyst after drainage was up to 100%[21], while open approach was always associated with significant morbidity and mortality postoperatively. Since the first performance of LF in a 73-year-old patient who presented with symptomatic uncomplicated liver cysts in 1991[22], an increasing number of successful reports on the management of laparoscopy have been published, including laparoscopic management for complex and parasitic cysts. Currently, laparoscopy is considered as a standard treatment for uncomplicated hepatic cysts. Parasitic liver cysts are not the review scope of this paper.

In this paper, we systematically reviewed the relevant literatures and conducted a meta-analysis of the measured outcomes of LF *vs* OF for congenital hepatic cysts, which demonstrated that symptom relief, recurrence rates, hospitalization cost, postoperative complications of the LF appear to be similar to the OF group, but the operative time, hospital stay, intraoperative bleeding and time to return to normal gastrointestinal functions and activities seem to be lower, it appears to be a safe and feasible alternative to open surgery for the management of congenital hepatic cyst, however, up to now, only a small number of comparative, non-randomized studies have been published which limited the extrapolation of the results to the clinical setting.

limitations and recommendations for future research

This meta-analysis of nonrandomized studies may have several limitations that must be taken into account when considering its results. First, there are fewer clinical RCTs of laparoscopic *vs* open approach in congenital hepatic cyst patients. Furthermore, due to the absence of blinding and high risk of bias, the overall methodological quality of all included studies was judged by the peers. Finally, the results from non-randomized controlled trails need to be evaluated, thus restricting its application in clinical practice.

This review included nine non-randomized controlled clinical trials, and a total of 657 patients were analyzed. The results demonstrated that the short-term outcomes of laparoscopic management seem to be successful in patients with congenital liver cysts if preoperative diagnosis is accurate. However, up to now, there are only a small number of comparative, nonrandomized studies published and many authors merely documented the technical feasibility of the procedure and did not present

the follow-up outcomes. Therefore, the above-mentioned outcomes should be used with caution, and extended follow-ups are required to assess the long-term survival rates before any definitive conclusions can be drawn.

COMMENTS

Background

An increasing number of studies have reported the benefits of laparoscopic fenestration (LF) for hepatic cyst patients, however, the majority of studies merely documented the technical feasibility of this procedure and did not compared its outcomes to conventional open gastric resection. In this paper, therefore, a systematic review and meta-analysis were performed to search for the best evidence for LF in the management of patients with congenital hepatic cysts.

Research frontiers

From a technical point of view, hepatic cysts may be treated interventionally by aspiration or surgical fenestration, enucleation and formal hepatic resection. Laparoscopic surgery is the most popular approach because this technique is safe, resulting in a shorter hospital stay and an early return to normal activities.

Innovations and breakthroughs

This review suggests that the short-term outcomes of LF were superior to open approach for patients with congenital hepatic cysts. To our knowledge, this is the first systematic review using the meta-analysis to study the benefit of LF in the management of congenital hepatic cysts.

Applications

The LF for congenital liver cysts is feasible and effective, with superior short-term outcomes as compared with the open fenestration (OF). Although randomized controlled trials with measured outcomes are not available, laparoscopic treatment of uncomplicated parenchymal liver cysts is considered as a standard treatment.

Peer review

This is a technically good study of OF *vs* LF.

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CASE REPORT

Anchor-wire technique for multiple plastic biliary stents to prevent stent dislocation

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Abstract

In endoscopic placement of multiple plastic biliary stents (PBSs), we sometimes experience proximal dislocation of the first PBS at the time of subsequent PBS insertion. We describe the case of a 79-year-old male with obstructive jaundice caused by cholangiocarcinoma who needed to receive multiple PBS placements for management of cholangitis. Although proximal dislocation of the first PBS was observed, we prevented the dislocation *via* our technique of using guidewire inserted from the distal end of the first PBS to the side hole as the anchor-wire. We could complete this technique only by inserting guidewire through the side hole of the first PBS during the process of releasing the first PBS and pulling out the guidewire and the inner sheath. It did not matter whether the anchor-wire went towards the third portion of the duodenum or the duodenal bulb. Here we introduce this "anchor-wire technique", which is useful for the prevention of PBS proximal dislocation in placing multiple PBSs.

Key words: Plastic biliary stent; Anchor-wire technique; Proximal dislocation; Prevention; Endoscopic retrograde cholangiography

Peer reviewer: Dr. Thiruvengadam Muniraj, MBBS, MD, PhD, MRCP(UK), University of Pittsburgh Medical Center, 100 Chatham Park Drive, Apt 511, Pittsburgh, PA 15220, United States

Hamada T, Nakai Y, Matsubara S, Isayama H, Narita A, Watanabe K, Koike Y, Matsukawa S, Kawase T, Koike K. Anchor-wire technique for multiple plastic biliary stents to prevent stent dislocation. *World J Gastroenterol* 2011; 17(28): 3366-3368 Available from: URL: http://www.wjgnet.com/1007-9327/full/v17/i28/3366.htm DOI: http://dx.doi.org/10.3748/wjg.v17.i28.3366

INTRODUCTION

Endoscopic placement of plastic biliary stents (PBSs) has become a widely accepted procedure for biliary obstruction. The utility of multiple PBSs was reported for the management of hilar biliary obstruction or the dilation of benign biliary stricture[1-3]. However, endoscopic placement of multiple PBSs is difficult and prolongs procedure time. One of the problems in endoscopic placement of multiple PBSs is proximal dislocation of the first PBS at the time of subsequent PBS insertions. A proximally dislocated PBS above the papilla may be very difficult to remove[4-6]. Here we report a technique to resolve this problem using an "anchor-wire".

CASE REPORT

A 79-year-old male was admitted with acute obstructive suppurative cholangitis (AOSC). The first endoscopic retrograde cholangiography (ERC) showed severe biliary stenosis with type Ⅲ hilar biliary stricture according to Bismuth's classification (Figure 1)[7]. We placed three 5 Fr PBSs endoscopically, one each in the left hepatic duct (LHD), the anterior branch of the right hepatic duct (a-RHD) and the posterior branch of the right hepatic duct (p-RHD). The diagnosis of cholangiocarcinoma was pathologically confirmed. Radiation therapy was started after obstructive jaundice was resolved.

Two months later, he was admitted with AOSC and the two PBSs were distally dislocated and disappeared. ERC was performed to insert 3 larger diameter (7 Fr) PBSs. However, we failed to lead a guidewire to the LHD, because of the more severe stenosis, which was probably caused by radiation therapy. We decided to insert two 7 Fr PBSs (Flexima™, Boston Scientific, Natick, Mass) to the a-RHD and p-RHD. First, we inserted a 0.035 inch guidewire (RevoWave™, Piolax Medical Devices, Kanagawa, Japan) into the p-RHD using a bendable cannula (SwingTip™, Olympus, Tokyo, Japan) and inserted a PBS (7 Fr, 15 cm) to the p-RHD across the papilla. When we tried to insert another PBS (7 Fr, 15 cm) to the a-RHD, interference of the two PBSs caused proximal dislocation of the first PBS.

To prevent proximal dislocation of the first PBS into the bile duct, we inserted the stiff guidewire (RevoWave™) from the distal end of the first PBS through the distal side hole, leading it towards the third portion of the duodenum and made it work as an "anchor-wire" (Figure 2). When we subsequently inserted the second PBS, the anchor-wire successfully prevented proximal dislocation of the first PBS inclined to migrate into the bile duct, by pulling it down.

At the time of subsequent stent exchange, the anchor-wire to the duodenal bulb could also work as the anchor (Figure 3). This time, we could complete this technique only by inserting the guidewire through the side hole in the process of releasing the first stent and pulling out the guidewire and the inner sheath.

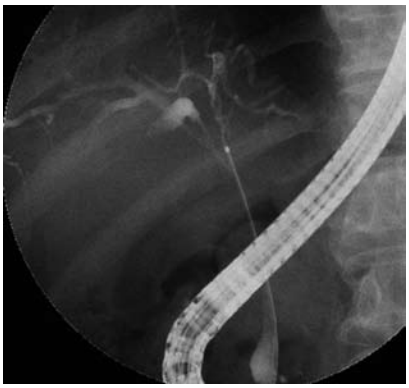

Figure 1 The first endoscopic retrograde cholangiography showed severe biliary stenosis with type Ⅲ hilar biliary stricture according to Bismuth's classification.

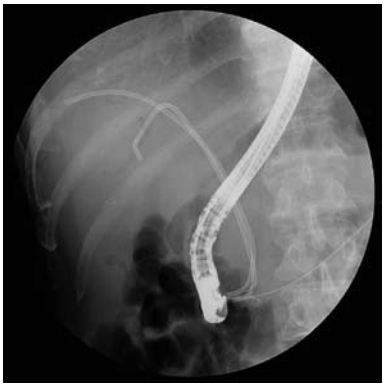

Figure 2 The second endoscopic retrograde cholangiography: the guidewire going through the side hole of the first plastic biliary stent worked as anchor-wire.

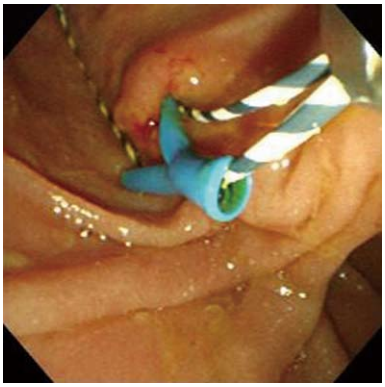

Figure 3 The endoscopic image at the third endoscopic retrograde cholangiography: the anchor-wire was inserted through the side hole of the first plastic biliary stent towards the duodenal bulb and another guidewire beside the plastic biliary stent.

DISCUSSION

We reported a technique named for "the anchor-wire technique", useful for preventing proximal dislocation of PBS in endoscopic placement of multiple PBSs.

Flexima™ is one of the most widely used PBSs, with a flap and a side hole on each end for preventing migration. However, we sometimes experience proximal dislocation of the first PBS at the time of subsequent PBS insertion when inserting multiple PBSs[3]. Proximal dislocation leads to stent dysfunction and PBSs dislocated above the papilla requires a particular technique for endoscopic removal[4-6]. Parlak *et al*[8] reported a technique for the prevention of this complication. They cannulated the distal side hole of the first PBS using a cannula and inserted a guidewire through the distal side of the PBS to the duodenum before they inserting the second PBS using the guidewire as the anchor. However, we experienced difficulty in realizing this technique for PBSs with side flaps, and distal migration of the first PBS could occur conversely, resulting from traction force of the anchor-

wire at the time of the second PBS insertion. Furthermore, repeated insertion of a guidewire with a cannula was necessary, leading to a prolonged procedure time. Our technique can be completed with ease and certainty because we can insert the guidewire through the side hole of the first PBS in the process of releasing the first stent and pulling out the guidewire and the inner sheath.

In conclusion, this anchor-wire technique can prevent proximal dislocation in multiple PBSs insertion.

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Events Calendar 2011

January 14-15, 2011
AGA Clinical Congress of Gastroenterology and Hepatology: Best Practices in 2011 Miami, FL 33101, United States

January 20-22, 2011
Gastrointestinal Cancers Symposium 2011, San Francisco, CA 94143, United States

January 27-28, 2011
Falk Workshop, Liver and Immunology, Medical University, Franz-Josef-Strauss-Allee 11, 93053 Regensburg, Germany

January 28-29, 2011
9. Gastro Forum München, Munich, Germany

February 4-5, 2011
13th Duesseldorf International Endoscopy Symposium, Duesseldorf, Germany

February 13-27, 2011
Gastroenterology: New Zealand CME Cruise Conference, Sydney, NSW, Australia

February 17-20, 2011
APASL 2011-The 21st Conference of the Asian Pacific Association for the Study of the Liver
Bangkok, Thailand

February 22, 2011-March 04, 2011
Canadian Digestive Diseases Week 2011, Vancouver, BC, Canada

February 24-26, 2011
Inflammatory Bowel Diseases 2011-6th Congress of the European Crohn's and Colitis Organisation, Dublin, Ireland

February 24-26, 2011
2nd International Congress on Abdominal Obesity, Buenos Aires, Brazil

February 24-26, 2011
International Colorectal Disease Symposium 2011, Hong Kong, China

February 26-March 1, 2011
Canadian Digestive Diseases Week, Westin Bayshore, Vancouver, British Columbia, Canada

February 28-March 1, 2011
Childhood & Adolescent Obesity: A whole-system strategic approach, Abu Dhabi, United Arab Emirates

March 3-5, 2011
42nd Annual Topics in Internal Medicine, Gainesville, FL 32614, United States

March 7-11, 2011
Infectious Diseases: Adult Issues in the Outpatient and Inpatient Settings, Sarasota, FL 34234, United States

March 14-17, 2011
British Society of Gastroenterology Annual Meeting 2011, Birmingham, England, United Kingdom

March 17-19, 2011
41. Kongress der Deutschen Gesellschaft für Endoskopie und Bildgebende Verfahren e.V., Munich, Germany

March 17-20, 2011
Mayo Clinic Gastroenterology & Hepatology 2011, Jacksonville, FL 34234, United States

March 18, 2011
UC Davis Health Informatics: Change Management and Health Informatics, The Keys to Health Reform, Sacramento, CA 94143, United States

March 25-27, 2011
MedicReS IC 2011 Good Medical Research, Istanbul, Turkey

March 26-27, 2011
26th Annual New Treatments in Chronic Liver Disease, San Diego, CA 94143, United States

April 6-7, 2011
IBS-A Global Perspective, Pfister Hotel, 424 East Wisconsin Avenue, Milwaukee, WI 53202, United States

April 7-9, 2011
International and Interdisciplinary Conference Excellence in Female Surgery, Florence, Italy

April 15-16, 2011
Falk Symposium 177, Endoscopy Live Berlin 2011 Intestinal Disease Meeting, Stauffenbergstr. 26, 10785 Berlin, Germany

April 18-22, 2011
Pediatric Emergency Medicine: Detection, Diagnosis and Developing Treatment Plans, Sarasota, FL 34234, United States

April 20-23, 2011
9th International Gastric Cancer Congress, COEX, World Trade Center, Samseong-dong, Gangnam-gu, Seoul 135-731, South Korea

April 25-27, 2011
The Second International Conference of the Saudi Society of Pediatric Gastroenterology, Hepatology & Nutrition, Riyadh, Saudi Arabia

April 25-29, 2011
Neurology Updates for Primary Care, Sarasota, FL 34230-6947, United States

April 28-30, 2011
4th Central European Congress of Surgery, Budapest, Hungary

May 7-10, 2011
Digestive Disease Week, Chicago, IL 60446, United States

May 12-13, 2011
2nd National Conference Clinical Advances in Cystic Fibrosis, London, England, United Kingdom

May 19-22, 2011
1st World Congress on Controversies in the Management of Viral Hepatitis (C-Hep), Palau de Congressos de Catalunya, Av. Diagonal, 661-671 Barcelona 08028, Spain

May 21-24, 2011
22nd European Society of Gastrointestinal and Abdominal Radiology Annual Meeting and Postgraduate Course, Venise, Italy

May 25-28, 2011
4th Congress of the Gastroenterology Association of Bosnia and Herzegovina with international participation, Hotel Holiday Inn, Sarajevo, Bosnia and Herzegovina

June 11-12, 2011
The International Digestive Disease Forum 2011, Hong Kong, China

June 13-16, 2011
Surgery and Disillusion XXIV SPIGC, II ESYS, Napoli, Italy

June 14-16, 2011
International Scientific Conference on Probiotics and Prebiotics-IPC2011, Kosice, Slovakia

June 22-25, 2011
ESMO Conference: 13th World Congress on Gastrointestinal Cancer, Barcelona, Spain

June 29-2, 2011
XI Congreso Interamericano de Pediatria "Monterrey 2011", Monterrey, Mexico

September 2-3, 2011 Falk Symposium 178, Diverticular Disease, A Fresh Approach to a Neglected Disease, Gürzenich Cologne, Martinstr. 29-37, 50667 Cologne, Germany

September 10-11, 2011
New Advances in Inflammatory Bowel Disease, La Jolla, CA 92093, United States

September 10-14, 2011
ICE 2011-International Congress of Endoscopy, Los Angeles Convention Center, 1201 South Figueroa Street Los Angeles, CA 90015, United States

September 30-October 1, 2011
Falk Symposium 179, Revisiting IBD Management: Dogmas to be Challenged, Sheraton Brussels Hotel, Place Rogier 3, 1210 Brussels, Belgium

October 19-29, 2011
Cardiology & Gastroenterology | Tahiti 10 night CME Cruise, Papeete, French Polynesia

October 22-26, 2011
19th United European Gastroenterology Week, Stockholm, Sweden

October 28-November 2, 2011
ACG Annual Scientific Meeting & Postgraduate Course, Washington, DC 20001, United States

November 11-12, 2011
Falk Symposium 180, IBD 2011: Progress and Future for Lifelong Management, ANA Interconti Hotel, 1-12-33 Akasaka, Minato-ku, Tokyo 107-0052, Japan

December 1-4, 2011
2011 Advances in Inflammatory Bowel Diseases/Crohn's & Colitis Foundation's Clinical & Research Conference, Hollywood, FL 34234, United States

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GENERAL INFORMATION

World Journal of Gastroenterology (*World J Gastroenterol*, *WJG*, print ISSN 1007-9327, online ISSN 2219-2840, DOI: 10.3748) is a weekly, open-access (OA), peer-reviewed journal supported by an editorial board of 1144 experts in gastroenterology and hepatology from 60 countries.

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Key words

Please list 5-10 key words, selected mainly from *Index Medicus*, which reflect the content of the study.

Text

For articles of these sections, original articles and brief articles, the main text should be structured into the following sections: INTRODUCTION, MATERIALS AND METHODS, RESULTS and DISCUSSION, and should include appropriate Figures and Tables. Data should be presented in the main text or in Figures and Tables, but not in both. The main text format of these sections, editorial, topic highlight, case report, letters to the editors, can be found at: http://www.wjgnet.com/1007-9327/g_info_20100315215714.htm.

Illustrations

Figures should be numbered as 1, 2, 3, *etc.*, and mentioned clearly in the main text. Provide a brief title for each figure on a separate page. Detailed legends should not be provided under the figures. This part should be added into the text where the figures are applicable. Figures should be either Photoshop or Illustrator files (in tiff, eps, jpeg formats) at high-resolution. Examples can be found at: http://www.wjgnet.com/1007-9327/13/4520.pdf; http://www.wjgnet.com/1007-9327/13/4554.pdf; http://www.wjgnet.com/1007-9327/13/4891.pdf; http://www.wjgnet.com/1007-9327/13/4986.pdf; http://www.wjgnet.com/1007-9327/13/4498.pdf. Keeping all elements compiled is necessary in line-art image. Scale bars should be

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Data that are not statistically significant should not be noted. $^{a}P < 0.05$, $^{b}P < 0.01$ should be noted ($P > 0.05$ should not be noted). If there are other series of P values, $^{c}P < 0.05$ and $^{d}P < 0.01$ are used. A third series of P values can be expressed as $^{e}P < 0.05$ and $^{f}P < 0.01$. Other notes in tables or under illustrations should be expressed as ^{1}F, ^{2}F, ^{3}F; or sometimes as other symbols with a superscript (Arabic numerals) in the upper left corner. In a multi-curve illustration, each curve should be labeled with ●, ○, ■, □, ▲, △, *etc.*, in a certain sequence.

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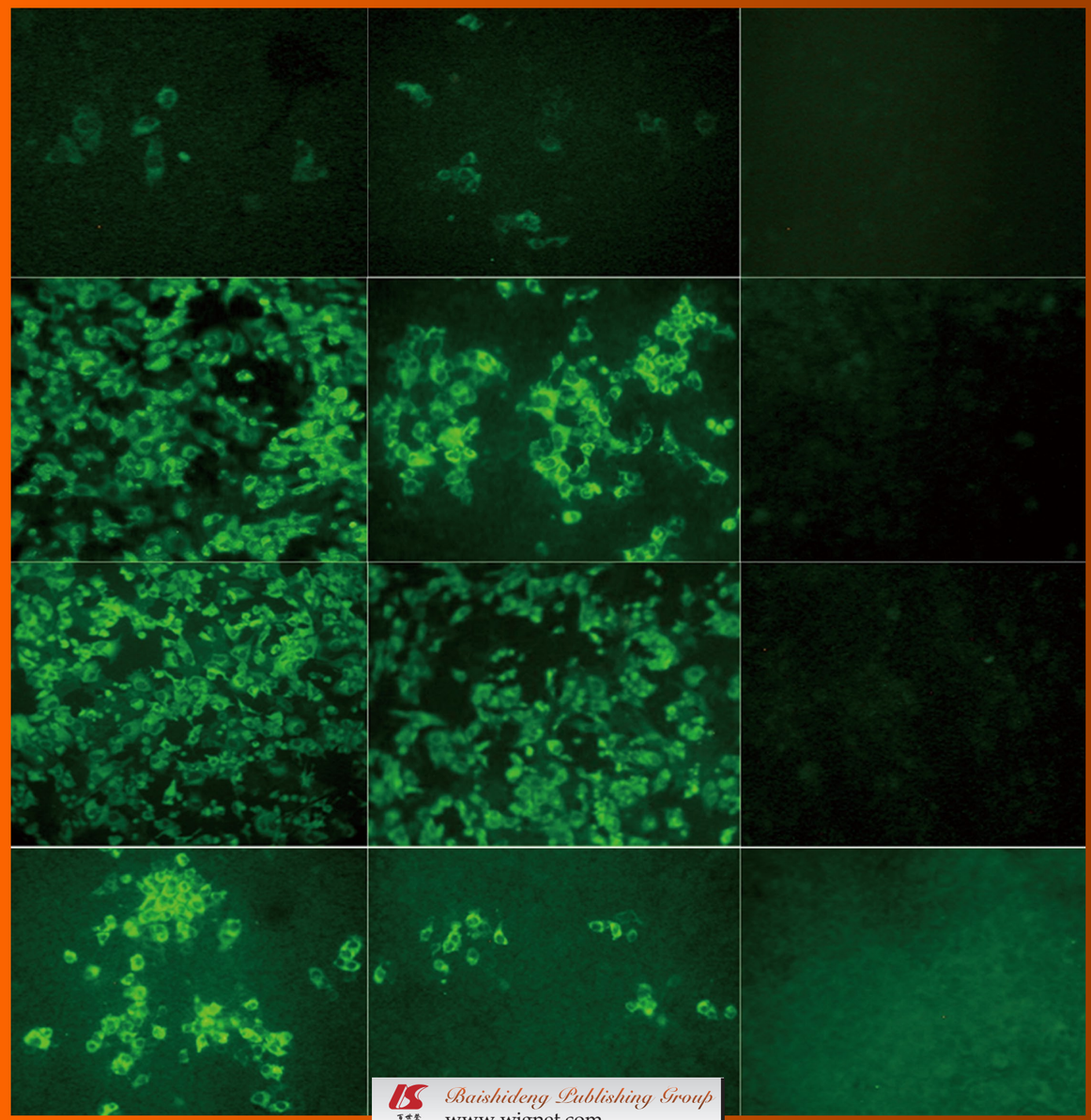

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EDITORIAL

Neuropsychological alterations in hepatitis C infection: The role of inflammation

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Abstract

About 50% of patients with hepatitis C virus (HCV) infection complain of neuropsychiatric symptoms, "brain fog", weakness, fatigue, and exhibit some degree of quality of life impairment, irrespective of the severity of liver disease. Since the first observation of HCV-related cognitive deficits, 10 studies have been published that have evaluated neuropsychiatric performance in patients with HCV infection and different degrees of hepatic impairment. Unfortunately, these have often included patients with cirrhosis, patients who had acquired the infection through previous intravenous drug misuse, who had a history of relatively recent treatment with interferon, or were on psychoactive medication. In addition, different neuropsychological batteries and tests that explored different cognitive domains were used, which makes the results of the studies difficult to compare. Finally, limited information is available on the pathogenesis of HCV-related cognitive impairment. Cerebral and/or systemic inflammation may be important players but their potential role has not been substantiated by experimental data. The present review outlines the available evidence of the presence of cognitive impairment in patients with HCV infection, with a focus on the potential relationship with cerebral and/or systemic inflammation.

Key words: Cognitive alterations; Hepatitis C virus; Inflammation

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INTRODUCTION

Hepatitis C virus (HCV) infection affects up to 2% of the world population and almost 4 million people in America. Although evolution to chronic HCV infection is extremely common, only 30% of chronically infected patients go on to develop end-stage liver disease and hepatocellular carcinoma.

The occurrence of hepatic encephalopathy is well

documented in patients with viral cirrhosis, as in patients with cirrhosis of other etiologies[1]. However, in recent years, there has been growing evidence that alterations in cerebral function in patients with chronic HCV infection may appear long before the development of severe liver fibrosis/cirrhosis. These alterations cannot be ascribed to hepatic encephalopathy. About 50% of patients with HCV infection complain of neuropsychiatric symptoms, "brain fog", weakness, fatigue, and exhibit some degree of quality of life impairment, irrespective of the severity of liver disease[2]. These alterations do not seem to relate to HCV genotype or replication[3]. Their etiology is unclear but it has been hypothesized that it is related to: (1) a direct effect of HCV on the brain; or (2) the neurotoxic effect of HCV-related systemic inflammation.

In the present review, we outline the available evidence of cognitive impairment in patients with HCV infection, and the possible role of cerebral and systemic inflammation.

COGNITIVE ALTERATIONS IN PATIENTS WITH HCV LIVER DISEASE

Early in the course of infection, patients with HCV infection report symptoms like fatigue, malaise, weakness and problems in maintaining attention and recalling information. These alterations can interfere with their ability to perform their activities, thus leading to impairment in health-related quality of life, which is well documented[4-12]. In addition, although treatment of chronic HCV infection can temporarily worsen health-related quality of life, the relationship between sustained viral response and improvement in quality of life is also well accepted[13].

The first significant evidence for a specific role of HCV in causing cerebral function abnormalities was produced in 2001 by Forton and colleagues, who detected cerebral metabolic abnormalities (elevated choline/creatine ratio) in the frontal white matter and basal ganglia of HCV-infected patients, using proton magnetic resonance spectroscopy (^{1}H MRS); these alterations were not present in either controls or patients with HBV infection[14]. In the following year, the same group showed significant impairment in concentration and working memory in 27 HCV-infected patients with active viral replication, compared to 20 controls and 16 anti-HCV-positive but HCV-RNA-negative patients[15].

Since these original observations, 10 studies have been published that have evaluated neuropsychiatric performance in patients with HCV infection and different degrees of hepatic impairment. Unfortunately, these have often included patients with cirrhosis (potentially, also those with minimal hepatic encephalopathy); patients who had acquired the infection by previous intravenous drug misuse; patients who had a history of relatively recent treatment with interferon; patients on psychoactive medication; or those who complained of significant fatigue; all of which could impinge on cognitive performance, in terms of motivation and psychomotor speed. In addition, different neuropsychological batteries and tests that explore different cognitive domains have been utilized, which makes it difficult to compare the results of the studies.

Hilsabeck and colleagues have documented a prevalence of cognitive dysfunction which ranged from 0% on a design copy task to 49% on a measure of sustained attention in a group of 66 HCV-infected patients; 44 (66%) of whom had cirrhosis[16]. The HCV-infected patients were compared to a cohort of 14 patients with liver disease of other etiology, who had normal cognitive performance. However, within the study group, there were factors that could have contributed to cognitive impairment, such as previous alcohol intake and HIV co-infection; the control group also included patients with previous alcohol misuse and those with cirrhosis, and possibly minimal hepatic encephalopathy. The authors were able to correlate the degree of fibrosis with that of cognitive impairment, and suggested that the latter might parallel progressive liver injury in HCV-infected patients. However, the inclusion of even a small number of patients with cirrhosis and hepatic encephalopathy might well be responsible for the correlations observed. In addition, sustained attention, which was found to be abnormal in almost half of the study group, is known to be impaired in patients with hepatic encephalopathy[17,18].

When the same authors administered a similar test battery to an independent group of HCV-infected patients, 33% of whom had cirrhosis, there was no correlation between perceived cognitive impairment, fatigue or depression and neuropsychological performance, which suggests that the latter might not be clinically relevant[19]. Similarly to the previous study, a correlation was observed between neuropsychological performance and the degree of fibrosis, which led the authors to suggest that progressive liver injury might result in worsening neuropsychiatric function in HCV-infected patients. Within this setting, it would be difficult to explain how a significant proportion of patients with HCV-related cirrhosis, just like those with cirrhosis of other etiology, would show completely normal neuropsychiatric function on extensive screening for hepatic encephalopathy.

The issue of the relationship between cognitive impairment and perceived fatigue in HCV-infected patients was subsequently addressed by Weissenborn and colleagues, who compared neuropsychological performance in 30 PCR-positive HCV-infected patients with normal liver function, 15 of whom reported moderate to severe fatigue[20]; patients with previous drug misuse, interferon treatment, psychiatric disease and patients on psychoactive drugs were excluded. The authors found a significant deficit in attention and higher executive function in patients compared to controls, in parallel with an increase in depression and anxiety. Patients with self-reported fatigue performed worse on the neuropsychological battery, whereas there was no correlation between anxiety/depression and cognitive performance. In the same

study, patients with HCV infection showed a significant decrease of the N-acetyl-aspartate/creatine ratio in the cerebral cortex on ^{1}H MRS, while the electroencephalogram was slowed in 25%[20]. In contrast, in a published abstract, Montagnese and colleagues have reported on an unexpectedly high prevalence of fast (β-dominated) electroencephalograms in a similarly well-selected population of HCV-infected patients. Similar features had been previously reported in HIV-infected individuals[21] and could be related to some degree of desynchronization of the cerebral electrical activity.

McAndrews and co-workers have confirmed the presence of minor attention deficits and impairment in verbal learning ability in their study of 37 well-selected HCV patients without disease-associated risk factors, such as substance misuse, cirrhosis or depression[22]. When compared with 46 age-matched controls, 13% of patients with HCV infection showed impairment in verbal learning ability; however, the chosen threshold for a pathological performance was 1.5 SDs below the norm, which is stricter than the usual 2 SDs. The authors themselves qualify the detected abnormalities as having limited clinical relevance. As in previous studies, McAndrews and colleagues also detected an increase in choline and a reduction in N-acetyl aspartate by MRS in the central white matter of patients compared to controls.

In contrast, Fontana and co-workers have found that 33% of 177 patients with HCV infection and advanced fibrosis who were enrolled in the HALT-C trial could be considered to have cognitive impairment (before interferon and ribavirin treatment), based on a composite score of 10 neuropsychological tests[23]. The most affected domains were verbal recall and working memory. However, 38% of patients had cirrhosis, and working memory is known to be impaired in patients with cirrhosis and hepatic encephalopathy[24], which was probably a significant confounder in this study. In addition, 50% of patients had been alcohol misusers and 46% had a history of intravenous drug abuse. In contrast with the findings by Hilsabeck and colleagues, Fontana and co-workers observed no relationship between cognitive alterations and the degree of fibrosis or mood disturbances[23].

More recently, Lowry and colleagues studied neuropsychiatric function in a well-selected, homogeneous cohort of 20 female, iatrogenically-infected patients; of whom, 11 were positive for HCV RNA and nine had spontaneously cleared the virus[25]. The authors showed that PCR-positive women had significantly poorer scores in the areas of memory, auditory recognition and sustained attention compared to a small group of nine healthy controls; these abnormalities were not present in the PCR-negative women.

To date, two studies that explored cognitive function in chronically HCV-infected patients were completely negative. The first was published by Cordoba and colleagues[26], who showed normal neuropsychiatric performance in 40 HCV patients with normal hepatic function; however, these individuals still exhibited some degree of quality of life impairment. In the same study, significant alterations in attention, executive function and motor performance were detected in a control group of patients with HCV-related cirrhosis. In contrast to most previous studies, Cordoba and co-workers selected their HCV-positive patients amongst healthy individuals screened for blood donation[26]; this is a fairly different population compared to patients with known chronic HCV infection.

The second negative study included 103 HCV-PCR-positive young patients (aged 6-19 years) who were studied with the Adaptive Behavioural and WAIS scales. In this group, the time lag between infection and cognitive assessment might have been significantly lower compared to the other, adult cohorts, thus possibly explaining, at least to some extent, the negative results[27].

One study that compared cognitive performance in 32 patients with chronic hepatitis C against 29 chronic hepatitis B showed than HCV patients had worse performance in verbal learning and memory compared to controls, but they did not differ from patients with hepatitis B virus liver disease[28]. However, about 20% of patients had liver cirrhosis in both groups. Moreover, only 50% of the study group had histological assessment and no clinical exclusion of cirrhosis was described by the authors.

INFLAMMATION AND HCV

The etiology of cognitive dysfunction in patients with chronic HCV infection remains unclear but two hypotheses have been put forward: (1) the virus infects the brain and has a direct neurotoxic effect; and (2) the virus is indirectly neurotoxic *via* cerebral and/or systemic inflammation.

A direct neurotoxic role for HCV is supported by reports of HCV replication within the central nervous system[29-31]. It has been suggested that the virus enters the brain by infecting peripheral blood mononuclear cells, which are precursors of the microglia and could act as a "Trojan horse"[32]. However, data on the association between the virus in the brain and impaired cognitive function are still lacking. Indeed, replication of quasispecies is very low within the brain; HCV RNA is almost undetectable in the cerebrospinal fluid[33,34] and there is no correlation between viral load and cognitive impairment in patients with HCV infection[20]. However, this is sometimes also the case for other HCV-related complications, such as cryoglobulinemia or vasculitis[35].

It is well known that the cytolytic effect of HCV within the liver relates to the activation of the immune system. Thus, chronic activation of the immune system could account, at least in part, for the observed cerebral alterations, due to increased systemic and/or local inflammation. A growing body of evidence supports immune system-to-brain communication, with peripheral immune activation being associated with behavioral, affective and cognitive disturbances. Peripheral proinflammatory

cytokines such as interleukin (IL)-1, and IL-6 are likely mediators of these effects, and penetrate the blood-brain barrier directly through active transport mechanisms, activation of the vagus nerve, stimulation of neurotransmitter systems, and therefore, modulation of brain activity. Most of the evidence that directly links peripheral proinflammatory cytokines with neurocognitive function is derived from animal models, in which increased peripheral IL-1 and IL-6 are associated with increased levels of these cytokines in the prefrontal cortex and hippocampus[36].

Increased levels of IL-6 have been associated with impairment in spatial learning and memory, which are prevented by the administration of specific antagonists. This suggests a primary role for inflammatory cytokines in mediating cognitive decline and deficits in chronic inflammation[37,38]. Likewise, peripheral markers of inflammation have been associated with cognitive decline in elderly patients. In a recent study that evaluated the correlation between IL-6 and cognitive performance in middle-aged volunteers, an inverse relationship was observed between circulating levels of IL-6 and auditory recognition memory, attention, working memory and executive function[39].

Once a patient has chronic HCV infection, proinflammatory cytokines such as IL-6, IL-4 and tumor necrosis factor (TNF)-α are produced and may be elevated for several decades. During this period, proinflammatory cytokines can cross the blood-brain barrier and therefore contribute to cognitive impairment.

Moreover, another possible contribution of inflammation to cognitive degeneration in HCV patients is local cerebral inflammation[34,40,41]. It has been shown that small amounts of HCV within the brain evoke a local inflammatory response, because macrophages infected with HCV *in vitro* can induce TNF-α and IL-8[32]. In addition, a recent study has shown activation of brain macrophages/microglia in autopsy brain tissue from HCV-positive patients[42]. Peripheral markers of the activation of cellular immunity have recently been assessed by Gess and colleagues in a group of 53 HCV-infected patients with mild liver disease. No association was observed between activated cellular immunity and subjectively perceived or objectively measured cognitive impairment[43].

FUTURE PERSPECTIVES

The studies that have explored cognitive function in patients with chronic HCV were extremely heterogeneous in terms of patient characteristics, confounding factors (e.g. intravenous drug misuse and previous alcohol intake), control groups, methodology and tests used to assess cognitive performance. An additional issue might be the fact that the study subjects ranged from patients who had cleared HCV to those with HCV-related cirrhosis, even within the same study group. Furthermore, the role of systemic inflammation in the pathogenesis of cognitive alterations in patients with HCV infection has never been directly explored.

It is possible that patients with chronic HCV infection and persistently normal transaminases for 6 mo (PNALT) could represent an extremely useful study group to provide additional information, particularly in relation to the role of HCV *per se* in causing neurocognitive dysfunction. When we evaluated systemic inflammation in this group of patients, no activation of systemic inflammation was observed[44]. This finding suggests that patients with normal transaminases have a different immunological response profile, compared to those whose transaminases remain elevated. In line with this hypothesis, previous studies have demonstrated an increase in HCV-specific $CD4^+CD25^+$ regulatory T cells and a decrease in $CD4^+$ response in patients with normal transaminases compared to patients with high transaminases[45].

Whether the absence of an activated systemic inflammatory response in PNALT patients also reflects better cognitive performance needs to be explored. In a preliminary study, we found that PNALT patients with normal serum levels of proinflammatory cytokines performed similarly to controls as far as memory, attention and cognitive evoked potential N400, which relates to semantic memory and verbal working memory. Patients with chronic hepatitis due to HCV had impairment in memory in 60% of cases, with concomitant increased amplitude of N400, which indicated the need for increased neuronal recruitment to perform the task[44].

In the two studies that did not demonstrate cognitive alterations in patients with HCV, HCV-positive individuals were selected from healthy volunteers screened for blood donation and young patients with hemophilia, respectively. These subjects were classified as HCV-positive individuals with normal transaminases but were not further characterized, and some might well have qualified as PNALT.

Future, prospective cohort studies should probably include patients with chronic HCV infection with minimal or no fibrosis, PNALT, hepatitis B surface antigen-positive patients with/without transaminitis and a control group with chronic systemic inflammation (i.e. inflammatory bowel disease). In addition, the neuropsychological evaluation should probably be conducted in a structured, comprehensive way, by cognitive domain, and test results scored against adequate, large and local normative databases, rather than simply compared to small internal control groups. Mood, fatigue and quality of life should also be assessed. This approach might provide more solid information on whether HCV-related cognitive impairment exists and, if so, on its clinical relevance.

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TOPIC HIGHLIGHT

Dr. Nimer Assy, MD, Assistant Professor, *Series Editor*

Nutritional recommendations for patients with non-alcoholic fatty liver diseases

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Abstract

Fatty liver is the most common liver disease worldwide. Patients with fatty liver disease die primarily from cardiovascular disease and not from chronic liver diseases. Hyperglycemia and hyperinsulinemia induce lipogenesis, thereby increasing the hepatic pool of fatty acids. This pool is also increased by increased delivery of fatty acids through the diet or lipolysis in adipose tissue. Nutritional consultations and lifestyle modification are important in the treatment of non-alcoholic fatty liver disease (NAFLD). Among the dietary constituents, combination of vitamin D, vitamin E, and omega-3 fatty acids shows promise for the treatment of NAFLD.

Key words: Weight reduction; Non-alcoholic fatty liver disease; Physical activity; Nutrition; Fat

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Fatty liver is the most common cause of liver diseases in adults and children[1]. Fatty liver disease in humans is an insulin-resistant condition and the liver over-produces glucose and triglycerides due to impaired insulin action[2]. Fatty liver is an independent predictor of diabetes and cardiovascular disease[3]. There are three major sources for increased liver fat accumulation: excessive delivery of free fatty acids from lipolysis of superficial and visceral fat depots (60%), increased *de novo* hepatic lipogenesis (30%), and increased nutritional intake (10%)[4]. Recently, an increase in dietary cholesterol has been suggested to induce *de novo* fatty acid synthesis in hepatocytes *via* the LXRa-SREBP-1c pathway[5]. The most common cause of death in patients with non-alcoholic fatty liver disease (NAFLD) is coronary artery disease (CAD), and not chronic liver disease[6]. Fatty liver increases cardiovascular risk by classical (dyslipidemia, hypertension or diabetes) and by less conventional mechanisms. New emerging risk factors include leptin, adiponectin, pro-inflammatory cytokines such as interleukin-6, C-reactive protein and plasminogen activator inhibitor-1, which together lead to increased oxidative stress, lipotoxicity and endothelial dysfunction, which finally promote CAD[7]. When classical risk factors are superimposed on fatty liver accumulation, they may further increase the new metabolic risk factors, thus exacerbating CAD.

Several changes in dietary intake have occurred in the past few years, including increased energy intake (24%), and increases in added sugars, flour and cereal products, fruit, added fats and total fat intake[8]. Use of high fructose corn syrup (HFCS), which is used as sweetener in

soft drinks, has increased to comprise 41% of total added sweeteners. Sucrose accounts for 45% of the remainder. These changes have certainly contributed to the increase in prevalence of NAFLD, by increasing obesity and by direct fructose ingestion from soft drinks[9].

The review by Zelberg-Sagi describes elegantly the data regarding the association between dietary intake and NAFLD, and has focused on the dietary treatment of NAFLD beyond weight loss and physical activity. She has shown clearly that "good food may be a good medicine". The dietary interventions that seem to be beneficial in NAFLD are: (1) nutritional counseling with a multidisciplinary team including a dietitian, psychologist, and physical activity supervisor (behavior, educational, and motivational therapy); (2) aerobic exercise (walking 30 min daily, or > 5 km/d three times weekly); (3) restriction of calorie intake to < 30 kcal/kg per day, with a balanced diet that includes low levels of saturated and *trans* fats and simple sugars; (4) gradual weight loss (10% within 6 mo); (5) avoid rapid weight loss (> 1.6 kg/wk) as this can increase the progression of NAFLD; (6) management of accompanying conditions such as diabetes, obesity, and metabolic syndrome; (7) avoid foods with HFCS (soft drink), fast food (trans fats, and reduce red and processed meats), and genetically modified crops; (8) morbid obese patients [body mass index (BMI) > 40 or BMI > 35 with comorbidity] may be considered for referral for bariatric surgery; (9) use of vitamin E (400-800 IU/d), vitamin D (1000 IU/d), omega-3 fatty acids (1 g/d fish oil), and omega-9 fatty acids (olive oil) is recommended; and (10) trial of orlistat in patients who fail diet therapy. Use of metformin/pioglitazone if insulin resistance index (HOMA) > 2, with or without ursodeoxycholic acid (15 mg/kg per day).

However, whether any type of diet including weight loss diets can prevent steatohepatitis or fibrosis is uncertain because data on histology before and after dietary intervention are lacking. It is also uncertain whether bariatric surgery can prevent fibrosis and decrease the metabolic risk factors for CAD. It is important to establish the effects of diet composition on the natural course of NAFLD. Such data are not available at present. Of the dietary constituents, combination of vitamin D, vitamin E, and omega-3 fatty acids shows promise for the treatment of NAFLD.

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TOPIC HIGHLIGHT

Dr. Assy Nimer, MD, Assistant Professor, *Series Editor*

Nutrition and physical activity in NAFLD: An overview of the epidemiological evidence

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Abstract

Nonalcoholic fatty liver disease (NAFLD) has been recognized as a major health burden. The high prevalence of NAFLD is probably due to the contemporary epidemics of obesity, unhealthy dietary pattern, and sedentary lifestyle. The efficacy and safety profile of pharmacotherapy in the treatment of NAFLD remains uncertain and obesity is strongly associated with hepatic steatosis; therefore, the first line of treatment is lifestyle modification. The usual management of NAFLD includes gradual weight reduction and increased physical activity (PA) leading to an improvement in serum liver enzymes, reduced hepatic fatty infiltration, and, in some cases, a reduced degree of hepatic inflammation and fibrosis. Nutrition has been demonstrated to be associated with NAFLD and Non-alcoholic steatohepatitis (NASH) in both animals and humans, and thus serves as a major route of prevention and treatment. However, most human studies are observational and retrospective, allowing limited inference about causal associations. Large prospective studies and clinical trials are now needed to establish a causal relationship. Based on available data, patients should optimally achieve a 5%-10% weight reduction. Setting realistic goals is essential for long-term successful lifestyle modification and more effort must be devoted to informing NAFLD patients of the health benefits of even a modest weight reduction. Furthermore, all NAFLD patients, whether obese or of normal weight, should be informed that a healthy diet has benefits beyond weight reduction. They should be advised to reduce saturated/trans fat and increase polyunsaturated fat, with special emphasize on omega-3 fatty acids. They should reduce added sugar to its minimum, try to avoid soft drinks containing sugar, including fruit juices that contain a lot of fructose, and increase their fiber intake. For the heavy meat eaters, especially those of red and processed meats, less meat and increased fish intake should be recommended. Minimizing fast food intake will also help maintain a healthy diet. PA should be integrated into behavioral therapy in NAFLD, as even small gains in PA and fitness may have significant health benefits. Potentially therapeutic dietary supplements are vitamin E and vitamin D, but both warrant further research. Unbalanced nutrition is not only strongly associated with NAFLD, but is also a risk factor that a large portion of the population is exposed to. Therefore, it is important to identify dietary patterns that will serve as modifiable risk factors for the prevention of NAFLD and its complications.

Key words: Nonalcoholic fatty liver disease; Nutrition; Physical activity; Weight reduction; Fat; Carbohydrates; Soft drinks; Nutrients

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NUTRITION AND OBESITY IN NAFLD PATHOGENESIS

Nonalcoholic fatty liver disease (NAFLD), which develops in the absence of alcohol abuse, has been recognized as a major health burden. The clinical implications of NAFLD are derived mostly from its common occurrence in the general population and its potential to progress to cirrhosis and liver failure[1-3]. Estimates suggest that about 20% to 30% of adults in developed countries have excess fat accumulation in the liver[4-8], 50% among people with diabetes, and about 80% in the obese and morbidly obese[6,9,10].

The high prevalence of NAFLD in Western countries is probably due to the contemporary epidemics of obesity and associated metabolic complications. Obesity, type 2 diabetes, and hyperlipidemia are recognized as risk factors for NAFLD[5,11-14]. Insulin resistance is frequently detected in patients with NAFLD, as it is in those without obesity and diabetes[14-19]. An increasing number of patients have been described with normal body mass index (BMI), although these individuals may have central adiposity and occult insulin resistance[17,18,20,21]. Moreover, epidemiological studies[22-24] indicate that this unique group of normal weight patients is characterized by an unhealthy dietary composition, as will be discussed later.

The efficacy and safety profile of pharmacotherapy in the treatment of NAFLD remains uncertain[25], and obesity is strongly associated with hepatic steatosis[26]; therefore, the first line of treatment is lifestyle modification. The usual management of NAFLD includes gradual weight reduction and increased physical activity, leading to an improvement in serum liver enzymes, reduced hepatic fatty infiltration, and, in some cases, a reduced degree of hepatic inflammation and fibrosis[27-33]. However, most studies did not include repeated liver biopsy, and thus histological improvement could not be determined.

Although research is emerging, it remains uncertain whether diets that are enriched with certain types of food or nutrients are more likely to cause fatty liver than other types of diets[26]. In light of the difficulty in reducing weight and maintaining the weight reduction in the long term[34], changing dietary composition without necessarily reducing calorific intake may offer a more realistic and feasible alternative to treat NAFLD patients. Therefore, exploring the association between specific nutrients and dietary composition and NAFLD is extremely important.

This review discusses the existing epidemiological evidence for the association between human NAFLD and dietary composition, weight reduction, and physical activity.

THE AMOUNT AND TYPE OF DIETARY FAT

Total dietary fat vs carbohydrates

There are three major sources for the increased triglyceride deposition in the liver: excessive influx of free fatty acids (FFA) from endogenous fat depots, increased *de novo* hepatic lipogenesis, and exogenous-nutritional fat. Recent human studies suggest that a significant fraction of fatty acids are taken up by the liver during the postprandial period[35,36]. Furthermore, NAFLD patients may exhibit alterations in postprandial hepatic lipid metabolism. In a study using an oral fat load test in 15 Nonalcoholic steatohepatitis (NASH) patients and 15 controls, total and very low density lipoproteins triglyceride in postprandial plasma were higher in NASH compared with controls, and postprandial plasma Apo B48 and Apo B100 responses in NASH were flat. This suggested an increased hepatic uptake of triglycerides in the postprandial period combined with reduced hepatic secretion of VLDL, which may promote liver steatosis[37].

Multiple studies in animals have documented that a high-fat diet rapidly induces hepatic steatosis[38-40], but data in humans are scarce.

The association between total dietary fat and hepatic fat content has been directly tested in humans by placing 10 obese women on two successive two-wk isocaloric diets, which contained either 16% or 56% of energy from fat, in randomized order using a crossover design and assessing the liver fat by proton spectroscopy. Liver fat decreased by 20% on the low-fat diet and increased by 35% on the high-fat diet. The changes in liver fat were paralleled by changes in fasting serum insulin concentrations. Importantly, these changes were independent of body weight, which did not change during the study[36].

In another study, 74 morbidly obese patients (90% of them with NAFLD) undergoing bariatric surgery underwent a preoperative dietary evaluation using a 24-h food recall. Food intake was compared to liver histopathology from biopsies obtained during surgery. There were no significant associations between total calorific intake or protein intake and either steatosis, fibrosis, or inflammation. However, higher carbohydrate intake (above 54% of calories) was associated with significantly higher odds of inflammation, while higher fat intake was associated with significantly lower odds of inflammation. However, this study was unable to discern any differences in specific dietary fat composition, perhaps due to insufficient power or misclassification of fat intake based on a single 24-h recall and, importantly, did not differentiate between simple *vs* complex carbohydrates[41]. The association with carbohydrates is supported and sharpened by a study from Japan comparing dietary habits between 28 patients with NASH and 18 with simple steatosis, indicating an excess intake of carbohydrates, especially of sweets and not cereals, in the NASH group[42].

Although the results appear conflicting, it would seem reasonable to say that over-consumption of either fat or carbohydrates is not recommended, and eventually all mac-

ronutrients should be consumed according to the accepted recommendations (e.g. those of the American Heart Association), as part of a balanced diet. Furthermore, as will be demonstrated later in this review, the specific subtypes of fat (saturated *vs* unsaturated and its subgroups) and carbohydrates (complex *vs* simple and its subgroups) may be more important than their total amount.

Type of dietary fat and other nutrients

In contrast to cardiovascular and metabolic diseases, there is little epidemiological evidence that the type of dietary fat is associated with fatty liver[5]. A small sample size study, but with meticulous dietary assessment based on 7-d alimentary record, evaluated 25 normal-weight NASH patients compared with age-, gender-, and BMI-matched controls. The dietary intake of NASH patients was richer in saturated fat and cholesterol, and was poorer in polyunsaturated fat, fiber, ascorbic acid, and tocopherol[37]. These results are supported by another study in which the ratio of polyunsaturated/saturated fatty acid intake in both the NASH and fatty liver patients was lower than the ratio in randomly selected controls[42].

The type of dietary fat has also been demonstrated to be associated with oxidative stress markers in non-alcoholic steatohepatitis. Analyzing dietary intake obtained by a food frequency questionnaire in 43 NASH patients and 33 healthy controls, a negative correlation was found with total and saturated fat intake, and with the ratio of reduced Plasma glutathione/oxidized glutathione, indicating an impaired glutathione metabolism and suggesting a pro-oxidant effect. Conversely, a positive correlation was found with carbohydrates, fiber, monounsaturated fatty acids (MUFA), and polyunsaturated fatty acids (PUFA), specifically n-3 polyunsaturated fatty acid (n-3 PUFA)[43].

Different types of fats can have a protective effect in NAFLD. The most established one is the n-3 PUFA. Experimental studies have shown that diets enriched with n-3 PUFA increase insulin sensitivity in rats[44], reduce intra-hepatic triglyceride content, and ameliorate steatohepatitis[45,46].

Two observational studies provide evidence of a lower consumption of omega-3 PUFA among NAFLD patients. The first is a case-control study of 45 NASH patients compared with a sample of 856 controls, matched for sex and age[47]. Diet history assessed by a food frequency questionnaire (FFQ) demonstrated a significantly higher intake of n-6 fatty acids and a higher n-6/n-3 ratio among NASH patients. These results suggest that the quality and combination of fat intake may be more relevant than its isolated amount; an excessive amount of n-6 fatty acids could be implicated in promoting necro-inflammation[47].

The second study was a cross sectional study in 349 volunteers from the general population. Diet history assessed by an FFQ demonstrated higher meat intake ($P < 0.001$) and a tendency ($P = 0.056$) to a lower intake of fish rich in omega-3 in NAFLD patients. n-6 fatty acids are abundant in meat; therefore, these data suggest a higher intake of n-6/n-3 ratio in NAFLD patients (Zelber-Sagi, Nitzan-Kaluski, Goldsmith, Webb, Blendis, Halpern *et al*, 2007).

Two pilot clinical trials support the protective role of omega-3 PUFA in NAFLD. The first was a nonrandomized open-label controlled trial that assessed the effect of a one-year n-3 PUFA supplementation (containing both eicosapentaenoic acid-EPA and docosahexaenoic acid-DHA) at a dose of 1000 mg/d in 42 NAFLD patients versus 14 patients that refused the treatment and were analysed as controls. PUFA supplementation significantly decreased serum liver enzymes (ALT, AST, and GGT) and reduced liver fat (as measured by ultrasonography) as compared to controls[48]. The second study was a non-controlled trial in 23 NASH patients that were supplemented with 2700 mg/d of EPA for one year. Serum ALT levels were significantly improved. Seven of the 23 patients underwent post-treatment liver biopsy, which showed improvement of hepatic steatosis and fibrosis, hepatocyte ballooning, and lobular inflammation in six patients[49]. In both trials, body weight remained unchanged.

There are two types of fat, trans fatty acids (TFA) and MUFA, which so far have not been tested or demonstrated to be associated with human NAFLD. However, based on their association with related diseases, such as diabetes and cardiovascular disease, should be considered in the nutritional recommendations of NAFLD.

Trans fatty acids

Little is known about the role of TFA in promoting liver injury in NAFLD. The association between TFA and increased risk of developing insulin resistance[50] and coronary heart disease by raising LDL cholesterol levels, lowering HDL cholesterol levels, raising triglyceride levels, and increasing CRP[51] suggest that it may be involved in NAFLD pathogenesis.

Compared with PUFA and saturated fatty acids-fed mice, TFA-fed mice had impaired glucose tolerance, characterized by greater homeostasis model assessment (HOMA), and NASH-like lesions due to greater hepatic lipogenesis[52]. In another experiment, the effect of a combination of features of a western lifestyle was tested. In mice fed TFA in a high-fat diet, high-fructose corn syrup, and interventions designed to promote sedentary behavior, isocaloric replacement of TFA with lard indicated that TFA played a major role in promoting hepatic steatosis and injury[53]. Therefore, the role of TFA in human NAFLD needs to be evaluated, which presents a challenge to nutritional epidemiologists, as information on TFA content in food is unknown in many cases.

Monounsaturated fatty acids

n-9 oleic acid is the most prevalent MUFA in the diet, and olive oil is one of its major sources (other sources are nuts and avocado). MUFA has been demonstrated to have a favorable effect on the lipid profile, with a reduction in both the LDL and total cholesterol to HDL ratio[54]. A meta-analysis of randomized, crossover trials comparing low-saturated-fat, high-carbohydrate diets or high-MUFA diets

in patients with type 2 diabetes revealed that high-MUFA diets improve lipoprotein profiles as well as glycemic control. High MUFA diets reduce fasting plasma triacylglycerol and VLDL-cholesterol concentrations by 19% and 22%, respectively, and cause a modest increase in HDL-cholesterol concentrations without adversely affecting LDL-cholesterol concentrations[55].

In rats with an MCD diet, olive oil was demonstrated to decrease the accumulation of triglycerides in the liver by 30% compared with the only MCD diet group. The serum triglycerides increase was 10% lower in the MCD diet + olive oil group compared with the MCD group. Olive oil improved insulin resistance, increased the release of Triglycerides from the liver, and decreased the flux of FFAs from peripheral adipose tissue back to the liver[56]. In rats, treatment with a balanced diet rich in olive oil contributed to the recovery of the liver from hepatic steatosis[57]. Olive oil, in contrast to polyunsaturated oils, was demonstrated to protect against the development of fibrosis[58].

However, it has not been demonstrated that NAFLD patients eat less MUFA as compared to controls[23,24,59], and the role of MUFA or olive oil in human NAFLD is yet to be demonstrated.

Cholesterol

With regard to cholesterol, results from observational studies have been conflicting. Some studies did not demonstrate different dietary intakes of cholesterol between NAFLD patients and controls[47,59]. However, Musso *et al*[23] did demonstrate a higher cholesterol consumption among normal weight NASH patients *vs* BMI matched controls. A recent study supported the role of dietary cholesterol in NAFLD. In this study, 12 normal weight NAFLD patients were compared to 44 obese NAFLD patients. A characteristic feature was that dietary cholesterol intake was significantly higher, while the intake of polyunsaturated fatty acids (PUFAs) was significantly lower, in the nonobese group. Similar differences were noted in comparison to 15 healthy non-obese controls. Therefore, this altered cholesterol and PUFA intake may be associated with the development of NAFLD in non-obese patients[24].

In addition, studies using non-obese animal models have confirmed that a hypercholesterol diet can induce NASH[60]. An increase in dietary cholesterol is suggested to induce *de novo* fatty acid synthesis in hepatocytes *via* the LXRa-SREBP-1c pathway[24].

In conclusion, studies testing the association with different types of dietary fats in normal weight NAFLD patients may clarify nutritional composition as a direct risk factor.

THE ASSOCIATION BETWEEN ADDED SUGAR AND SOFT DRINKS CONSUMPTION AND NAFLD

Soft drinks are a leading source of artificially added sugar in the world[61].

In recent decades, intake of sugar-sweetened beverages has increased around the globe[62]. Recent data (2005-2006) show that children and adults in the United States consume about 172 and 175 kcal/d, respectively, per capita from sugar-sweetened beverages[63]. The consumption of sugar-sweetened beverages has been linked to risks for obesity, diabetes, metabolic syndrome, fatty liver, and heart disease, possibly by providing excess calories and large amounts of rapidly absorbable sugars[59,64-69]. In a recently published health policy report, taxation of sugar-sweetened soft drinks has been proposed as a means of reducing the intake of these beverages and thereby lowering disease burden and health care costs[62].

A sucrose-rich diet increases the hepatic synthesis of triglycerides. Rats and humans that are fed either sucrose- or fructose enriched diets develop fatty livers[65,70]. Therefore, it is reasonable to suggest that NAFLD patients should limit their fructose consumption[71]. In addition, cola soft drinks contain caramel coloring, which is rich in advanced glycation end products (AGEs), which can increase insulin resistance and inflammation[61].

In recent years, several studies have been published on the association between soft drinks consumption and NAFLD, demonstrating a positive association[22,59,72,73]. The first was a cross-sectional study of a sub-sample (n = 375) of the Israeli National Health and Nutrition Survey (MABAT 1999-2001). A semi-quantitative food-frequency questionnaire was administered and showed that NAFLD patients have a higher intake of soft drinks. Moreover, the higher intake of soft drinks was associated with an increased risk of NAFLD, independently of age, gender, BMI, and total calories[59].

In a study on 31 normal weight NAFLD patients with no obvious classic risk factors and 30 healthy controls matched for gender and age, it was found that NAFLD patients consume significantly higher amounts of added sugar and that most of it (43%) comes from soft drinks and juices, compared to only 8% in the controls[22]. Another study by the same group demonstrated similar results; 80% of patients with NAFLD had excessive soft drinks intake (> 500 cc/d) as compared to 17% in controls[73].

In a recent study, the consumption of fructose containing beverages was compared in NAFLD patients and controls matched for gender, age, and BMI. It was demonstrated that consumption of fructose in patients with NAFLD was two-fold higher compared to matched controls[72].

Recently, a large-scale study of 427 NAFLD patients expanded the understanding of the hepatic damage that may be related to over-consumption of fructose-containing beverages. After controlling for age, sex, BMI, and total calorie intake, daily fructose-containing drinks consumption was significantly associated with higher fibrosis stage (OR = 3.2, 1.4-7.4 95% CI for $\geq$ 7 servings *vs* < 7 per week) in both younger and older age groups, and a lower steatosis grade, but only in the older group of patients[74].

Thus, these studies identified an important modifiable risk factor. Physicians and dietitians should routinely in-

clude questions regarding soft-drink consumption as part of the patient's history and advise patients to avoid it.

WESTERN DIETARY PATTERN AND FAST FOOD

Examination of overall dietary patterns would more closely parallel the real world, where people eat meals consisting of a variety of foods with complex combinations of nutrients that may be interactive or synergistic[75]. The studies presented above regarding dietary composition usually provide indications for several nutrients or foods that characterize the dietary intake of NAFLD patients, and may be looked at as an unhealthy pattern or western dietary pattern. This pattern seems to include overconsumption of fructose and soft drinks[22,59,72], lower consumption of fiber[47], overconsumption of meat[59] or saturated fat and cholesterol[23,24], lower consumption of fish or omega-3 fatty acids[47,59] or PUFA[24], and lower consumption of some vitamins[23], which may indicate a below the recommended consumption of vegetables and an unbalanced diet in general.

Fast-food consumption has strong positive associations with weight gain and insulin resistance in humans. In the CARDIA study, a 15-year prospective follow up of 3031 young adults, those with frequent (more than twice a week) visits to fast-food restaurants gained an extra 4.5 kg of bodyweight and had a two-fold greater increase in insulin resistance compared to participants with infrequent (less than once a week) fast-food consumption[76]. Furthermore, feeding experimental animals with the "cafeteria diet" (a feeding regimen similar to fast food) leads to liver damage[53].

What happens if we do the same experiment in humans? 18 healthy, young students were put on a fast food diet that included at least two fast food meals a day for four weeks. They increased their caloric intake and body weight and their HOMA values doubled. Hepatic triglyceride content increased, as did serum ALT levels. After the intervention started, 11 out of 15 with normal ALT levels at baseline had elevated ALT levels at one week; eight had persistent elevation during the intervention; and two had persistent elevation even at six months follow up. Thus, in clinical evaluations of subjects with elevated ALT levels, medical history should include not only questions about alcohol and soft drinks intake, but should also explore whether recent excessive intake of fast food has occurred[77].

Potential mechanisms of hepatotoxicity are high energy density & portion size, high fat & saturated fat, high refined carbohydrate, low fiber, high fructose corn syrup, caramel coloring, red meat, industrially produced trans fatty acids, promoting free fatty acid overflow to the liver and local inflammation[78].

WEIGHT REDUCTION

Throughout the past decades, three types of trials have dealt with weight reduction in NAFLD. Historically, the first ones, in the 1960s, tested very low calorie diets (VLCD) leading to drastic weight reduction. These diets were based on fasting and formulas and did not discuss behavioral therapy at all. Thereafter, a second wave of trials explored a more balanced diet, sometimes combined with physical activity, only mildly referring to behavioral therapy and sometimes with short-term follow up. More recently, structured balanced diets combined with a detailed behavioral therapy program aiming at a long-term lifestyle modification have been evaluated.

Examples of the first generation are small sample trials from the 1960s[79] and 1970s[80] that included fasting or very low calorie diet (about 500 kcal), leading to drastic weight reduction. Steatosis was reduced in all patients, but liver damage, as indicated by fibrosis and focal necrosis, was observed in some patients during the acute weight loss. In a later study from 1991, Andersen *et al*[81] provided 41 morbidly obese patients with a 400 kcal formula-based diet, again leading to improved steatosis. However, 24% developed slight portal inflammation ($P = 0.039$) or slight portal fibrosis ($P = 0.063$). This study helps in setting the upper limit for the rate of weight reduction in NAFLD patients, as none of the patients who lost less than 1.6 kg/wk developed fibrosis. Interestingly, liver biochemistry improved regardless of the histological changes[81]. In another VLCD study, a weight reduction of greater than or equal to 10% resulted in normalized abnormal hepatic test results in most patients; however, liver biopsies were not obtained[82].

Two small sample size studies tested the effect of a balanced diet and gradual weight reduction on liver histology. Ueno *et al*[83] demonstrated significant reductions in hepatic steatosis after only three months on treatment. Hepatic inflammation and fibrosis also improved, although not significantly, probably because of the short follow up. The one year long term trial included behavioral therapy, with regular meetings with a dietitian and group sessions, and weekly food records. Nine out of 15 patients, who lost an average of 7% of their body weight, had an improved NASH score, and the remaining six, who had no weight loss, had stable scores[84].

The next study is one of a few RCT's testing weight reduction in NAFLD. In a 48-wk intervention, 32 NASH patients were randomized to receive intensive lifestyle intervention or basic education about healthy lifestyle (controls). A moderate, balanced diet was combined with moderate-intensity activities, with particular emphasis on walking with pedometers. Classical behavioral strategies were also extensively applied: self-monitoring of eating and exercise, stimulus control techniques, problem solving *etc*. NASH histological activity score (NAS) improved significantly in the treatment arm in comparison with the control group. Participants who achieved weight loss of > 7% compared with those who lost less than 7%, had significant improvements in steatosis, lobular inflammation, ballooning injury, and NAS[85]. In the Orlistat trial by Harrison *et al*[86], a somewhat bigger weight reduction of at least 9% was necessary to achieve sig-

nificant improvement in NAS, although 5% reduction was sufficient for improving steatosis. Recently, another RCT tested the effect of a 12-mo intensive lifestyle intervention on hepatic steatosis in a specific subgroup of patients with type 2 diabetes. The intervention included a moderate calorific restriction plus increased physical activity and weekly meetings, whereas the control group received only general information on nutrition and physical activity. After 12 mo, participants assigned to the intensive intervention, as compared to controls, lost more weight (-8.5% *vs* -0.05%; $P < 0.01$) and had a greater decline in steatosis measured by H-MRS (-50.8% *vs* -22.8%; $P = 0.04$). The intervention was also beneficial in prevention of NAFLD, as 26% of controls *vs* 3% of intervention participants, without NAFLD at baseline, developed NAFLD at 12 mo[87].

Three recent, relatively large sample size studies addressed the effect of diet, provided in different settings, on ALT levels[88-90]. In the trial by Suzuki *et al*[89] 348 male subjects with elevated ALT were recruited from annual health checkups, and were given health care instructions using customized brochures and then followed at health checkups three times a year. At one year follow up, all subjects achieving ⩾ 5% weight reduction showed improvement in serum ALT and 136 subjects had ALT normalization. In the second trial 152 patients with elevated liver enzymes were randomized to either a moderate (6 sessions/10 wk) or low-intensity (3 sessions/4 wk) lifestyle counseling intervention or control group. Reduction in liver enzymes was greatest in the moderate-intensity intervention group and least in the control group, in parallel to the proportion of subjects achieving weight loss[88]. In the third trial, with a smaller sample size, 67 patients with NAFLD were enrolled into a 6-mo home-based lifestyle modification intervention, which included monthly visits with a physician and nutritional counselling every three months. At six months, there were significant improvements in terms of body weight, liver/spleen ratio, and liver enzymes. This study's flaw was a large attrition rate, with only 22 patients (33%) completing the 6-month intervention[90], perhaps indicating that patients require a more intensive follow up.

The challenge in demonstrating the therapeutic efficacy of weight reduction in NAFLD has been the lack of liver histology as an outcome in most studies, or on the other hand, the limited sample size and statistical power whenever liver biopsy is undertaken because of its invasive nature. Liver biopsy is necessary for the evaluation of therapeutic effects beyond reduction in ALT and regression of steatosis on imaging (or only disappearance of steatosis when simple ultrasound is applied).

This is especially important because certain diets may seem beneficial according to reduction of liver enzymes, while actually leading to liver damage that can only be observed by liver biopsy. Future advances in identification and validation of non-invasive methods for hepatic fibrosis, inflammation, and quantification of steatosis should help determine if weight reduction is effective for treating all the features of NAFLD, at which stages of the disease should weight reduction be introduced, the optimal weight reduction rate and nutrient composition. According to research so far, although mostly small sample size trials have been performed, the results are consistent and indicate that weight reduction can be considered as an established treatment.

PHYSICAL ACTIVITY

From the perspective of NAFLD patients, weekly or daily performance of walking, swimming, or cycling might seem as simple as jumping of the cliff.

As with diet, low long-term compliance is also the rule for increased physical activity: on average 20% after two years follow up[91]. Despite the difficulties, increased physical activity (PA) is highly beneficial. Indeed, PA has been shown to reduce the risk of T2DM, insulin resistance, hypertension, dyslipidemia, impaired fasting glucose (IFG), and the metabolic syndrome[92-95]. This indicates that PA could play a role in the treatment of patients with NAFLD.

Several observational studies indicated an inverse association between reported leisure time PA, or cardiorespiratory fitness, and the prevalence of NAFLD. In a large-scale study ($n = 349$) of the general population, the NAFLD group engaged in less reported leisure time PA, including total, aerobic, and resistance. Engaging in any kind of PA remained significant after adjusting for insulin resistance and circulating adiponectin plus nutritional factors, but not BMI. Only the association with resistance PA remained significant with further adjustment for BMI[96]. A large-scale study ($n = 218$ men) demonstrated an inverse association between fitness categories and the prevalence of NAFLD, regardless of BMI[97]. In a small study on 37 NAFLD patients with liver biopsy, there was a lower cardiorespiratory fitness among patients with higher NAFLD activity score and NASH versus no NASH[98].

The beneficial effect of exercise is supported by recent clinical trials. The first[99] included 141 patients with suspected NAFLD based on abnormal liver enzymes and exclusion of other causes of liver disease who were randomized to either the intervention arms (three months physical activity counseling delivered at three intensity levels) or a control arm. Patients who increased their PA by ⩾ 60 min per week ($n = 85$) significantly reduced their weight (-2.4 kg on average), HOMA, and all liver enzymes. Importantly, these improvements were independent of the change in weight. These results are supported by a previous pilot trial demonstrating that moderate intensity aerobic exercise helped to normalize ALT levels in 65 NASH patients receiving moderately energy-restricted diet[100], although this improvement cannot be regarded as independent of weight loss. Thus, it seems that among NAFLD patients, even small increments in regular PA can improve liver enzymes; encouraging information that can be provided to patients. Another recent trial assessed the effect of short-term (four weeks) aerobic exercise training

on hepatic, blood, abdominal, and muscle lipids in 19 sedentary obese men and women using magnetic resonance imaging and proton magnetic resonance spectroscopy (1H-MRS). Four weeks of aerobic cycling exercise (three cycle sessions per week (30-45 min) significantly reduced mean hepatic triglyceride concentration by 21%, along with a 12% reduction in visceral adipose tissue volume and a 14% reduction in plasma free fatty acids. Importantly, no change in weight or dietary intake was noted, thus isolating the net effect of aerobic exercise[101].

Higher cardiorespiratory fitness at baseline may contribute to a successful hepatic outcome during lifestyle modification that includes dietary counseling and exercise. Among the parameters predicting the change in liver fat, fitness at baseline emerged as the strongest factor, independently of exercise intensity during the intervention. However, it should be remembered that cardiorespiratory fitness reflects not only recent physical activity habits, but also genetics[102].

In a recent study, 12 obese adolescents underwent a three-month resistance exercise program consisting of 2 × 1 h/wk, exercising all major muscle groups. The exercise program resulted in significant strength and lean body mass gain. Although hepatic fat content remained unchanged, hepatic insulin sensitivity increased and glucose production rate decreased, without weight loss[103]. Although aerobic exercise seems to have more extensive effects, a longer duration and/or a more intensive resistance exercise program may be required for reduction of hepatic fat content. For those who have physical limitations or low motivation that prevents them from performance of aerobic PA, resistance exercise can serve as an alternative option.

PA benefits NAFLD beyond encouraging weight reduction. Exercise alone, in the absence of any change in body weight or composition, may enhance insulin sensitivity and glucose homeostasis[104]. PA appears to result in insulin-receptor upregulation in muscle tissue and hence increased delivery of glucose and insulin to the muscles[105]. Exercise also has a beneficial effect on FFA metabolism, by enhancing whole-body lipid oxidation[106]. Hepatic triglyceride accumulation was shown to decrease with exercise intervention[107] and hepatic FFA uptake was lower in trained (endurance training) compared to untrained male subjects[108]. Similar findings were demonstrated in comparing monozygotic male twins that had a marked difference in leisure-time physical activity and aerobic fitness, where, in the absence of the confounding effects of genetic factors, the active twin had decreased hepatic FFA uptake[106].

In recent years, increasing attention has been paid to resistance training as a useful adjunctive tool of exercise[109,110]. A recent study showed that resistance training, without a concomitant weight loss diet, significantly improved insulin sensitivity and fasting glycemia and decreased abdominal fat[111]. Tsuzuku *et al*[112] demonstrated that non-instrumental resistance training, using body weight as a load, appears to be effective in decreasing visceral fat and improving metabolic profiles, without weight loss. The results of a randomized trial comparing the effect of aerobic *vs* resistance training on coronary risk factors, demonstrated that only the resistance training group showed a reduction in total body fat, with an associated increase in lean body mass[113]. A meta-analysis comparing aerobic training with weight training concluded that weight training resulted in greater increases in fat-free mass[114]. An increase in muscle mass may improve insulin sensitivity by increasing the available glucose storage area, thereby reducing the amount of insulin required to maintain a normal glucose tolerance[115].

The Centers for Disease Control and Prevention (CDC), the American Heart Association (AHA), and the Healthy People 2010 Objectives recommend adults to attain ⩾ 30 min of moderate-intensity physical activity on most, and preferably all, days of the week, or vigorous-intensity physical activity ⩾ 3 times per week for ⩾ 20 min each time. Although these recommendations have been widely publicized, only 27.7% US adults meet recommended levels of either moderate or vigorous physical activity, whereas 29.2% report no regular physical activity outside of their work[116,117]. Moreover, the prevalence of physically active adults among patients with diabetes is lower than in those without diabetes[118] and subjects with diabetes are less likely to meet physical activity recommendations[119].

In NAFLD patients, compliance may be even lower because fatigue has been demonstrated to be markedly higher in NAFLD patients compared to controls, and is associated with inactivity and excessive daytime sleepiness[120].

Apparently, the empty half of the glass - sedentary time - is by itself associated with metabolic status. Time spent sedentary, measured objectively by individually calibrated heart rate monitoring, predicted higher levels of fasting insulin, independent of the amount of time spent at moderate- and vigorous-intensity activity levels. This highlights the importance of reducing sedentary time in order to improve metabolic status, in addition to the benefits associated with a physically active lifestyle[121].

Environmental factors that discourage physical activity include an environment that encourages automobile use rather than walking (like lack of sidewalks), and that has few cues to promote activity and numerous cues that discourage activity (television, computers *etc*)[122].

POTENTIALLY THERAPEUTIC DIETARY SUPPLEMENTS

Vitamin E

Treatment with vitamin E (α-tocopherol) at high doses of 300-1000 IU/day (about 30 IU is the Recommended Dietary Allowance) has demonstrated conflicting results when leading to reduction of liver enzymes in an uncontrolled trial[123], but failed to show significant added value over lifestyle modification in controlled trials[124,125].

In a recent randomized, large long-term clinical trial, 247 NASH patients without diabetes were randomized to three arms: pioglitazone at a dose of 30 mg/d, vitamin E at a dose of 800 IU daily (84 subjects), or placebo

(83 subjects), for two years. Only vitamin E therapy, as compared with placebo, was associated with a significantly higher rate of improvement in nonalcoholic steatohepatitis (43% *vs* 19%, $P = 0.001$). Serum alanine and aspartate aminotransferase levels were reduced with vitamin E and with pioglitazone, as compared with placebo and both agents were associated with reductions in hepatic steatosis, but not with improvement in fibrosis scores[126]. Despite these promising results, treatment with high dose vitamin E should be carefully considered due to its troubling association with increased risk for hemorrhagic stroke[127] and all-cause mortality[128] in randomized controlled trials. Indeed, the authors of the study indicate that cardiovascular events occurred with equal frequency in all three study groups; however, the trial was too small to detect meaningful differences in the incidence of cardiovascular events[126].

Vitamin D

Increasing evidence suggests that vitamin D may have an important role in modifying risk for cardiometabolic outcomes, including type 2 diabetes, hypertension, and cardiovascular disease[129-131]. Serum 25(OH)D levels were demonstrated to be independently associated with both insulin sensitivity and beta-cell function among individuals at risk of type 2 diabetes[132].

Recently, serum 25(OH) Vitamin D concentrations have been shown to be associated with NAFLD[133]. Targher[133] *et al* studied circulating 25(OH)D3 in 60 consecutive patients with biopsy-proven NAFLD, and 60 healthy controls of comparable age, sex, and BMI, and found reduced levels in those with NAFLD (51.0 ± 22 nmol/L *vs* 74.5 ± 15 nmol/L, $P < 0.001$). The differences in 25(OH)D concentrations observed between the groups were little affected by adjustment for age, sex, BMI, creatinine, calcium, HOMA-insulin resistance, and the presence of the metabolic syndrome. Furthermore, among NAFLD patients, decreased 25(OH)D concentrations were closely associated with the histological severity of hepatic steatosis, necroinflammation, and fibrosis ($P < 0.001$ for all). In a recent abstract, the association between vitamin D concentration, fatty liver, and coronary artery disease (defined as a stenosis of > 50% in at least one major coronary artery by cardiac CT) was tested in 60 patients with NAFLD compared to 30 sex, age matched healthy controls. Patients with NAFLD showed lower vitamin D concentration (13 ± 8 ng/mL *vs* 31 ± 4 ng/mL, $P < 0.001$) and severe vitamin D deficiency (< 12 ng/mL, OR 2.5, 95% CI 1.5-4.6, $P < 0.01$) predicted coronary artery disease independent of metabolic syndrome[134].

Currently, the association between vitamin D status and NAFLD and its potential therapeutic role warrants further research.

CONCLUSION

NAFLD is not only a cause of chronic liver disease and a component of the metabolic syndrome, but might also predict the tendency to develop diabetes mellitus[135-137] and has also been suggested to be associated with coronary artery disease[138-144]. In terms of public health, it will be important to detect NAFLD at a relatively young age, prior to other metabolic complications, because the treatment of NAFLD will be part of the primary prevention of type-2 diabetes and coronary artery disease.

Identifying modifiable risk factors for prevention and treatment of NAFLD is therefore important. Nutrition has been demonstrated to be associated with NAFLD and NASH in both animals[65,145] and humans[22,36,47,59,73,85], and thus serves as a major route of prevention and treatment. However, most human studies are observational and retrospective, allowing limited inference about causal associations. Furthermore, nutritional studies that rely on reported recall of diet are prone to information bias that could weaken existing associations and underestimate the contribution of certain nutrients to the pathogenesis of NAFLD. This limitation can be minimized by meticulous methods of dietary assessment (e.g. obtaining more repeated dietary recalls from each patient) to reduce measurement error, and using a larger sample size that will provide sufficient statistical power, which might uncover associations between nutrients and NAFLD. Large prospective studies and clinical trials are now needed to establish a causal relationship.

Currently no firm recommendations can be formulated, because of the lack of high quality, evidence-based data with hepatic histological outcomes. However, based on available data, patients should optimally achieve a 5%-10% weight reduction. A recent position statement on NAFLD/NASH[146] recommended on a weight loss of 7%, as proposed by International Societies on the basis of an extensive body of literature. Setting realistic goals is essential for long-term successful lifestyle modification[147,148], because obese patients tend to have unrealistic weight loss expectations (about 25%-35%) that if unmet, lead to adverse effects, such as lower satisfaction with treatment and a lower self-esteem[149]. More effort must be devoted to informing NAFLD patients of the health benefits of even a modest weight reduction, and feedback should be provided not only on weight loss, but also on individual changes in behavior and risk factors[148].

Furthermore, all NAFLD patients, whether obese or of normal weight, should be informed that a healthy diet has benefits beyond weight reduction. They should be advised to reduce saturated/trans fat and increase polyunsaturated fat with special emphasize on omega-3 fatty acids. They should reduce added sugar to its minimum, try to avoid soft drinks containing sugar (including fruit juices that contain a lot of fructose) and increase fiber intake. For the heavy meat eaters, especially those of red and processed meats, less meat and increased fish intake should be recommended. Minimizing fast food intake will also help maintain a healthy diet. Physical activity should be integrated into behavioral therapy in NAFLD, as even small gains in PA and fitness may have significant health benefits. A combination of educational, behavioral, and motivational strategies is required to help patients achieve lifestyle change[148]. Preferably, this should be

provided by multidisciplinary teams including dietitians, psychologists, and physical activity supervisors[150,151]. "Let food be your medicine" said Hippocrates; so should say more and more physicians to their NAFLD patients.

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ORIGINAL ARTICLE

Trend in gastric cancer: 35 years of surgical experience in Japan

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Abstract

AIM: To investigate the trend in gastric cancer surgery in the context of rapid therapeutic advancement in Japan and East Asia.

METHODS: A retrospective analysis was performed on 4163 patients who underwent gastric resection for gastric cancer with histological confirmation between 1971 and 2007 at the surgical unit in Kitasato University Hospital, to determine the trend in gastric cancer requiring surgery.

RESULTS: Gastric cancer requiring surgical resection increased in our hospital, but the incidence adjusted for population was constant during the observed period. Interestingly, the ratio of diffuse type/intestinal type gastric cancer was unexpectedly unchanged, and that of advanced/early gastric cancer (EGC) was, however, markedly reduced, while the actual incidence of potentially curative advanced gastric cancer tended to decrease. The incidence of EGC requiring surgery tended to increase as a whole, which is consistent with increased prevalence of endoscopic surveillance. As a result, overall survival and mortality of gastric cancer requiring gastric resection has recently markedly improved.

CONCLUSION: In Japan, planned interventions may improve surgical gastric cancer mortality, but an unexpected trend of persistent existence of intestinal type cancer suggests the need for more robust medical intervention.

Key words: Histology; Age factors; Clinical classification; Prognosis; Disease progression; Gastric cancer

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INTRODUCTION

Gastric cancer was newly diagnosed in 934 000 people worldwide in 2002, accounting for 8.6% of new cancers.

It was the fourth leading cancer, following lung, breast, and colorectal cancer. In addition, it was the second leading cause of cancer deaths worldwide, with approximately 700 000 deaths reported in 2002, indicating that gastric cancer had a poorer prognosis than breast and colorectal cancer. Because of improvements in food storage methods putatively associated with infection-related gastric cancer, gastric cancer incidence and mortality have fallen dramatically over the past 70 years worldwide. Although its prevalence is being reduced in most advanced countries, the number of patients is expected to increase up to 1 100 000 in 2010 as the population ages worldwide[1].

Demographic trends in gastric cancer differ by tumor location and histology. While there has been a marked decline in distal, intestinal type gastric cancers, the incidence of proximal, diffuse type adenocarcinomas of the gastric cardia has been increasing, particularly in Western countries. For example, in the USA, gastric cancer was the leading cause of death in the 1930s, but mortality rates have fallen dramatically despite no planned prevention, while diffuse type, proximal gastric cancer is sharply increasing[2]. Diverging trends in the incidence of gastric cancer by tumor location or histology suggest that they may represent two diseases with different etiologies. The main risk factors for distal gastric cancer include *Helicobacter pylori* (*H. pylori*) infection and dietary factors, whereas gastroesophageal reflux disease and obesity play important roles in the development of proximal stomach cancer. Intestinal type tumors predominate in high risk geographical areas, such as East Asia including Japan, and a decline of the intestinal type gastric cancer in the distal stomach is believed to account for most of the decrease in gastric cancer rates worldwide[3]. Detailed epidemiological data will help guide future cancer control strategies, and we herein investigate the recent Japanese trend in gastric cancer requiring surgery to provide useful cancer information.

MATERIALS AND METHODS

The current study was conducted as a retrospective analysis of 4163 gastric cancer patients who underwent gastrectomy at the Kitasato University Hospital between September 1971 and March 2007 and had proven histology of adenocarcinoma of the stomach. The patients who underwent surgery without removal of stomach tissue, and had no histology were initially ruled out (almost stage Ⅳ disease or any stage with invasion of the pancreas head). Histological information was collected from the formal documents of the pathological division in the Kitasato University Hospital (the East and Main hospital). Incidence and mortality corrected for the population in the Sagamihara city were calculated between 1972 and 2006 (35 years), because the years 1971 and 2007 did not have full annual data. Histology was shown as pap, tub1, tub2, por1, por2, sci, and muc for adenocarcinoma. Intestinal type gastric cancer was defined as pap, tub1, and tub2, and diffuse type as por1, por2, sci, and muc. Among the cases, some cancers were included in both cancers, and such cases were defined as mixed type. Early gastric cancer (EGC) was defined as mucosal or submucosal cancers irrespective of lymph node metastasis status. Advanced gastric cancer (AGC) was defined as cancers invading beyond the muscularis proprior.

For all EGC, curative operations were successfully performed, while lymph node dissection was predominant for D2 during the early phase. According to the guideline for gastric cancer in 2001, lymph node dissection had gradually transitioned from D2 to D1+α (No. 7) or β (No. 7, 8, 9) dissection. For AGC stages Ⅰ to Ⅲ, D2 dissection had been the standard option for surgery, if severe complications were not present or in very elderly patients. R1 or R2 surgery was performed in 3% of stage Ⅰ to Ⅲ patients, who largely had positive margins of the esophagus or duodenum. For stage Ⅳ AGC, various operative procedures were selected according to the individual surgeons. As a whole, the earlier operations tended to be extended to include D2, D3 or even D4 lymph node dissection, while more recently D0 or D1 lymph node dissection became prevalent for cases with peritoneal dissemination and/or positive cytology (CY1) or distant metastasis. The staging system used was the 13th Japanese Classification of Gastric Cancer in all recent cases, but before 2000, there was no consistent information on CY status. CY1 was the initial staging, which could affect operation performance, and we also have to allow for changes in staging classification before and after 2000.

The population of Sagamihara city was determined from published city data (http://www.city.sagamihara.kanagawa.jp/toukei/gaiyo/001902.html). The incidence of disease was calculated by the following formula: number of gastric cancer patients requiring surgery in the surgical unit of Kitasato University/population in Sagamihara city × 100 000.

Statistical computations were performed using SAS software package (SAS Institute, Cary, NC), Stat View version 5.0. The Kaplan-Meier method was used to estimate cumulative survival rates, and differences in survival rates were assessed using the log rank test. A P value < 0.05 was considered statistically significant. The time of follow-up was calculated from the date of surgery. Mortality rates were calculated by the following formula: total deaths during the observed term/sum of the population in Sagamihara city during the same term. Cases were censored within 5 years, although these were below 1%, and were not judged as deaths.

RESULTS

Incidence of gastric cancer requiring surgical resection is constant in Kitasato University Hospital

The surgical unit of Kitasato University Hospital was opened in 1971, and the number of gastric cancer patients requiring surgical resection has been increasing until recently as shown in Figure 1A. Intriguingly, the ratio of intestinal type gastric cancer to diffuse type gastric cancer has not declined like the trend worldwide or in Western countries (Figure 1A and B). The majority of patients requiring surgery had been referred by neighbor-

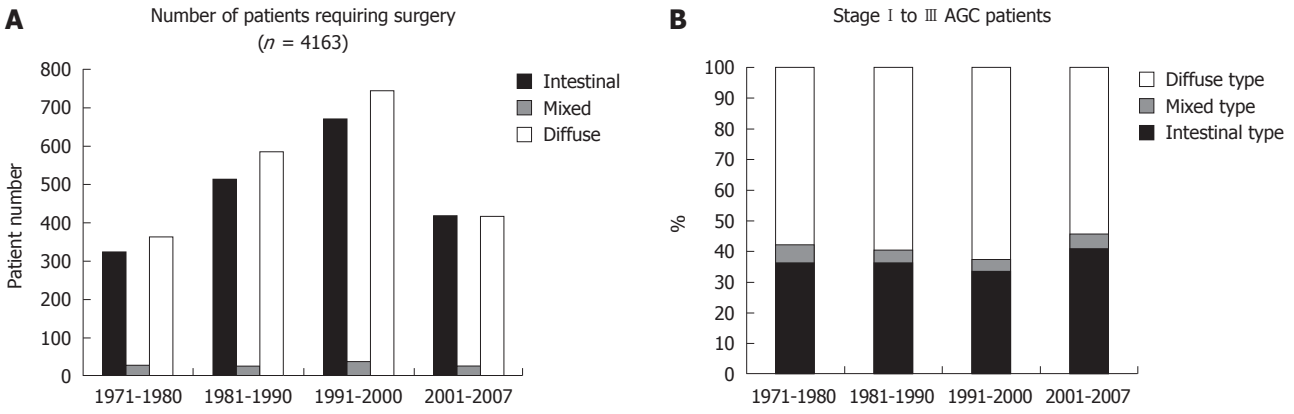

Figure 1 Trends in gastric cancer patients in Kitasato University Hospital. A: Trend in the numbers of patients requiring surgery, according to 3 histology categories, namely intestinal, mixed, and diffuse type; B: Trend in the proportion of intestinal type advanced gastric cancer (AGC) of stages Ⅰ to Ⅲ. Note that the proportion of intestinal type AGC did not decline during the time period (the final time period was 7 years only).

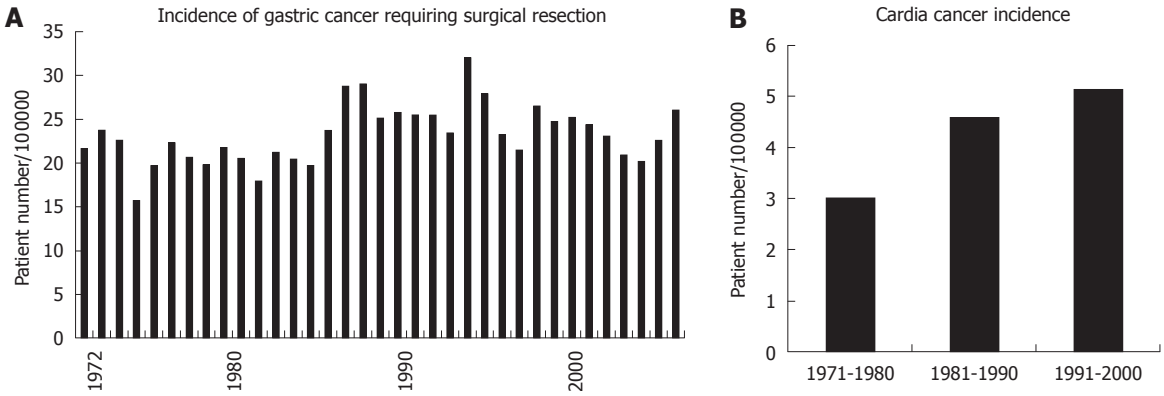

Figure 2 Incidence of gastric cancer treated in Kitasato University Hospital, according to the population of Sagamihara city. A: Incidence is shown between 1972 and 2006, because full annual data for 1971 and 2007 was not available. The incidence of gastric cancer requiring surgery did not decline; B: Incidence of gastric cardia cancer has been increasing until recently. Incidence is per 100 000 population.

ing clinics or middle-scale hospitals or the Gastrointestinal Division of Internal Medicine at Kitasato University. The hospital is located in Sagamihara city (population about 700 000 in 2010), Kanagawa Prefecture, Japan, and is a dormitory town for the Tokyo and Yokohama area, with a markedly increasing population in recent years. The incidence of gastric cancer requiring surgery largely ranged between 20 and 30 per 100 000 population, and it was unexpectedly constant during the nearly 35 years of the data (Figure 2A), which is consistent with the Japanese trend as a whole. The incidence of gastric cancer in Japan is the highest in the world, and it was calculated to be 69.2 in men and 28.6 in women per 100 000 population[4]. Allowing for inoperable patients or those who were treated by endoscopic resection, a considerable proportion of patients requiring surgery in the Sagamihara city were sent to our hospital, putatively representing the Japanese trend of gastric cancer requiring surgical resection. This trend is very different from that worldwide or in other developed countries.

Altered distribution of EGC and AGC requiring surgical resection

Despite a constant incidence of gastric cancer requiring surgery, the ratio of EGC to AGC persistently increased from low rates (about 30%) to similar rates (about 50%) (Figure 3). The actual numbers of gastric cancer cases are shown in Figure 4. We subdivided gastric cancer requiring surgical resection into 4 categories, namely mucosal EGC (Early-M), submucosal EGC (Early-SM), AGC stage Ⅰ to Ⅲ, and AGC stage Ⅳ. Although very small in number, EGC stage Ⅳ cases were either Early-M or Early-SM according to our classification. The number of Early-M cases increased until 1994, and afterwards decreased probably due to the extended indication for endoscopic resection of mucosal EGC. On the other hand, the number of Early-SM cases persistently increased until recently, probably because it is inevitably an indication for surgical resection as it has potential for lymph node metastasis. As endoscopic screening has become more prevalent than ever, early detection of SM cancers have also increased. On the other hand, the number of AGC stage Ⅰ to Ⅲ (potentially curative) and stage Ⅳ was relatively constant, while the population increased in Sagamihara city. After calculating the incidence of each gastric cancer group according to the population in Sagamihara, the incidence of stage Ⅰ to Ⅲ and stage Ⅳ AGC showed a tendency to decrease (Figure 5). On the other hand, the incidence of

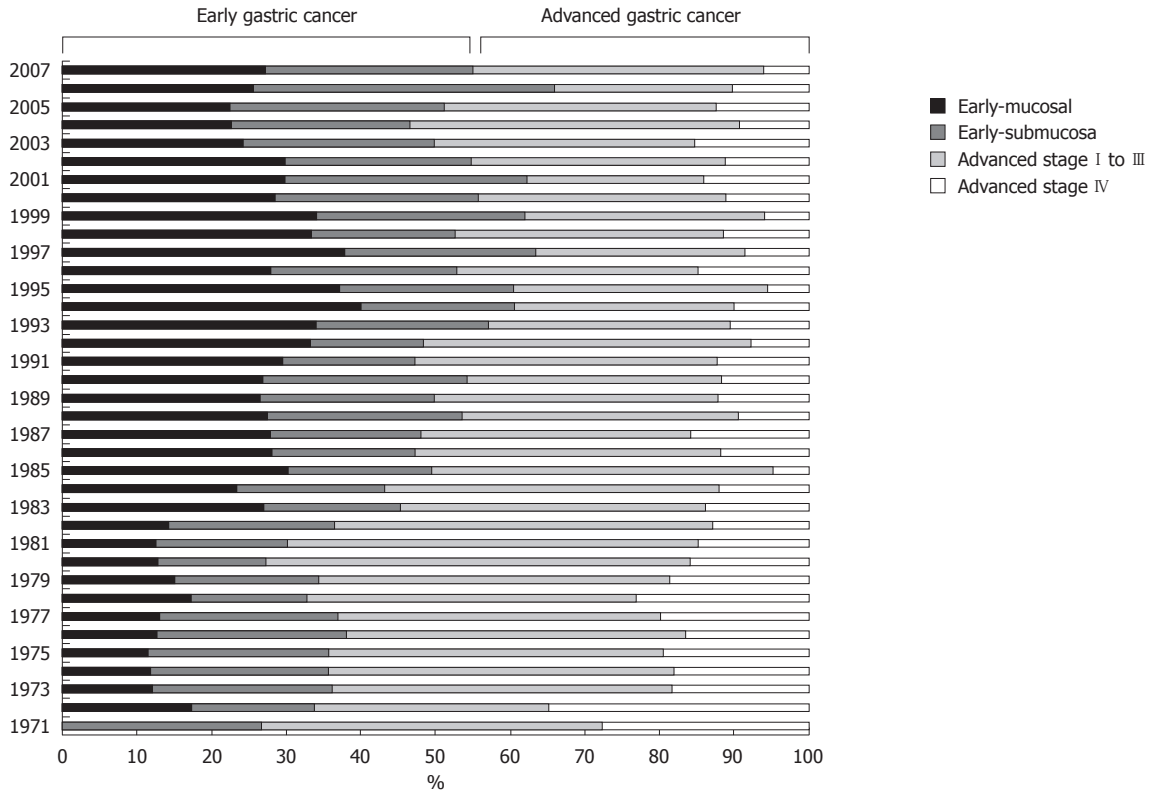

Figure 3 Proportion of early gastric cancer and advanced gastric cancer. Early gastric cancer (EGC) was further subdivided into mucosal EGC (Early-mucosal) and submucosal EGC (Early-submucosa), while advanced gastric cancer was subdivided into stage Ⅰ-Ⅲ and stage Ⅳ. Note that the proportion of EGC increased over time.

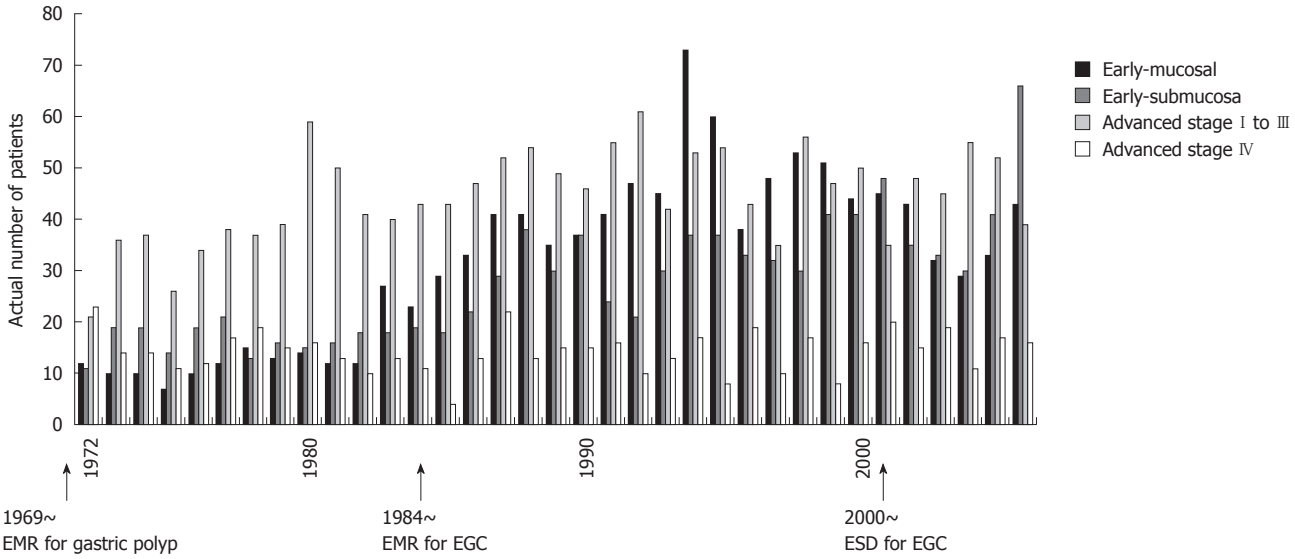

Figure 4 Trend in the number of patients according to the 4 gastric cancer categories in Kitasato University Hospital. The initial year of the critical events in endoscopic resection in Japan is designated by arrows. EMR: Endoscopic mucosal resection; ESD: Endoscopic submucosal dissection; EGC: Early gastric cancer.

EGC markedly increased as a whole. After the recent increased prevalence of endoscopic submucosal dissection and its potential extended application, Early-M and Early-SM exhibited a fluctuating ratio (Figure 6).

Prognosis and mortality of gastric cancer

Overall survival of gastric cancer requiring surgical resection markedly improved until recently (Figure 7A). This may be due to the change in the distribution of stage of gastric cancer requiring surgical resection by planned intervention (early detection by endoscopy or double-contrast barium imaging and endoscopic resection). To confirm whether a long-term reduction in the mortality of gastric cancer was observed, we also examined 5-year mortality, and histological distribution of AGC requiring surgical resection. The numbers of deaths at 5 years were

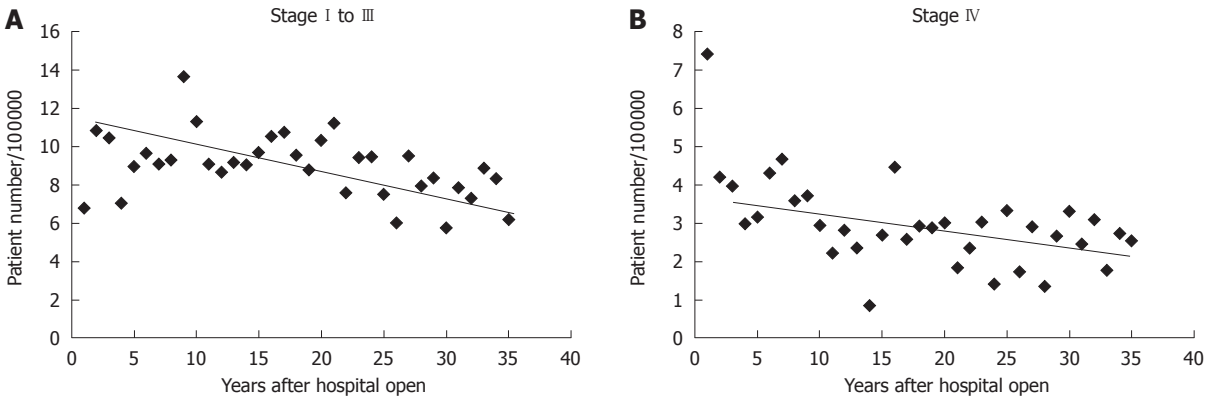

Figure 5 Incidence of advanced gastric cancer treated in Kitasato University Hospital per 100 000 population of Sagamihara city. A: Incidence of advanced gastric cancer (AGC) stage Ⅰ to Ⅲ the observed 35 years between 1972 (corresponding to 1) and 2006 (corresponding to 35). Note that the incidence of AGC stage Ⅰ to Ⅲ declined during the time period; B: Incidence of AGC stage Ⅳ during the same 35 years.

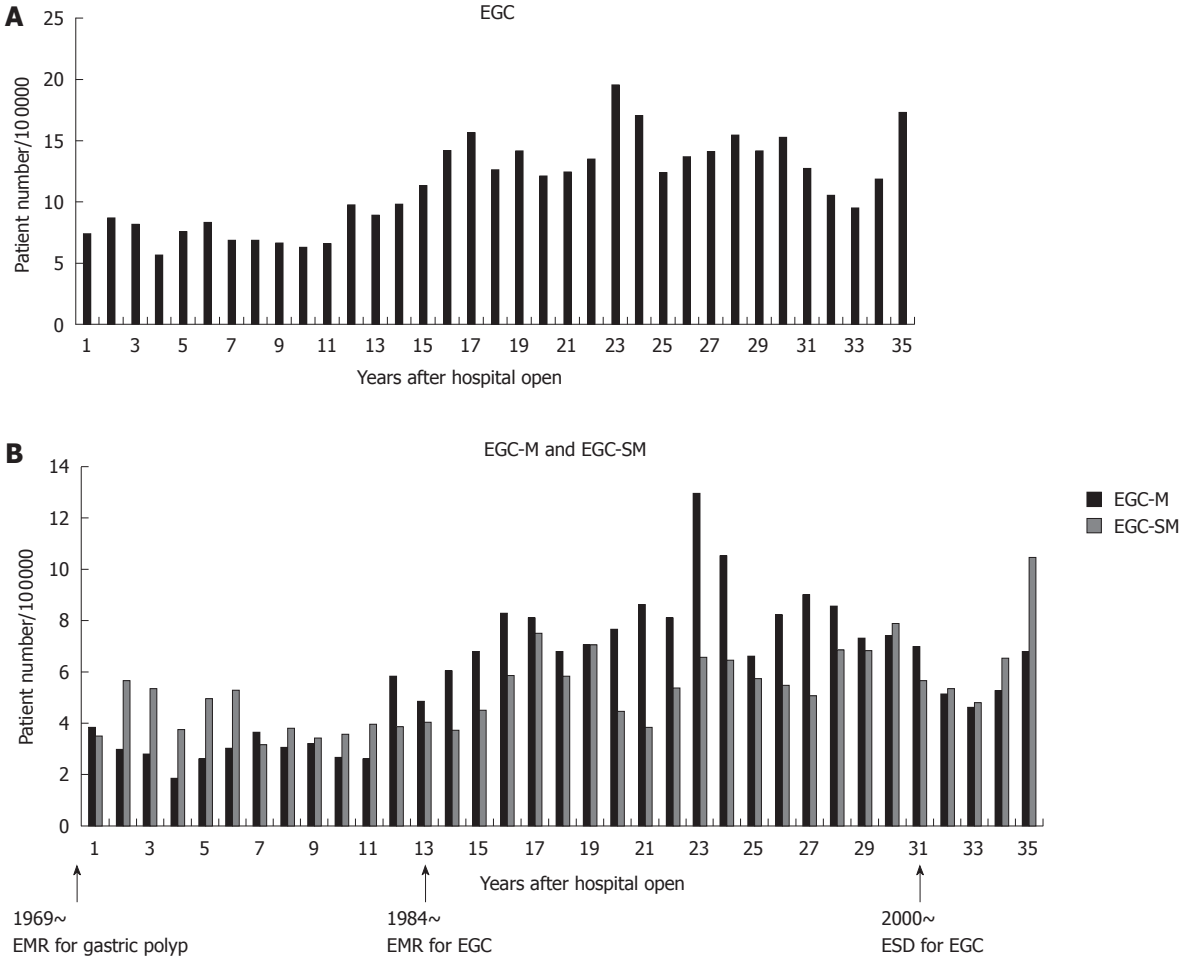

Figure 6 Incidence of early gastric cancer treated in Kitasato University Hospital per 100 000 population in Sagamihara city. A: Incidence of early gastric cancer (EGC) in the 35 years between 1972 and 2006. Note that the incidence of EGC increased during the time period; B: Incidence of EGC-M and EGC-SM during the observed 35 years. The initial year of the critical events in endoscopic resection in Japan is designated by arrows. EMR: Endoscopic mucosal resection; ESD: Endoscopic submucosal dissection; M: Mucosa; SM: Submucosa.

307, 354, and 323 according to the time period (1971-1980, 1981-1990, 1991-2000), and mortality was calculated according to the population in Sagamihara. The mortality of gastric cancer requiring surgical resection at 5 years after surgery gradually decreased (Figure 7B). In order to determine the effect against reduced mortality by introduction of interventions, we examined the ratio of intestinal type AGC to diffuse type AGC stage Ⅰ to Ⅲ (Figure 1B). As a result, we could not confirm a changing distribution of intestinal type and diffuse type AGC stage Ⅰ to Ⅲ as in

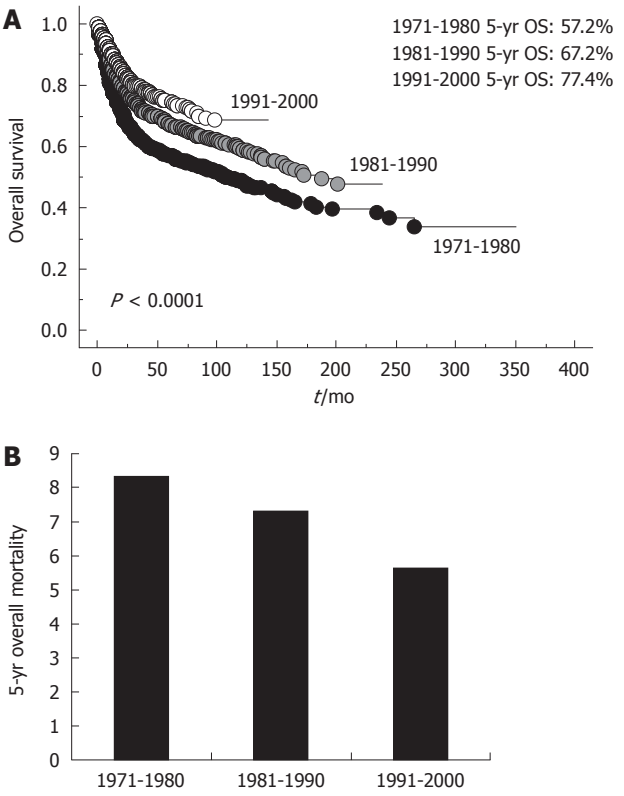

Figure 7 Clinical outcome of gastric cancer requiring surgery in the Kitasato University Hospital. A: Overall survival (OS) of gastric cancer requiring surgery shown according to time period (1971-1980, 1981-1990, 1991-2000). Five-year OS was 57.2%, 67.2%, and 77.4% according to time period, and has remarkably improved; B: Mortality of gastric cancer requiring surgery shown according to time period (1971-1980, 1981-1990, 1991-2000), which is consistent with (A).

other developed countries, in spite of rigorous planned intervention, suggesting that early resection of intestinal type EGC did not necessarily result in a reduced incidence of intestinal type AGC.

DISCUSSION

In the current study, we did not observe a declining trend of intestinal type gastric cancer in contrast to other developed countries[2]. The incidence of gastric cancer requiring surgical resection was unchanged in our hospital over the observed time period (35 years), which is consistent with the nationwide statistics that show a constant incidence of gastric cancer in Japanese people in recent years[5]. Our data therefore suggested that intestinal type gastric cancer has not changed in our country in spite of planned interventions such as rigorous screening for EGC, in which annual screening with a double-contrast barium technique and endoscopy is recommended for persons over the age of 40 years with subsequent endoscopic resection as necessary[6]. Recent introduction of endoscopic resection for a proportion of intestinal type mucosal EGC may have been expected to result in a reduced incidence of AGC or even EGC requiring surgery, but a reduced rate of intestinal type was not observed. Intestinal type gastric cancer is associated with *H. pylori* infection, which may result from a poor hygienic environment in early childhood[7]. Elderly Japanese had been living through World War II, when there was likely to be a poor hygienic environment in their childhood, which may be reflected in the high incidence of *H. pylori* infection in the elderly in Japan[8]. From such considerations, a decreasing trend in intestinal type gastric cancer as in other advanced countries may be expected 10 to 20 years later in Japan. Careful observation is needed to determine the future trend in gastric cancer in Japan.

On the other hand, our current data obviously demonstrated that the clinical outcome of gastric cancer requiring surgery has greatly improved recently (Figure 7A), and that mortality after surgery has declined (Figure 7B). Recent statistics in Japan also showed that mortality of gastric cancer exhibited a decreasing trend[5]. Gastric cancer consists of either EGC or AGC, but mortality largely reflects the prognostic outcome of AGC, and a reduced incidence of AGC must be associated with reduced mortality (Figures 3 and 5). Although AGC stage Ⅳ showed a similar tendency as other AGC, we could not conclude whether stage Ⅳ disease had actually declined, because far advanced AGC (stage Ⅳ) has usually been treated in the Gastrointestinal Department of Internal Medicine. Internal Medicine in our university rigorously screens for EGC and treats with endoscopic resection[9,10] as well as chemotherapy for far AGC[11,12]. Such active interventions for EGC may result in a reduction in mortality of gastric cancer requiring surgery. This also reflects previous investigations that indicate the critical significance of large-scale screening for gastric cancer in reducing mortality[13,14].

We were disappointed at the lack of change in intestinal type gastric cancer rates in this study. A recent report about the effect of eradication of *H. pylori* infection on metachronous gastric cancer after endoscopic resection of EGC suggested that the eradication itself may reduce the incidence of intestinal type gastric cancer[15]. In that randomized trial of 544 patients with positive *H. pylori* infection, metachronous gastric cancer after endoscopic resection for EGC largely included intestinal type gastric cancer (32/33), which may suggest that further planned intervention including eradication of *H. pylori* infection can reduce the incidence of intestinal type gastric cancer requiring surgery. Moreover, our current analysis included remnant gastric cancer, and intestinal type predominates in remnant cancer (about 70%)[16], indicating that eradication of *H. pylori* would have great preventive potential not only for primary gastric cancer, but also the secondary remnant gastric cancer in terms of intestinal type gastric cancer. Thus we should investigate the clinical significance of eradication of *H. pylori* infection to reduce the incidence of intestinal type gastric cancer in a population-based study. In Japan, a potent toxic strain of *H. pylori* with *cagA* positivity is more prevalent (almost 100%) than in other countries (about 60%)[17-19]. Thus further more general intervention may be needed to further reduce the incidence and mortality of gastric cancer in Japan.

On the other hand, a recent increase in cardia cancer is an emerging threat in gastric cancer worldwide[20,21]. There has also been a rising trend in esophageal adenocarcinoma, in which obesity, gastroesphageal reflux disease (GERD), and Barrett's esophagus are major etiologic factors, and cardia cancers share certain epidemiologic features with adenocarcinomas of the distal esophagus and gastroesophageal junction, suggesting that they represent a similar disease. In the past 30 years, the incidence of gastric cardia adenocarcinoma rose by 5- to 6-fold in developed countries[22], but not in the developing countries or East Asia[2]. Gastric cardia tumors now account for nearly half of all stomach cancers among men from the Western world[21]. In Japan, foods have become westernized with an increase in obesity in the population, resulting in a rapidly increasing incidence of GERD[23], and cardia cancer is also increasing as we elucidated in this current study (Figure 2B). In Japan, the proportion of gastric cardia tumors remains relatively small among the total gastric cancer as compared to that in Western countries, but as it is supposed to be unrelated to *H. pylori* infection[24], identification of methods other than eradication of *H. pylori* infection are necessary to reduce the incidence of cardia cancer.

In conclusion, we revealed both the recent trend of gastric cancer requiring surgery in Japan and the difference from that in other developed countries. We found that intestinal type gastric cancer has not declined in Japan, and thus additional strategies beyond the present interventions such as mass eradication of *H. pylori* infection in the population may be needed to further reduce the incidence. On the other hand, gastric cardia cancer remains a small proportion of the gastric cancers in Japan, but its incidence is increasing as in Western countries. This finding warrants urgent development of novel strategies to reduce the risk of cardia cancer.

COMMENTS

Background

The reduction in gastric cancer observed over many years in the Western world without a planned intervention is unique in the history of malignant disease, but the trend of gastric cancer in Japan remains largely unknown in the rapid course of therapeutic advancement.

Research frontiers

The trend in an urban single high-volume institute would reveal the most recent trend in gastric cancer requiring surgery, and elucidate critical clinical concerns.

Innovations and breakthroughs

The study revealed that the ratio of diffuse type/intestinal type gastric cancer was unexpectedly unchanged despite planned and rigorous interventions. However, the actual incidence of potentially curative advanced gastric cancer showed a tendency to decrease. The incidence of early gastric cancer requiring surgery tended to increase as a whole, consistent with the increased prevalence of endoscopic surveillance. As a result, overall survival and mortality of gastric cancer requiring gastric resection drastically improved recently as in other areas of Japan.

Applications

In Japan, planned interventions by early detection of gastric cancer requiring surgery may improve mortality of the disease, but an unexpected trend of persistent existence of intestinal type gastric cancer suggested that a more robust planned medical intervention (removal of *Helicobacter pylori* for high risk patients) or intensive causative research (identification of disease etiology) in addition to the present interventions may be needed to reduce the incidence as in Western countries.

Peer review

The work is an important retrospective analysis of 4163 gastric cancer patients with proven histology of adenocarcinoma of the stomach, who underwent gastrectomy during a long period and at one institution. The authors analyzed the trends of gastric cancer, reporting interesting data. The work is a well designed and well written study. I think that this study is of interest for the readers of the journal.

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ORIGINAL ARTICLE

Function of nonstructural 5A protein of genotype 2a in replication and infection of HCV with gene substitution

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Abstract

AIM: To explore the function of Nonstructural 5A (NS5A) protein of genotype 2a (JFH1) in the replication and infection of hepatitis C virus (HCV).

METHODS: Intergenotypic chimera FL-J6JFH/J4NS5A was constructed by inserting *NS5A* gene from 1b stain HC-J4 by the overlapping polymerase chain reaction (PCR) method and the restriction enzyme reaction. *In vitro* RNA transcripts of chimera, prototype J6JFH and negative control J6JFH1 (GND) were prepared and transfected into Huh-7.5 cells with liposomes. Immunofluorescence assay (IFA), fluorescence quantitative PCR and infection assay were performed to determine the protein expression and gene replication in Huh-7.5 cells.

RESULTS: The HCV RNA levels in FL-J6JFH/J4NS5A chimera RNA transfected cells were significantly lower than the wild type at any indicated time point ($2.58 \pm 5.97 \times 10^6$ *vs* $4.27 \pm 1.72 \times 10^4$, $P = 0.032$). The maximal level of HCV RNA in chimera was $5.6 \pm 1.8 \times 10^4$ GE/μg RNA at day 34 after transfection, while the wild type reached a peak level at day 13 which was 126 folds higher ($70.65 \pm 14.11 \times 10^5$ *vs* $0.56 \pm 0.90 \times 10^5$, $P = 0.028$). HCV proteins could also be detected by IFA in chimera-transfected cells with an obviously low level. Infection assay showed that FL-J6JFH/J4NS5A chimera could produce infectious virus particles, ranging from 10 ± 5 ffu/mL to 78.3 ± 23.6 ffu/mL, while that of FL-J6JFH1 ranged from $5.8 \pm 1.5 \times 10^2$ ffu/mL to $2.5 \pm 1.4 \times 10^4$ ffu/mL.

CONCLUSION: JFH1 NS5A might play an important role in the robust replication of J6JFH1. The establishment of FL-J6JFH/J4NS5A provided a useful platform for studying the function of other proteins of HCV.

Key words: Hepatitis C virus; Nonstructural 5A; Chimera; Cell culture-produced virus; Replication; Infection

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INTRODUCTION

Hepatitis C virus (HCV) is an enveloped, positive-stranded RNA virus classified in the hepacivirus genus of the

Flaviviridae family[1]. HCV infection is a major cause of chronic hepatitis, liver cirrhosis and hepatocellular carcinoma[2]. Interferon (IFN) and the nucleotide analogue ribavirin were used as the basic treatment, and sustained virologic response (SVR) rate was achieved in only 38%-63% of the treated patients although substantial improvements have been made[3]. The response to antiviral therapy depends highly on the HCV genotype. HCV is classified into six major genotypes and 30 subtypes, of which, 1a and 1b are distributed most frequently and studied intensively[4,5]. Patients infected with HCV genotype 1 showed a response rate of 38%-52% to IFN therapy, whereas patients infected with genotypes 2 or 3, achieved a SVR in up to 90% of treated patients[6-8]. The HCV genome is about 9600 nucleotides in length, encoding a single polyprotein that is processed by host and viral proteases into at least 10 distinct structural and nonstructural proteins in the order of C-E1-E2-P7-NS2-NS3-NS4A-NS4B-NS5A-NS5B[9]. The major structural proteins (Core, E1 and E2), together with a host derived lipid bilayer and the viral RNA, comprise the viron. p7 and NS2 are located within the polyprotein between these structural and nonstructural proteins. p7 is a small membrane protein with ion channel activity, NS2 and NS3 contain proteases that cleave the junction between NS2 and NS3. The nonstructural proteins NS3-NS5B are considered to assemble into membrane-associated HCV replication complex.

In the last decade, studies of HCV replication and translation have been conducted using subgenomic or full-length genomic replicon culture system[10-12]. Until recently, studies about the entire life cycle of HCV, especially the processes involved in virus entry, assembly and release, have been advanced with the development of genotype 2a JFH1 based cell culture systems, which could produce infectious HCV particles[13-15]. However, robust cell culture systems for HCV genotypes 1a and 1b, the most prevalent genotypes in the world, have not yet been developed successfully, with the exception for strains H77 and H77-S of genotype 1a[16,17], although several groups have constructed intragenotypic and intergenotypic chimeras[18-21]. What renders the JFH1 genome so special, and which element affects viral particle production and release? Wakita's work suggested that NS5B, 3'UTR and NS3 helicase activity of JFH-1 contribute to the efficient replication of JFH-1[13]. Since the NS5A protein has been the subject of intensive research over the last decade, in this paper, we focused on the NS5A protein and tried to elucidate its functions in the production of infectious virus[22].

NS5A is part of a multi-protein, membrane-bound replication complex, and plays a key role in both viral RNA replication and modulation of the physiology of the host cell, and in the establishment and maintenance of persistent infection as well[23-25]. NS5A has been shown to interact with a number of proteins which contribute to the replication of HCV, including grb2, PI3 kinase, hVAP, FBL2, and FKBP8[26-29]. NS5A is a phosphoprotein that can be found in basally phosphorylated (56 kDa) and hyperphosphorylated forms (58 kDa)[30,31]. As observed in HCV genotype 1a and 1b, the adaptive mutations of NS5A region related to phosphorylation form could efficiently promote the replication of HCV, whereas, in the JFH1 isolate, the robust replication does not need the adaptive mutation of NS5A. In order to clarify the function of NS5A domain, especially that of JFH1 isolate in the replication and infection of HCV, a chimera containing genotype 1b NS5A domain on the background of FL-J6JFH1 was constructed to study the effects of gene substitution. The results indicate that the intergenotypic chimera FL-J6JFH/J4NS5A did replicate and produce infectious viral particles *in vitro*, however, with much lower titers than the prototype FL-J6JFH, which suggested that JFH1 NS5A plays an important role in the replication and cell culture-produced HCV (HCVcc) production.

MATERIALS AND METHODS

Construction of chimera

pFL-J6JFH1 and pFL-J6JFH1 (GND) were generous gifts from Rice CM (Rockefeller University, USA). Full-length clone FL-J6JFH/J4NS5A chimera was constructed as follows. First, HCV 1b *NS5A* gene fragment was amplified from pHCV-J4[32] with the primers FJ45A-JFH (5'-GCCCCATCCCATGCTCCGGCTCGTGGCT-3') and RJ45A-JFH (5'-GAGTATGACATGGAGCAG-CAGACGACATC-3'). Then, overlapping polymerase chain reaction (PCR) was carried out to amplify the close sequences in the following steps. The junction of J6JFH1 NS4B with J4-NS5A was amplified with the primers F-BamJFH (5'-CTACTGCCTGGGATCCTGTCTC-3') and RJFH4B (5'-GCATGGGATGGGGCAGTCC-3'). The two PCR products were gel-purified and extended using the primers F-BamJFH and RJFH4B to get J6JFH1 NS4B-J4NS5A. JFHNS5B was amplified with the primers FJFH5B (5'-TCCATGTCATACTCCTGGAC-3') and RJFH-Bsp (5'-GATGTTGTACAGT ACACCTTG-3'). The fragment of JFHNS4B-J4NS5A-JFHNS5B was amplified using JFHNS4B-J4NS5A and JFHNS5B as templates with the primers F-BamJFH and RJFH-Bsp, and the PCR product was cloned into pMD18-T vector (TaKaRa, Dalian, China) and digested with Mfe I + Kpn I. The vector was gel purified and ligated with 4960bp fragment from pFL-J6JFH/ Mfe I + Kpn I to get subclone pT-I. Finally, the full-length pJ6JFH/J4-NS5A was constructed by ligating the 6506bp fragment from pT-I/Kpn I + Bsp1407 I with 5866bp fragment from pFL-J6JFH/ Kpn I + Bsp1407 I. All cloned PCR products were verified by DNA sequencing.

Cells

Huh7.5 cells (a generous gift from Rice CM) were cultured in complete Dulbecco's modified Eagle medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum, 10 mmol/L Hepes, 100 units/mL penicil-

lin, 100 mg/mL streptomycin, 2 mmol/L L-glutamine (Invitrogen) at 37℃ in a 5% CO_2 incubator. Cells were split every 3 to 4 d at a ratio of 1:3 to 1:4.

In vitro transcription and transfection

Plasmid DNA was linearized with Xba I (TaKaRa, Dalian, China), and transcribed using the RiboMAXTM Large Scale RNA Production System (Promega, CA, USA) according to the manufacturer's instructions. The RNA transcripts were treated with RNase-free *DNA*se I (TaKaRa, Dalian, China) and purified, and the RNA concentrations were checked by spectrophotometry. Twenty-four hours prior to transfection, the Huh-7.5 cells were plated at 3×10^5 per well to a 12-well plate with fresh medium without antibiotics. For the transfection, a total of 5 μg RNA transcripts were mixed with Lipofectamine TM 2000 (Invitrogen, CA, USA), and the transfection was carried out according to the manufacturer's instructions.

Quantitation of HCV RNA

At different time points after transfection, cells were collected and total RNAs were extracted with RNAex reagent (Watson, Shanghai, China) according to the manufacturer's instructions. The RNAs were reversely transcribed into cDNA with random primer and then quantitated by real-time PCR using SYBR Premix Ex Taq (TaKaRa, Dalian, China). In brief, 1 μg RNA was reversely transcribed in a 25 μL reaction mixture at 37℃ for 30 min, followed by inactivation of the reverse transcriptase at 95℃ for 15 min. Products were then amplified by PCR for 35 cycles each, at 95℃ for 10 s, 60℃ for 15 s and 72℃ for 5 s. Primer sets targeting the 5'-non coding regions of J6JFH1 included forward primer F-HCVN: 5'-GCGTTAGTATGAGTGTCGTG-3', and reverse primer R-HCVN: 5'-TCGCAAGCACCCTACAG-3'. Reverse transcription PCR (RT-PCR) amplification of cellular glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in the parallel control with the primer sets (forward primer, 5'-TGGGCTACACTGAGCACCAG-3'; reverse primer, 5'-AAGTGGTCGTTGAGGGCAAT-3')[33]. RNA levels of HCV and GAPDH were determined according to a standard curve consisting of serial dilutions of the plasmid containing the full-length HCV cDNA or human GAPDH PCR products.

Immunofluorescence assay for HCV-specific proteins

Cells were fixed for 20 min with methanol (-20℃), and then permeabilized with 0.1% tritonX-100(v/v) for 30 min at room temperature. After washing with PBS, cells were blocked for 1 h with normal sera. HCV-specific proteins were checked by incubation with sera from hepatitis C patients (HCV-positive sera collected from Changhai Hospital, Shanghai, China) as primary antibody followed by incubation with a 1:1000 dilution of FITC-conjugated goat anti-human IgG (Jackson Immunology Institute, USA) for 1 h at room temperature. The percentage of HCV-positive cells was evaluated under microscopy as 0% (no cell infected), 5%, 10%-90%, 95% and 100% (all cells infected).

Titration of HCVcc infection

At indicated time points after transfection, cell culture supernatants were harvested and cleared with low-speed centrifugation and 0.45 μm filter. Cell supernatants were serially diluted at 10 folds with complete DMEM and used to infect 10^4 naïve Huh-7.5 cells per well in 96-well plates. The inoculums were removed after 1 h incubation at 37℃, and cells were supplemented with fresh complete DMEM. The level of HCV infection was determined 3 d after infection by immunofluorescence assay (IFA) for HCV proteins. The viral titer was calculated at focus forming units per milliliter of supernatant (ffu/mL), determined by the average number of positive foci at the highest dilutions.

Sequence analysis of HCVcc NS5A

HCV RNA was isolated from cell supernatants harvested at day 13, 34 and 59 after transfection using RNAex LS reagent (Watson, Shanghai, China) and then used as template to generate cDNA in a reverse transcription reaction. The primer sets (forward primer: 5'-CGTCGACAAATGTCCGGCTCGTGGCTAAG-3'; reverse primer: 5'-GGTACCGCTCGAGTGCAGCAGACGACATCC-3') for full-length HC-J4 *NS5A* gene were used for PCR with PrimeSTARTM HS DNA polymerase (TaKaRa, Dalian, China). Nucleotide sequencing of the products was performed by Invitrogen Biotechnology Corporation (Shanghai, China).

Statistical analysis

All results were expressed as the mean ± SD. Statistical comparison was made using Student's *t* test after analysis of variance. Differences were considered statistically significant at $P < 0.05$.

RESULTS

The intergenotypic chimera FL-J6JFH/J4NS5A was generated and confirmed by DNA sequencing and restriction enzyme reaction (Figure 1). Cells transfected with genomic RNA transcripts of FL-J6JFH1, FL-J6JFH/J4NS5A and FL-J6JFH1 (GND) were cultured and passaged for more than 59 d. At the indicated time points, total RNA was isolated from the transfected Huh-7.5 cells, and the levels of HCV RNA were determined by HCV-specific fluorescent quantitive RT-PCR. In the prototype FL-J6JFH1 transfected cells, the minimum level of cellular RNA, $8.2 \pm 1.5 \times 10^5$ copies/μg HCV RNA, was detected at day 9 after transfection (Figure 2). Thereafter, the intracellular HCV RNA levels began to increase, reaching a maximal level of $7.1 \times 10^6 \pm 7.2 \times 10^5$ GE/μg RNA by day 13 after transfection, and similar levels were maintained until the experiment was

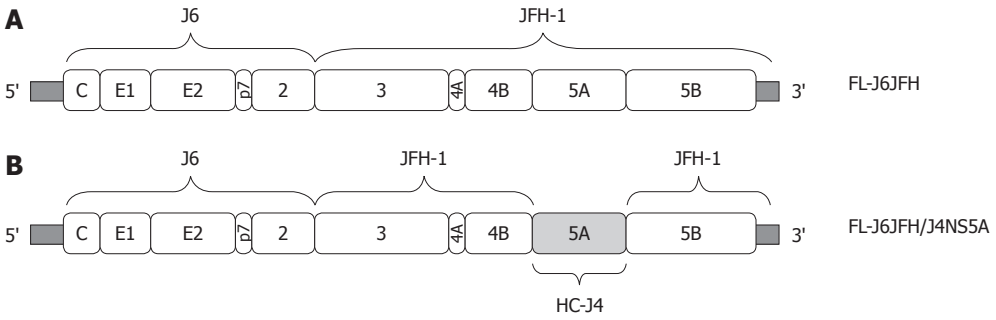

Figure 1 Construction of chimeric FL-J6JFH/J4NS5A genome containing genotype 1b (HC-J4) NS5A domain. FL-J6JFH/J4NS5A was constructed by inserting full length gene of HC-J4 NS5A into FL-J6JFH1 using overlapping polymerase chain reaction combined with enzyme restriction reaction. A: Prototype FL-J6JFH1; B: Chimera FL-J6JFH/J4NS5A. Vertical bars: HC-J4 NS5A.

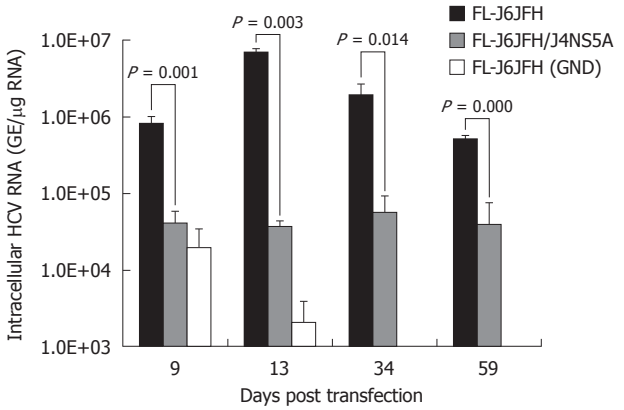

Figure 2 Fluorescence quantitative reverse transcription polymerase chain reaction assay for hepatitis C virus RNAs in cell lysis of RNA transcripts transfected Huh-7.5 cells. Five micrograms *in vitro* transcribed RNAs were electroporated into 3 × 10^5 Huh-7.5 cells. Transfected cells were harvested at the indicated time points after transfection. Intracellular hepatitis C virus (HCV) RNA was analyzed by fluorescence quantitative reverse transcription polymerase chain reaction and displayed as genome equivalents per microgram total RNA (GE/μg RNA).

terminated on day 59. While in the chimera FL-J6JFH/J4NS5A RNA transfected cells, the RNA level was significantly lower than that of prototype at any indicated time point (2.58 ± 5.97 × 10^6 *vs* 4.27 ± 1.72 × 10^4, P = 0.032). The maximal level of HCV RNA in chimera was 5.6 ± 1.8 × 10^4 GE/μg RNA at day 34 after transfection, while the prototype reached a peak by 126 folds at day 13 (70.65 ± 14.11 × 10^5 *vs* 0.56 ± 0.90 × 10^5, P = 0.028). As a negative control, FL-J6JFH1 (GND) showed a rapid disappearance of HCV intracellular RNA after transfection (Figure 2).

When the transfected cells were passaged, parts of them were collected, and the expression of HCV proteins was examined by IFA with sera from hepatitis C patients. HCV-specific proteins were detectable in both prototype and chimera RNA transfected cells. The Huh7.5 cells transfected with FL-J6JFH1 (GND) and FL-J6JFH1 incubated with normal sera were negative for HCV proteins (Figure 3). The IFA results indicated that the percentage of HCV protein-positive cells in the prototype-transfected cell cultures increased from 5% on day 9 to almost 100% on days 13 and 34. On day 59, it declined to about 50% (Figure 3). On the other hand, the proportion of positive cells in the chimera-transfected cells increased from 5% on day 9 to 50% on day 13, and then to nearly 70% on day 34. About 10% of the cells were still positive for HCV protein staining on day 59 when the experiment was terminated (Figure 3).

To determine whether FL-J6JFH/J4NS5A produced HCVcc in the transfected Huh-7.5 cells, naïve Huh-7.5 cells were inoculated with cell supernatants harvested from cultured Huh-7.5 cells at days 9, 13, 34 and 59 after transfection. The IFA results revealed that HCV protein-positive cells were detectable at day 3 after inoculation (Figure 4). To further assess the infectivity titers of HCVcc collected at different time points, the supernatants were serially diluted, and the discrete foci of HCV protein-positive cells were counted (Figure 5). HCVcc of FL-J6JFH transfected cells was first detectable with 5.8 ± 1.5 × 10^2 ffu/mL at day 9 after transfection, and then reached a maximum of 2.4 ± 1.3 × 10^4 ffu/mL by day 25 after transfection, which is consistent with the levels of intracellular J6JFH1 RNA (Figure 2). The chimera FL-J6JFH/J4NS5A was replicated with slower kinetics than the prototype (Figure 2 and 5). The HCVcc produced by chimera was lower than that of the prototype at any time point (1.96 ± 1.52 × 10^6 *vs* 5.56 ± 8.99 × 10^4, P = 0.014), the peak titer of 78.3 ± 23.6 ffu/mL appeared to be delayed and was 300 folds lower than the prototype(2.39 ± 0.18 × 10^4 *vs* 64.17 ± 39.26, P < 0.001). Collectively, the substitution of JFH1 *NS5A* gene with that of HC-J4 disrupted the high efficient production of HCV virions of FL-J6JFH1.

Since *NS5A* gene of HCV genotype 1a and 1b is easy to introduce compensatory mutations during the evolution with the hosts, the variation of *NS5A* gene in the nascent recombinant viruses was analyzed by sequencing to investigate if any mutation occurred in the HC-J4 *NS5A* genes in the backbone of FL-J6JFH1. The viral RNAs were extracted from the cell culture supernatants of days 13, 34 and 59 after transfection and were subsequently used to amplify the whole gene of NS5A by RT-PCR. At the same time, the *NS5A* gene of prototype was also checked for the mutation. Nucleotide sequencing of the products did not reveal any mutation in the sequence of NS5A region in both the chimera and prototype dur-

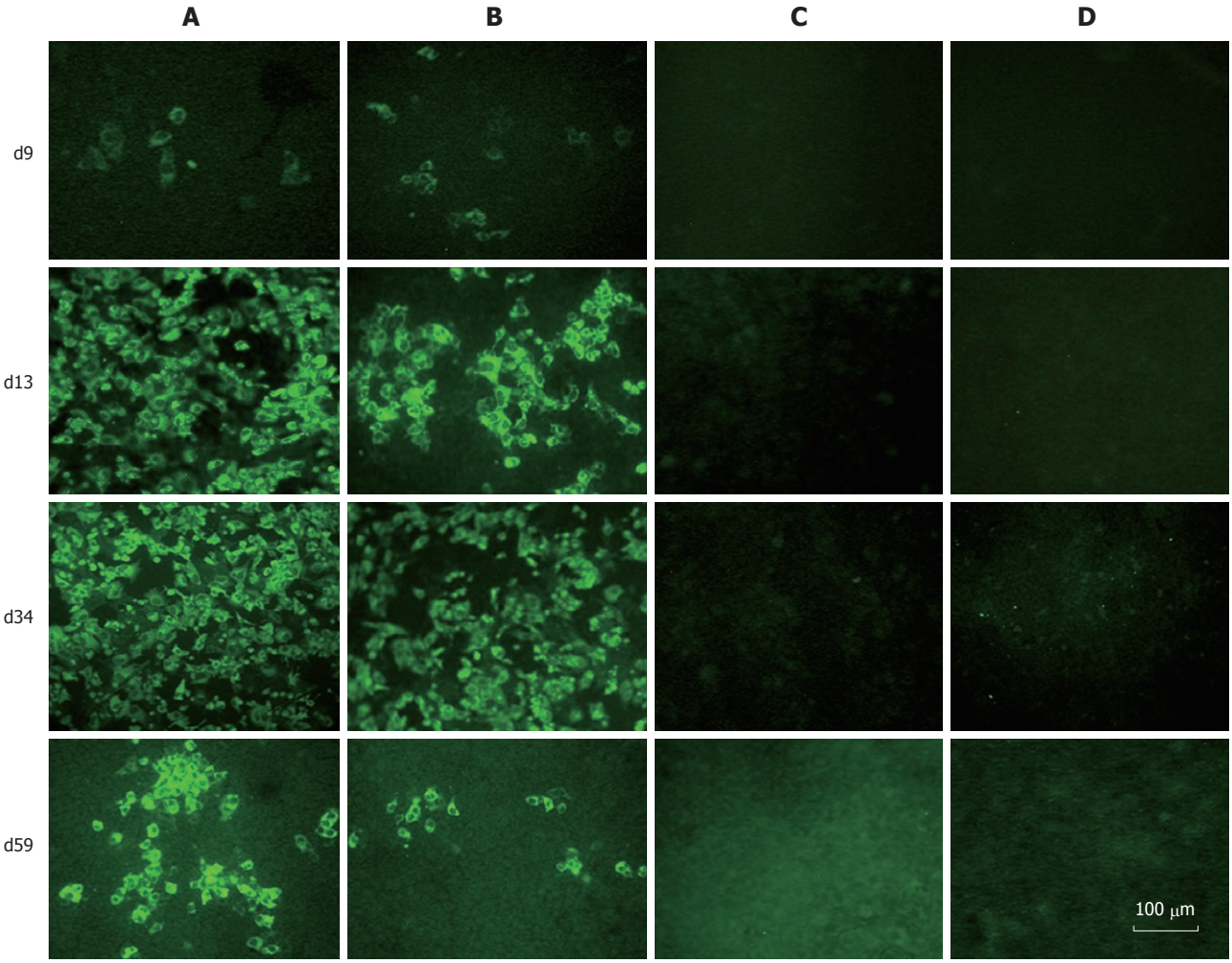

Figure 3 Immunofluorescence assay of hepatitis C virus proteins in RNAs transfected Huh-7.5 cells. Huh-7.5 cells were passaged at day 9, 13, 34 and 59 after transfection, and cultured in 96-well plates for the detection of hepatitis C virus (HCV) proteins using sera from HCV patients as primary antibody. A: FL-J6JFH1-transfected cells; B: FL-J6JFH/J4NS5A-transfected cells; C: FL-J6JFH1 (GND)-transfected cells; D: FL-J6JFH1-transfected cells incubated with normal sera as primary antibody.

ing the culture (data not shown).

We observed that FL-J6JFH/J4NS5A and FL-J6JFH1 had different cell cytopathogenicities. At day 13 after transfection, a high number of non-adherent cells were observed in the cell culture transfected with the prototype, possibly due to a high titer of HCVcc produced by FL-J6JFH1 (Figure 6). Thirteen days after transfection, the levels of HCV RNA and HCVcc produced by prototype reached a maximum (Figures 2 and 5), and cell growth recovered to normal around day 30 after transfection. In contrast, the chimera RNA transfected cells showed no cytopathic effect (CPE) until the experiment was terminated at day 59, which also indicated that virus production was much less than the prototype.

DISCUSSION

The discovery of high efficiently replicative JFH1 strain enabled us to develop an authentic *in vitro* cell culture system of HCV, however, other natural HCV strains have not yet been shown to replicate efficiently and demonstrate robust infectivity in cell culture without adaptive mutations. Since JFH1 isolate is unique among the HCV strains and not necessarily representative of HCV biology, there is an urgent demand for establishing cell culture systems for all the HCV genotypes, especially for genotype 1b, the major genotype of human infections in most Asian countries[34-36].

A growing body of evidences showed that NS5A protein possesses multiple and diverse functions in RNA replication, interferon resistance, and viral pathogenesis of HCV. In this study, the intergenotypic chimera constructed with gene substitution was studied to elucidate the roles of NS5A in both HCV replication and virus particle production. *In vitro* transcripts of FL-J6JFH/J4NS5A were prepared and transfected into Huh-7.5 cells, the influence of substitution of JFH1 NS5A with J4 NS5A on the replication and infection of HCV was observed. In a long culture period, the chimeric genome did replicate and produce infectious virus *in vitro*, but the levels of RNA replication, protein expression and HCVcc production decreased significantly compared

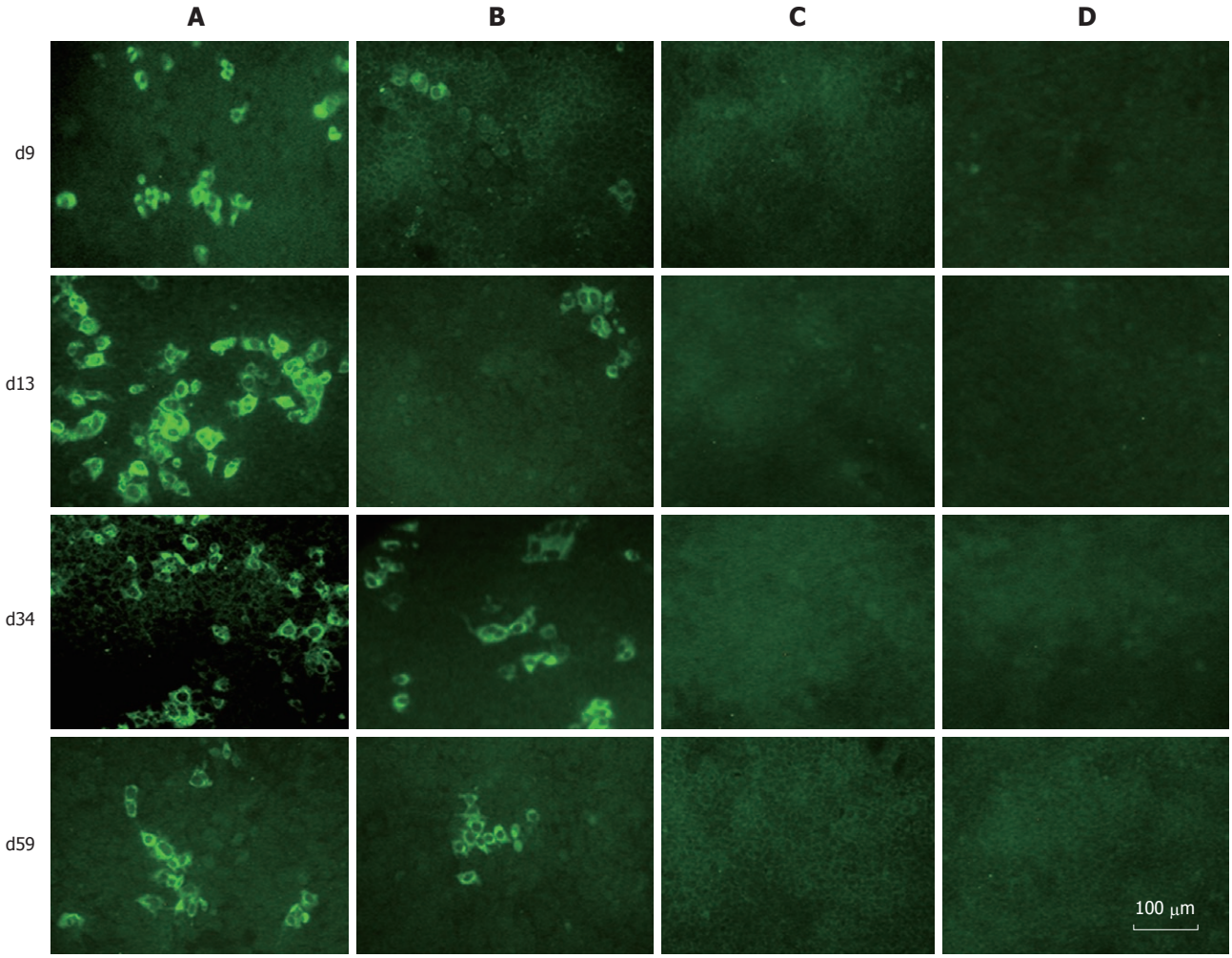

Figure 4 Immunofluorescence assay of hepatitis C virus proteins in naïve Huh-7.5 cells infected with cell supernatants from prototype and chimera transfected Huh-7.5 cells. The naïve Huh-7.5 cells were inoculated with supernatants collected at different time points during the experiment. Three days after transfection, the cells were subjected to immunofluorescence assay using sera from hepatitis C virus patients as primary antibody. A: Cells inoculated with supernatants from FL-J6JFH1-transfected cells; B: Cells inoculated with supernatants from FL-J6JFH/J4NS5A-transfected cells; C: Cells inoculated with supernatants from FL-J6JFH1 (GND)-transfected cells; D: Cells inoculated with supernatants from FL-J6JFH1-transfected cells incubated with normal sera as primary antibody.

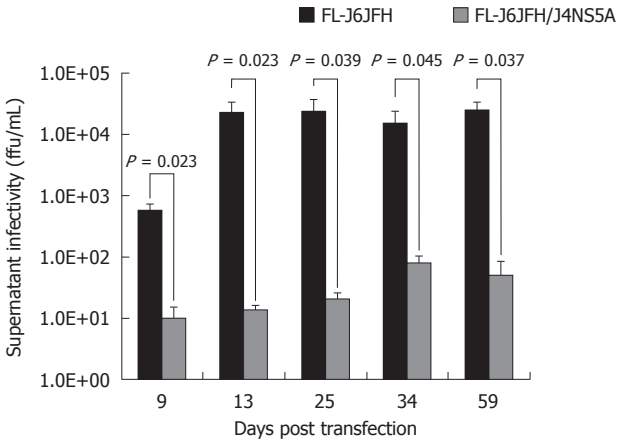

Figure 5 Comparison of cell culture-produced hepatitis C virus produced by chimera and prototype. The full-length RNA transcripts of the prototype and chimera were transfected into Huh-7.5 cells. The supernatants were harvested at the indicated time points after transfection. The infectivity titers of the supernatants were determined, and the values were presented as focus forming unit per milliliter (ffu/mL).

with the prototype. The above results suggested that the NS5A domain of JFH1 isolate played an important role in the replication of J6JFH1 and the release of infectious particles, and may be one of the determinants to establish robust *in vitro* infection system based on the JFH1 consensus clone. The growth kinetics of the cells transfected with both chimera and prototype were also observed. At day 13 after transfection, a large number of non-adherent cells were noted continuously in the culture transfected with the prototype, which may be caused by robust replication of J6JFH1. In contrast, the chimeric RNA transfected cells showed no CPE during the whole experiment, which also indicated that virus production was much less than the prototype. Recent studies suggest that NS5A is involved in the assembly and maturation of infectious viral particles. Three studies have confirmed the participation of NS5A in the assembly of HCV particles[37-39].

In addition, a recent study suggested that the interaction between the structural and nonstructural genes

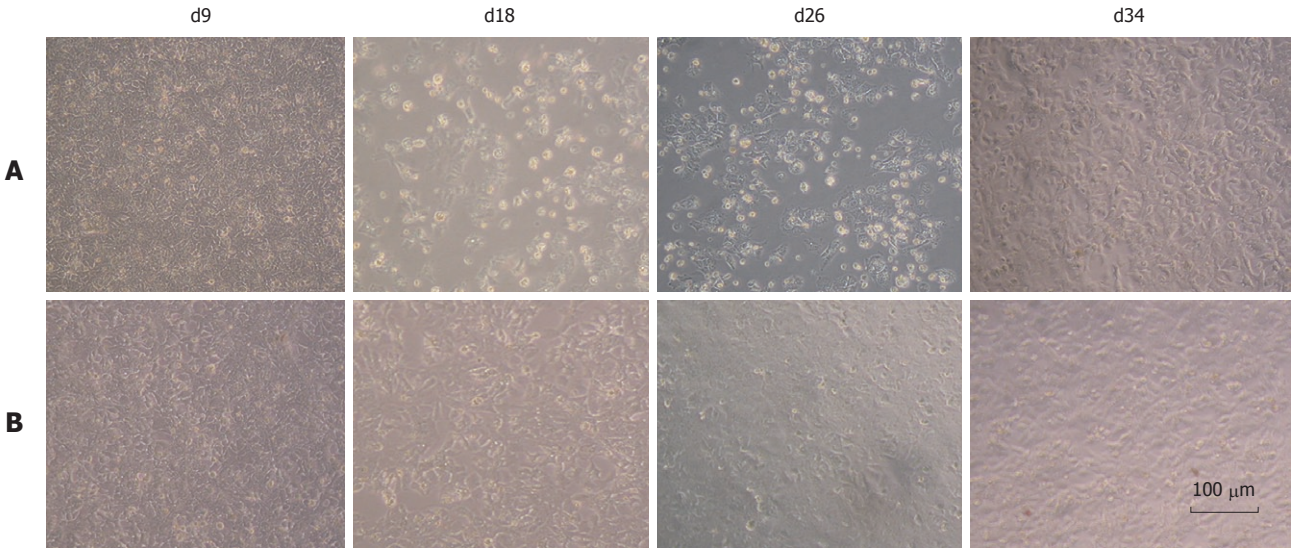

Figure 6 Cell morphology of chimera and prototype genome RNA transfected cells. The cytopathic effect was observed in prototype RNA tansfected Huh-7.5 cells under phase-contrast microscopy from day 9 to day 34 after transfection. No cytopathic effect was observed in the chimeric RNA transfected cells. A: FL-J6JFH1-transfected cells; B: FL-J6JFH/J4NS5A-transfected cells.

of the same genotype may be important for infectious particle formation, and Linderbach[14] generated chimeras comprising the core to NS2 region of either the infectious genotype 2a strain J6 or the genotype 1a H77 and the nonstructural region of JFH-1. Transfection of the J6/JFH1 chimera into Huh-7.5 cells resulted in efficient virus replication and release of viral particles, while the H77/JFH1 chimera was only detectable in virus replication but not in infectious particle formation. Several other studies showed that other proteins such as core, p7 and NS2 proteins were involved in the process of virus particle production either in the early step or in the late step[39,40]. How the related proteins are regulated remains an interesting question to be addressed. And the answers will be greatly help establish cell culture systems for all the other HCV genotypes and search for efficient antiviral therapies for HCV infection.

In conclusion, a chimeric FL-J6JFH/J4NS5A genome by replacement of the entire *NS5A* gene of genotype 2a with that from genotype 1b was constructed. The replication efficiency and virus production of HCV chimera significantly decreased compared with the prototype. Since the *NS5A* gene is the only difference between chimera and prototype, JFH1 NS5A plays a critical role in robust replication of JFH1-based isolate or chimeras. Moreover, intergenotypic chimera FL-J6JFH/J4NS5A showed lower replication and infectivity than FL-J6JFH1 without CPE, which might be similar to natural HCV infection, and could be used as a valuable tool for comparative studies on the functions of NS5A in a genuine cell culture system and for the *in vitro* examination of the effects of potential anti-viral drugs targeting NS5A.

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COMMENTS

Background

Hepatitis C virus (HCV) is a major cause of chronic hepatitis, liver cirrhosis, and even hepatocellular carcinoma. Lack of *in vitro* cell culture system hindered the development of new preventive and therapeutic methods for HCV. Recently, the establishment of cell culture system for HCV genotype 2a JFH1 and JFH1 based chimera genome, greatly promoted the study of life cycle and antiviral drugs of HCV. However, this model is only limited to HCV genotype 2a JFH1. Why the JFH1 genome is so special, and which protein affects virus production and release? Since HCV Nonstructural 5A (NS5A) is supposed to be pleiotropic protein playing a key role in both viral replication and virus production, the role of NS5A of JFH1 should be further illustrated, which will help establish cell culture models for all the other HCV genotypes.

Research frontiers

The establishment of JFH1-based cell culture models is a milestone of HCV research. Recent studies showed that genotype 1-7 cell culture was developed with intragenotypic and intergenotypic chimeras of JFH1, and these cell culture systems could be used for the selection of antiviral drugs or compounds and the search for new antiviral therapies targeting viral or host factors.

Innovations and breakthroughs

Recent reports have used the JFH1-based *in vitro* cell culture model to study the cell cycle and the function of structural and nonstructural proteins of HCV. Since the *in vitro* cell culture model is only limited to JFH1, it is necessary to explore the important proteins contributed to the production of cell culture-produced HCV (HCVcc). This paper focused on the NS5A protein of JFH1, and FL-J6JFH/J4NS5A chimera was constructed. Since *NS5A* gene is the only difference between the chimera and the prototype, the data reflecting the role of JFH1 NS5A in the robust replication of JFH1 are required in the future studies.

Applications

In this study, a 2a/1b chimera FL-J6JFH/J4NS5A was established by the method of gene substitution, and the function of JFH1 NS5A in the replication and infection was studied. By using this platform, other key proteins responsible for HCV replication and infection could be studied alone or in combination so as to establish *in vitro* cell models for other genotypes of HCV.

Terminology

Gene substitution is usually used to construct chimera to study the function of

particular genes. The part or whole of the target gene can be replaced by related or unrelated genes.

Peer review

Wang *et al* investigated the function of NS5A protein of genotype 2a from JFH1 strain in replication and infection of HCV. They demonstrated that NS5A protein from JFH1 strain play an important role in the replication and production of HCVcc. Experimental design and methods seem appropriate and they presented convincing evidence, although some issues need to be addressed.

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ORIGINAL ARTICLE

Identification of methylation profile of *HOX* genes in extrahepatic cholangiocarcinoma

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Abstract

AIM: To identify methylation profile and novel tumor marker of extrahepatic cholangiocarcinoma (CCA) with high throughout microarray.

METHODS: Differential methylation profile was compared between normal bile duct epithelial cell lines and CCA cell lines by methyl-DNA immunoprecipitation (MeDIP) microarray. Bisulfite-polymerase chain reaction (BSP) was performed to identify the methylated allels of target genes. Expression of target genes was investigated before and after the treatment with DNA demethylating agent. Expression of candidate genes was also evaluated by immunofluorescence in 30 specimens of CCA tissues and 9 normal bile duct tissues.

RESULTS: Methylation profile of CCA was identified with MeDIP microarray in the respects of different gene functions and signaling pathways. Interestingly, 97 genes with hypermethylated CpG islands in the promoter region were homeobox genes. The top 5 hypermethylated homeobox genes validated by BSP were HOXA2 (94.29%), HOXA5 (95.38%), HOXA11 (91.67%), HOXB4 (90.56%) and HOXD13 (94.38%). Expression of these genes was reactivated with 5'-aza-2'-deoxycytidine. Significant expression differences were found between normal bile duct and extrahepatic CCA tissues (66.67%-100% *vs* 3.33%-10%).

CONCLUSION: HOXA2, HOXA5, HOXA11, HOXB4 and HOXD13 may work as differential epigenetic biomarkers between malignant and benign biliary tissues.

Key words: DNA methylation; Epigenetic; Promoter microarray; Cholangiocarcinoma

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INTRODUCTION

Cholangiocarcinoma (CCA), including intra-hepatic cholangiocarcinoma (ICC) and extra-hepatic cholangio-

carcinoma (ECC), is a cancer arising from the neoplastic transformation of cholangiocytes. Several epidemiological studies show that the incidence and mortality rates of CCA have been increasing worldwide in the past decades. In Europe, approximately 50 000 new cases of primary liver cancer are diagnosed each year. Data from the Cancer Incidence in Five Continents indicates that approximately 20% of those cases are attributed to CCA[1]. Although surgical excision is considered as the most effective therapeutic approach, the 5-year survival rate is still lower than 5%. CCA is characterized by late diagnosis, poor prognosis and lack of response to both chemotherapy and radiation therapy. In addition, the only effective surgical excision is not frequently applicable because of delayed diagnosis[2]. For these reasons, specific and sensitive biomarkers for CCA will facilitate early detection and surgical treatment of CCA.

In recent years, much has been learned about epigenetic changes which were confirmed to be an important mechanism in multiple tumors. Epigenetic is defined as heritable modifications of the genome that are not accompanied by changes in DNA sequence[3]. Aberrant promoter methylation is initiated at about 1% of all CpG islands, and as much as 10% CpG islands become methylated CpG islands during multistep processes of tumorigenesis[4]. Methylation is restricted to CpG dinucleotides which are largely depleted from the genomes excepted at short genomic regions called CpG islands, which commonly represent promoters[5]. Promoter CpG islands hypermethylation can result in gene silencing, which is an alternative mechanism of gene inactivation and contributes to tumor formation, including CCA. One of the most promising molecular markers for CCA is aberrant DNA methylation marker[6].

For decades, scientists have been engaged in methylation profile of CCA. Lots of epigenetically silenced genes have already been identified in CCA, such as p14ARF, RASSFIA, TMS1/ASC, APC, E-cadherin, DAPK, and RUNX3. These silenced genes are involved in important molecular pathways, such as cell cycle regulation, apoptosis, DNA repair and cell adhesion[7-9]. Although the list of epigenetically silenced genes is increasing, it is still the iceberg of the whole methylation profile of CCA, and only a few genes show promise as tumor biomarkers for early diagnosis and prognosis. One reason for this status is that most of the studies focused on the limited tumor suppressor genes that have historically been shown to be inactivated by classical mutations/deletions[10]. Thus, investigation of aberrant DNA methylation of CCA with a high throughput assay will contribute to present the whole and complete methylation pattern of CCA.

In this study, we compared the differential methylation pattern between normal epithelial cell line of bile duct and ECC cell line by genome-wide CpG methylation profiling to discover candidate genes. We also investigated and evaluated the promising candidate genes as differential epigenetic biomarkers between malignant and benign biliary tissues.

MATERIALS AND METHODS

Cell lines and tissue samples

ECC cell line TFK-1 was purchased from the official cell bank (DSMZ, Germany). Normal bile duct epithelial cell line bile duct epithelial cells (BEC) was generously provided by Hiromi Ishibashi (Japan).

TFK-1 cells were cultured in RPMI-1640 medium (GIBCO, USA) with the presence of 10% FBS (GIBCO, USA) at 37℃ in a humidified atmosphere containing 5% CO_2. According to the previous description[11], BEC cells were resuspended and cultured in BEC medium (1:1 mixture of Ham's F12 and DMEM, supplemented with 5% fetal calf serum, epithelial growth factor 10 ng/mL, cholera toxin 10 ng/mL, hydrocortisone 0.4 μg/mL, triiodo-thyronine 1.3 μg/L, transferring 5 μg/mL, insulin 5 μg/mL, adenine 24.3 μg/mL, and hepatocyte growth factor 10 ng/mL) (all from GIBCO, USA).

Normal and neoplastic specimens were obtained from resected ECC samples at the Tongji Hospital. Nine normal extrahepatic bile duct samples were obtained from discarded tissues from non-invasive CCA patients who underwent bile duct resections. Thirty extrahepatic bile duct samples were obtained from ECC at different pathologic staging. Of these patients, 3 patients were at stage Ⅰ/Ⅱ and 27 patients were at stage Ⅲ/Ⅳ. And 11 were younger than 60 years and 28 were ⩾ 60 years old (range, 49-74 years, mean age at diagnosis, 63.7 years).

DNA extraction and bisulfate treatment

DNA was isolated as described before. Briefly, total DNA was extracted and purified by DNeasy Blood and Tissue Kit (Qiagen, Germany) according to the manufacturer's instructions. Concentration of DNA was assessed by spectrophotometry, confirmed by gel electrophoresis and stored at -20℃. In order to convert all unmethylated cytosine to uracil, genomic DNA (2 μg) from cells was subjected to sodium bisulfate using the EpiTect Bisulfite Kit (Qiagen, Germany) according the manufacturer's instructions.

Methyl-DNA immunoprecipitation and microarray hybridization

Methyl-DNA immunoprecipitation (MeDIP) was performed as described previously[12-14]. Genomic DNA was sonicated to produce random fragments in size of 200-600 bp. Four micrograms of fragmented DNA was used for a standard MeDIP assay as described. After denaturation at 95℃ for 10 min, immunoprecipitation was performed using 10 μg monoclonal antibody against 5-methylcytidine in a final volume of 500 μL IP buffer (10 mmol/L sodium phosphate, pH 7.0), 140 mmol/L NaCl, 0.05% Triton X-100) at 4℃ for 2 h. Immunoprecipitated complexes were collected with Dynabeads Protein A and M-280 sheep anti-mouse IgG (Roche, Germany) at 4℃ for 12 h, washed with 1 × IP buffer for 4 times, treated with Proteinase K at 50℃ for 4 h, and purified by phenol-chloroform extraction and isopropanol precipitation.

Table 1 Primer sequences, fragments and annealing temperature used for bisulfite-polymerase chain reaction and reverse transcription-polymerase chain reaction

Genes	Primer 5'→3'	Annealing temperature (℃)	Size (bp)
BSP			
HOXA2	L: TGTTTTAATAGAATTTATGTGGTTGG R: ATAACTACCCTCTACCTCCCCC	56	190
HOXA5	L: GTTTTGGAGAAATATTATATAAAAGTTATT R: CAATTAAAAAAATAAATCCTACCC	50	145
HOXA11	L: TGAGTATAAGTATGTTGTATGGGGG R: TTATAACCACCTCAAAAAAAACAAC	60	148
HOXB4	L: TAGAGGTGAGGTAGAATAGGAGGGT R: ACCCAACACCAAAATTTACATAAAA	60	204
HOXD13	L: GTGGGTTTAGTTAGGTTTGGGT R: TCTAACCCTCTCTCCCTCTATAAAC	60	239
RT-PCR			
HOXA2	F: GTCACTCTTTGAGCAAGCCC R: TAGGCCAGCTCCACAGTTCT	59	345
HOXA5	F: ACTCCGGCAGGTACGGCTACG R: -CCGCTGGAGTTGCTTAGGGAG	62	259
HOXA11	F: TGCCAAGTTGTACTTACTACGTC R: GTTGGAGGAGTAGGAGTATGTA	61	181
HOXB4	F: GTGCAAAGAGCCCGTCGTCTA CC R: CGTGTCAGGTAGCGGTTGTAGTG	65	161
HOXD13	F: TGCTCCTCTTCTGCCGTTGT R: CCTGTGGCTGGTCCTTGGT	55	467

BSP: Bisulfite-polymerase chain reaction; RT-PCR: Reverse transcription-polymerase chain reaction.

Immunoprecipitated methylated DNA was labeled with Cy5 fluorophere and the input genomic DNA was labeled with Cy3 fluorophere. Labeled DNA from the enriched and the input pools was combined (1-2 μg) and hybridized to NimbleGen HG18CpG promoter (Roche, Germany), which contained 28226 all known CpG islands annotated by UCSC (University of California Santa Cruz) and all well-characterized RefSeq promoter regions [from -800 bp to +200 bp transcription start sites (TSSs)] totally covered by 385000 probes. Arrays were then washed and scanned with NimbleScanTM2.2 (NimbleGen) microarray scanner. After normalization, raw data was input into SignalMap software (v1.9, Roche-NimbleGen) to observe and evaluate the differential methylation peaks between cell lines.

A customized peak-finding algorithm provided by NimbleGen was applied to analyze methylation data from MeDIP-microarray (NimbleScan v2.5; Roche-NimbleGen) as previously described. The algorithm was used to perform the modified Kolmogorov-Smirnov test on several adjacent probes using sliding windows to predict enriched regions across the array. We filtered the differential methylation peaks according to the principles suggested by Pälmke *et al*[15]: (1) At least one coverage of probes is located inside the peak; and (2) The mean log-ratio (> 2) across the peak has to be positive for at least one of the two samples.

Bisulfite-polymerase chain reaction

The bisulfate modified DNA was amplified with forward and reverse primers for target genes. Table 1 shows the sequences of primers and annealing temperature used in the polymerase chain reaction (PCR) reaction. One microliter PCR products were cloned into pCR-TOPO using the TOPO TA cloning kit (Invitrogen, USA) according to the manufacturer's instructions. One microliter reaction products were transformed into One Shot TOP 10 chemically competent cells and cultured for 1 h in 250 μL Super Optimal Broth with Catabolite Repression medium at 225 r/min in the incubator shaker. After overnight growth on Luria-Bertani agar plates containing 50 μg/mL kanamycin, plasmid DNA of 10 positive clones of each gene was extracted by EaZy nucleic acid TM Plasmid Mini-Kit Ⅱ (OMEGA, USA) and sequenced using T7 or M13 forward and reverse primers.

Treatment with 5-Aza-2'-deoxycytidine

1×10^5 TFK-1 cells were seeded into each well of a 6-well plate and cultured for 24 h. In the following 3 d, cells were treated continuously with 1 μmol/L (refreshed daily) 5-Aza-2'-deoxycytidine (Sigma, USA). Cells without drug treatment were used as control.

RNA purification and reverse transcription-PCR

Total RNA from cells was extracted using the TRIZOL reagent (Invitrogen, USA). Reverse transcription reaction was performed using MMLV-RT (TOYOBA, Japan). cDNA was amplified by PCR according to the manufacturer's instructions. The primer sequences for target genes used in the reaction are shown in Table 1.

Immunofluorescence

Forty micrometers-thick sections of tissue samples were cut and mounted on slides. The sections were subsequently rinsed with phosphate-buffered saline (PBS) for

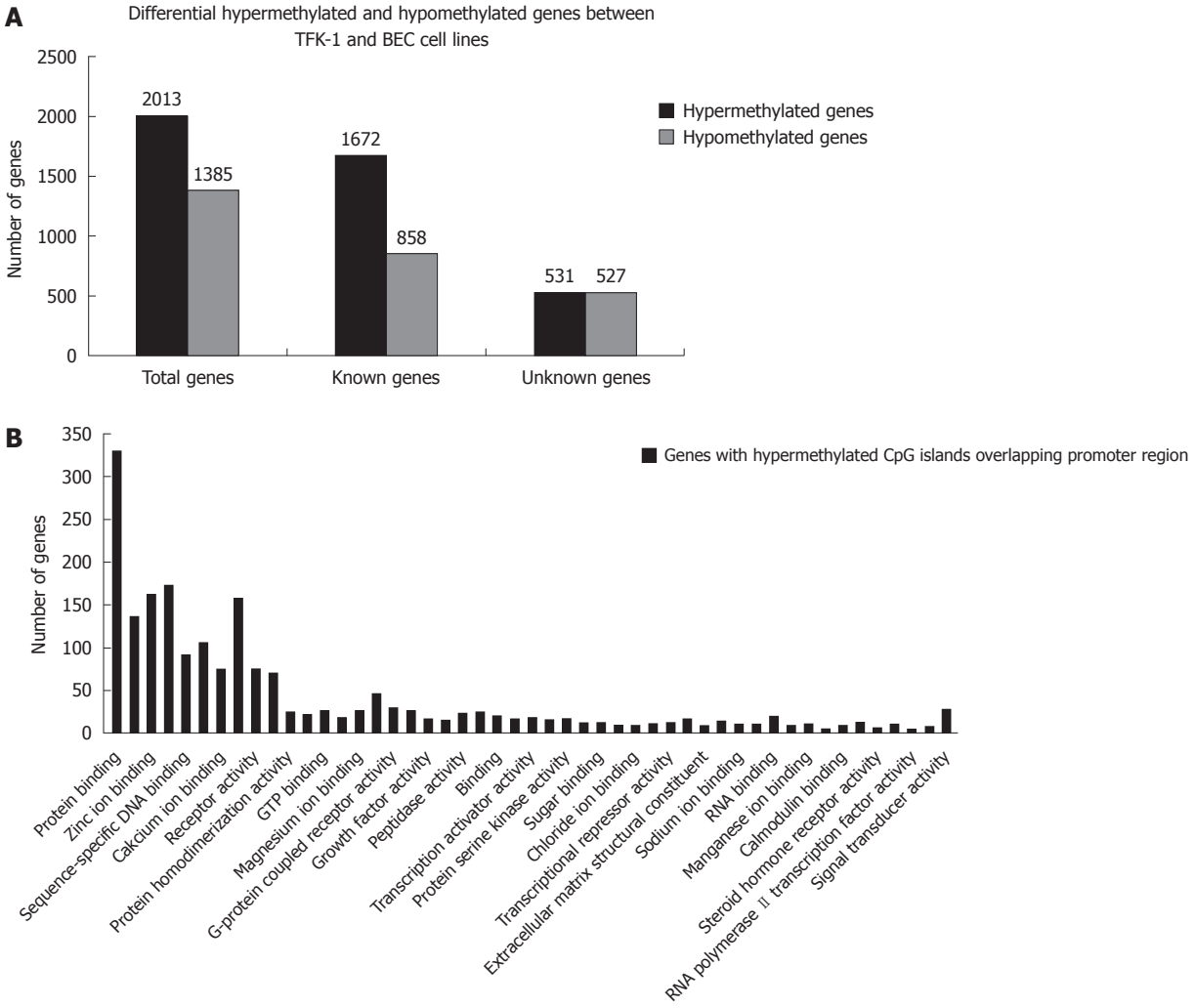


Figure 1 Identification of differentially hypo- or hypermethylated genes by Methylated DNA Immunoprecipitation and functional analysis of differentially hypermethylated genes by Molecule Annotation System. A: Differentially hypermethylated and hypomethylated genes between TFK-1 and bile duct epithelial cells (BEC) cell lines; B: Functional categories of differentially hypermethylated genes identified by the Molecule Annotation System.

10 min, incubated with 0.5% Triton X-100 for 60 min, and with block solution (5% cattle serum + 0.5% Triton X-100) at room temperature for 1.5 h. After aspiration of block solution, sections were incubated with diluted rabbit polyclonal antibody (Santa Cruz, USA) in block solution overnight at 4℃. The reaction was stopped by three washes with PBS. Then sections were incubated with diluted secondary antibodies (goat anti-rabbit 1:400) conjugated with CY3 NHS Ester for 2 h at 37℃. Slides were washed in PBS and counterstained with 1 μg/mL 4',6-diamidiono-2-phenylindole for 10 min. The slides were finally mounted with aqueous mounting medium containing anti-fade. Images were obtained by fluorescent microscopy (OLYMPUS IX70).

Statistical analysis

Values were presented as mean ± SD. The mean values of the two subgroups were compared by Student's *t* test. For each *HOX* gene, the median expression was noted as a percentage of immunoreactive samples. Fisher exact test was performed to assess the differences in the median expression values between tissue specimens. All *P* values were two-sided and $P < 0.05$ was considered statistically significant. All experiments were performed at least 3 times independently.

RESULTS

MeDIP

We identified 2013 differential hypermethylated CpG islands including 1672 known CpG islands and 531 unknown CpG islands, and 1385 hypomethylated CpG islands including 858 known CpG islands and 527 unknown CpG islands (Figure 1A). Although they spread on different chromosomes, the cluster tendency was obvious that hypermethylated regions preferred to cluster on specific chromosomes, including Chr2, Chr17 and Chr7. The result was different from the former reports that 3p21.3 was methylation hot spot of ECC, but stood in line with other reports that Chr17 and Chr2 were methylation hot spots of myeloid leukemia, bladder cancer and lung cancer[16-18]. Lots of genes have been identified related to tumorigenesis of CCA, such as BCL2, COX2, IGF2, NEUROG1, RAR DAPK1, CDH1[19].

Table 2 Methylated *HOX* genes identified by methyl-DNA immunoprecipitation microarray

No.	Genes	Description	CpG location	Strand	Position of gene
1	*HOXA2*	HomeoboxA2	chr7:27109706-27110004	-	Promoter
2	*HOXA5*	HomeoboxA5	chr7:27149138-27152087	-	Promoter
3	*HOXA11*	HomeoboxA11	chr7:27191575-27192154	-	Promoter
4	*HOXB2*	HomeoboxB2	chr17:43982786-43983443	-	Promoter
5	*HOXB4*	HomeoboxB4	chr17:44010214-44010603	-	Promoter
6	*HOXB5*	HomeoboxB5	chr17:44025521-44026457	-	Promoter
7	*HOXC10*	HomeoboxC10	chr12:52664963-52666369	+	Promoter
8	*HOXD1*	HomeoboxD1	chr2:176761203-176762596	+	Promoter
9	*HOXD9*	HomeoboxD9	chr2:176694670-176696537	+	Promoter
10	*HOXD12*	HomeoboxD12	chr2:176672308-176673755	+	Promoter
11	*HOXD13*	HomeoboxD13	chr2:176665300-176666525	+	Promoter

It is well known that aberrant promoter hypermethylation of TSS is an alternative mechanism of gene inactivation and contributes to the carcinogenesis of human neoplasmas, so we investigated the methylation pattern of CpG islands in TSS. We further filtered the experimental data by screening differential hypermethylation peaks overlapping the promoter region of the relevant transcript (-800 to +200 bp). We identified 970 genes with hypermethylated CpG islands overlapping promoter region, which included 317 unknown genes and 653 known genes. We then checked the function of these genes annotated in UCSC and RefSeq gene database, and found that these genes were involved in different cellular process, such as cell-cell adhesion, cell migration, signal transduction and cell repair (Figure 1B).

Interestingly, we also observed a phenomenon that 97 of 970 genes with hypermethylated CpG islands in the promoter region belonged to homeobox gene clusters. These homeobox genes were mainly distributed on HOX, ANTP, PRD, LIM and SINE subclasses. However, aberrant epigenetic changes of *HOX* genes would disrupt the *HOX* gene expression and affect various pathways that promote tumorigenesis and metastasis in different cancers, such as breast cancer, lung cancer, ovarian cancer and prostate cancer[20-22], and research of *HOX* genes was limited in choangiocarcinoma. To further evaluate the influence of DNA methylation changes on the expression of *HOX* genes, we picked 11 homeobox genes showing hypermethylated status for further analysis (Table 2).

Bisulfite-PCR

To further verify the hypermethylation of *HOX* genes, we performed bisulfite-PCR (BSP) to validate the results of MeDIP. We observed that HOXA2, HOXA5, HOXA11, HOAB4 and HOXD13 were the top 5 methylated genes in TFK-1 cells. As shown in Figure 2B, the frequency of promoter hypermethylation was 94.29% for HOXA2, 95.38% for HOXA5, 91.67% for HOXA11, 90.56% for HOXB4 and 94.38% for HOXD13 in TFK-1 cells. In contrast, promoter profiles of HOXA2, HOXA5, HOXA11, HOXB4 and HOXD13 were unmethylated in BEC. The graphical MeDIP-microarray data of the top 5 *HOX* genes is presented in Figure 2A.

Treatment with 5-Aza-2'-deoxycytidine

Methylation is a reversible process that DNA methyltransferase inhibitors can restore the original expression and function of epigenetically silenced genes *in vitro* and *in vivo*. To determine if the lack of transcription of these genes may be influenced by aberrant methylation, we treated the TFK-1 cells with demethylating agent 5-Aza-2'-deoxycytidine. Reverse transcription-PCR (RT-PCR) was carried out on the top 5 candidate *HOX* genes. The results showed that the expression of HOXA2, HOXA5, HOXA11, HOXB4 and HOXD13 was rare in TFK-1, whereas it was restored after the treatment with 5-Aza-2'-deoxycytidine (Figure 3).

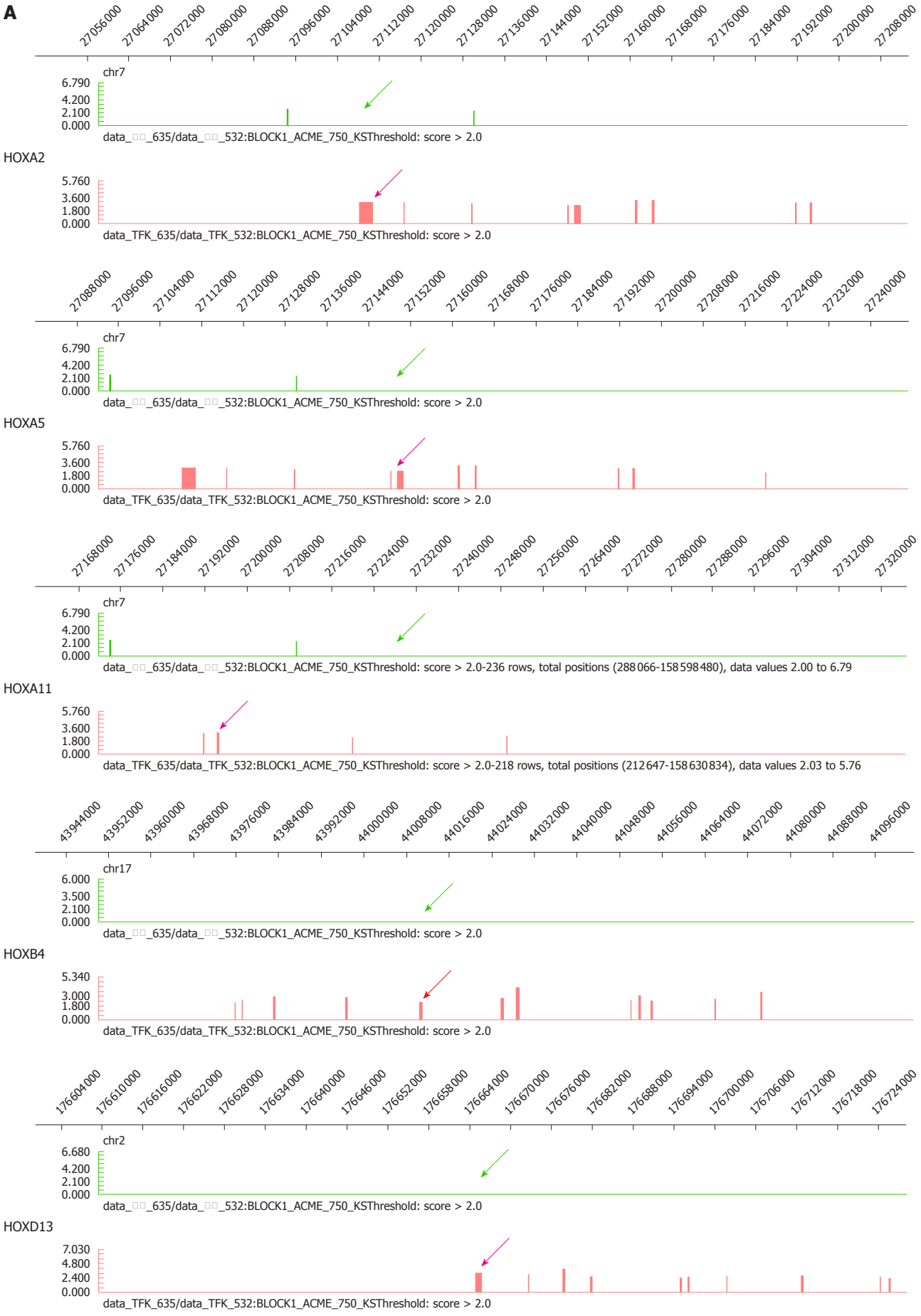
Expression of candidate genes in fresh tumors

Genes can acquire extra DNA methylation changes in the cell culture process and methylation profile of cell lines may not accurately reflect the methylation profile of tumors *in vivo*[23]. Besides, only one ECC cell line TFK -1 was used in this research. To compensate for this limitation, we assayed the expression of target *HOX* genes in primary ECC and normal bile duct samples using immunofluorescence.

Representative immunofluorescemce photo images of the top 5 *HOX* genes which have been validated by BSP are shown in Figure 4. Strong positive staining for *HOX* genes was found in normal bile duct samples while tumor samples showed negative staining for *HOX* gene expression. The frequency of positive staining of HOXA2, HOXA5, HOXA11, HOXB4 and HOXD13 was 77.78% (7/9), 100% (9/9), 100% (9/9), 66.67% (6/9) and 88.89% (8/9), respectively in normal bile duct samples, while it was only 10% (3/30), 3.33% (1/30), 3.33% (1/30), 6.67% (2/30) and 6.67% (2/30), respectively in ECC samples (Table 3).

Aging is one of the most important risk factors in the development of neoplasia and methylation of genes increases with age[24]. There was no significant difference between the lower age group and advanced age group (age > 60 years) in our research. However, there was a tendency that *HOX* genes mostly expressed in early pathological stages but not in advanced pathological stages, the difference being not statistically significant.

A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chr2
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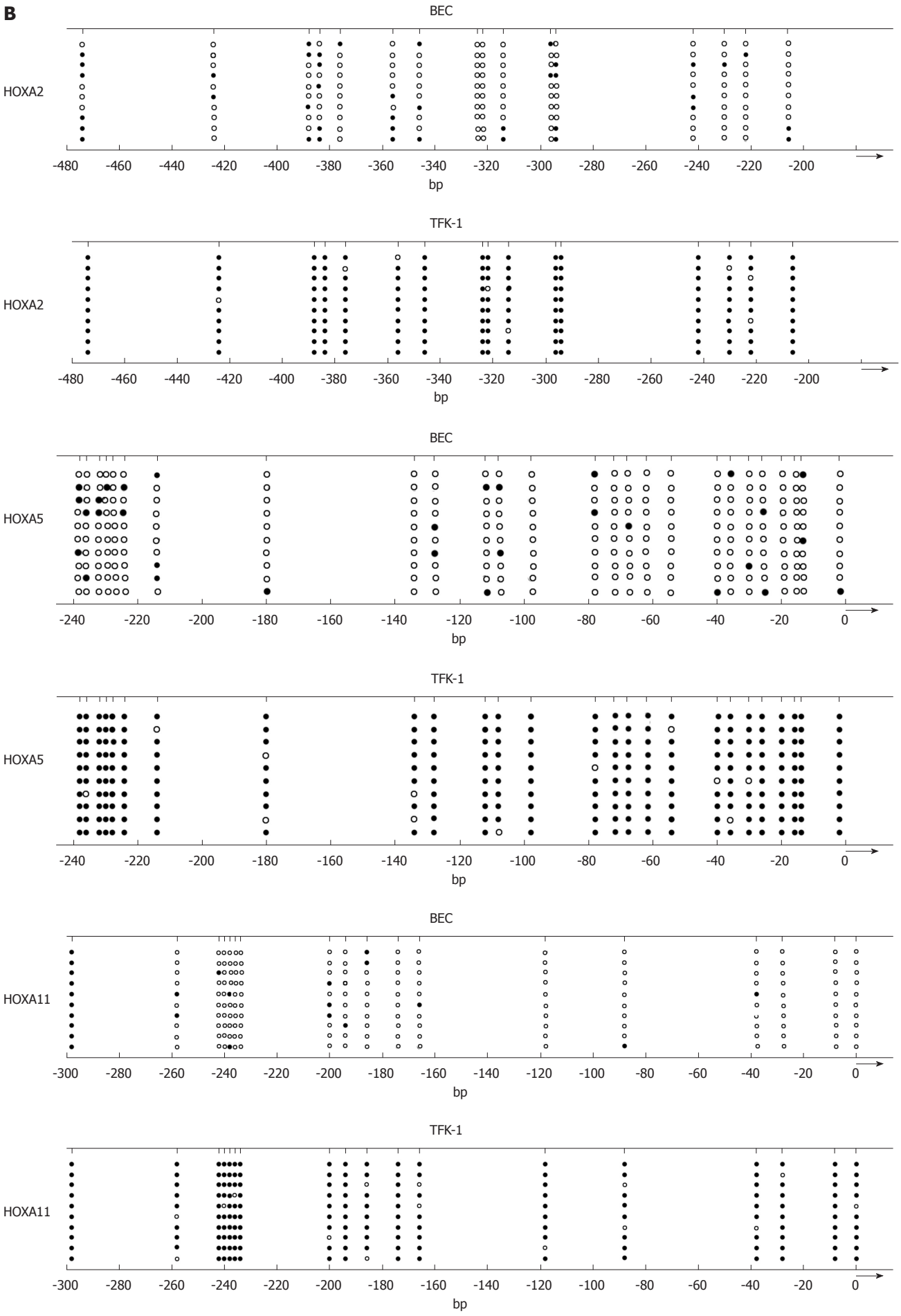
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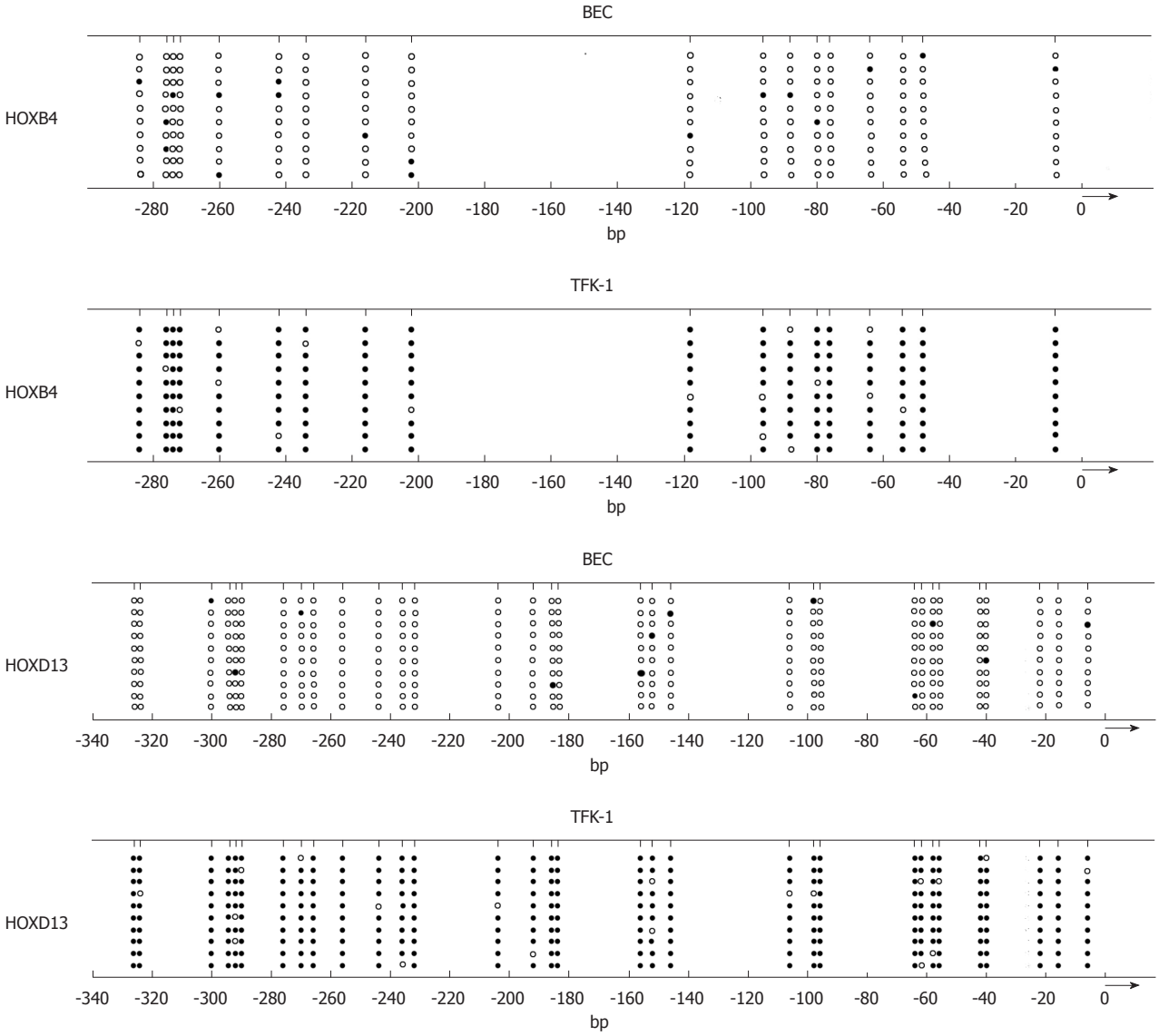

Figure 2 DNA methylation analyses of CpG islands of hypermethylated *HOX* genes. A: Graphical representation of Methylated DNA Immunoprecipitation microarray (Signalmap software, NimbleGen). The panels show the DNA methylation profile at homeobox cluster genes in normal epithelial cell of bile duct cell line bile duct epithelial cells (BEC) and cholangiocarcinoma cell line TFK-1. The methylated CpG islands are indicated by bar and arrow in TFK-1 (red) and BEC (green). Chromosomal location is indicated at the top of the diagram; B: Bisulfate-sequencing of HOXA2, HOXA5, HOXA11, HOXB4 and HOXD13. Each vertical line represents a single CpG site on the top. The regions were analyzed by bisulfate-sequencing. The transcription start site and location of exon 1 are shown by thick bars on the bottom. Each row represents an individual cloned allele. Circles represent CpG sites and their spacing accurately reflects the CpG density of the region. Black circles, methylated CpG site; white circles, unmethylated CpG site. Dense methylation at the promoters was found in TFK-1. In contrast, promoter hypomethylation found in BEC among all the genes.

DISCUSSION

The objective of this study was to identify novel genes inactivated by promoter methylation in ECC. We used a microarray-based strategy as an initial screening approach to identify the differential methylation pattern of ECC.

We then validated methylation status of candidate genes in cells and identified the expression of candidate genes in ECC samples. Kim *et al*[7] reported that there was a statistically significant difference between gene methylation frequencies of ECC and ICC. Yang *et al*[8] demonstrated that ICC and ECC tissues exhibited overlapping but distinct methylation profiles. Some hypermethylated genes that have been confirmed in these literatures were also identified in our study, including RASSF1A, $P15^{INK4b}$, $P16^{INK4a}$, NEUROG1, CDH1, IGF2, GSTP1, APC and RUNX3. However, previous studies have explored methylation profile of ECC, and only CpG islands that have been demonstrated to be hypermethylated in other human cancer tissue types were investigated in ECC later. Our present study applied microarray to investigate 28 226 CpG islands and demonstrated abundant differentially hypermethylated genes that were not previously known in ECC.

We found that 97 genes with hypermethylated promoter CpG islands belonged to homeobox gene clusters. *Hox* genes are one of the master regulators of morphogenesis and cell differentiation embryogenesis of

Table 3 HOXA2, HOXA5, HOXA11, HOXB4, and HOXD13 methylation status of 30 cholangiocarcinoma patients and 9 normal controls *n* (%)

Genes	Age (*n* = 39)			Normal (*n* = 9)	Patients (*n* = 30)			*P* value[1]
	< 60 yr (*n* = 11)	> 60 yr (*n* = 28)	*P* value		Tumor stage Ⅰ/Ⅱ (*n* = 3)	Tumor stage Ⅲ/Ⅳ (*n* = 27)	*P* value	
HOXA2	4 (7.69)	6 (15.39)	0.424	7 (77.78)	1 (33.33)	2 (7.41)	0.511	< 0.001
HOXA5	3 (7.69)	7 (17.95)	1.0	9 (100)	1 (33.33)	0 (0)	0.206	< 0.001
HOXA11	2 (5.13)	7 (17.95)	1.0	9 (100)	1 (33.33)	0 (0)	0.206	< 0.001
HOXB 4	3 (7.69)	5 (12.82)	0.663	6 (66.67)	2 (66.67)	0 (0)	0.007	0.001
HOXD13	4 (7.69)	6 (15.39)	0.424	8 (88.89)	1 (33.33)	1 (3.70)	0.193	< 0.001

Fisher exact test was performed to identify differences in median expression values within the tissue samples. [1]Fisher test between normal controls and cholangiocarcinoma patients.

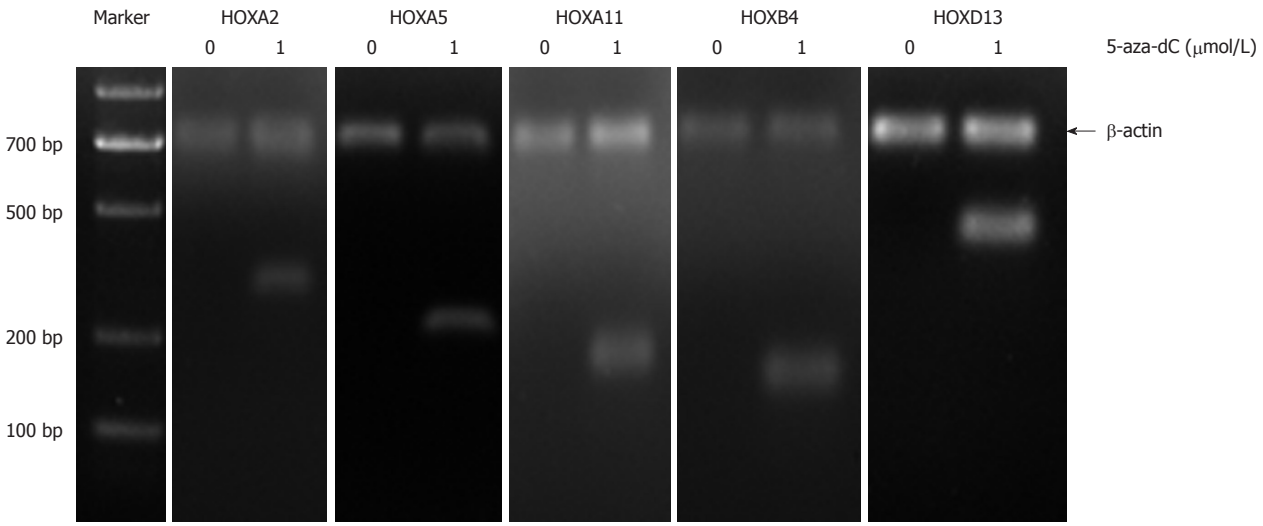

Figure 3 Reverse transcription-polymerase chain reaction analysis for expression profile of HOXA2, HOXA5, HOXA11, HOXB4 and HOXD13 in TFK-1 cells before and after the treatment with demethylating agent 5-aza-2-deoxycytidine (5-aza-dC). Loss of expression of genes was observed before the treatment in TFK-1. Re-expression of genes was observed upon the treatment with 1 μmol/L demethylating agent 5-aza-2-deoxycytidine (5-aza-dC). **TFK-1 cells were subjected to** the 1 μmol/L **5-aza-dC treatment for 3 d before RNA prepared for reverse transcription-polymerase chain reaction assessment.** β-actin 700 bp.

animals[25]. Numerous examples of aberrant *Hox* gene expression have been found in cancer. Abate-Shen[26] proposed three mechanisms, including temporospatial deregulation, gene dominance and epigenetic deregulation, to classify aberrant changes of *Hox* genes[26]. The third mechanism, epigenetic deregulation, can modify or silence the expression of *Hox* genes in tumor tissues. However, lots of hypermethylated *Hox* genes have been identified at different stages of primary squamous cell carcinomas, such as lung carcinoma, ovarian carcinoma, breast carcinoma and cervical carcinoma[27,28], and little is known about methylation status of *Hox* gene in CCA.

We picked 11 hypermethylated homeobox genes according to the filter principles and mapped the biological relationship between the 11 *HOX* genes and their related genes to analyze in-depth their roles in tumorigenesis of ECC (Figure 5). EED-EZH2 and PRC2 complex had an intrinsic histone methyltransferase activity to H3K27 and silenced some *HOX* genes[29]. Besides, Hphf1 could associate with the core components of EED-EZH2 complex to modulate its enzymatic activity and *HOX* gene expression[30]. MeDIP microarray indicated that EZH2 was also methylated in TFK-1 cells and we therefore, speculated that the expression of *HOX* gene was down-regulated by epigenetically silenced core components of EED-EZH2 complex. However, more studies remain to be conducted to find out their relationship.

BSP confirmed that HOXA2, HOXA5, HOXA11, HOXB4 and HOXD13 methylated by MeDIP microarray were densely hypermethylated in TFK-1 cells, but fully unmethylated in BEC cells. The 5-Aza-dC method is very useful for identifying the genes reactivated by treatment with DNA demethylating agent. One can also prioritize the reactivated genes by gene expression analysis to identify the genes that are normally expressed but undergo silencing in cancers[31]. In our research, TFK-1 cells showed loss of expression of HOXA2, HOXA5, HOXA11, HOXB4, and HOXD13. After the treatment with demethylating agent 5-aza-2-deoxycytidine, the expression was restored in TFK-1 cells. The association between DNA methylation and expression agreed with the theory that epigenetic deregulation would silence the expression of *Hox* genes in tumor tissues proposed by Abate-Shen[26]. Drugs that modulate DNA methylation

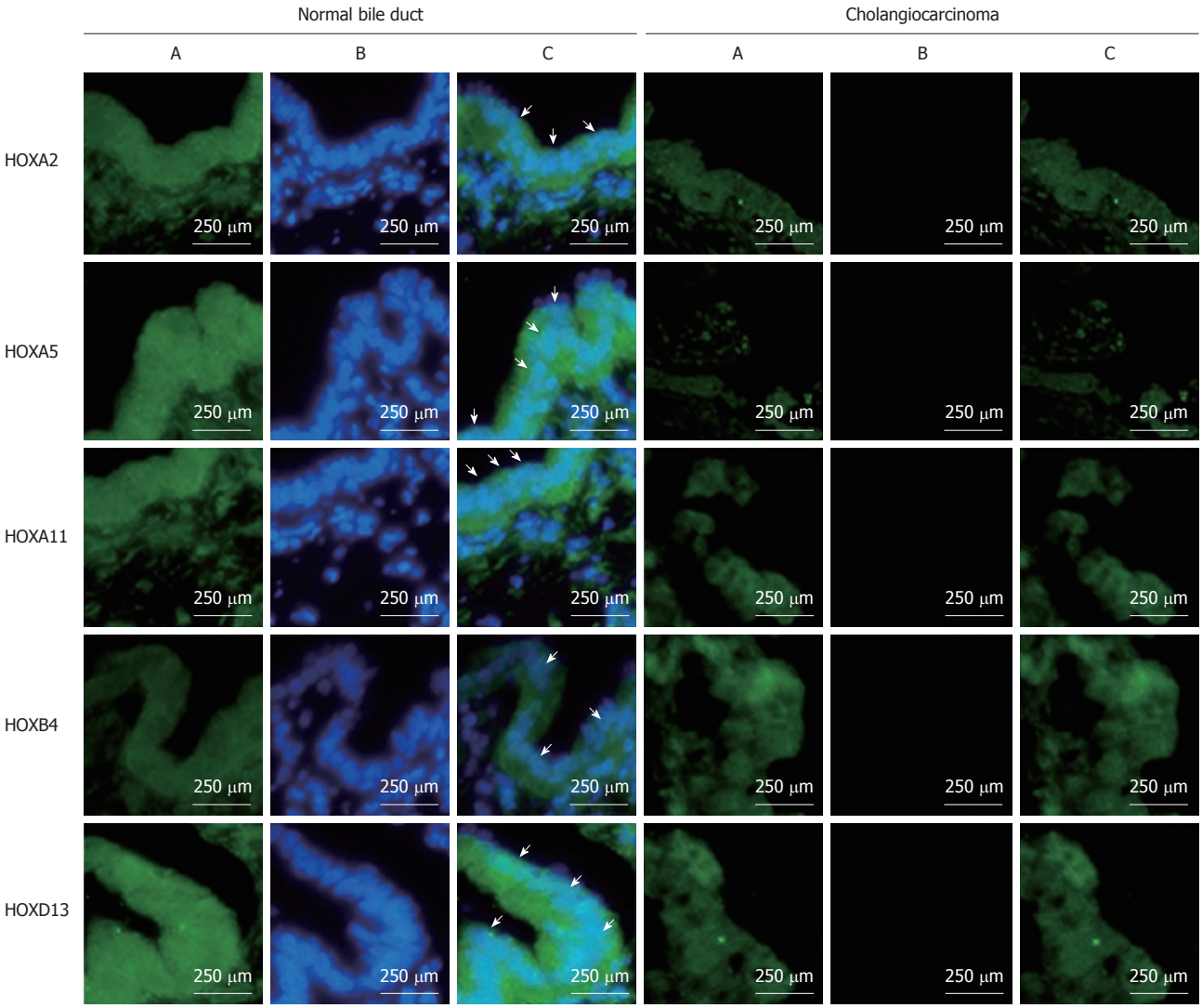

Figure 4 Expression of HOXA2, HOXA5, HOXA11, HOAB4, HOXD13 in extra-hepatic cholangiocarcinoma by immunofluorescence. Green channel is nuclear staining by 4',6-diamidiono-2-phenylindole (DAPI) (A), blue channel is the target gene (B), and overlay of DAPI and target genes (C). Arrows indicate the positive target genes in specimens.

are in clinical trials and have been shown to affect gene expression *in vivo*. Although there is considerable literature on the possible antitumor mode of action of DNA methylation, such as 5-aza-2-deoxycytidine drugs, their exact mechanism in CCA remains unclear and needs further investigations.

We have shown that the CpG islands hypermethylation of *HOX* genes is not a unique feature of TFK-1 cells, but common among ECC samples. However, the expression of *HOX* genes was detected more frequently in low-stage ECCs than in high-stage ECCs, the difference being not statistically significant. Our results were in line with the research published by Kim *et al*[7] that no association was found between CpG island hypermethylation and histologic classification in terms of hypermethylation of methylated CpG island locus or the total number of methylated CpG island loci. Jacinto *et al*[32] evaluated the aberrant hypermethylation changes in the benign colorectal adenomas, a precursor to invasive colorectal tumors, and suggested that hypermethylation of genes was an early event in the pathway to a full-blown tumor. To date, there have been few studies on the methylation patterns of precancerous lesions of CCA. We did not assess the association between DNA methylation changes and precancerous lesions of CCA in the present study.

As important members of the homeobox superfamily, *HOX* genes encode transcription factors and contribute to oncogenesis by allowing activation of anti-apoptotic pathways. HOXA5 has been identified to directly regulate the expression of *p53* and *hMLH1* genes as a transcription factor in breast cancer cells, and contribute to safeguarding the cells against malignant transformation. Moreover, its expression can induce the apoptosis in breast cancer through p53-independent apoptotic pathway mediated by caspases2 and 8[33]. Our results were in agreement with the results by others authors that the frequency of hypermethylated HOXA5 was high in ECC. However, additional studies are still needed before exact mechanism can be concluded.

DNA methylation of polycomb group target genes

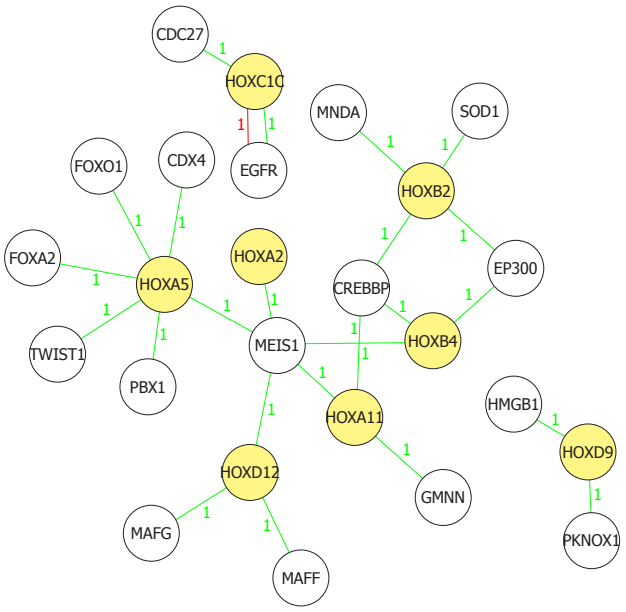

Figure 5 Network analysis of known biological relationships between *HOX* genes and their related genes. Network analysis was performed and produced by Molecule Annotation System. Yellow icons indicate methylated *Hox* genes and colorless icons indicate methylated non-*HOX* genes, which have been demonstrated to be related to *Hox* genes in other studies. Green lines indicate high correlation and red lines indicate low correlation. All present hypermethylated status in the microarray results. Number between icons indicates the relationship mentioned in the reports.

is an early step in tumorigenesis and can potentially be assayed to predict cancer risk. Fiegl *et al*[34] pointed out that DNA methylation of *HOXA11* gene, a member of PGGT genes, was frequently present in ovarian cancer and HOXA11 methylation status was a prognostic marker. In our research, expression of HOXA11 was rare in TFK-1 cell lines and ECC. Silenced *HOX* genes in ECC, which might be induced by hypermethylation, might work as differential epigenetic biomarkers between malignant and benign biliary tissues.

HOXD13, binding origins primarily during G1 phase of the cell cycle, promotes the assembly of pre-replication complex proteins at replication origins and stimulates DNA synthesis *in vivo* in a transient DNA replication assay[35,36]. According to the literature, the expression of HOXD13 always increased in malignant tumors, such as brain, lung, and prostate carcinoma. However, our result that HOXD13 was methylated in TFK-1 cell lines with decreased expression, was opposite to the results from other studies. Besides, the expression frequency of HOXD13 was only 6.67% in ECC. Our results agreed with a recent work which demonstrated that HOXD13 expression decreased in pancreas and stomach tumor subtypes[37].

HOXA2 is regulated by growth hormone and growth factors. Kazuhiko Maeda has demonstrated that the expression frequency of HOXA2 in melanoma with distant metastasis was higher than that in melanoma without metastasis[38]. We gained an opposite result in ECC. The expression of HOXA2 was only 10% in ECC.

We speculated that tissue and cancer specificity might be responsible for the differential expression of HOXA2 and HOXD13 in ECC compared with other cancers. Several recent genome studies show that DNA methylation profiles in mammals are tissue specific[39,40]. Doi *et al*[41] demonstrated that differentially methylated regions in the reprogrammed cells completely distinguished brain from liver and spleen tissues and largely distinguished colon cancer from normal colon tissues. The studies also demonstrated that methylation pattern of *HOX* genes also differed greatly among various kinds of cancers, such as hypermethylated HOXA5, HOXA10 and HOXB7 in breast, hypermethylated HOXB13 and HOXC8 in prostate, and hypermethylated HOXD1, HOXD8, HOXC6 and HOXC11 in neuroblastoma. However, our understanding of tissue-specific DNA methylation of *HOX* genes in cancer is still limited and many questions remain to be answered.

HOXB4 is always considered as a catalyst for leukemia development[42]. To our knowledge, this is the first study to identify hypermethylated HOXB4 in the solid tumor. The expression frequency of HOXB4 was 6.67% in ECC samples.

Although MeDIP assay demonstrated that HOXC1, HOXD9, HOXB2, HOXB5 and HOXD1 were methylated in TFK-1 cells, BSP assay suggested that the hypermethylation was not significant. The artificial methylation induced during the culture might be responsible for the positive results of microarray. Besides, the discrepancy might be attributed to the different methods used in the methylation assay. BSP is a DNA sequencing approach, which has a higher sensitivity for detecting allelic hypermethylation in target sequences than the MeDIP.

In conclusion, we used the high throughput MeDIP microarray to investigate methylation profile of ECC. We identified 2013 differentially hypermethylated CpG islands that were involved in various cellular processes, such as cell-cell adhesion, cell migration, signal transduction and cell repair. The results provided a foundation for further studies about the mechanism of ECC. HOXA2, HOXA5, HOXA11, HOXB4 and HOXD13 showed a high frequency of hypermethylation in ECC cells and loss of expression in ECC samples, which raised the possibility that *HOX* genes might work as differential epigenetic biomarkers between malignant and benign biliary tissues.

COMMENTS

Background

Extrahepatic cholangiocarcinoma (ECC) is a malignant cancer with ineffective treatment and poor prognosis. It has been confirmed that aberrant epigenetic alterations contribute to cancer formation in multiple of cancers. DNA hypermethylation is the most common epigenetic abnormality in cancer.

Research frontiers

Aberrant promoter hypermethylation is an important mechanism of gene inactivation and contributes to the carcinogenesis of ECC. However, many epigenetically silenced genes have already been identified in cholangiocarcinoma, methylation file of ECC is still unclear. In this study, the authors compared differential methylation profile between normal bile duct epithelial cell and ECC cell lines

by genome-wide CpG methylation profiling to discover candidate methylated genes.

Innovations and breakthroughs

The advent of microarray technologies that enables the analysis of a large number of DNA/RNA fragments in a high throughput way has opened new opportunities for epigenetic studies. This is the first study to utilize the high throughput Methylated DNA Immunoprecipitation (MeDIP) microarray to investigate methylation file of ECC. The authors identified 2013 differential hypermethylated CpG islands that were involved in various cellular processes. Furthermore, the authors validated that HOXA2, HOXA5, HOXA11, HOXB4 and HOXD13 showed high frequency of hypermethylation and loss expression in ECC.

Applications

By understanding the differential methylation profile of HOXA2, HOXA5, HOXA11, HOXB4 and HOXD13 in ECC, this study may raises the possibility that *HOX* genes may work as differential epigenetic biomarkers between malignant and benign biliary tissues for diagnosis and treatment.

Terminology

In mammals, 39 human *HOX* genes are located in four clusters (A-D) on different chromosomes at 7p15, 17q21.2, 12q13, and 2q31 respectively. *HOX* genes are evolutionarily high conserved. HOX proteins can function as monomers or homodimers to directly drive the transcription of downstream targets, and sequester other proteins to enhance or repress gene expression. *HOX* genes are integral to normal temporospatial limb and organ development along the anterior-posterior axis.

Peer review

The manuscript of Shu *et al* entitled "Identification of methylation profile and novel tumor marker of cholangiocarcinoma with MeDIP microarray" adds new evidence to the potential role of DNA methylation in ccholangiocarcinoma development and progression.

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ORIGINAL ARTICLE

Late SV40 factor: A key mediator of Notch signaling in human hepatocarcinogenesis

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Abstract

AIM: To investigate the relationship between late SV40 factor (LSF) and Notch signaling in the development and progress of hepatocellular carcinoma (HCC).

METHODS: Liver cancer tissue specimens from 25 patients were analyzed for Notch-1 and LSF expression by immunohistochemistry. The correlation between expression and the biological effects of Notch-1 and LSF were analyzed using genetic and pharmacological strategies in HCC cell lines and human normal cell lines, including hepatic stellate cells (HSC) and human embryonic kidney epithelial cells (HEK).

RESULTS: Immunohistochemistry showed that both Notch-1 and LSF were significantly upregulated in HCC samples (76%, 19/25, $P < 0.0001$ and 84%, 21/25, $P < 0.0001$, respectively) compared with non-cancer samples. Activation of Notch-1 by exogenous transfection of Notch1 intracellular domain increased LSF expression in HSC and HEK cells to levels similar to those seen in HepG2 cells. Furthermore, blocking Notch-1 activation with a γ-secretase inhibitor, DAPT, downregulated LSF expression in HepG2 cells. Additionally, a biological behavior assay showed that forced overexpression of LSF promoted HepG2 cell proliferation and invasion.

CONCLUSION: LSF is a key mediator of the Notch signaling pathway, suggesting that it might be a novel therapeutic target for the treatment of HCC.

Key words: Notch receptor; Late SV40 factor; Signal transduction; Hepatocellular carcinoma

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INTRODUCTION

Hepatocellular carcinoma (HCC) is the third leading cause of cancer-related death in the world[1]. In China, the incidence of HCC has nearly doubled over the past 2 decades, and it is now second only to lung cancer in terms of total deaths[2]. The high mortality rate of HCC is due to the fact that no systemic therapy is available for advanced cases of the disease[3]. A previous study suggested that deregulated Notch signaling plays a crucial role in the transformation and neoplastic proliferation of human malignancies[4]. We showed that the transcriptional factor, late SV40 factor (LSF), is overexpressed in the liver of > 80% of HCC patients compared with normal liver. How-

ever, the exact role played by aberrant Notch signaling in hepatocarcinogenesis has not been elucidated. Here, we identified LSF as a downstream mediator of Notch1 signaling and showed that LSF mediates, at least in part, Notch1-induced carcinogenesis.

Notch genes encode heterodimeric transmembrane receptors, which play a critical role in maintaining the balance between cell proliferation, differentiation, and apoptosis. Aberrant expression of wild-type Notch receptors, ligands and downstream targets has been reported in cervical carcinoma[5,6], lung[7], colon[8], head and neck[9] and renal carcinomas[10], acute myeloid leukemia[11] and Hodgkin's and large-cell lymphomas[12]. Hence, aberrant Notch signaling may contribute to carcinogenesis.

LSF, also known as LBP-1c and TFCP2, is a ubiquitously expressed mammalian transcription factor that regulates diverse cellular and viral promoters[13,14]. A major cellular target of LSF is the thymidylate synthase gene, which encodes the rate limiting enzyme in the production of dTTP, required for DNA synthesis[15]. Deregulated LSF expression might facilitate entry into the G1/S phase of the cell cycle, promote DNA synthesis, stimulate transformation, and facilitate cancer cell survival. However, there is little evidence to suggest a potential role for LSF in HCC. Moreover, the relationship between Notch-1 and LSF in human hepatocarcinogenesis is unknown.

In this study, we identified LSF as a novel mediator of dysregulated Notch signaling, and showed that activation of Notch-1 in human Ras-transformed cells upregulated the expression of LSF. We also performed a detailed experimental analysis to elucidate the relationship between Notch signaling and LSF expression in hepatocarcinogenesis and to identify signaling pathways that may be targeted for the treatment of highly aggressive HCC.

MATERIALS AND METHODS

Tissue specimens

Archived liver cancer specimens were obtained from the Department of Pathology, Nanjing First Hospital, from November 2008 to January 2010. Twenty-five patients (20 men and 5 women) undergoing surgery for HCC were enrolled in the study. All diagnoses were based on pathological and/or cytological evidence. Paraffin sections were tested by histopathological examination and immunohistochemistry. Histological classification was conducted according to the criteria set out by Edmonson and Steiner[16]. Ethical approval was obtained from the hospital and informed consent was given by all patients prior to sample collection.

Cell lines and drugs

The human HCC cell lines, HepG2, Bel-7402, and QGY-7703 were obtained from the Chinese Academy of Sciences (Institute of Shanghai Cell Biology and Chinese Type Culture Collection, China). Human embryonic kidney epithelial cells (HEK), hepatic stellate cells (HSC) and hepatocytes (L02 cells) were preserved in our laboratory. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, USA) containing 10% fetal bovine serum (FBS) (Wisent, Canada) and maintained in humidified air containing 5% CO_2 at 37℃ and 95% humidity. HepG2 cells and hepatocytes (negative controls) were treated with 50 μmol/L DAPT (Sigma, USA) to block Notch signaling.

Construction of the H-Ras-pEGFP-C_1, NICD1-pEGFP-C_1 and LSF-pEGFP-C_1 recombinant vector and cell transfections

Reverse transcription-polymerase chain reaction (RT-PCR) was carried out using human HCC cell RNA as the template (Table 1). After incubation with specific restriction endonucleases (*Bam*H Ⅰ and *Xho* Ⅰ for hRAS, Takara, Japan; *Bgl*Ⅱ and *Hin*dⅢ for Notch-ICD1, Takara; and *Bgl*Ⅱ and *Hin*dⅢ for LSF, Takara) the PCR products were inserted into the corresponding sites of the pEGFP-C1 vector. Notch-ICD1 (bp 5309-7655 bp of the Notch-1 intracellular domain), the open-reading frame of H-Ras, and LSF were cloned into pEGFP-C1 (Clontech, Palo Alto, California, USA) to generate the H-Ras-pEGFP-C_1, NICD1-pEGFP-C_1, LSF-pEGFP-C_1 recombinant vectors (for expression of green fluorescent protein), respectively. Transfection efficiency was estimated from the percentage of GFP-positive cells.

Successful construction of the recombinant vectors was confirmed by enzyme digestion and gene sequencing. The recombinant vectors were then transiently transfected into HEK cells and stably transfected to HSC cells using Lipofectamine 2000 (Invitrogen). NICD1-pEGFP-C_1 and LSF-pEGFP-C_1 were stably transfected to HepG2 cells after screening with 450 mg/L G418 (Sigma).

Western blotting analysis

Immunoblotting of cellular extracts was performed using antibodies to Notch1-ICD (Cell Signaling Technology, MA, USA), LSF (Abcam Incorporation, MA, USA), H-Ras (Oncogene Research Products, CA, USA) and β-actin (Santa Cruz Biotechnology, CA, USA). The secondary antibodies used were HRP-conjugated anti-rabbit and anti-mouse Ig (Santa Cruz Biotechnology). Cells were harvested using NE-PER™ Nuclear and Cytoplasmic Extraction Reagents (Pierce, Rockford, USA) and equal amounts of cellular proteins were separated on 8% SDS-PAGE gels. Proteins were transferred to PVDF membranes, and the blots were probed with primary antibodies to Notch1-ICD (intracellular domain) and LSF. β-actin was used as the internal control protein. Blots were washed 3 times for 5 min with phosphate buffered saline (PBS) containing 0.1% Tween-20, and incubated with the HRP-conjugated secondary antibody (1:5000) for 1 h. The membranes were washed as described above and the bands detected by enhanced chemiluminescence (Pierce, Rockford, USA).

Table 1 Reverse transcription-polymerase chain reaction primer sequences

Primer	Sequence (5′-3′)	Product size (bp)	Temperature (℃)
Notch1-ICD	Forward: CTAAGATCTCCTGAGGGCTTCAAAGTGTC Reverse: GCGAATTCCTTGAAGGCCTCCGGAAT	2375	63
H-Ras (ORF)	Forward: GAGGATCCATGACGGAATATAAGCTGG Reverse: GTGAATTCTCAGGAGAGCACACACTTG	668	61
Hes-1	Forward: ATGAACGAGGTGACCCGCTT Reverse: CTGGAAGGTGACACTGCGTT	443	62
LSF (ORF)	Forward: GGAAAGATCTAGGATGGCCTGGGCTCTGAAG Reverse: CAAAGCTTGGGCACGAAACGCCGCACTCCT	1547	59
LSF (promoter analysis)	Forward: CTAGGTACCCAACATGGTAAGATCCTGTCTCT Reverse: GGAGATCTTCTCATCCCTGCTTTCTGTTTCCT	2020	56
GAPDH	Forward: GGTGGAGGTCGGAGTCAACGGA Reverse: GAGGGATCTCGCTCCTGGAGGA	240	60

ORF: Open-reading frame; ICD: Intracellular domain; LSF: Late SV40 factor; LSF (promoter analysis): Upstream sequence before start code.

Immunohistochemistry

Immunohistochemistry was performed on formalin-fixed, paraffin-embedded surgical liver cancer specimens from 25 patients. Detection of the antigen-antibody complex was performed using the Mouse ABC staining kit (Santa Cruz Biotechnology, USA). Staining of sections was assessed in 10 consecutive fields (× 20 magnification) using a validated semi-quantitative scale where - denotes absence of staining; +/- denotes occasional, weak hepatocytic staining; + denotes staining of > 5% hepatocytes; ++ denotes staining of 6%-30% of hepatocytes, and +++ denotes staining of > 30% hepatocytes (high expression).

Luciferase reporter gene assay

LSF DNA sequences between nucleotides (nt) 51 475 800 and 51 478 000 of GenBank accession GCF_000001305.13 were cloned into the pGL-3 vector (Premerger, USA) to generate LSF-pGL-3. Transient co-transfection into HSC was performed using a control plasmid (pSV-βgal) as an internal control in 96-well plates using the Lipofectamine 2000 transfection reagent. Dual luciferase activity was measured after 48 h using a kit from Promega (Madison, Wisconsin) and values were normalized for protein content and transfection efficiency (established from the percentage of GFP-positive cells).

Flow cytometric analysis

The HepG2 cell cycle was assessed by flow cytometric analysis. Briefly, HepG2 cells were harvested and immediately fixed in 70% ethanol at 4℃ overnight. Cells were then treated with 50 mg/L RNaseA (Sigma) for 30 min at 37℃ and stained for 10 min with 50 mg/L PI (Sigma). Samples were then analyzed for their DNA content using a FACSAria Cell Cytometer (BD Biosciences, San Jose, CA, USA). Data were analyzed using Cell Quest software (BD Biosciences).

Cell proliferation and viability assay

To assess cell growth, HepG2 cells infected with LSF-pEGFP-C (1×10^6 cells/mL) were seeded into 25 cm^2 plates. The cells were cultured for 24 h in the serum-free DMEM supplemented with insulin (10 mg/mL) for synchronization. The cells were then washed three times in PBS (pH 7.4), digested with 0.25% trypsin, and prepared as a single-cell suspension. The cells were then plated into 96-well plates at a concentration of 2×10^3 cells/mL. The cells were cultured at 37℃ in 5% CO_2 and 95% humidity for 24, 48, 72, and 96 h. A thiazolyl blue test was performed to measure the absorbance at 492 nm. Four hours prior to each time point, MTT (20 μL) was added to each well at 37℃ for 4 h. After removing the medium, 150 μL dimethylsulfoxide was added to each well and the A was measured using a microplate reader (Multiskan MK 3, Thermo, Germany).

Colony formation assay

HepG2 cells were pre-treated by infection with LSF-pEGFP-C_1. One thousand cells were seeded into six-well plates with 2 mL culture medium containing 10% FBS. After culture in DMEM containing 10% FBS at 37℃ in a humidified, 5% CO_2 atmosphere the colonies were counted. Cells were washed twice with PBS, stained with Giemsa, and colonies containing > 50 cells were counted. The cloning efficiency (%) = (the number of clones / the number of seed cells) × 100%.

Invasion assay

Cell invasion assays were performed in 6.5 mm Transwells (8.0 μm pore size) (Coring Incorporation, USA). Prior to each experiment, the polycarbonate filters were coated with diluted Matrigel (BD Bioscience). Untreated and treated HepG2 cells were added to the coated filters (5×10^4 cells/filter) in 200 μL of serum-free DMEM in triplicate wells. DMEM medium containing 10% FBS was added to the lower chambers. After 24 h at 37℃ in a 5% CO_2 and 95% humidity incubator, the non-invading cells on the upper surface of the filter were wiped off using a cotton swab. Invading cells were fixed in 95% alcohol for 10 min and stained with Giemsa for 12 min. The number of cells in five randomly selected fields was counted under a microscope (× 200 magnification).

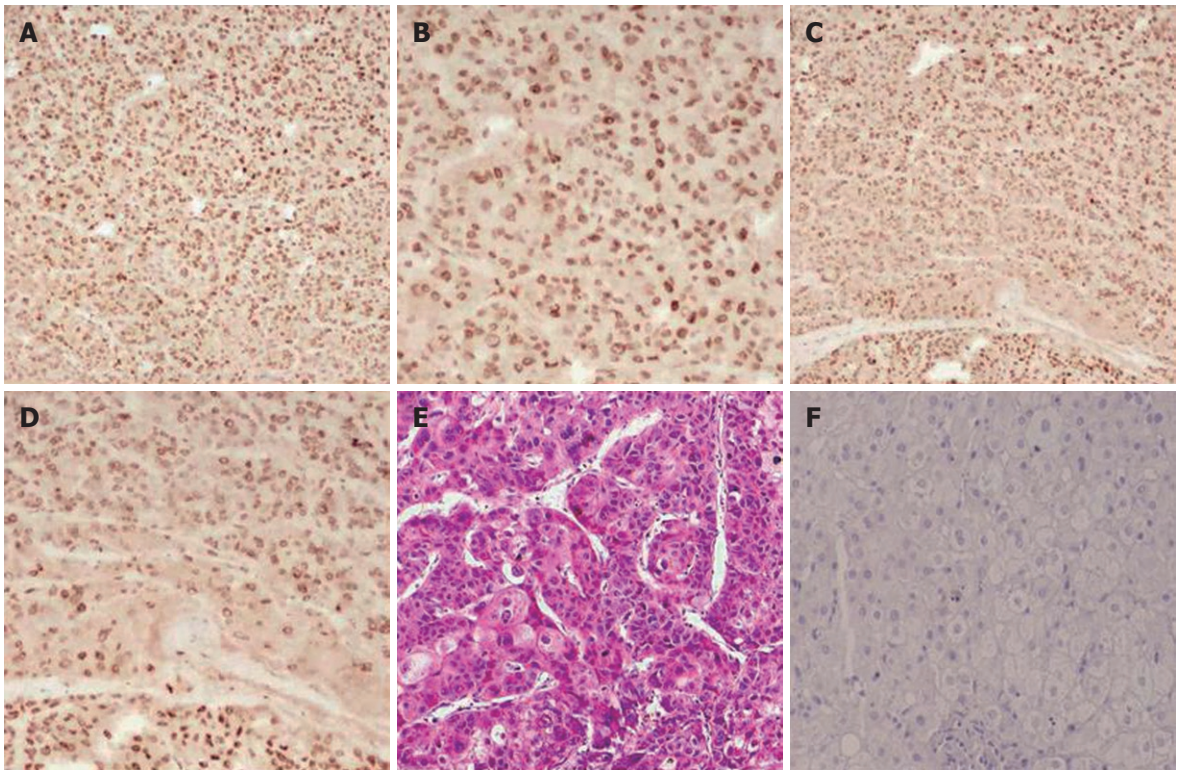

Figure 1 Correlation between late SV40 factor overexpression and aberrant Notch-1 activation in liver cancer. Liver cancer tissue specimens from 25 patients were analyzed for Notch-1 and late SV40 factor (LSF) expression by immunochemistry. A: Representative sample showing the strong correlation between Notch-1 expression and LSF expression; A and B were labeled with Notch-1 antibody (magnification: A × 10 and B × 20); C and D were labeled with an anti-LSF antibody (magnification: C × 10 and D × 20); E: Liver cancer specimen stained with hematoxylin and eosin; F: Human normal liver tissue specimen labeled with Notch1 and LSF antibodies. Expression is negative (control).

In vivo tumorigenicity assays

For the *in vivo* tumorigenicity assays, SCID/NOD mice (Shanghai, China) were housed under specific pathogen-free conditions. Mice (n = 8) were injected subcutaneously in the left flank with 3 × 10^6 HepG2 cells transfected with LSF-pEGFP-C_1 or with an empty vector. Tumor growth was measured every 3-4 d in a 3-dimensional fashion using a caliper. All animal studies were conducted under IACUC-approved protocols at Southeast University, Nanjing, China.

Statistical analysis

All results were expressed as the mean ± SD or as percentages where appropriate. Significant differences were tested using SPSS 12.0 (SPSS Inc., Chicago, IL, USA) and a 2-tailed t-test or Fisher's exact test. $P < 0.05$ was determined to be statistically significant.

RESULTS

Notch-1 and LSF expression are associated with HCC

Notch-1 interacts with many downstream effectors that regulate complex cytoplasmic signaling networks. We studied the expression of Notch1-ICD and LSF in 25 cases of human primary HCC using immunochemistry (Figure 1). Notch1-ICD was detectable in 19/25 cases (Figure 1A and B). Twenty-one out of 25 cancers were positive for LSF (Figure 1C and D). Liver cancer specimens stained with hematoxylin and eosin are shown in Figure 1E. Normal human liver tissue specimens were also labeled with Notch1 and LSF antibodies. The results showed negative expression in normal tissue (Figure 1F). Tumors positive for Notch1-ICD showed strong, condensed nuclear staining for LSF.

Next, we investigated the correlation between Notch1-ICD and LSF expression and HCC stage. As shown in Table 2, high expression of Notch1-ICD and LSF was observed during the advanced pathological stages of HCC [Notch1-ICD was positive in 3/8 (37.5%) of stage Ⅰ/Ⅱ, and in 16/17 (94.1%) of stage Ⅲ/Ⅳ tumors, P = 0.006. LSF was positive in 4/8 (50%) of stage Ⅰ/Ⅱ and in 17/17 (100%) of stage Ⅲ/Ⅳ tumors, P = 0.006. Notch1-ICD and LSF were both positive in 2/8 (25%) of stage Ⅰ/Ⅱ stage and 14/17 (82.5%) of stage Ⅲ/Ⅳ tumors, P = 0.01]. However, there was no correlation with patient gender, age, histological type and cellular differentiation. Although the expression of Notch1-ICD and LSF was more frequent in HCC samples [Notch1-ICD was positive in 15/25 (60%); LSF was positive in 16/25 (64%); and Notch1-ICD and LSF were both positive in 13/17 (76.5%)] than in the other histological types, no statistically significant difference was found (P = 0.059, P = 0.081 and P = 0.359, respectively).

Taken together, these results suggest that activation of Notch-1 signaling and elevated LSF expression play a key role in the pathogenesis of HCC.

Table 2 Association between the activation Notch-1 and late SV40 factor in liver cancer specimens and clinicopathological features

Patients	*n*	Notch[1]	LSF[2]	Notch1 and LSF[3]	*P* value
Gender					0.562[1], 1.000[2], 0.312[3]
Male	20	16	17	14	
Female	5	3	4	2	
Age (yr)					0.194[1], 0.548[2], 0.075[3]
< 50	7	4	5	2	
⩾ 50	18	15	16	13	
Histological type					0.059[1], 0.081[2], 0.359[3]
HCC	17	15	16	13	
Bile duct cell carcinoma	1	0	0		
Nodular hepatocellular carcinoma	6	4	5	4	
Other	1	0	0		
Cellular differentiation					0.137[1], 0.294[2], 1.000[3]
Well/moderately	18	12	14	11	
Poor/undifferentiated	7	7	7	5	
Stage					0.006[1], 0.006[2], 0.01[3]
Ⅰ/Ⅱ	8	3	4	2	
Ⅲ/Ⅳ	17	16	17	14	

[1]Notch1 *P* value; [2]LSF *P* value; [3]Notch1 and LSF *P* values; Fisher's exact test. HCC: Hepatocellular carcinoma; LSF: Late SV40 factor.

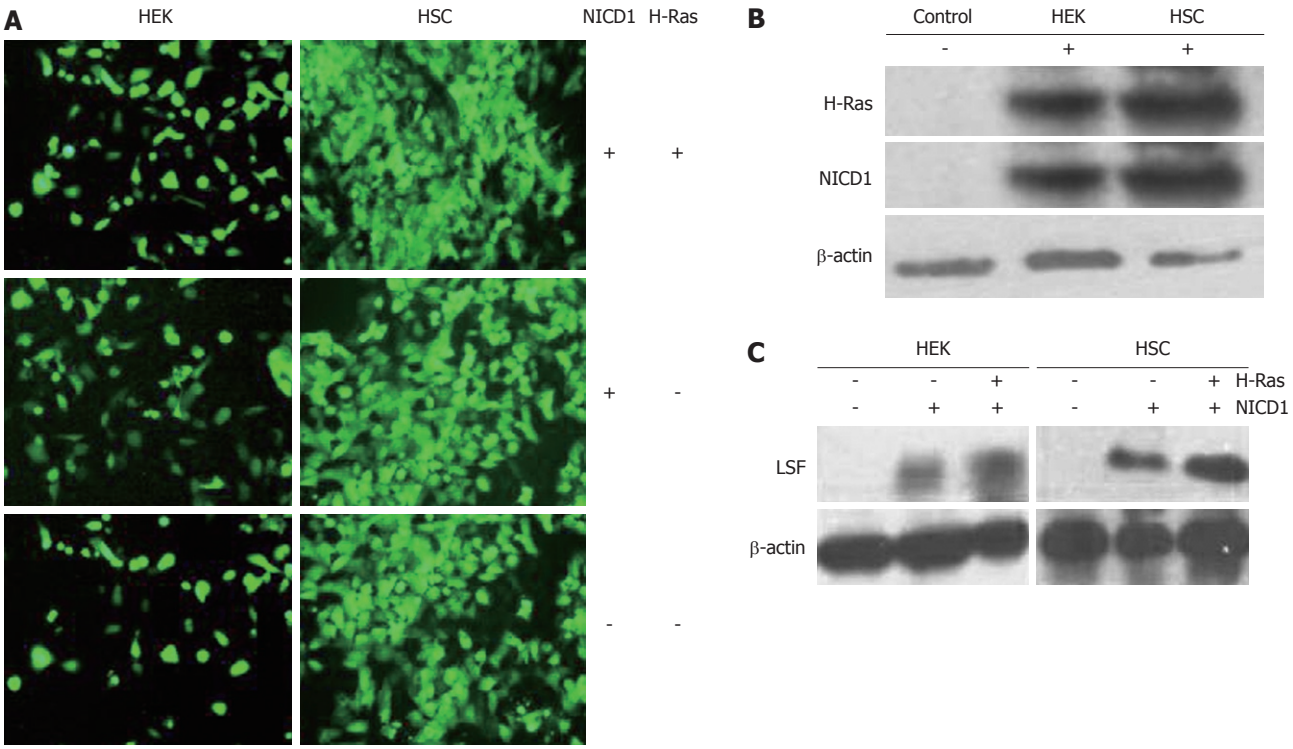

Figure 2 Late SV40 factor expression is increased in human normal cells showing forced overexpression of exogenous Notch1-ICD. A: Transient transfection of H-Ras and/or NICD1 and stable transfection of H-Ras and/or NICD1 into hepatic stellate cells (HSC); B: Western-blotting analysis of H-Ras and NICD1 from human embryonic kidney (HEK) cells and human HSC; C: Late SV40 factor (LSF)-dependent expression correlates with Notch1-ICD protein levels in human normal cells after forced overexpression of exogenous Notch1-ICD. Co-expression of H-Ras and Notch1-ICD significantly increases LSF levels.

LSF is upregulated in normal human cells after forced overexpression of exogenous Notch1-ICD

LSF is an important mammalian transcription factor that binds cellular promoters modulated by cell growth signals[13,14]. In this study, we examined the role of LSF and Notch-1 in 2 human H-Ras-transformed cell lines, HSC and HEK (Figure 2A). Western blotting with antibodies against intracellular Notch-1 revealed one major band with an apparent molecular mass of 110kD, corresponding to the intracellular cleavage product, Notch1-ICD. Western blotting also detected H-Ras (21kD, Figure 2B). Western blotting with antibodies to LSF revealed one major band with an apparent molecular mass of 63kD (Figure 2C). These results show that cells expressing exogenous Notch1-ICD can augment the expression of LSF protein. Furthermore, we observed that LSF

was robustly up-regulated in both HSC and HEK cells after co-transfection of H-Ras-pEGFP-C1 and NICD1-pEGFP-C1. This suggests that LSF is involved in Notch signaling and establishes a regulatory role for LSF in the pathogenesis of HCC.

LSF, a new mediator of deregulated Notch signaling in HCC

We next explored the relationship between Notch-1 and LSF in HCC cell lines from human primary tumors using pharmacological and genetic approaches. Western blotting analysis of Notch1-ICD and LSF expression in 3 HCC cell lines showed that Notch1-ICD and LSF levels were upregulated (hepatocytes were used as a negative control, Figure 3A). HepG2 cells were then treated with DAPT, which blocks endogenous Notch-1 activation. This resulted in decreased expression of Notch1-ICD and downregulation of LSF (Figure 3B). Next, NICD1-pEGFP-C1 was transfected into HepG2 cells expressing constitutively activated Notch-1 (Figure 3B). LSF-pEGFP-C1 was also introduced into HepG2 cells (Figure 3C). The expression levels of Notch-ICD and LSF protein were both elevated (Figure 3B-E). Hes-1 mRNA levels were also increased in NICD1-pEGFP-C1/HepG2 cells compared with that in the controls (untreated HepG2 cells) (Figure 3F and Table 1). We determined that perturbed Notch signaling significantly upregulated LSF expression in the HCC cell lines. We confirmed these results by stably introducing Notch1-ICD into HSC cells and by transient transfection in HEK cells as described above. Activation of LSF-dependent transcription is an indicator of LSF activity. The promoter assay showed that LSF reporter activity in HSC transfected with NICD1-pEGFP-C1 was directly correlated with LSF protein levels (Figure 3G), showing an approximately 60-fold increase above that in the controls. Taken together, these data show that LSF is a novel mediator of dysregulated Notch signaling in HCC.

LSF stimulates cell proliferation and promotes cell cycle progression and invasion of HepG2 cells in vitro

We next explored the biological role of LSF in the progression of HCC. As cell proliferation is closely linked to progression through the cell cycle, we analyzed the cell cycle of cultured cells by flow cytometry (Figure 4A). We first looked at the cell cycle in HepG2 cells transfected with LSF-pEGFP-C1. The data showed a decreasing proportion of cells in G0/G1 and an increasing proportion of cells in S phase compared with the controls (Figure 4B, $P < 0.01$). No obvious differences were observed at G2/M in any of the groups. The MTT assay showed that forced overexpression of LSF promoted proliferation and growth above that in untreated HepG2 cells (Figure 4C, $P < 0.01$), while there was no statistical significance between the growth of HepG2 cells and pEGFP-C1/HepG2 cells. However, the colony formation assay showed that the number of HepG2 cell clones overexpressing LSF was significantly increased compared with that in the controls (Figure 5A-D, $P < 0.05$); a similar result was shown when calculating the clone efficiency (Figure 5E, $P < 0.05$). However, in this study, the invasion assay showed that the invasive ability of LSF-transfected HepG2 cells was increased compared with that of the controls (Figure 6, $P < 0.05$). Taken together these observations suggest that LSF accelerates the development and progression of HCC.

LSF increases the growth of tumors derived from HepG2 cells in vivo

In light of the pro-proliferative effects of LSF *in vivo*, we tested whether LSF could promote the proliferation of HCC cells *in vivo*. The tumorigenicity of HepG2 cells was examined in eight mice inoculated with either the empty vector or with the exogenous LSF expression vector. Representative tumor growth curves are shown in Figure 7A. The mean tumor volume was significantly larger in LSF-transfected nude mice than in those transfected with the empty vector (Figure 7B, $P < 0.01$).

DISCUSSION

HCC is a highly aggressive cancer for which there is no currently available effective treatment and it is the third most frequent cause of cancer deaths[17,18]. Therefore, identifying signaling pathways that could be targeted to enhance the sensitivity of a therapy is of great importance. Mounting evidence shows that constitutively active Notch receptors induce proliferative activity[19-22] and that deregulated expression and/or activity of wild-type Notch receptors occurs frequently in human malignancies, including T-cell acute lymphoblastic leukemias[11,21-26], HCC[27,28], non-small cell lung cancer[29], breast cancers[30], colon cancer[8], and Hodgkin's and large-cell lymphomas[12]. However, the possible molecular mechanisms underlying deregulated Notch signaling and its involvement in the development and progress of human HCC are unknown. Immunohistochemistry of liver cancer tissue specimens showed that Notch-ICD1 (76%, 19/25), LSF (84%, 21/25) and both LSF and Notch1-ICD (76.5%, 13/17) were abundantly expressed in HCC, but were either undetectable or only very occasionally expressed in non-tumor liver tissues. This suggests that their overexpression may be linked to cancer development and/or progression. Western blotting showed that cell lines derived from HCCs spontaneously overexpressed Notch-1 and LSF compared with normal hepatocytes. We also found increased LSF protein expression in HepG2 cells transfected with NICD1-pEGFP-C1. We used a γ-secretase inhibitor, DAPT, to block Notch signaling and found that, when Notch signaling was inhibited in HCC cell lines, LSF protein levels decreased in a time dependent manner. These observations suggest that overexpression of Notch1-ICD protein plays an important role in human hepatocarcinogenesis. However, we also observed a new phenomenon: that the expression of LSF increased along with upregulated Notch1-ICD protein levels in HepG2 cells. So, we investigated the relationship between LSF and

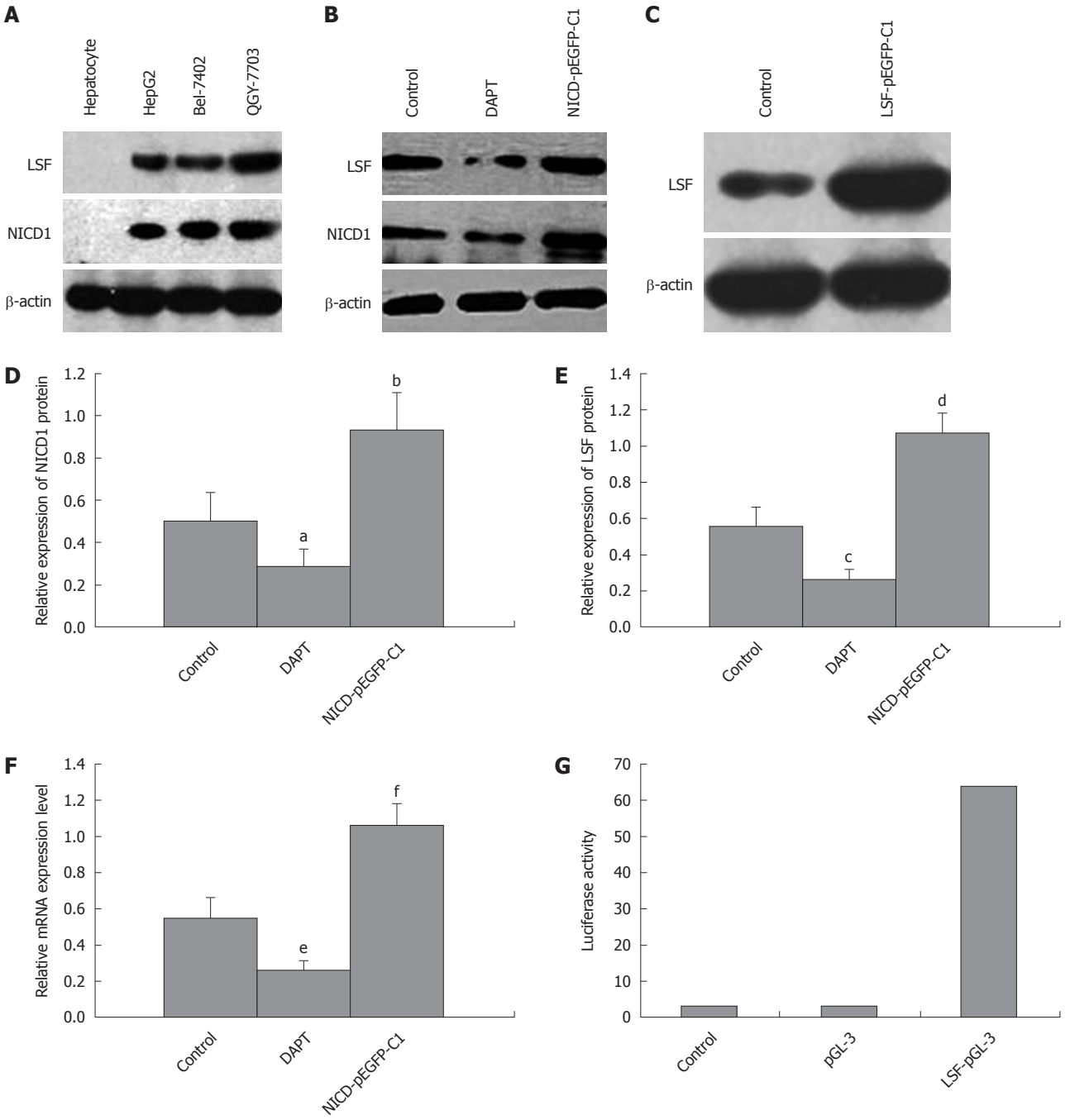

Figure 3 Aberrant Notch-1 activation upregulates late SV40 factor levels in hepatocellular carcinoma cells. A: Western-blotting analysis of Notch1 and late SV40 factor (LSF) expression in 3 hepatocellular carcinoma (HCC) cell lines. Notch1 and LSF levels are upregulated (hepatocytes were used as the negative control); B: Inhibition of Notch-1 signaling by 50 μmol/L DAPT (a γ secretase inhibitor). Notch1-ICD and LSF levels are significantly decreased compared with those in untreated HepG2 cells; C: Overexpression of exogenous LSF in HepG2 cells. LSF levels are significantly increased compared with those in untreated HepG2 cells. Representative blots are shown from three independent experiments with identical results. β-actin was used as an internal control for equal loading of samples and the relative ratios of each band were normalized to β-actin; D: The mean ± SE of 3 experiments analyzing the relative expression of Notch1-ICD (DAPT group: $^{a}P < 0.05$, Notch-ICD1 group: $^{b}P < 0.05$); E: DAPT group: $^{c}P < 0.05$, Notch-ICD1 group: $^{d}P < 0.05$; F: Hes-1 mRNA expression levels were determined by semi-quantitative reverse transcription-polymerase chain reaction and mRNA levels were normalized to those of GADPH. Results represent the mean ± SE of 3 independent experiments (DAPT group: $^{e}P < 0.05$, NICD1 group: $^{f}P < 0.05$); G: Promoter assay showing an approximately 60-fold increase in LSF reporter activity in hepatic stellate cells (HSC) transfected with NICD1-pEGFP-C1 compared with controls.

the Notch signaling pathway. We first performed a series of experiments involving 2 human tumor cell lines (HSC and HEK cells) expressing the human telomerase reverse transcriptase subunit and oncogenic H-Ras to explore the relationship between them. First, we determined that LSF expression was upregulated in NICD1-transformed human normal cell lines (such as HEK and HSC). Next, we showed that forced overexpression of exogenous Notch1-ICD in Ras-transformed cells robustly increased LSF expression. Furthermore, a promoter assay showed that

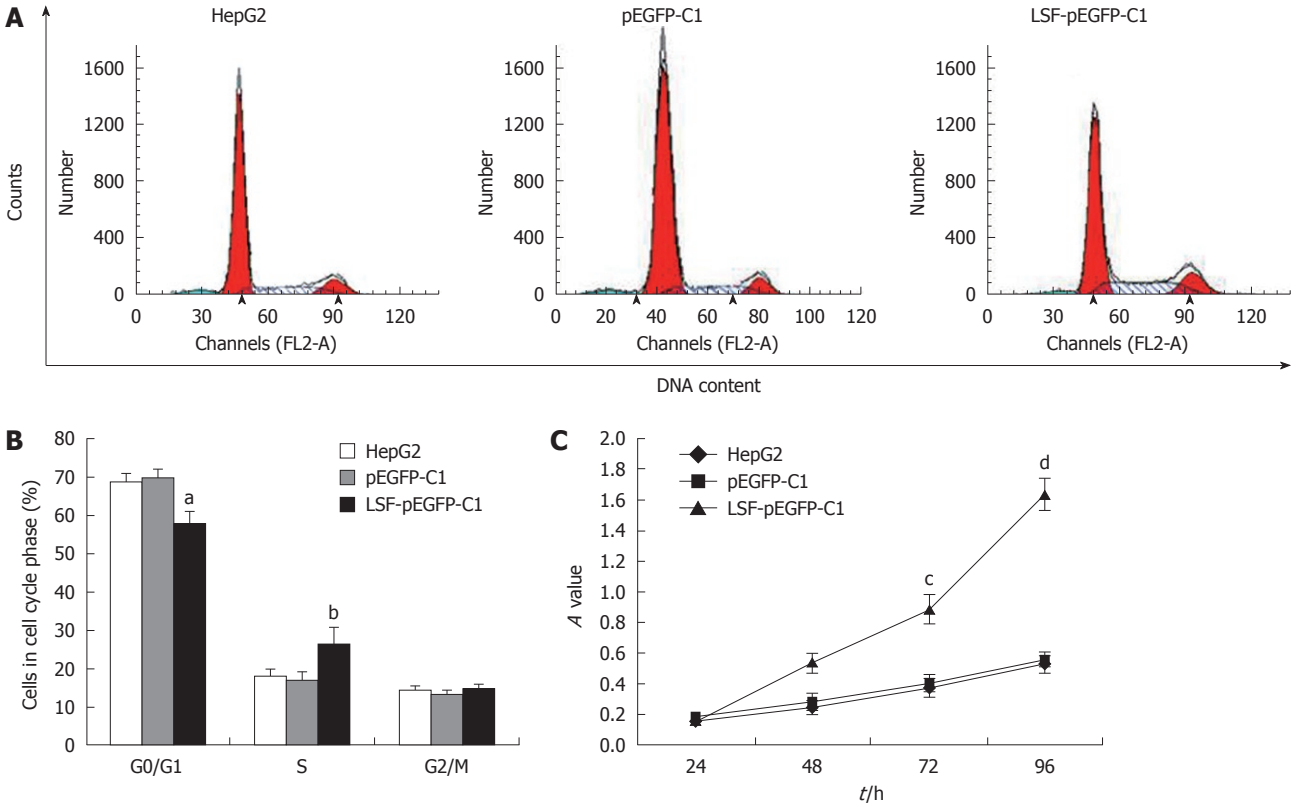

Figure 4 Effects of late SV40 factor overexpression on cell growth and the cell cycle of HepG2 cells. The cell-cycle profile was determined as the percentage of cells in the G0/G1 stage of the cell-cycle. Untransfected and transfected cells were harvested by trypsinization and fixed in 70% ethanol. A: Results of a representative experiment; B: Mean ± SE of 3 independent experiments ($^{a}P < 0.01$, $^{b}P < 0.01$ *vs* the other groups); C: Cell viability of the indicated cells at the indicated time points measured using a standard 3- (4,5-cimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay. Data represent the mean ± SE ($^{c}P < 0.01$, $^{d}P < 0.01$ *vs* the other

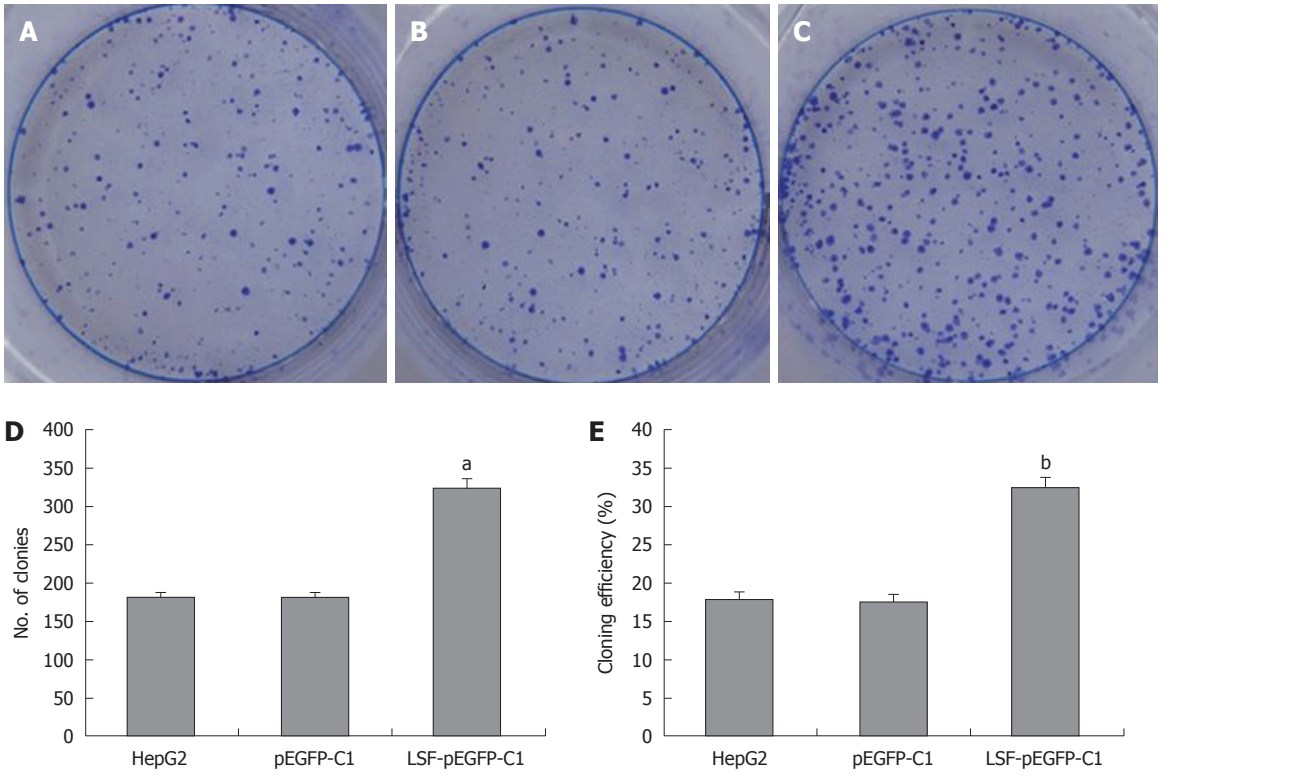

Figure 5 Exogenous late SV40 factor expression increases the proliferation of HepG2 cells. A: Colony formation assay for uninfected HepG2 cells; B: HepG2 cells transfected with pEGFP-C1; C: HepG2 cells transfected with late SV40 factor (LSF)-pEGFP-C1; D: Colony data represent the mean ± SE ($^{a}P < 0.05$); E: Clone efficiency data (mean ± SE; $^{b}P < 0.05$).

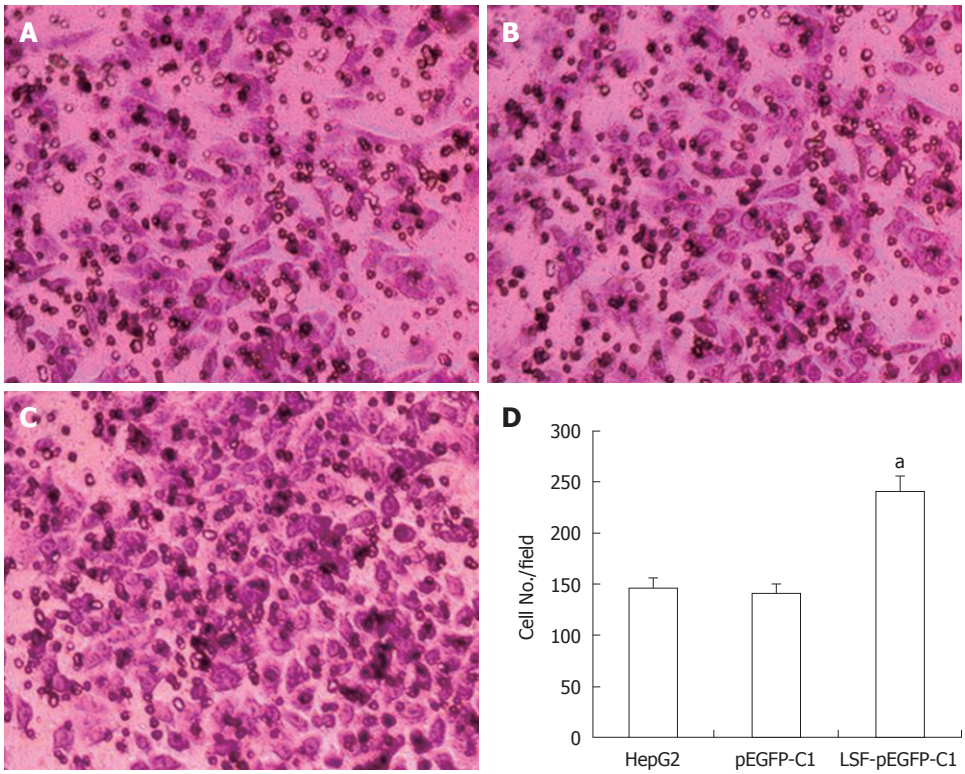

Figure 6 Effects of exogenous late SV40 factor expression on cell invasiveness. A: Matrigel invasion assay using uninfected HepG2 cells; B: HepG2 cells transfected with pEGFP-C1; C: HepG2 cells transfected with late SV40 factor (LSF)-pEGFP-C1; D: Data represent the mean ± SE ($^aP < 0.05$).

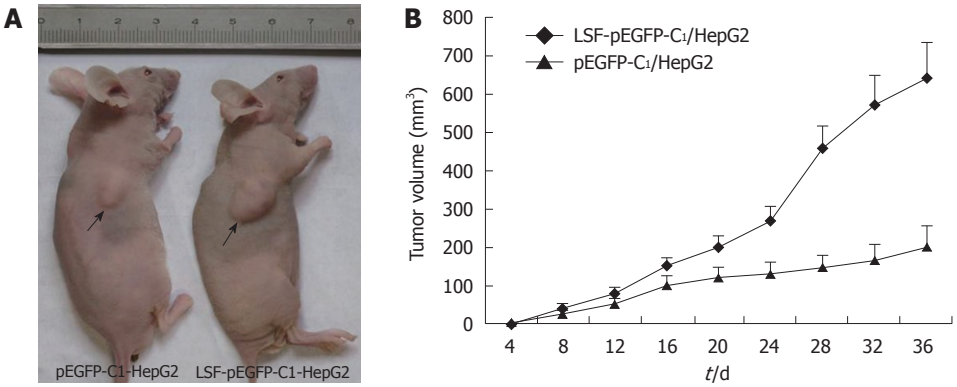

Figure 7 Overexpression of exogenous late SV40 factor increases the growth of HepG2 tumors *in vivo*. A: 2×10^6 cells were transfected with the late SV40 factor (LSF)-pEGFP-C1 recombinant vector or the empty vector and injected into nude mice ($n = 8$). Representative picture of nude mice injected with LSF-pEGFP-C1/HepG2 cells and pEGFP-C1/HepG2 cells (arrow) at week 5; B: The mean tumor volume ± SE (mm^3) within each group. The mean tumor volume in the LSF-pEGFP-C1/HepG2 mice ($P < 0.01$) was significantly larger than that in the empty-vector group.

LSF reporter activity in HSC transfected with NICD1-pEGFP-C1 was directly correlated with LSF protein levels. Thus, we speculate that LSF is a key mediator of the Notch signaling pathway.

LSF is directly targeted in diverse cell types by the MEK/ERK kinase pathway, which is central to growth factor signaling[31-33]. Recent data show that LSF acts as an oncogene in HCC[34]. However, any relationship between LSF signaling and its biological and tissue-specific functions is largely speculative at present. Our data suggest that LSF participates in dysregulated Notch signaling in human hepatocarcinogenesis. As described above, our observations also indicate that constitutively active Notch-1 increases LSF expression in liver cancer and in human HCC cell lines, such as HepG2. In fact, the exogenous Notch-1 activation we observed in normal human cells with upregulated LSF might underlie the molecular mechanisms involved in the pathogenesis of human HCC. In this study, the biological behavior assay suggested that LSF plays an important role in hepatocarcinogenesis. LSF is essential for cell cycle progression at the G1/S transition after reentry of quiescent cells into the cell cycle[13,14]. Our data suggest that LSF plays an important role in DNA synthesis and cell survival. Forced

overexpression of LSF in HepG2 cells resulted in highly aggressive tumors in nude mice. Our results also showed that pathological activation of Notch signaling and elevation of LSF is necessary for neoplastic proliferation, which facilitates entry into G1/S phase of the cell cycle, promotes DNA synthesis, and functions as an anti-apoptotic factor. These observations indicate the need for detailed investigations into the role of Notch signaling and LSF and Ras mutations, increased expression of wild-type Ras isoforms, and other mechanisms involved in the formation of human HCC.

Plausible mechanisms that perturb Notch signaling mediated by LSF effects in human hepatocarcinogenesis involved in growth promotion and inhibition of apoptosis. Further study of how LSF affects Notch signaling pathways should be performed in deficient mice and/or in cell cultures in which signaling is knocked down. It would be highly informative to tease out the distinct contribution of Notch signaling to the overall function of LSF. In further studies, we will investigate the role of LSF in dysregulated Notch signaling in HCC in more detail.

The results of this study indicate that LSF is a novel mediator of Notch signaling and plays a crucial role in the neoplastic proliferation, invasion, development and progression of human HCC. Our observations place LSF among the key mediators of Notch-1 signaling, and suggest that it might be a novel therapeutic target for the treatment of highly aggressive HCC.

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COMMENTS

Background

Although alterations in Notch1 and late SV40 factor (LSF) have been observed in hepatocellular carcinoma (HCC), little is known about their relationship during the development and progression of liver cancer. LSF has been identified as an important oncogene in human HCC. However, the mechanisms underlying tumorigenesis mediated by Notch1 and LSF are controversial and the relationship between them remains unclear.

Research frontiers

The Notch family, including Notch1, is associated with the development and progression of HCC, and LSF acts as a key oncogene in this disease. However, the mechanism by which LSF is involved in Notch signaling in human hepatocarcinogenesis has not been addressed. In this study, the authors demonstrate the relationship between Notch signaling and LSF, and its biological role in human hepatocarcinogenesis.

Innovations and breakthroughs

Abnormal expression of Notch1 and LSF in human HCC suggests that LSF may interact with the Notch signaling pathway during human hepatocarcinogenesis. This is the first report showing that LSF is a downstream effector of dysregulated Notch signaling in human HCC. The *in vitro* and *in vivo* data suggest that LSF might be a potential therapeutic target.

Applications

This study identifies a signaling pathway involved in hepatocarcinogenesis that may be a novel therapeutic target for the treatment of HCC.

Terminology

Notch was initially identified as being responsible for a specific phenotype that manifested as "notches" in the wing blades of *Drosophila melanogaster*. The Notch family comprises single-pass transmembrane proteins consisting of extracellular, transmembrane, and intracellular domains that correlate with different cellular functions, including cell-fate determination, tissue patterning and morphogenesis, cell differentiation, proliferation and death. LSF, also known as LBP-1c and TFCP2, regulates diverse cellular and viral promoters.

Peer review

In this study, Fan *et al* show that LSF acts as a downstream mediator of the Notch signaling network, thus playing a crucial role in the neoplastic proliferation and invasion of human HCC.

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BRIEF ARTICLE

Laparoscopic calibrated total *vs* partial fundoplication following Heller myotomy for oesophageal achalasia

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Abstract

AIM: To compare the mid-term outcomes of laparoscopic calibrated Nissen-Rossetti fundoplication with Dor fundoplication performed after Heller myotomy for oesophageal achalasia.

METHODS: Fifty-six patients (26 men, 30 women; mean age 42.8 ± 14.7 years) presenting for minimally invasive surgery for oesophageal achalasia, were enrolled. All patients underwent laparoscopic Heller myotomy followed by a 180° anterior partial fundoplication in 30 cases (group 1) and calibrated Nissen-Rossetti fundoplication in 26 (group 2). Intraoperative endoscopy and manometry were used to calibrate the myotomy and fundoplication. A 6-mo follow-up period with symptomatic evaluation and barium swallow was undertaken. One and two years after surgery, the patients underwent symptom questionnaires, endoscopy, oesophageal manometry and 24 h oesophago-gastric pH monitoring.

RESULTS: At the 2-year follow-up, no significant difference in the median symptom score was observed between the 2 groups (P = 0.66; Mann-Whitney U-test). The median percentage time with oesophageal pH < 4 was significantly higher in the Dor group compared to the Nissen-Rossetti group (2; range 0.8-10 *vs* 0.35; range 0-2) (P < 0.0001; Mann-Whitney U-test).

CONCLUSION: Laparoscopic Dor and calibrated Nissen-Rossetti fundoplication achieved similar results in the resolution of dysphagia. Nissen-Rossetti fundoplication seems to be more effective in suppressing oesophageal acid exposure.

Key words: Achalasia; Dor fundoplication; Dysphagia; Gastroesophageal reflux; Laparoscopy; Nissen-Rossetti fundoplication

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INTRODUCTION

Oesophageal achalasia is the best understood and best

characterised oesophageal motility disorder[1]. Of unknown aetiology, it is a functional disease secondary to irreversible degeneration of oesophageal myenteric plexus neurons causing aperistalsis or uncoordinated contractions of the oesophageal body and incomplete or absent post-deglutitive relaxation of the lower oesophageal sphincter (LES)[2,3]. All the available treatments for achalasia are palliative, being directed toward elimination of the outflow resistance caused by abnormal LES function and aiming to improve the symptoms related to oesophageal stasis, such as dysphagia and regurgitation[4,5].

According to the literature, surgical therapy, relying on an oesophago-gastric extramucosal myotomy with fundoplication, seems the treatment of choice for achalasia, being more effective in improving symptoms than both endoscopic pneumatic dilation and endoscopic botulinum toxin injection into the LES, especially in the long-term[6-11]. Furthermore, the advent of laparoscopic techniques has rekindled interest in the surgical management of this disease, decreasing the morbidity associated with thoracotomic or laparotomic myotomy which for several years indicated that endoscopic pneumatic dilation was the first line therapy for oesophageal achalasia[3].

However, many issues regarding the surgical technique are still debated such as the length of myotomy, the association of an anti-reflux procedure with myotomy and the type of fundoplication to perform. Concerning the latter question, both a 180° anterior and 270° posterior partial fundoplication represent the most frequently performed anti-reflux procedures after myotomy.

Although the total 360° wrap is generally considered an obstacle to normal oesophago-gastric transit in the presence of defective peristaltic activity, some authors showed that the Nissen-Rossetti fundoplication is not an obstacle to oesophageal emptying after Heller myotomy, achieving excellent results in terms of dysphagia and providing total protection from gastroesophageal reflux (GER)[10].

The present study aimed to compare the surgical and mid-term outcomes of laparoscopic calibrated Nissen-Rossetti fundoplication with laparoscopic Dor fundoplication, performed after oesophago-gastric myotomy, for the treatment of oesophageal achalasia.

MATERIALS AND METHODS

All patients referred between September 2002 and March 2007 for primary oesophageal achalasia were inserted in a prospective database including the results of symptoms evaluation and of oesophageal instrumental studies. Using this database, the results of fifty-six patients (26 men, 30 women; mean age 42.8 ± 14.7), who had undergone minimally invasive surgical treatment, were analyzed in this study.

All patients enrolled in this study were between 16 and 70 years old, had a preference for surgical treatment and had no absolute contraindications for laparoscopic surgery.

We excluded patients with central nervous system diseases, mental disorders, connective system diseases, diabetes mellitus, neoplastic diseases, inflammatory bowel diseases, pregnancy, previous gastro-intestinal diseases and those who were on medications that may have influenced gastric acidity or motility. Patients presenting with achalasia associated with other oesophageal diseases were also excluded. In the same way, patients who had undergone previous surgical treatment for achalasia and who had previous oesophageal, gastric or biliary surgery were excluded.

Preoperative evaluation of the patients was performed using symptom questionnaires (composite symptom score combining severity and frequency of symptoms[12] and SF-36 questionnaire[13,14]), barium swallow, upper gastrointestinal endoscopy and stationary oesophageal manometry.

All enrolled patients underwent laparoscopic extramucosal Heller myotomy associated with an anti-reflux procedure.

Depending on the type of fundoplication performed after myotomy, the study was divided in two periods: during the first 2 years (from April 2003 to April 2005), the authors performed a total calibrated (Nissen-Rossetti) fundoplication, and from May 2005 to May 2007 they performed an anterior 180° (Dor) fundoplication. A 6-mo follow-up period with symptomatic evaluation and barium swallow was undertaken. Twelve and twenty-four months after surgery, all patients were invited to repeat the symptomatic evaluation and were interviewed about the persistence of preoperative symptoms and about the presence of symptoms related to the GER disease (GERD) (heartburn, acid regurgitation and atypical symptoms).

Data regarding symptoms and patients' general health (GH) were collected *via* the same questionnaires used for the preoperative evaluation. Furthermore, at the same follow-up points (12 and 24 mo), each patient was asked to undergo barium swallow, endoscopy, oesophageal manometry and 24 h oesophago-gastric pH monitoring, to evaluate any abnormal GER, as part of the study protocol.

The study was approved by the ethics committee of Second University of Naples and conducted according to the ethical standards of the Helsinki declaration.

Each patient gave informed written consent.

Symptoms and quality of life

A composite symptom score, combining severity and frequency of dysphagia, regurgitation and chest pain, was used to evaluate patients' symptoms, the range varying from 0 (no symptoms) to 33 (maximum symptoms)[12].

Quality of life (QoL) was evaluated by means of the SF-36 questionnaire[14], which measures eight domains of health-related quality of life (HRQL) using 36 items. These include physical functioning, physical role, bodily pain, emotional role, GH, social functioning, mental health and vitality. The SF-36 scores range from 0 to 100,

with low scores representing poorer HRQL and a score of 100 representing the best possible HRQL.

Barium swallow

In all patients a standard oesophageal radiological examination after swallowing a bolus of contrast (Prontobario HD-Bracco, Milan, Italy) was obtained before surgery and at 6, 12 and 24 mo follow-up.

The presence of hold-up in the lower two-thirds of the oesophagus, bird-beak appearance, scarce and slow oesophageal body clearance and dilated and atonic oesophagus were considered suggestive of achalasia.

The maximum oesophageal diameter was measured in the antero-posterior projection at the site of the barium-air level and was recorded to grade the severity of achalasia as follows: stage I < 4 cm; stage II 4-6 cm; stage III > 6 cm; stage IV, any diameter with sigmoid morphologic appearance of the oesophagus[10].

Upper gastrointestinal endoscopy

Endoscopy was performed in all patients to rule out any malignancies before surgery and, 1 and 2 years after surgery, to evaluate the presence of reflux oesophagitis or stenosis.

The presence of atonic and dilated oesophageal body, food stagnation in the oesophagus, spastic oesophago-gastric junction (EGJ) and difficult crossing through the EGJ in the absence of any malignancies were considered suggestive of achalasia. The presence of oesophagitis was graded according to the Los Angeles classification[15].

Oesophageal manometry

All subjects underwent stationary oesophageal manometry with an eight channel, multiple-lumen catheter (4 open tips at the same level and oriented radially at 90° intervals and the other 4 extending proximally at 5 cm intervals) (Menfis Biomedica Inc. Bologna, Italy), perfused with a pneumo-hydraulic capillary infusion system (Menfis Biomedica Inc., Bologna, Italy).

Each channel was connected to an external pressure transducer (Menfis Biomedica Inc. Bologna, Italy) and the electric signal was sent to an acquisition/amplification module that subsequently directed the processed signal to a digital system for data acquisition, storing and analysis.

Each manometric evaluation was performed in all patients after 12-h fasting and after discontinuation of all medication affecting the gastroesophageal tract for at least 1 wk.

The catheter was passed through the nose until all the channels were placed into the stomach. The gastric pressure at the end of expiration was recorded and used as a reference point. If the catheter did not pass the EGJ, a guidewire placed in the stomach during endoscopy was used.

Manometric evaluation of the LES and of the neo-sphincter was performed using the stationary and motorized pull-through techniques, according to Gruppo Italiano Studio Motilità Apparato Digerente guidelines[16]. The parameters used for LES and neo-sphincter evaluation were resting pressure, total length and percentage of post-deglutitive relaxation.

Oesophageal motor activity (amplitude and duration of waves, percentage of peristaltic and simultaneous post-deglutitive sequences) was evaluated with stationary pull-through after 20 dry swallows.

Incomplete relaxation of the LES and aperistalsis of the oesophageal body (characterised either by simultaneous oesophageal contractions or no apparent contractions) were the manometric diagnostic criteria for achalasia[1].

Twenty-four-hour oesophago-gastric pH monitoring

At 12 and 24-mo follow-up, following manometric evaluation, 24-h ambulatory combined oesophageal and gastric pH monitoring was performed. Two glass pH catheters (Telemedicine s.r.l., Naples, Italy) were passed through the nose, positioning the proximal pH sensor 5 cm above the upper border of the LES, defined by previous oesophageal manometry. The distal pH sensor was located in the stomach, 5 cm distal to the lower border of the LES. Before each study, the pH probes were calibrated in buffer solutions of pH 7 and 1. The patients were instructed to remain in an upright or seated position during the daytime, to take three meals and to keep a diary of food intake, symptoms, and the time of the supine and upright position.

The data were registered and stored on a portable digital recorder (Menfis Biomedica Inc. Bologna, Italy) for 24 h and then the sensors were removed and the data downloaded into a personal computer for analysis. Gastroesophageal acid reflux was defined as a drop in oesophageal pH below 4.

Number of reflux episodes (normal value < 50), number of reflux episodes longer than 5 min (normal value < 3.1), percentage of total, upright and supine time with intraesophageal pH < 4 (normal values < 4.2%, < 6.3%, < 1.2%, respectively), longest reflux episode (normal value < 9.2 min) and DeMeester score (normal value < 17.92), were the parameters used for computerized analysis of acid reflux[17].

Operative technique

All patients were operated using the same technique and by the same surgeon.

Briefly, surgery was performed using a five-port technique with 4 trocars of 10 mm in diameter and 1 of 5 mm. Pneumoperitoneum at 12 mmHg was induced through the open laparoscopy technique. The patient was placed in a 20° reverse-Trendelemburg position.

The surgeon was placed between the patient's legs, an assistant on the right side of the patient and another assistant on the left side. With the left hepatic lobe raised, using a grasper and a vessel-sealing system (Ligasure^{1100} Atlas™ 10 mm; Valleylab/Tyco Healthcare UK Ltd.) the Laimer-Bertelli membrane was divided to expose the diaphragmatic pillars and the oesophageal anterior wall. When a Nissen-Rossetti fundoplication was performed, a wide dissection of the diaphragmatic crus was carried

out to achieve a window, at least 5 cm in length, behind the lower oesophagus. Thus, the right diaphragmatic pillar was dissected from top to bottom exposing the deep portion of the left pillar that was subsequently dissected from bottom to top achieving a wide mobilization of the oesophagus on its lower mediastinal and abdominal portion. During dissection, the anterior and posterior vagus nerves were identified and preserved. Subsequently, after identification of the squamo-columnar junction (SCJ) by means of the endoscope, an oesophago-gastric myotomy, 5-6 cm long, extending 3-3.5 cm on the gastric side and 2-2.5 cm on the oesophageal tract, was performed. The anterior 180° fundoplication was performed with three non-absorbable stitches on each side suturing the gastric wall to the edge of the myotomy.

Total fundoplication was performed with two non-absorbable stitches, using the anterior wall of the gastric fundus, not incorporating the anterior wall of the oesophagus.

In each case, division of the short gastric vessels was not necessary. During the surgical procedures, the myotomy and the fundoplication were calibrated through endoscopy and manometry, by means of the same instruments used for patients' preoperative evaluation. In particular, the endoscope was inserted transorally at the beginning of the surgical procedure. Identification of the SCJ, using the transillumination properties of the endoscope, facilitated dissection of the lower oesophagus and guided extension of the myotomy.

At endoscopy, the myotomy was considered adequate if no mucosal tears were found and when the appearance of a complete opening of the EGJ was achieved. Intraoperative manometry was performed placing the catheter in the stomach by means of a guidewire. The myotomy was considered adequate if a residual LES resting pressure less than 4 mmHg was registered[10].

With regard to the endoscopic and manometric calibration of the oesophageal wrap, the fundoplication was considered inadequate (too tight, misplaced or asymmetric) when a difficult transit of the endoscope through the wrap occurred, when the position of the wrap in relation to the SCJ was not correct (less than 1 cm above the SCJ), the internal aspect of the wrap seemed irregular and interrupted on the retroversion views and when the neo-sphincter resting pressure exceeded 40 mmHg[10]. According to intraoperative endoscopy and manometry, whenever the fundoplication was not effectively calibrated, the surgeon refashioned it correctly.

Statistical analysis

Statistical analysis was carried out using the programs In-Stat Graph-Pad Prism® 5 and Graph-Pad StatMate® (San Diego, California, USA). Values are expressed as mean ± SD or medians (25th, 75th percentiles, range). Continuous data were compared between each group using the Unpaired *t*-test, the Paired *t*-test or the Mann-Whitney *U*-test, when indicated, according to distribution. Prevalence data were compared between groups using Fisher's exact test. A probability value of less than 0.05 was considered significant. The primary end point of the study was the incidence of pathological clinical and instrumental GER in the Heller plus Dor, and Heller plus Nissen-Rossetti groups. Secondary end points included symptom score and dysphagia recurrence rate, QoL and postoperative neo-sphincter pressure at oesophageal manometry. Outcome variables were analyzed on an intention-to-treat basis.

Table 1 Preoperative characteristics of the 2 patient groups

Features	Total fundoplication	Partial fundoplication	*P*-value
Sex	12 M; 14 F	14 M; 16 F	
Age (yr, mean ± SD)	42.4 ± 14.3	43.3 ± 15.4	0.82[1]
Weight (kg, mean ± SD)	71 ± 7.7	70.3 ± 7.14	0.71[1]
Previous pneumatic dilation	2	2	
Previous botulinum toxin injection	1	1	
Duration of symptoms (mo), median (interquartile range)	7.5 (3.7-15.7)	7.5 (4.7-27.7)	0.43[2]

[1]Unpaired *t*-test; [2]Mann-Whitney *U*-test. M: Male; F: Female.

RESULTS

Of the 56 patients who underwent laparoscopic Heller myotomy, 30 (14 men, 16 women; mean age 42.4 ± 14.3) received an anterior 180° partial fundoplication and 26 (12 men, 14 women; mean age 43.3 ± 15.4) received a Nissen-Rossetti fundoplication. Table 1 shows the preoperative characteristics of the two groups of patients.

There were not significant differences in age, gender, weight distribution and duration of symptoms between the two groups.

Four patients, 2 in the total and 2 in the anterior fundoplication group, had undergone previous pneumatic dilation of the cardia, whereas 2 patients (1 in the Dor group and 1 in the Nissen-Rossetti group) had undergone endoscopic injection of botulinum toxin into the LES before surgery.

Preoperative assessment

All patients had dysphagia and no acid regurgitation, whereas 12 patients (21.4%) reported chest pain (Figure 1). Median symptom score was 22.5 (range 12-33) [21 (range 12-33) in the Dor group and 23 (range 12-33) in the Nissen-Rossetti group (P = 0.40; Mann Whitney *U*-test)]. At oesophagogram, the median of the maximum oesophageal diameter was 4.75 cm (range 3.5-10 cm).

Based on the results of barium oesophagogram, achalasia severity was stage Ⅰ in 18 (32.14%) patients (10 in the Dor group and 8 in the Nissen-Rossetti group), stage Ⅱ in 24 (42.8%) patients (14 in the Dor group and 10 in the Nissen-Rossetti group), stage Ⅲ in 10 (17.8%) patients (4 in the Dor group and 6 in the Nissen-Rossetti group) and stage Ⅳ in 4 (7.1%) patients (3 in the Dor group and 1 in the Nissen-Rossetti group).

Table 2 Surgical and early postoperative outcome in the 2 patient groups

Variables	Total fundoplication	Partial fundoplication	*P*-value
Median operating time (min), median (range)	90 (75-100)	90 (75-150)	0.02[1]
Major intraoperative complications	1	1	
Minor intraoperative complications	0	2	
Lower oesophageal sphincter pressure (mean ± SD)	29.38 ± 0.5	24.1 ± 0.5	0.001[2]
Postoperative complications	1	2	
Transfusions	0	0	

[1]Mann-Whitney *U*-test; [2]Unpaired *t*-test.

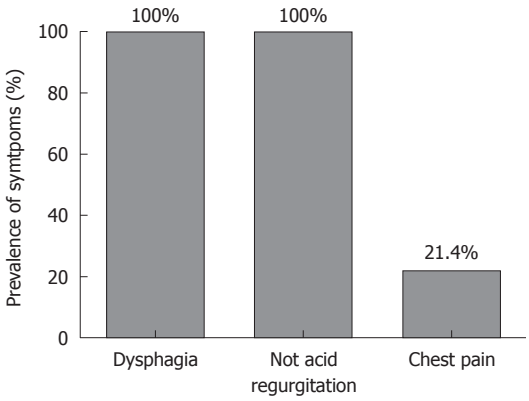

Figure 1 Preoperative symptoms. Prevalence of dysphagia, regurgitation and heartburn in patients enrolled in the study.

Upper gastrointestinal endoscopy showed, in all cases, an atonic and dilated oesophageal body and a spastic EGJ, whereas grade A oesophagitis was found in 7 patients (12.2%).

At manometry, simultaneous oesophageal contractions were found in 20 patients (35.7%), whereas in 36 cases (64.2%) no apparent contractions of the oesophageal body were recorded. Evaluation of the LES showed incomplete relaxations in all patients. Median LES-P was 22 mmHg (interquartile range 15-30).

The prevalence of symptoms, the presence of oesophagitis, and the manometric and radiological features did not differ significantly between the 2 groups of patients.

Surgery and early postoperative outcome

All surgical procedures were completed laparoscopically. No mortality was observed. Table 2 shows the results of surgery and early postoperative outcome.

Median duration of surgery was 90 min (range, 75-100 min) in the Dor group and 90 min (range 75-150 min) in the Nissen-Rossetti group ($P = 0.67$; Mann-Whitney *U*-test).

One major intraoperative complication was observed both in the Dor and Nissen-Rossetti group.

In the first case, an intraoperative mucosal tear occurred and was immediately repaired by placing 1 stitch and abdominal drainage. The other patient developed an intraoperative pneumothorax which required thoracic drainage. In another two patients in the Dor group, a minor intraoperative complication occurred, represented by an episode of intraoperative cervical subcutaneous emphysema. Blood transfusions were not required in any of the patients. The myotomy and fundoplication were calibrated by intraoperative endoscopy and manometry in both groups. In each case, residual LES pressure lower than 4 mmHg after myotomy, as measured by intraoperative manometry, was considered satisfactory.

The intraoperative median resting pressure of the new valve was significantly higher in the Nissen-Rossetti group, compared with the Dor group [29 (range 25-32) in the Nissen-Rossetti and 22.5 (range 20-29) in the Dor group ($P < 0.001$; Mann Whitney *U*-test)].

In the early postoperative period, two patients in the Dor group developed pulmonary atelectasis, promptly resolved with conservative treatment, and one patient in the Nissen-Rossetti group had urinary retention.

Median hospital stay was 3 d (range 2-7 d) in the Nissen group and 3 d (range 2-7 d) in the Dor group, no significant differences between the groups were observed ($P = 0.81$; Mann-Whitney *U*-test).

Follow-up assessment

Follow-up data were available for all patients at 6 and 12 mo follow-up, whereas, at 24 mo follow-up, the clinical and instrumental data of two patients with a Nissen-Rossetti fundoplication were missing.

These two patients showed a good outcome at 6 and 12 mo, and although they underwent symptomatic evaluation at 24 mo follow-up, they rejected invasive investigations (endoscopy, manometry and pH-metry).

Consequently, 24 mo after surgery, complete instrumental data were available for 54 patients (96.4%), and based on the intention-to-treat analysis, the patients without 24-h oesophago-gastric pH monitoring were considered not to show pathological GER.

At 24 mo follow-up, 4 patients in the Dor group (4/30 = 13.3%) reported heartburn and acid regurgitation, whereas no patients in the Nissen-Rossetti group had GERD symptoms ($P = 0.11$; Fisher's exact test).

Four patients (2 with stage Ⅳ and 2 with stage Ⅲ achalasia) (4/56 = 7.1%) had moderate and occasional dysphagia; of these patients, 2 received a Nissen-Rossetti (2/26 = 7.7%) and 2 a Dor fundoplication (2/30 = 6.6%) ($P = 1.00$; Fisher's exact test).

The postoperative median symptom score decreased from 21 (range 12-33) to 3 (range 3-12) in the Dor group ($P < 0.0001$; Mann-Whitney *U*-test) and from 23 (range 12-33) to 3 (range 3-12) in the Nissen-Rossetti group ($P < 0.0001$; Mann-Whitney *U*-test), without any significant difference between the 2 groups.

In particular, the median dysphagia score did not differ significantly between the 2 groups [3 (range 0-5) in

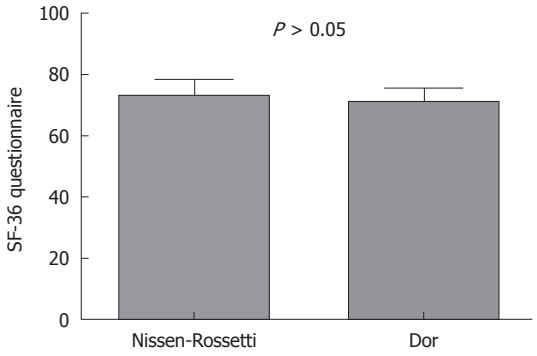

Figure 2 Quality of life at 24 mo follow-up. Postoperative QoI (as measured by the SF-36 questionnaire) in patients with a partial anterior fundoplication and with a total fundoplication ($P > 0.05$; unpaired *t*-test).

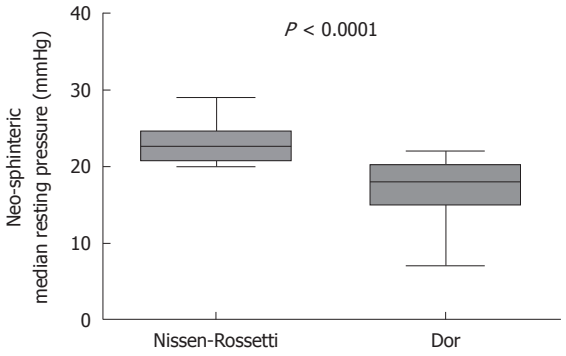

Figure 3 Postoperative oesophageal manometry. Neo-sphinteric median resting pressure in patients with a Dor and with a Nissen-Rossetti fundoplication ($P < 0.0001$; Mann-Whitney *U*-test).

the Nissen-Rossetti group and 3 (range 0-5) in the Dor group ($P = 0.91$; Mann Whitney *U*-test)].

Although the total mean health-related QoL score was significantly higher than the preoperative score (83.6 ± 4.2 *vs* 71.3 ± 4.7: $P < 0.0001$; paired *t*-test), there was no significant difference in the QoL between the patients with Dor and Nissen-Rossetti fundoplication (70.5 ± 4.06 *vs* 72.3 ± 45.3: $P = 0.16$; unpaired *t*-test) (Figure 2).

At radiological examination, none of the patients showed hold-up in the lower two-thirds of the oesophagus or bird-beak appearance.

Furthermore, the median of the maximum oesophageal diameter decreased from 4.75 (range 3.5-10) to 4 (range 3-10) ($P = 0.01$; Mann-Whitney *U*-test), with no significant differences between the Nissen-Rossetti and Dor groups ($P = 0.93$; Mann-Whitney *U*-test).

Two years after surgery, upper gastrointestinal endoscopy showed grade A-B oesophagitis in 3 patients in the Dor group who did not show oesophagitis preoperatively. Normal endoscopic features were found in all other patients.

At manometry, the median neo-sphincter resting pressure in the Nissen-Rossetti group was significantly higher than that in patients with Dor fundoplication [22.5 (range 20-29) in the Nissen-Rossetti *vs* 18 (range 7-22) in the Dor group ($P = 0.91$; Mann Whitney *U*-test)] (Figure 3). In all cases, the neo-sphincter showed a complete post-deglutitive relaxation.

Manometric evaluation in 8 patients with stage II achalasia, 4 in the Dor group (4/30 = 13.3%) and 4 in the Nissen-Rossetti group (4/24 = 16.6%), showed complete peristaltic contractions of the oesophageal body in 35.6% of dry swallows.

At pH-metry, 4 patients with an anterior fundoplication (4/30 = 13.3%) showed pathological acid reflux, whereas none of the 26 patients in the Nissen-Rossetti group had abnormal oesophageal acid exposure ($P = 0.11$; Fisher exact test).

The median percentage time with oesophageal pH less than 4 was significantly higher in the Dor group compared to the Nissen-Rossetti group (2; range 0.8-10 *vs* 0.35; range 0-2) ($P < 0.0001$; Mann-Whitney *U*-test) (Figure 4).

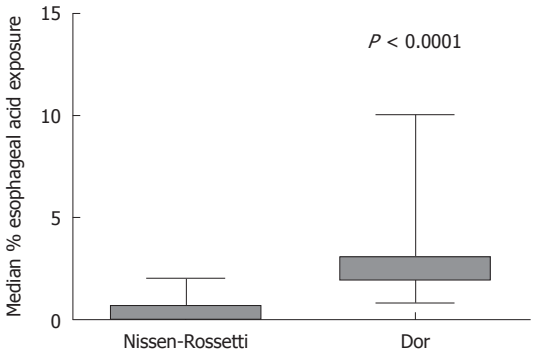

Figure 4 Oesophago-gastric pH monitoring at 24-mo follow-up. Median percentage of total time with oesophageal acid exposure in patients with partial and total fundoplication ($P < 0.0001$; Mann-Whitney *U*-test).

DISCUSSION

At present, the aetiology of primary achalasia is still not clear, and only palliative and not curative treatments for this disease are available. The goal of the current therapeutic options is the long-term relief of dysphagia, preventing recurrences and improving QoL. The surgical management of achalasia seems to achieve, among the various treatment options, the best short and long-term clinical outcome, especially a minimally invasive approach, currently considered the treatment of choice for patients with idiopathic achalasia[6-9,18-23].

However, although laparoscopic Heller myotomy has become an established therapeutic method and has achieved rapid and widespread diffusion, some points regarding the surgical procedure are still controversial.

The length of myotomy is the first matter of debate.

Although some authors have proposed a limited myotomy on the lower oesophagus, preserving a small portion of the LES to prevent postoperative reflux[24,25], most authors have recommended a myotomy extending 4-6 cm on the oesophagus and 1-2 cm on the gastric side followed by an anti-reflux procedure[4,5,10,18].

In this study, we performed an oesophago-gastric myotomy 5-6 cm long, with a proximal extension of 2-2.5 cm above the Z-line and a distal extension of 3-3.5 cm below the same landmark.

In a previous experimental study with intraoperative computerized manometry, we observed that myotomy of the oesophageal portion of the LES (without dissection of the gastric fibres) did not lead to a significant variation in the sphincteric pressure or vector volume. Instead, dissection of the gastric fibres for at least 2-2.5 cm on the anterior gastric wall, created a significant modification of the LES pressure profile[26]. This may be due to destruction of the anterior portion of the semicircular clasps and of the gastric sling fibres, which once disconnected from the posterior branches, lose their hook properties decompressing the posterior fibres[27].

These findings led us not to perform a long oesophageal myotomy, reducing damage to the oesophageal musculature and extending the myotomy for 3-3.5 cm on the gastric side. This point seems particularly relevant if we consider that some studies reported, by means of 24-h ambulatory oesophageal manometry, signals of reappearance of peristaltic activity after Heller's myotomy[28-31]. On the other hand, in 8 patients in the present study at manometric follow-up, complete peristaltic contractions of the oesophageal body were found. The association of an anti-reflux procedure with myotomy is still a controversial issue.

Ellis, based on a case series, performed a thoracoscopic limited oesophago-myotomy without a fundoplication, obtaining good results for relief of dysphagia with a low incidence of postoperative GER[24,25]. However, according to the literature, the thoracoscopic approach carries both technical disadvantages and poorer postoperative outcome, compared to laparoscopic access[32-35].

Other authors, based on retrospective studies, reported excellent outcomes by adding an anti-reflux procedure (Dor or Toupet) to either laparoscopic or laparotomic myotomy, showing a 5.7% to 10% incidence of pathological GER at short- and long-term follow-up[36-39].

On the other hand, some authors proposed a limited mobilization of the lower oesophagus and preservation of the oesophageal lateral and posterior attachments, with the intention of preserving the native anti-reflux mechanism, avoiding an anti-reflux procedure after the myotomy[40].

The first and the only prospective randomized double-blind clinical trial comparing Heller myotomy *vs* Heller myotomy with an anti-reflux procedure was designed by Richards, an historic supporter of laparoscopic Heller myotomy without fundoplication[41]. The same author reported a 47.6% incidence of pathological acid reflux in the group with Heller myotomy alone, *vs* 9.1% in the group with associated anterior fundoplication ($P = 0.005$), whereas no significant differences in surgical outcome and postoperative dysphagia were found. The author concluded that the addition of a Dor fundoplication to Heller myotomy provided more reliable protection from postoperative pathological GER.

Furthermore, recent retrospective studies evaluating the long-term follow-up of laparoscopic and trans-thoracic Heller myotomy, despite effective relief of dysphagia, showed an incidence of pathological GER between 11.3% and 80%[42-44].

Moreover, the results of a recent meta-analysis suggest that adding a fundoplication decreases pathologic acid exposure and GER symptoms after myotomy, and that the resolution of dysphagia is independent of whether a fundoplication is performed[11].

There is no agreement on the type of fundoplication to carry out.

The proponents of partial fundoplication, such as anterior 180° and 270° posterior, argue that the addition of a total fundoplication on the myotomized oesophagus may impair the oesophageal clearance, retarding oesophageal emptying and resulting in a higher rate of recurrent dysphagia[41]. However, few available data support this point of view.

Topart *et al*[45]showed a recurrence and re-operation rate of 82.4% associated with progressive oesophageal dilation in a series of 17 patients undergoing complete myotomy and short floppy Nissen fundoplication.

Wills demonstrated a similar incidence of postoperative dysphagia, regurgitation and heartburn between patients with partial and total fundoplication after Heller myotomy[46].

Falkenback, based on a prospective randomized trial comparing Heller myotomy alone with Heller plus Nissen fundoplication, found no significant difference at long-term follow-up in the rate of postoperative dysphagia, in contrast to the incidence of postoperative pathological GER, which was significantly higher in the group with Heller myotomy alone (13% *vs* 0.15%)[47].

Recently, Rossetti *et al*[10] reported the long-term follow-up of 195 consecutive laparoscopic procedures for the treatment of oesophageal achalasia with Heller myotomy plus Nissen-Rossetti fundoplication. This approach achieved good results with a 2.2% incidence of postoperative dysphagia and an absence of pathological GER in all 75 patients undergoing instrumental follow-up.

The first prospective randomized trial, comparing total Nissen fundoplication with a 180° anterior Dor fundoplication after myotomy for grade I and II achalasia, has recently been published[48]. The authors reported a 15% incidence of dysphagia in the group with Heller myotomy plus total fundoplication *vs* 2.8% in the group with anterior fundoplication ($P < 0.001$), whereas no significant difference in GER control was found, even when the incidence of pathological GER was higher in the Dor group compared to the Nissen group (5.6% *vs* 0%). Furthermore, the authors found a correlation between the grade of oesophageal dilation and recurrence of dysphagia, concluding that the Nissen fundoplication could be considered in selected cases such as younger patients with grade I achalasia.

The present study aimed to compare the surgical and mid-term outcome of total fundoplication with anterior partial fundoplication following myotomy in the treatment of primary achalasia. Unlike the previous study[48], based on the results of barium oesophagogram, patients

with stage 3 achalasia were not excluded. Moreover, the similar characteristics between the 2 groups of patients and the strict exclusion criteria allowed a reliable comparison between the two groups.

Our data show firstly, a good and similar immediate postoperative outcome for both Nissen-Rossetti and Dor fundoplication.

Indeed, the two major intraoperative complications did not lead to a poorer postoperative outcome, and no significant difference in hospitalization between the two patient groups was observed. On the other hand, only one of these complications, pneumothorax, was probably related to the type of fundoplication, which occurred during mobilization of the lower mediastinal oesophagus, which in this case, appeared to be strongly adherent to the pleura. Furthermore, at 24 mo follow-up, there was no significant difference in the symptom score and QoL between the patients with partial and total fundoplication. Only 4 patients with grade 3 achalasia had occasional and moderate postoperative dysphagia and were equally distributed in the 2 groups.

In our opinion, these data, in contrast to a previous study[48], reflect both our surgical technique and evaluation of the clinical results using a score which combined the severity and frequency of symptoms. The use of the anterior wall of the stomach in fashioning the wrap allows the construction of a neo-sphincter that adequately relaxes during swallowing, thus allowing normal bolus transit through the gastroesophageal junction even in patients with impaired oesophageal motility[49,50].

Moreover, endoscopic and manometric calibration of the wrap leads to an objective evaluation of the fundoplication, avoiding a too tight or too long wrap that may increase the incidence of postoperative dysphagia[10].

Concerning the evaluation of symptoms, dysphagia is a symptom which may indicate a sensation of impaired, difficult or obstructed bolus transit through the oesophagus and may occur with variable frequency. In patients with achalasia treated surgically, although dysphagia may dramatically improve, the impaired oesophageal motility persists despite treatment.

Occasionally, after surgery, some patients may have rapid and excessive food intake which, in the presence of inadequate oesophageal clearance, may increase the risk of postoperative dysphagia. Furthermore, episodic emotional stress and psychological abnormalities may influence the subjective perception of bolus transit.

For these reasons, only a score which combines severity with frequency of symptoms may lead to an adequate evaluation of postoperative dysphagia.

This study, in contrast to other reports[41,51], seems to indicate that total fundoplication on a myotomized oesophagus, does not impair oesophageal bolus transit more than an anterior partial fundoplication.

Similarly, our data, according to Rossetti *et al*[10], also suggest that at mid-term follow-up, the calibrated Nissen-Rossetti fundoplication, performed according to our technique, does not represent an obstacle to oesophageal emptying after Heller myotomy. Obviously, further research to confirm these results at long-term follow-up with larger sample sizes are needed.

At 24 mo follow-up, although the difference in the incidence of pathological reflux was not statistically significant (the power of the study was low for the limited number of patients), none of the patients with Nissen-Rossetti fundoplication showed pathological oesophageal acid exposure, in contrast to four patients with abnormal oesophageal pH-monitoring results in the Dor group. Furthermore, the median percentage time with oesophageal pH less than 4 was significantly higher in the Dor group compared to the Nissen-Rossetti group.

Similarly, the mean resting pressure of the neo-sphincter was significantly higher in patients with total fundoplication than in those with partial fundoplication.

These findings seem to indicate that total fundoplication allows more reliable protection from acid GER, compared to anterior partial fundoplication. On the other hand, the literature suggests that Nissen fundoplication is effective in reversing not only acid but also non-acid reflux[52].

Interestingly, at 2 years follow-up, we found an incidence of pathological reflux of 13.3% in patients with partial fundoplication which was higher than that reported at 6-mo follow-up in a previous prospective randomized study[41].

Moreover, other recent retrospective analyses suggest a progressive increase in oesophageal acid exposure at long-term follow-up in achalasic patients treated with myotomy plus Dor fundoplication[6,53].

These data raise the question of whether partial anterior fundoplication is able to prevent pathological oesophageal exposure over time.

However, this is beyond the scope of this study and long-term follow-up results are needed to verify the effect of pathological reflux on the clinical outcome of patients undergoing surgery for oesophageal achalasia. The higher incidence of reflux symptoms and pathological reflux in the Dor group did not lead to worsening of the symptom score and QoL. This probably reflects the fact that, after surgical treatment, the patients with abnormal oesophageal acid exposure reported only mild heartburn and regurgitation and were euphoric about their ability to eat.

According to Ponce, patient satisfaction was more related to improvement in dysphagia, than to the absence of reflux symptoms[54]. However, other authors have emphasized the harm of GER in patients with impaired oesophageal clearance and myotomized oesophagus[10]. In our study, 24 mo after surgery, patients with pathological oesophageal acid reflux showed, at most, grade A oesophagitis and underwent standard dose PPI therapy.

In conclusion, our study seems to indicate that total and anterior partial fundoplication, performed after Heller myotomy for oesophageal achalasia, showed similar mid-term results in term of dysphagia and QoL. Furthermore, Nissen-Rossetti fundoplication seems superior to

Dor fundoplication in preventing postoperative oesophageal acid exposure, even if the clinical and statistical relevance of this finding was not clearly evident at the mid-term follow-up.

COMMENTS

Background

Oesophageal achalasia is the best characterised oesophageal motility disorder and is caused by irreversible degeneration of oesophageal myenteric plexus neurons. It is characterised by aperistalsis or uncoordinated contractions of the oesophageal body and incomplete or absent post-deglutitive relaxation of the lower oesophageal sphincter.

Research frontiers

Although the surgical therapeutic option is considered the gold standard, many issues regarding the surgical technique are still debated such as the length of myotomy, the association of an anti-reflux procedure with myotomy and the type of fundoplication to perform.

Innovations and breakthroughs

Although the total 360° wrap is generally considered an obstacle to normal oesophago-gastric transit in the presence of defective peristaltic activity, some authors have shown that Nissen-Rossetti fundoplication is not an obstacle to oesophageal emptying after Heller myotomy, achieving excellent results in terms of dysphagia and providing total protection from gastroesophageal reflux. Moreover, the only prospective randomized trial comparing total Nissen fundoplication with the 180° anterior Dor fundoplication after myotomy, was carried out for grade I and II achalasia only, evaluating dysphagia recurrences using a non specific/dedicated score.

Applications

The study seems to indicate that total and anterior partial fundoplication, performed after Heller myotomy for oesophageal achalasia, shows similar mid-term results in term of dysphagia and quality of life. Furthermore, Nissen-Rossetti fundoplication seems superior to Dor fundoplication in preventing postoperative oesophageal acid exposure, even if the clinical and statistical relevance of this finding was not clearly evident at the mid-term follow-up. In other words, not only partial fundoplication, but also the total wrap may be considered when choosing the best anti-reflux procedure after Heller myotomy for oesophageal achalasia. Obviously, our data should be confirmed by further randomized controlled trials with larger series and longer follow-up.

Terminology

Nissen, Dor and Toupet fundoplications represent anti-reflux procedures which can be performed after myotomy in the treatment of oesophageal achalasia. The first, is a total (360°) wrap, whereas, the remaining techniques are partial wraps (180° anterior and 180° posterior, respectively).

Peer review

This is an excellent, provocative study that needs to be confirmed with a randomized trial.

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BRIEF ARTICLE

Somatostatin adjunctive therapy for non-variceal upper gastrointestinal rebleeding after endoscopic therapy

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Abstract

AIM: To evaluate the effect of pantoprazole with a somatostatin adjunct in patients with acute non-variceal upper gastrointestinal bleeding (NVUGIB).

METHODS: We performed a retrospective analysis of a prospective database in a tertiary care university hospital. From October 2006 to October 2008, we enrolled 101 patients with NVUGIB that had a high-risk stigma on endoscopy. Within 24 h of hospital admission, all patients underwent endoscopic therapy. After successful endoscopic hemostasis, all patients received an 80-mg bolus of pantoprazole followed by continuous intravenous infusion (8 mg/h for 72 h). The somatostatin adjunct group (n = 49) also received a 250-μg bolus of somatostatin, followed by continuous infusion (250 μg/h for 72 h). Early rebleeding rates, disappearance of endoscopic stigma and risk factors associated with early rebleeding were examined.

RESULTS: Early rebleeding rates were not significantly different between treatment groups (12.2% *vs* 14.3%, P = 0.766). Disappearance of endoscopic stigma on the second endoscopy was not significantly different between treatment groups (94.2% *vs* 95.9%, P = 0.696). Multivariate analysis showed that the complete Rockall score was a significant risk factor for early rebleeding (P = 0.044, OR: 9.080, 95% CI: 1.062-77.595).

CONCLUSION: The adjunctive use of somatostatin was not superior to pantoprazole monotherapy after successful endoscopic hemostasis in patients with NVUGIB.

Key words: Somatostatin; Pantoprazole; Gastrointestinal bleeding; Rebleeding

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INTRODUCTION

The prevalence of non-variceal upper gastrointestinal bleeding (NVUGIB) is > 100 per 100 000 people yearly[1,2].

The number of NVUGIB cases has increased over recent years, due to the increasing use of non-steroidal anti-inflammatory drugs (NSAIDs) and antiplatelet drugs[3]. Among the conditions that lead to NVUGIB episodes, the most common is peptic ulcer disease[4]. Despite recent advances in endoscopic management of patients with NVUGIB, the overall mortality rate has remained at 5%-10% for several decades[4,5]. Therefore, there is a need to develop additional medical therapies that will improve the maintenance of hemostasis.

Data from *in vitro* studies have shown that platelet aggregation, the initial step of hemostasis, proceeds optimally at neutral pH. In a slightly acidic environment, platelet aggregation is impaired, and at pH < 6, it is virtually abolished. In acidic gastric juice, pepsinogen is processed to activated pepsin, which readily digests freshly formed blood clots within minutes. Furthermore, plasmin-mediated fibrinolysis impairs fibrin reinforcement of the initial platelet clot. It is important to understand these aspects, because ulcer rebleeding may be caused by early dissolution of the blood clot[6,7]. Thus, maintaining intragastric pH above 6 is important in the management of patients with NVUGIB. The use of a proton pump inhibitor (PPI), like omeprazole or pantoprazole, reduces the risk of rebleeding and death; thus, this has become the standard of care in patients with NVUGIB after endoscopic hemostasis[8-11].

Somatostatin and its analogs have been shown to induce hemostasis in variceal bleeding[12]. Somatostatin inhibits the release of vasodilator hormones, such as glucagon, indirectly causing splanchnic vasoconstriction and decreased portal inflow. It has a short half-life and disappears within minutes of bolus infusion[13]. Somatostatin exerts profound inhibitory effects in several gastrointestinal functions, including the secretion of gastric acid, gastrin, and pepsin[14]. The inhibition of pepsin secretion can stabilize clots or fibrin plugs that are readily digested by proteolytic activity[15,16]. also, it might offer an advantage over drugs that only inhibit gastric acid secretion, such as histamine 2 receptor antagonists and PPIs. In addition, without altering renal hemodynamics, somatostatin also induces reductions in portal venous volume, superior mesenteric blood flow, and gastric blood flow, which are positively correlated with rebleeding rates in patients with peptic ulcer bleeding[17,18]. Previously, Jenkins *et al*[19] have reported that somatostatin is an effective treatment for the control of NVUGIB in high-risk patients, i.e. those in whom hemorrhage does not cease spontaneously or is likely to recur. In a meta-analysis that compared somatostatin to histamine 2 receptor antagonists and placebo, somatostatin was more effective at reducing the risk for continued bleeding or rebleeding and at reducing peptic ulcer bleeding[20]. In addition, somatostatin has been suggested to be more effective than pantoprazole in maintaining high gastric pH during the first 12 h of infusion[21]. Rebleeding episodes often occur within 24 h in the majority of patients[22], therefore, we hypothesized that the use of somatostatin as an adjunct to pantoprazole potentiates hemostasis in patients at high risk for rebleeding.

There have been no reports about the use of somatostatin as an adjunct to a PPI in patients with NVUGIB. This retrospective report of prospectively collected data investigated the effect of using a somatostatin adjunct in patients with NVUGIB under high-risk conditions. We also analyzed risk factors for early rebleeding.

MATERIALS AND METHODS

Patients

We reviewed the medical records of 205 patients who were admitted for NVUGIB to the emergency room at the Pusan National University Hospital in South Korea, from October 2006 to October 2008. We maintained a prospective database of patients investigated for NVUGIB. These data was analyzed retrospectively. This was not a blinded study.

The clinical Rockall score was calculated at the time of admission. Thereafter, the complete Rockall score was determined according to endoscopic findings[23]. A Forrest classification was also described according to endoscopic findings[24]. Patient demographic details, including symptoms of gastrointestinal hemorrhage, comorbidity, relevant drug history, initial biochemistry, and hematological profiles were recorded at admission (Table 1).

Patients who had endoscopic high-risk stigma (spurting, oozing and visible vessel) were included. Patients were excluded when they presented with an esophageal or gastric varix, pregnancy, < 18 years old, previous history of gastric surgery, a known allergy to somatostatin or pantoprazole, renal failure (creatinine > 2 mg/dL), bleeding from gastrointestinal cancer, or deficient hemostasis (platelet count < 50000/mL and international normalized ratio of the prothrombin time > 1.5). Finally, a total of 101 patients were enrolled.

All patients gave informed consent before the initiation of endoscopic procedures and somatostatin administration. The study was approved by the ethics committee of the Institutional Review Board.

Procedures

Any use of antiplatelet agents, NSAIDs, or anticoagulants was discontinued after admission. All endoscopy procedures, including thermal techniques and mechanical hemostasis with clipping devices, were performed by experts that had > 3 years experience in performing therapeutic endoscopy. Endoscopic procedures were performed within 24 h after hospital admission with an Olympus GIF Q260 endoscope. If adherent clots were observed, they were removed by endoscopic forceps. During endoscopy, when a stigma of a recent hemorrhage was observed, endoscopic injection therapy (epinephrine diluted 1:10000 in 0.9% saline) was performed with either hemoclips or monopolar coagulation with coagulation forceps, depending on the preference of the endoscopist.

Table 1 Clinical characteristics of treatment groups (mean ± SD) *n* (%)

	Pantoprazole group (*n* = 52)	Somatostatin group (*n* = 49)	Total cohort (*n* = 101)	*P* value
Sex (male)	19 (36.5)	13 (26.5)	32 (31.7)	0.280
Age (yr)	65.44 ± 19.46	64.24 ± 14.13	64.86 ± 17.01	0.735
Hemodynamic shock	26 (50.0)	27 (55.1)	53 (52.5)	0.608
Helicobacter pylori infection	14 (26.9)	8 (16.3)	22 (21.8)	0.197
Hemoglobin (g/dL)	8.56 ± 2.84	8.26 ± 2.61	8.41 ± 2.72	0.857
Hemoglobin < 7 g/dL	17 (32.7)	16 (32.7)	33 (32.7)	0.997
Blood urea nitrogen (mg/dL)	40.20 ± 27.06	39.47 ± 26.83	39.84 ± 26.82	0.920
Creatinine (mg/dL)	1.17 ± 0.80	1.29 ± 1.33	1.23 ± 1.09	0.187
Albumin (g/dL)	3.12 ± 0.54	2.79 ± 0.59	2.96 ± 0.59	0.173
Type 2 diabetes mellitus	12 (23.1)	16 (32.7)	28 (27.7)	0.283
Hypertension	22 (43.3)	19 (38.8)	41 (40.6)	0.718
Heart failure	7 (13.5)	4 (8.2)	11 (10.9)	0.393
Ischemic heart disease	15 (28.8)	11 (22.4)	26 (25.7)	0.462
Antiplatelet medication	24 (46.2)	20 (40.8)	44 (43.6)	0.589
NSAID	6 (11.5)	3 (6.1)	9 (8.9)	0.340
Multiple antiplatelet medications	5 (9.6)	2 (4.1)	7 (6.9)	0.274
Steroids	2 (3.8)	4 (8.2)	6 (5.9)	0.359
Melena	31 (59.6)	28 (57.1)	59 (58.4)	0.801
Hematemesis	28 (53.8)	32 (65.3)	60 (59.4)	0.241
Hematochezia	2 (3.8)	5 (10.2)	7 (6.9)	0.209
Complete Rockall score	6.84 ± 1.47	6.87 ± 1.31	6.86 ± 1.39	0.911
Rockall score > 6	26 (50.0)	29 (59.2)	55 (54.5)	0.354
Early rebleeding	6 (12.2)	7 (14.3)	13 (13.3)	0.766

NSAID: Non-steroidal anti-inflammatory drug.

Enrolled patients were assigned to one of two groups. After the initial endoscopy, both groups received an 80-mg bolus of pantoprazole, followed by continuous intravenous (IV) infusion at 8 mg/h for a total of 72 h. The pantoprazole alone group received only pantoprazole for 72 h. The somatostatin adjunctive group, in addition to the pantoprazole for 72 h, received a 250-μg bolus of somatostatin, followed by continuous IV infusion of 250 μg/h for a total of 72 h. No other anti-ulcer medication was administered. At 48 h after initial endoscopy, repeat endoscopy was performed to investigate the presence of hemorrhagic stigma. When a remnant stigma was observed, an additional endoscopic procedure was performed, if deemed necessary clinically. After the 72-h infusion, patients were given one of the following, orally, each day, for 8 wk: 40 mg pantoprazole; 20 mg rabeprazole; 30 mg lansoprazole; or 40 mg esomeprazole.

Outcomes and measurement

The primary end point was the rate of clinically significant early rebleeding, as defined below. Secondary outcomes were the loss of endoscopic high-risk stigma on subsequent endoscopy and the associated risk factors for early rebleeding.

Definition

Hemodynamically, shock was defined as systolic pressure < 90 mm Hg or heart rate > 110 bpm. Stigma of recent hemorrhage or high-risk ulcer stigma was defined as spurting (Forrest classification I a), oozing (Forrest classification I b) and visible vessel (Forrest classification Ⅱ a)[24]. Rebleeding was defined as: (1) fresh hematemesis or fresh blood in the nasogastric tube; (2) passage of fresh melena or hematochezia with additional evidence of recurrent bleeding (a drop in hemoglobin of ⩾ 2 g/dL within 24 h after endoscopy); and (3) bleeding observed by endoscopy[25]. Early rebleeding was defined as rebleeding within 7 d of the endoscopic interventions.

Statistical analysis

Univariate analyses were performed with a χ^2 test or Fisher's exact test for categorical variables and Student's *t* test for continuous variables. Variables with $P < 0.25$ in the univariate analysis were included in a multiple logistic regression model to identify independent risk factors for early rebleeding. $P < 0.05$ indicated statistical significance. Statistical calculations were performed with SPSS for Windows version 12.0 (SPSS Inc., Chicago, IL, USA).

RESULTS

Among 205 patients who were admitted due to NVUGIB episodes, 104 were excludes as follows: endoscopic hemostasis not achieved successfully (*n* = 15); bleeding from gastrointestinal cancer (*n* = 15); and no high-risk bleeding stigma (*n* = 74). Finally, a total of 101 patients with NVUGIB were enrolled.

The treatment groups were not significantly different in the clinical characteristics including Rockall scores (Table 1). The mean patient age (SD) was 64.86 ± 17.01 years and 31.7% (32/101) were male. The mean complete Rockall score (SD) was 6.86 ± 1.39 (Table 1). Thirteen patients (13.3%) experienced rebleeding within 7 d after endoscopic intervention. Between treatment

Table 2 Endoscopic findings between treatment groups *n* (%)

	Pantoprazole group (*n* = 52)	Somatostatin group (*n* = 49)	Total cohort (*n* = 101)	*P* value
Cause of bleeding				0.177
Gastric ulcer	27 (51.9)	29 (59.2)	56 (55.4)	
Duodenal ulcer	11 (21.2)	8 (16.3)	19 (18.8)	
Dieulafoy lesion	13 (25.0)	7 (14.3)	20 (19.8)	
Mallory-Weiss syndrome	1 (1.9)	5 (10.2)	6 (5.9)	
Forrest type				0.894
Ⅰa	3 (5.8)	4 (8.2)	7 (6.9)	
Ⅰb	24 (46.2)	22 (44.9)	46 (45.5)	
Ⅱa	25 (48.1)	23 (46.9)	48 (47.5)	
Loss of stigma	49 (94.2)	47 (95.9)	96 (95.0)	0.696

Table 3 Rebleeding according to endoscopic findings *n* (%)

	No rebleeding (*n* = 88)	Rebleeding (*n* = 13)	Total (*n* = 101)	*P* value
Forrest type				0.990
Ⅰa	6 (6.8)	1 (7.7)	7 (6.9)	
Ⅰb	40 (45.5)	6 (46.2)	46 (45.5)	
Ⅱa	42 (47.7)	6 (46.2)	48 (47.5)	
Loss of stigma	84 (95.5)	12 (92.3)	96 (95.0)	0.625

groups, the rebleeding rates were not significantly different ($P = 0.766$) (Table 1). A second endoscopic intervention was successful in most patients that experienced rebleeding (11/13, 84.6%). Two cases received an angiographic embolization because endoscopic intervention failed to stop bleeding. There was no bleeding-related death during the study period. The most common cause of NVUGIB was gastric ulcer (55.4%, 56/101) (Table 2). The treatment groups were not significantly different for endoscopic Forrest classification ($P = 0.894$) and loss of endoscopic high-risk stigma ($P = 0.696$) (Table 2). The early rebleeding rate according to endoscopic Forrest classification was not significantly different ($P = 0.990$) (Table 3).

For risk factor analysis for early rebleeding, univariate analysis showed that complete Rockall score > 6 was a significant indicator ($P = 0.003$) of early rebleeding (Table 4). Multivariate analysis showed that only the complete Rockall score was significantly associated with early rebleeding ($P = 0.044$, OR: 9.080, 95% CI: 1.062-77.595) (Table 5).

There were no serious adverse events related to the drugs used in this study, and no serious drug interactions were noted between pantoprazole and somatostatin during the infusion period.

DISCUSSION

NVUGIB is a serious medical disorder. Although endoscopic therapy is a highly effective treatment method, successful endoscopic treatment is largely dependent upon the expertise of the endoscopist[26,27]. After endoscopic hemostasis, the use of a PPI has become the standard of care in patients with NVUGIB[8-11]. However, a recent study has shown that a high-dose, continuous infusion of PPIs may not be sufficient to sustain an intragastric pH $\geqslant$ 6[28]. Somatostatin has been used in variceal bleeding[12], and it has been suggested to be more effective than pantoprazole in maintaining high gastric pH during the first 12 h of infusion[21]. If endoscopy is contraindicated or unavailable, somatostatin might be a reasonable alternative solution. In clinical practice, patients likely to have bleeding might be considered for somatostatin treatment before definitive endoscopy[29].

From a theoretical point of view, somatostatin has the advantage of reducing gastroduodenal blood flow and pepsin secretion, in addition to inhibiting gastric acid secretion[14,17,21]. These effects may be of value for patients with NVUGIB; particularly in patients with high-risk endoscopic findings. Therefore, we hypothesized that adjunctive use of somatostatin with pantoprazole could prove effective in reducing early rebleeding in patients treated for NVUGIB. The present study focused on the effects of infusing somatostatin as an adjunct to pantoprazole after a successful endoscopic procedure in patients with endoscopic high-risk stigma. Although this was not a randomized study, the clinical baseline characteristics were not significantly different between the treatment groups, including hemodynamic shock, endoscopic findings and Rockall scores (Tables 1 and 2). The results showed that the adjunctive use of somatostatin was not superior to pantoprazole infusion alone in preventing rebleeding ($P = 0.766$) (Table 1). We enrolled patients with endoscopic high-risk stigma who were treated with endoscopy; 48 h after initial endoscopy, a second endoscopy was performed to confirm the absence of the hemorrhagic stigma. The result was not significantly different between treatment groups ($P = 0.696$) (Table 3). A previous meta-analysis[30] has shown that all endoscopic therapies (including clips and thermal therapy) reduce the risk of rebleeding compared with pharmacotherapy alone. The present study enrolled patients in whom therapeutic interventions were successfully performed at initial endoscopy, therefore, it is not surprising that differences in endoscopic findings were not identified as important risk factors for rebleeding. When a high-risk hemorrhagic stigma could be eradicated by endoscopic intervention, gastric acid inhibition with a high dose of PPI alone appeared to be sufficient for maintaining hemostasis. Among patients that experienced rebleeding, two required angiographic embolization because endoscopic intervention was unsuccessful. Only a small number of cases required additional angiographic embolization, therefore, statistical analysis was limited.

Optimal acid suppression facilitates clot formation over arteries in bleeding peptic ulcers. A previous study has reported that infusion of high-dose omeprazole before endoscopy accelerated the resolution of signs of bleeding in ulcers and reduces the need for endoscopic therapy[31]. If infusion of high-dose omeprazole after hemostasis had been administered, the rates of recurrent

Table 4 Clinical characteristics according to occurrence of early rebleeding event (mean ± SD) *n* (%)

	No rebleeding (*n* = 88)	Rebleeding (*n* = 13)	Total (*n* = 101)	*P* value
Sex (male)	27 (30.7)	5 (38.5)	32 (31.7)	0.574
Age (yr)	64.25 ± 17.40	69.0 ± 13.97	64.86 ± 17.01	0.350
Hemodynamic shock	46 (52.3)	7 (53.8)	53 (52.5)	0.916
Helicobacter infection	19 (21.6)	3 (23.1)	22 (21.8)	0.904
Hemoglobin (g/dL)	8.61 ± 2.77	7.09 ± 2.02	8.41 ± 2.72	0.060
Hemoglobin < 7 g/dL	26 (29.5)	7 (53.8)	33 (32.7)	0.081
Blood urea nitrogen (mg/dL)	39.81 ± 25.92	40.09 ± 33.50	39.84 ± 26.82	0.972
Creatinine (mg/dL)	1.26 ± 1.12	1.03 ± 0.83	1.23 ± 1.09	0.473
Albumin (g/dL)	2.97 ± 0.60	2.86 ± 0.47	2.96 ± 0.59	0.532
Type 2 diabetes mellitus	21 (23.9)	3 (23.1)	24 (23.8)	0.950
Hypertension	38 (43.2)	3 (23.1)	41 (40.6)	0.168
Heart failure	9 (10.2)	2 (15.4)	11 (10.9)	0.577
Ischemic heart disease	17 (19.3)	1 (7.7)	18 (17.8)	0.454
Antiplatelet medication	37 (42.0)	7 (53.8)	44 (43.6)	0.423
NSAID	7 (8.0)	2 (15.4)	9 (8.9)	0.380
Multiple antiplatelet medications	5 (5.7)	2 (15.4)	7 (6.9)	0.221
Steroid	5 (5.7)	1 (7.7)	6 (5.9)	0.572
Melena	50 (56.8)	9 (69.2)	59 (58.4)	0.397
Hematemesis	51 (58.0)	4 (30.8)	55 (54.5)	0.066
Hematochezia	5 (5.7)	2 (15.4)	7 (6.9)	0.199
Complete Rockall score	6.73 ± 1.40	7.69 ± 1.03	6.86 ± 1.39	0.020
Rockall score > 6	43 (48.9)	12 (92.3)	55 (54.5)	0.003
Somatostatin use	41 (46.6)	8 (61.5)	49 (48.5)	0.314

NSAID: Non-steroidal anti-inflammatory drug.

Table 5 Predictors of early rebleeding on multivariate analysis

	P value	Exp (B)	95% CI
Adjunct somatostatin use	0.374	0.527	0.128-2.164
Hypertension	0.175	2.864	0.627-13.086
Multiple antiplatelet medication	0.421	2.351	0.239-18.879
Hemoglobin < 7 g/dL	0.402	1.768	0.466-6.704
Hematemesis	0.072	3.672	0.889-15.179
Hematochezia	0.614	0.593	0.078-4.517
Complete Rockall score > 6	0.044	9.080	1.062-77.595

bleeding did not differ between the groups[31]. In high-risk patients, early endoscopy involving therapy stops bleeding and potentially saves lives. Early endoscopy also permits low-risk patients to be discharged early from hospital. The use of high-dose PPIs cannot replace the need for early endoscopy. Our study group had stigmata of recent hemorrhage and most of the endoscopy was performed within 4 h. The effect of preemptive PPI on rebleeding might be negligible. It may be different between arterial and venous bleeding (such as varix or telangiectasia). In this study, most of the lesions were arterial bleeding. Variceal bleeding was excluded and no telangiectatic lesions were included.

In a large epidemiological study, increased risk of gastrointestinal bleeding was significantly associated with low-dose aspirin use (< 100 mg/d)[32]. In the present study, 43.6% (44/101) of patients were using antiplatelet agents (including aspirin). However, we found that the risk of early rebleeding was not significantly associated with antiplatelet agents (P = 0.423) (Table 4). The risk of gastrointestinal bleeding due to antiplatelet drugs persists as long as therapy continues, but declines within 7 d of withdrawal; a time comparable to the life of the platelet[32]. Thus, although the use of antiplatelet drugs is a principal risk factor for gastrointestinal bleeding, the risk of rebleeding might be associated with the time after antiplatelet withdrawal.

Several scoring systems have been developed to assess the risk of recurrent bleeding and death in patients with upper gastrointestinal bleeding. Although endoscopic findings may identify individuals who are at high risk for rebleeding, other factors such as age and comorbidity may affect overall mortality. Of the scoring systems that include endoscopic findings, the Rockall scoring system[23] is most commonly used. The Rockall scoring system takes into account age, presence of shock, comorbidity, source of bleeding, and major stigmata from recent hemorrhage. We found that the complete Rockall score was a significant predictor of early rebleeding (P = 0.044, OR: 9.080, 95% CI: 1.062-77.595).

There were some limitations to the current study. Although the study data were collected prospectively, it was not a randomized study, and the doctor responsible for ordering medication was not blinded to the patient's condition. Although the mean Rockall score, an extensively validated measure of the risk for morbidity, was not significantly different between the two groups, it may not have been sufficiently comprehensive. It is possible that somatostatin treatment was associated with other, unmeasured clinical and demographic variable, and these may have confounded our results. The rebleeding rate might have been affected (somatostatin group was higher than control group, 14.3% *vs* 12.2%, respectively, P = 0.766).

Moreover, we did not measure intragastric pH; therefore, we could not precisely determine the efficacy of adjunctive somatostatin for maintaining intragastric pH. Finally, because all enrolled patients were treated with endoscopy and therapeutic interventions, a definitive comparison between the medications might not have been possible.

In conclusion, we believe that this is the first study to focus on the adjunctive effect of somatostatin with PPI in acute NVUGIB patients with high-risk endoscopic lesions. Adjunctive somatostatin for management of NVUGIB did not show an additive effect in reducing early rebleeding. Complete Rockall score can predict early rebleeding for patients who have high-risk endoscopic stigma after successful endoscopic management.

COMMENTS

Background

Proton pump inhibitors (PPIs) and somatostatin are suggested to be effective treatments for non-variceal upper gastrointestinal bleeding (NVUGIB). However, the clinical effect of a PPI with a somatostatin adjunct has not been established. We hypothesized that the use of somatostatin as an adjunct to pantoprazole may potentiate hemostasis in patients at high risk for rebleeding.

Research frontiers

NVUGIB is a serious medical disorder. After endoscopic hemostasis, the use of a PPI has become the standard of care in patients with NVUGIB. However, a recent study has shown that high-dose, continuous infusion of PPIs may not be sufficient to sustain an intragastric pH $\geqslant$ 6. Somatostatin has been suggested to be more effective than pantoprazole in maintaining high gastric pH during the first 12 h of infusion. From a theoretical point of view, somatostatin has the advantage of reducing gastroduodenal blood flow and pepsin secretion in addition to inhibiting gastric acid secretion. These effects may be of value for patients with NVUGIB, particularly in patients with high-risk endoscopic findings.

Innovations and breakthroughs

Adjunctive use of somatostatin was not superior to pantoprazole monotherapy after successful endoscopic hemostasis in patients with NVUGIB.

Applications

Adjunctive use of somatostatin was not superior to pantoprazole monotherapy after successful endoscopic hemostasis in patients with NVUGIB. Complete Rockall score can predict early rebleeding for patients who have high-risk endoscopic stigmata after successful endoscopic management.

Terminology

NVUGIB means bleeding from non-variceal origins such as peptic ulcer, Dieulafoy lesion and Mallory-Weiss syndrome. High-risk ulcer stigma is defined as spurting (Forrest classification Ⅰa), oozing (Forrest classification Ⅰb) and visible vessels (Forrest classification Ⅱa).

Peer review

Choi *et al* have performed a study to establish the effect of adjunctive somatostatin for prevention of NVUGIB after endoscopic therapy. This paper is interesting and it could be valuable for other researchers.

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BRIEF ARTICLE

Survival and prognostic factors in hepatitis B virus-related acute-on-chronic liver failure

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Abstract

AIM: To investigate the survival rates and prognostic factors in patients with hepatitis B virus-related acute-on-chronic liver failure (HBV-ACLF).

METHODS: Clinical data in hospitalized patients with HBV-ACLF admitted from 2006 to 2009 were retrospectively analyzed. Their general conditions and survival were analyzed by survival analysis and Cox regression analysis.

RESULTS: A total of 190 patients were included in this study. The overall 1-year survival rate was 57.6%. Patients not treated with antiviral drugs had a significantly higher mortality [relative risk (RR) = 0.609, P = 0.014]. The highest risk of death in patients with ACLF was associated with hepatorenal syndrome (HRS) (RR = 2.084, P =0.026), while other significant factors were electrolyte disturbances (RR = 2.062, P = 0.010), and hepatic encephalopathy (HE) (RR = 1.879, P < 0.001).

CONCLUSION: Antiviral therapy has a strong effect on the prognosis of the patients with HBV-ACLF by improving their 1-year survival rate. HRS, electrolyte disturbances, and HE also affect patient survival.

Key words: Hepatitis B virus; Acute-on-chronic liver failure; Antiviral therapy; Nucleosides; Survival analysis

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INTRODUCTION

Hepatitis B virus (HBV) infection is an established cause of liver-related morbidity and mortality, and has been described as a global public health problem. Some patients had abnormal liver functions or even liver failure during the lengthy course of chronic HBV infection[1]. Liver failure is characterized by severe deterioration in liver function. This clinical syndrome involves the development of hepatic encephalopathy (HE), jaundice, coagulopathy, and hepatorenal syndrome (HRS). Acute-on-chronic liver failure (ACLF) refers to acute deterioration in liver function in a patient with previously well-compensated chronic liver disease, as a result of a precipitating event

such as sepsis or upper gastrointestinal bleeding. HBV infection is the most common cause of ACLF in China[2,3]. If the relevant prognostic factors for HBV-related ACLF could be clarified in the course of chronic hepatitis B, corresponding measures could be adopted to reduce the associated mortality.

In the present study, we analyzed the outcomes of the patients with HBV-ACLF using survival analysis, and the factors affecting mortality using a Cox regression model. The results of this study will further the understanding of the prognosis of the patients with HBV-ACLF, thus helping in the management of the condition.

MATERIALS AND METHODS

Patients

One hundred and ninety patients admitted to the 302 Military Hospital from September 2006 to January 2009 and diagnosed with HBV-ACLF were included in this retrospective study. Exclusion criteria included concurrent viral infections (e.g. HAV, HCV, HDV, HEV, human immunodeficiency virus, cytomegalovirus, Epstein-Barr virus); liver failure by other causes (including autoimmune, alcohol- or drug-related diseases); malignant tumors; and patients with other severe systematic or mental diseases. The research protocol was approved by the Ethics Committee of the 302 Military Hospital.

Clinical diagnosis and definitions

HBV-ACLF was defined as acute hepatic insult manifesting as jaundice and coagulopathy, complicated within 4 wk with ascites and/or HE in a patient with previously diagnosed or undiagnosed chronic hepatitis B, which includes compensated liver cirrhosis, with total bilirubin $\geqslant$ 171 μmol/L or an increase $\geqslant$ 17.1 μmol/L, and prothrombin activity (PTA) $\leqslant$ 40%[4].

The diagnosis of liver cirrhosis was based on medical history, physical examination, biochemical parameters, ultrasound findings and/or liver biopsy. The diagnostic criteria included the presence of hepatic stigmata, spider angioma or splenomegaly on physical examination, together with laboratory abnormalities (e.g. low serum albumin, high serum globulin, high serum γ-globulin, low platelet count) and esophagogastroduodenal endoscopic signs of portal hypertension. The occurrence of liver cirrhosis during follow-up was also defined by ultrasonographic features suggestive of cirrhosis, based on a quantitative scoring system derived from the appearance of the liver margins, parenchymal echotexture, portal vein caliber, and spleen diameter[5,6], supplemented with the presence of esophageal varices or ascites.

The severity of HE was graded as: (1) slowness of mentation, mildly disturbed sleep-awake cycle; (2) drowsiness, confusion, inappropriate behavior, disorientation, apathy, mood swings, anxiety, restlessness; (3) very sleepy but rousable, unresponsive to verbal commands, markedly confused, combative and hyper-reflexic; and (4) unconscious, decerebrate or decorticate posturing[7].

HRS is a potentially reversible syndrome that occurs in patients with cirrhosis, ascites, and liver failure. It is diagnosed by presence of cirrhosis with ascites; absence of shock; serum creatinine > 133 mmol/L (1.5 mg/dL); no improvement in serum creatinine (decrease to a level of $\leqslant$ 133 mmol/L) at least 2 d after diuretic withdrawal and volume expansion with albumin, and no current or recent treatment with nephrotoxic drugs; absence of parenchymal kidney disease as indicated by proteinuria > 500 mg/d; microhematuria (> 50 red blood cells per high-power field); and/or abnormal renal ultrasonography[8].

Abnormal serum levels of sodium or potassium were considered as electrolyte disturbances. The normal serum levels of sodium and potassium were 136-145 mmol/L and 3.5-5.2 mmol/L, respectively.

Follow-up of patients to monitor mortality

All patients with HBV-ACLF were followed up for at least 12 mo. The outcome (recovery, bridging to liver transplantation, or death) was recorded for each patient. Data for patients who underwent liver transplantation or death were considered as censored data.

Statistical analysis

Statistical analyses were performed using SPSS software version 13.0 (SPSS Inc., Chicago, IL, USA). Patients' clinical and biochemical indices were expressed as mean ± SD. Analysis of variance was used for comparisons between different treatment groups, and significant differences were assessed using F-tests and Student-Newman-Keuls tests. χ^2 tests were used for comparisons between the rates of liver cirrhosis, complications, and sex. The Kaplan-Meier method was used to estimate the overall survival rates. The log-rank test was used to compare the death rate between groups.

We performed univariate and multivariate analyses of the associations between the 12-mo mortality (dependent variable) and independent variables, and then multivariate Cox regression analysis and calculated the relative risk ratios and 95% CI. P value < 0.05 was considered statistically significant.

RESULTS

Patient characteristics

The clinical and biochemical characteristics of the 190 patients (159 men and 31 women, mean ± SD age, 45.7 ± 11.1 years) with HBV-ACLF are presented in Table 1. Of the 190 patients, 141 had received antiviral therapy (nucleoside analogues: lamivudine 100 mg/d or entecavir 0.5 mg/d), and 11 patients had undergone liver transplantation. There were no significant differences in clinical or biochemical characteristics among the three patients who received entecavir, lamivudine, or no antiviral treatment). One hundred and twenty patients had liver cirrhosis (Child-Turcotte-Pugh score < 7), 64 had HE, 16 had HRS, 63 had spontaneous peritonitis (SBP), and 144 had electrolyte disturbances. The overall 12-mo

Table 1 Complications and laboratory tests in patients with hepatitis B virus-related acute-on-chronic liver failure (mean ± SD) *n* (%)

Complications and biochemical characteristics	Entecavir group	Lamivudine group	Non-antiviral group
No. of patients	77	64	49
Gender (M/F)	66/11	56/8	37/12
Age (yr)	45.90 ± 11.33	44.38 ± 10.39	47.20 ± 11
Ascites	64 (83.1)	53 (82.8)	35 (71.4)
HRS	7 (9.1)	6 (9.4)	3 (6.1)
HE	23 (29.9)	22 (34.4)	19 (38.8)
Ⅰ-Ⅱ	19 (24.7)	18 (28.1)	16 (32.7)
Ⅲ-Ⅳ	4 (5.2)	4 (6.3)	3 (6.1)
SBP	23 (29.9)	21 (32.8)	19 (38.8)
Liver cirrhosis	48 (62.3)	38 (59.4)	34 (69.4)
Electrolyte disturbances	54 (70.1)	49 (76.6)	41 (83.7)
Alanine transaminase (U/L)	412.84 ± 605.62	271.14 ± 304.24	180.96 ± 222.50
Total bilirubin (μmol/L)	311.34 ± 120.99	340.81 ± 123.38	265.51 ± 121.88
Albumin (g/L)	37.25 ± 11.84	34.09 ± 10.90	35.76 ± 12.34
Prothrombin activity (%)	32.16 ± 9.21	31.36 ± 10.16	31.64 ± 10.59
International normalized ratio	2.46 ± 0.69	2.47 ± 0.61	2.58 ± 0.86
Creatinine (μmol/L)	97.78 ± 55.42	88.85 ± 25.08	96.94 ± 38.16
Sodium (mmol/L)	133.57 ± 13.37	133.52 ± 6.47	132.57 ± 5.61
HBeAg (+/-)	33/44	27/37	22/27
HBV DNA (IU/mL)	(1.00 ± 8.51) × 10^8	(4.00 ± 26.69) × 10^7	(4.41 ± 1.08) × 10^7
HBV DNA level (< 10^3/10^3-10^7/> 10^7)	4/61/11	3/52/10	4/39/6
MELD score	29.07 ± 4.02	28.69 ± 4.43	28.61 ± 3.49
MELD score (< 30/≥ 30)	47/30	41/23	34/15

HRS: Hepatorenal syndrome; HE: Hepatic encephalopathy; SBP: Spontaneous bacterial peritonitis; HBV: Hepatitis B virus; MELD: Model for end-stage liver disease.

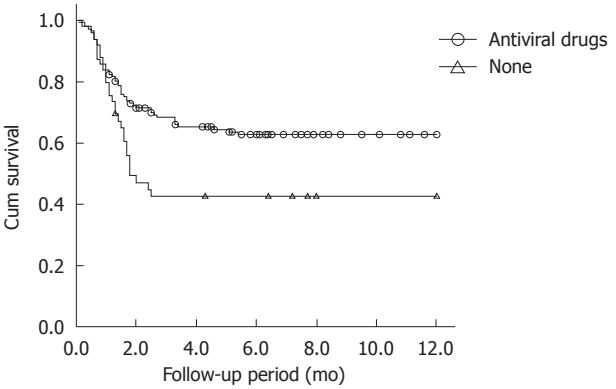

Figure 1 Survival curves for patients taking or not taking antiviral drugs by Kaplan-Meier method. Total, *n* = 190; antiviral drugs, *n* = 141; no antiviral drugs, *n* = 49.

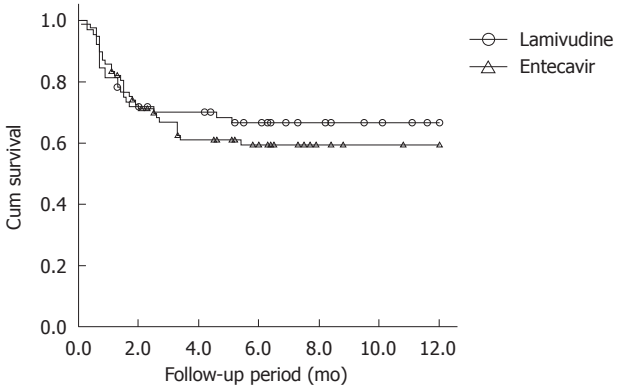

Figure 2 Survival curves for the entecavir and lamivudine groups by Kaplain-Meier method. Total, *n* = 141; entecavir group, *n* = 77; lamivudine group, *n* = 64.

survival rate was 57.6%.

Survival analysis of patients taking and not taking antiviral drugs

Patients not taking antiviral drugs had significantly higher mortality than those taking antivirals (χ^2 = 8.050, *P* = 0.005). The mean (SE) 1-year survival rates of patients who did and did not receive antiviral treatment were 62.7% (0.042) and 42.5% (0.073), respectively (Figure 1). There was no significant difference in survival rates between the entecavir group (*n* = 77) and the lamivudine group (*n* = 64) (χ^2 = 0.399, *P* = 0.527) (Figure 2).

Analysis of prognostic factors in patients with HBV-ACLF

Univariate analysis identified eight factors significantly associated with long-term patient survival: age, cirrhosis, electrolyte disturbances, HE, SBP, HRS, PTA, and the use of antiviral drugs (Table 2). However, the levels of HBV DNA before treatment and model for end-stage liver disease (MELD) score had no significant effects on the survival rate, and were not significantly different among the three groups (*P* = 0.383 and *P* = 0.053).

Forward Cox regression analysis identified antiviral therapy, HRS, HE, and electrolyte disturbances to be independently associated with the mortality (Table 3).

DISCUSSION

HBV-ACLF is associated with a high mortality[9-11], although liver transplantation can significantly improve the

Table 2 Factors affecting prognosis based on single-factor analysis

	Cases	Median time (mo)	1-yr survival rate (%)	χ^2 value	*P* value
Age (yr)					
20-45	88	6.7	69.9	9.047	0.003
> 45	102	2.5	46.5		
Fundamental disease					
Cirrhosis	120	2.6	49.1	8.026	0.005
No cirrhosis	69	8.4	71.8		
Antiviral therapy					
No	49	1.8	42.5	8.050	0.005
Yes	141	5.5	62.7		
Electrolyte disturbances					
No	46	11.8	84.1	14.397	< 0.001
Yes	144	2.6	49.3		
HE grade					
No	126	7.1	70.7	38.596	< 0.001
Ⅰ-Ⅱ	53	1.6	35.1		
Ⅲ-Ⅳ	11	1.5	9.1		
SBP					
No	127	6.0	64.3	5.345	0.021
Yes	63	2.5	44.3		
HRS					
No	174	5.7	60.9	18.403	< 0.001
Yes	16	0.9	20.8		
PTA groups (%)					
< 20	18	1.2	22.2	22.619	< 0.001
20-30	77	5.1	58.6		
30-40	66	6.7	70.6		
> 40	29	3.3	47.1		

HE: Hepatic encephalopathy; SBP: Spontaneous bacterial peritonitis; HRS: Hepatorenal syndrome; PTA: Prothrombin activity.

Table 3 Results of multivariable Cox regression analysis

Variables	Relative risk ratio	95% CI	Wald value	*P* value
Age	1.322	0.792-2.208	1.139	0.286
Cirrhosis	1.144	0.648-2.021	0.215	0.643
Antiviral therapy	0.609	0.430-1.067	6.809	0.014
Electrolyte disturbances	2.062	1.282-6.433	6.571	0.010
HE	1.879	1.335-2.646	13.065	< 0.001
SBP	1.295	0.798-2.103	1.095	0.295
HRS	2.084	1.090-3.984	4.928	0.026
PTA	0.940	0.699-1.264	0.168	0.682

HE: Hepatic encephalopathy; SBP: Spontaneous bacterial peritonitis; HRS: Hepatorenal syndrome; PTA: Prothrombin activity.

survival rate[12,13]. However, liver transplantation is limited by many factors, especially donor shortages; only 11 patients in the current study received a transplant. Improved medical treatment is the key to prolonging the survival of patients with HBV-ACLF. The effects of antiviral treatment with nucleoside analogs on hepatitis B related liver failure is currently a focus of clinical research, but their efficacy remains controversial. Several reports have suggested that lamivudine could significantly improve the prognosis of patients with liver failure[14-17], but Kumar *et al*[18] reported that, although lamivudine significantly decreased the levels of HBV DNA in patients with acute hepatitis B, it did not result in any significant biochemical or clinical improvement, compared with patients receiving a placebo. In the current study, survival analysis showed that the mortality of patients who received nucleoside analog (entecavir or lamivudine) therapy was significantly lower than that of patients who did not receive antiviral drugs. This indicates that treatment with nucleoside analogs (lamivudine/entecavir) could improve the prognosis of patients with HBV-ACLF, and suggests that nucleoside analog therapy should be implemented in these patients as soon as possible.

In addition to antiviral therapy, other factors were found to significantly influence the prognosis, including HE, electrolyte imbalance, and HRS. Methods for treating and preventing the complications of HBV-ACLF remain important research topics. Yu *et al*[19] found that, in HBV-ACLF patients treated with lamivudine and plasma exchange, multivariate analysis identified a MELD score of 30-40 or > 40 to be a good predictor of treatment outcome. The present study, however, found no significant effect of MELD score on prognosis. However, the *P* value of 0.053 suggests that a MELD score > 30 might predict a poorer prognosis in patients with HBV-ACLF if a larger sample size was analyzed. Thus, although pretreatment HBV DNA levels and MELD score had no significant effects on patient survival in this study, further studies using larger samples, or multicenter trials, are required to confirm these results.

HBV-ACLF, although rare, remains a rapidly progressive and frequently fatal condition. Traditional treatment is generally supportive, and HE, HRS and electrolyte disturbances remain the leading causes of death. In China, liver injury is caused mostly by hepaciviruses (especially HBV)[20] and may therefore be preventable. Clinicians should be aware of the rapid evolution of liver failure, and the possible risks for patients who develop any degree of coagulopathy and encephalopathy. Because the outcome is unpredictable, early transfer to a transplantation facility should be considered before the onset of advanced grades of coma, after which transfer becomes impossible. Further understanding of the pathophysiologic characteristics of this multisystemic condition and the development of better supportive therapies should improve the outcome of patients with HBV-ACLF.

COMMENTS

Background

Hepatitis B virus related acute-on-chronic liver failure (HBV-ACLF) is associated with a high mortality, although liver transplantation can significantly improve the survival rate. However, liver transplantation is limited by many factors, especially donor shortages. Improved medical treatment is the key to prolonging the survival of patients with HBV-ACLF. If the related prognostic factors are clarified in the course of HBV-ACLF, corresponding measures can be taken to reduce the mortality.

Research frontiers

Although antiviral therapy using lamivudine or entecavir have shown promising results in chronic hepatitis B and liver cirrhosis, the effects of antiviral treatment with nucleoside analogs on hepatitis B related liver failure remains controversial. And it has become currently a focus of clinical research.

Innovations and breakthroughs

The present study demonstrates that antiviral therapy had a strong effect on the prognosis of patients with HBV-ACLF by improving their 1-year survival rate. Hepatorenal syndrome, electrolyte disturbances, and hepatic encephalopathy also affected patient survival.

Applications

The results of study indicate that antiviral therapy and preventing complications [especially hepatic encephalopathy (HE), hepatorenal syndrome and electrolyte disturbances] may serve as a favorable alternative to reduce the mortality of the patients with HBV-ACLF.

Terminology

HBV-ACLF is defined as acute hepatic insult manifesting as jaundice and coagulopathy, complicated by ascites and/or HE in a patient with previously diagnosed or undiagnosed chronic hepatitis B, which includes compensated liver cirrhosis, with total bilirubin (TBiL) $\geqslant$ 171 μmol/L or an increase in TBiL $\geqslant$ 17.1 μmol/L, and prothrombin activity $\leqslant$ 40%.

Peer review

The definition of ACLF should be clarified in no uncertain terms so that it is clear to the reader of the article. Language also needs to be taken care of.

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BRIEF ARTICLE

Long-term results of small-diameter proximal splenorenal venous shunt: A retrospective study

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Abstract

AIM: To investigate recurrent variceal hemorrhage and long-term survival rates of patients treated with partial proximal splenorenal venous shunt.

METHODS: Patients with variceal hemorrhage who were treated with small-diameter proximal splenorenal venous shunt in Ruijin Hospital between 1996 and 2009 were included in this study. Shunt diameter was determined before operation using Duplex Doppler ultrasonography. Peri-operative and long-term results in term of rehemorrhage, encephalopathy and mortality were followed up.

RESULTS: Ninety-eight patients with Child A and B variceal hemorrhage received small-diameter proximal splenorenal venous shunt with a diameter of 7-10 mm. After operation, the patients' mean free portal pressure ($P < 0.01$) and the flow rate of main portal vein ($P < 0.01$) decreased significantly compared with that before operation. The rates of rebleeding and mortality were 6.12% (6 cases) and 2.04% (2 cases), respectively. Ninety-one patients were followed up for 7 mo-14 years (median, 48.57 mo). Long-term rates of rehemorrhage and encephalopathy were 4.40% (4 cases) and 3.30% (3 cases), respectively. Thirteen patients (14.29%) died mainly due to progressive hepatic dysfunction. Five- and ten-year survival rates were 82.12% and 71.24%, respectively.

CONCLUSION: Small-diameter proximal splenorenal venous shunt affords protection against variceal rehemorrhage with a low occurrence of encephalopathy in patients with normal liver function.

Key words: Partial portacaval shunt; Hemorrhage; Esophageal varices; Shunt diameter; Encephalopathy

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INTRODUCTION

Total portacaval shunt provides an excellent therapeutic effect for hemorrhage due to esophageal and/or gastric varices as portal venous pressure decreases to a normal range. However, there is a deleterious effect on liver function due to the complete loss of prograde portal flow which is accompanied with a high encephalopathy rate[1].

To reduce the complications caused by total portacaval shunt, partial portacaval shunt has been developed since the 1980s in an attempt to effectively decompress the portal vein while maintaining prograde portal flow to the liver to diminish postoperative encephalopathy. Several centers have validated the hemodynamic advantage of partial portacaval shunts, which significantly reduced the episodes of encephalopathy compared with the shunts that totally divert portal flow[2-5]. The theory of the small-diameter portacaval shunt is based on that variceal hemorrhage will not occur if the pressure gradient between the portal system and the systemic caval system is about 12 mmHg (about 16.3 cmH_2O)[6,7]. The most popular small-diameter portacaval H-graft decompressive shunt proposed by Sarfeh *et al*[1] involves placement of an 8-mm diameter polytetrafluoroethylene (Gore-Tex) graft between the portal vein and inferior vena cava.

The operation of partial portosystemic shunt was initiated in the 1990s in our center. In 1991, we firstly investigated the relationship between portal venous diameter (PVD), free portal pressure (FPP) and collateral venous diameters in percutaneous transhepatic portography (PTP)[8], and found that FPP could decrease to 2.64 kPa (26.94 cmH_2O) when the portosystemic shunt diameter (SD) was 67% of PVD. In the studies of Rousselot *et al*[9] and Burcharth *et al*[10], no bleeding episodes due to gastroesophageal varices occurred if FPP was 2.64 kPa (26.94 cmH_2O). This data outclasses the normal range of PVP (1-5 mmHg, 6.79-13.58 cmH_2O). According to the data from PTP, the suitable SD could be determined before operation. However, PTP is a kind of traumatic examination and not suitable for every patient. Thus, Duplex Doppler ultrasonography was introduced to determine SD before the shunting[11]. We had established an equation to calculate SD based on portal venous flow, superior mesenteric venous flow and PVD[11]. Since 1996, proximal splenorenal venous shunting with predicted portal SD has been carried out in 98 patients with hypersplenism and esophageal variceal hemorrhage. This is a retrospective analysis of our experience with proximal splenorenal venous shunting with small stoma.

MATERIALS AND METHODS

Patients with hepatic cirrhosis and portal hypertension received proximal splenorenal venous shunt in the Department of Surgery of Ruijin Hospital, Shanghai Jiaotong University School of Medicine. The study design was approved by the independent ethics committee of Ruijin Hospital. Inclusion criteria consisted of Child A or B (score less than 7 points)[12], prograde hepatic portal flow, splenomegaly and hypersplenism, absence of refractory ascites, absence of encephalopathy, absence of portal thrombosis, and hemorrhage due to esophageal and/or gastric varices or portal gastropathy.

Preoperational examinations for clinical conditions and laboratory tests including blood cell counts, hepatic function and blood coagulation, were made in all patients. Esophageal variceal hemorrhage was confirmed by endoscopic examination. Encephalopathy was assessed from stage 0 to 4 by West Haven classification system[13]. Ascites was classified as absent, moderate (clinically evident, but well-controlled with fluid restriction and oral diuretics), or severe (abdominal distention refractory to fluid restriction and maximal diuretic therapy). The diagnosis of hypersplenism was established with the presence of splenomegaly and significant reduction of blood cell counts. The patients were staged by Child classification and preshunt model for end-stage liver disease (MELD) score. Hemodynamics of portal venous system was documented by Duplex Doppler ultrasonography including portal vein, splenic vein and superior mesenteric vein.

In our previous study[11], an equation was established to calculate the SD according to PVD, PVF and splenic venous flow (SVF) of Duplex Doppler ultrasonography before operation, i.e. SD (mm) = PVD (mm) × [1 - SVF (mL/min)/PVF (mL/min)]$^{1/4}$ × 67%.

The procedure of operation included splenectomy and small-diameter proximal splenorenal venous shunting. In brief, left Kocher incision at upper abdomen was undertaken in all patients. Splenectomy was performed and followed by the exposure of splenic vein and left renal vein. The diameter of anastomosis was determined by the equation mentioned above. If the diameter of splenic veous stump was equal to pre-calculated SD, end-to-side anastomosis would be performed, otherwise, side-to-side anastomosis would be performed. Continuous suture was adopted using 5-0 Gore-Tex or 5-0 polypropolene. Free portal venous pressure was determined before splenectomy, and before and after shunting.

Surgical complication, rehemorrhage, encephalopathy and operative mortality were recorded within 30 d after operation. Duplex Doppler ultrasonography was used to examine the portal venous system just before hospital discharge. After discharge, the following clinical manifestations were monitored: rehemorrhage, encephalopathy, hepatic dysfunction or failure and the occurrence of hepatocellular carcinoma. Hematemesis and/or melana were considered as rehemorrhage. Encephalopathy was assessed as mentioned above.

Statistical analysis

SPSS 13.0 software for Windows was used for statistical analysis. Data were expressed as the mean ± SD and differences were considered significant at $P < 0.05$. Repeated measure analysis was performed to compare the change of FPP before and after shunting. Statistical significance of hemodynamic changes of portal vein before and after shunting was determined using paired t test. Survival probabilities of patients and survival curves were determined by life table analysis using the Kaplan-Meier method. A comparison of survival probabilities between Child A and Child B, or MELD (4-6) and MELD (7-10) was made using Wilcoxon (Gehan) statistics.

Table 1 Patients' demographics and laboratory tests (mean ± SD) *n* (%)

Items	Total No. of patients (98)
Age (yr)	45.1 ± 9.1 (19-73)
Sex	
Male	80 (71.43)
Female	18 (18.37)
Cause of cirrhosis	
Hepatitis B	87 (88.78)
Others	11 (11.22)
Esophageal and/or gastric variceal bleeding	98 (100)
White cell count (× 10^9/L)	2.7 ± 1.3
Red cell count (× 10^{12}/L)	3.63 ± 0.68
Hemoglobin (g/L)	97.58 ± 22.55
Blood platelets count (× 10^9/L)	57.53 ± 26.28
Serum total bilirubin (mmol/L)	20.47 ± 8.05
Serum albumin (g/L)	36.23 ± 4.14
Child classification	
A	75 (76.53)
B	23 (23.47)
MELD score	6.87 ± 1.25

MELD: Model for end-stage liver disease.

Table 2 Change of free portal pressure during shunt operation (*n* = 98) (mean ± SD)

	Before splenectomy	Before shunting	After shunting
FPP (cmH_2O)	42.62 ± 5.90	36.24 ± 5.23[1]	29.18 ± 3.69[2,3]

[1]Before splenectomy *vs* before shunt, $P = 0.000$; [2]Before splenectomy *vs* after shunting, $P = 0.000$; [3]Before shunting *vs* after shunting, $P = 0.000$. FPP: Free portal pressure.

RESULTS

From May 1996 through March 2009, 98 patients, aged 19-73 years, received small-diameter proximal splenorenal venous shunt in our department. The preoperative clinical data of the patients and laboratory tests are shown in Table 1. As a result, 88.78% of hepatic cirrhosis was due to hepatitis B infection, and the rest was due to various reasons including blood fluke (5), cryptogenic causes (3), alcohol intake (2) and hepatitis C (1). Splenomegaly and hypersplenism were observed in all patients while no ascites and encephalopathy were found before shunting.

Pre-calculated SD was 7 mm in 3 patients, 8 mm in 54 patients, 9 mm in 23 patients and 10 mm in 18 patients, respectively. After splenectomy, reduction of mean FPP was 14.97% ($P < 0.01$) (Table 2). After shunting, the mean of free portal pressure decreased to 29.18 cmH_2O (range, 21-39 cmH_2O) with a rate of 31.53% ($P < 0.01$) (Table 2, Figure 1).

After operation, Duplex Doppler ultrasonography was undertaken in 50 patients. The diameter and flow rate of main portal vein were decreased significantly after shunting compared with that before operation ($P < 0.01$) (Table 3). The flow velocity of portal vein was also reduced but not significantly ($P = 0.088$).

Table 3 Hemodynamic changes of portal vein before and after shunting by Duplex Doppler ultrasonography (*n* = 50) (mean ± SD)

Portal vein	Before shunting	After shunting
Diameter (mm)	13.91 ± 1.61	12.38 ± 1.82[a]
Flow velocity (cm/s)	17.79 ± 8.28	15.41 ± 7.55
Flow rate (mL/min)	1561.58 ± 582.64	965.27 ± 512.14[a]

[a]$P = 0.000$.

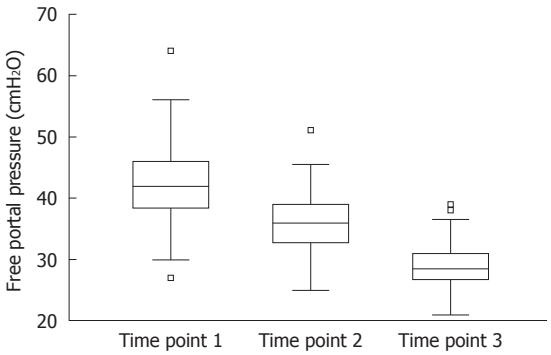

Figure 1 Change of portal pressure during operation (*n* = 98). Time point 1: Before splenectomy; Time point 2: After splenectomy and before shunting; Time point 3: After shunting.

Postoperative complications were observed in 13 patients (13.54%) during hospitalization. Six patients (6.12%) had gastrointestinal bleeding after shunting, including hematemesis in three and melana in three. The cause of rehemorrhage was portal venous thrombosis in 4 patients. Rebleeding was controlled by conservative treatment or endoscopic sclerosing therapy, and venous thrombosis was treated by thrombolytic treatment. Abdominal bleeding occurred in 5 patients (5.1%). Among them, one patient received surgical treatment while the others recovered after conservative treatment. Spontaneous hemothorax and severe ascites were observed each in one patient (1.02%), who was recovered after expectant therapy.

Seven patients were lost to follow-up after discharge. The other 91 patients were followed up for a median period of 48.57 mo (range, 7 mo to 14 years).

After discharge, 4 patients (4.40%) had rehemorrhage including hematemesis in two and melana in two. Encephalopathy was observed in three patients (3.30%), one of them received liver transplantation and the others received conservative treatment.

The 30-d perioperative mortality was 2.04% (2). One patient died of hematemesis after 28 d and another one died of abdominal bleeding and disseminated intravascular coagulopathy. Seven patients (7.27%) died 1 year after operation. The 5-year and 10-year mortality rates increased to 16.57% (15) and 27.64% (25) respectively after operation (Table 4, Figure 2). Among the 13 deaths, 8 patients died of progressive hepatic failure, and 5 died of hepatocellular carcinoma. One patient died of uncontrolled gastrointestinal rehemorrhage, and one died of unknown

Table 4 Actual cumulative survival and mortality after shunting (n = 91)

Years after shunting	Cumulative survival (%)	Cumulative mortality (%)	Death (n)
0	95.43	4.57	4
1	92.81	7.19	2
2	88.21	11.79	3
3	86.45	13.55	1
4	84.44	15.56	1
5	82.12	17.88	1
6	79.29	20.71	1
7	75.69	24.31	1
8	71.24	28.76	1
9	71.24	28.76	0
10	71.24	28.76	0
11	71.24	28.76	0
12	71.24	28.76	0
13	71.24	28.76	0

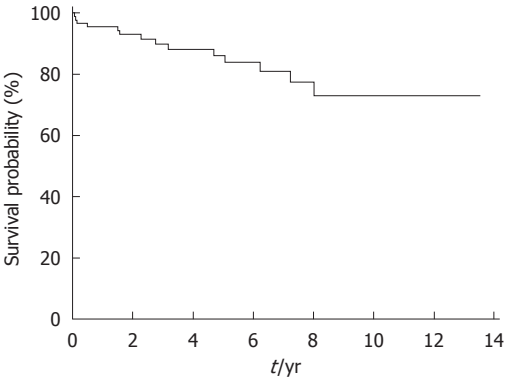

Figure 2 Survival curve for 91 patients undergoing small-diameter proximal splenorenal venous shunting.

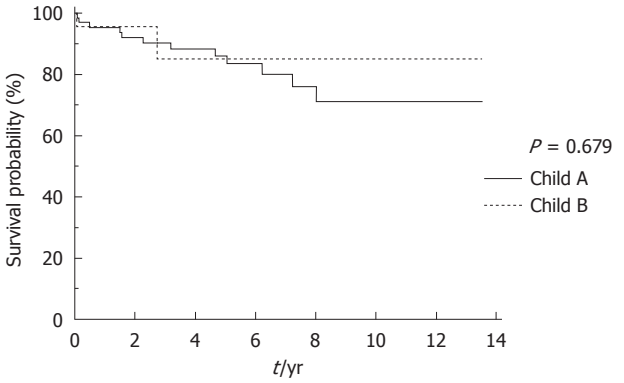

Figure 3 Comparison of survival curve for patients with Child A and Child B variceal hemorrhage undergoing small-diameter proximal splenorenal venous shunting.

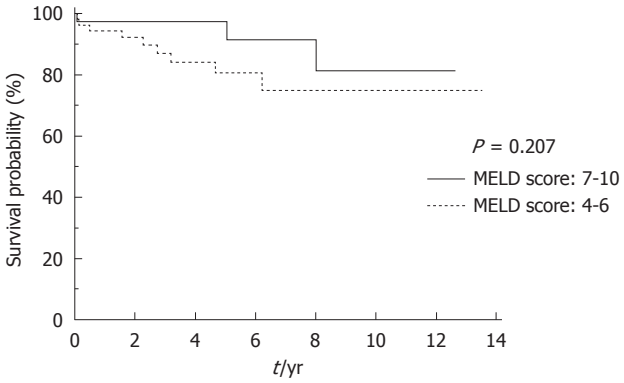

Figure 4 Comparison of survival curve by model for end-stage liver disease scoring in patients undergoing small-diameter proximal splenorenal venous shunting. MELD: Model for end-stage liver disease.

cause. For patients with Child A variceal hemorrhage, the survival after shunting was similar to those with Child B variceal hemorrhage ($P > 0.05$) (Figure 3). The long-term survival rate in patients with variceal hemorrhage with MELD score 7-10 was similar to those with MELD score 4-6 as well (Figure 4).

DISCUSSION

Since liver transplantation becomes the most promising treatment for advanced hepatic diseases in terminal stage, surgical shunting has been much less practiced in treating cirrhotic patients with portal hypertension. However, not all cirrhotic patients, especially those with better liver function and variceal hemorrhage, have chance to receive liver transplantation in China. Therefore, surgical shunting is still the main treatment for controlling variceal hemorrhage. The present study was conducted to investigate the long-term effectiveness of small-diameter proximal splenorenal venous shunt for patients with portal hypertensive bleeding.

The main etiology of hepatic cirrhosis is hepatitis B in China. Splenomegaly and hypersplenism exist in almost all patients with portal hypertension simultaneously. Splenectomy and proximal splenorenal venous shunt became the first modus operandi in our study. In addition, a high incidence of rebleeding and encephalopathy was observed in patients with Child C variceal hemorrhage who received small-diameter portacaval shunt[3]. There are also higher operative mortality and the lower long-term survival rate observed in patients with Child C variceal hemorrhage after partial portacaval shunting than those with Child A and B variceal hemorrhage[14]. So it has been suggested that the indication for partial portacaval shunting is Child A and some selected Child B variceal hemorrhage patients and the anticipated outcome of shunting is at least equivalent to that of liver transplantation in these patients[14]. Thus only patients with Child A or B variceal hemorrhage were enrolled for shunting in our center and selective operation was adopted instead of urgent shunting.

It was confirmed that small-diameter (mostly 8-10 mm) proximal splenorenal venous shunt could effectively prevent variceal rehemorrhage in our study. Rebleeding rate in the peri-operative period (6.02%) was reasonable considering that the main cause of bleeding was the thrombosis. When compared with the results from other types of small-diameter portacaval shunt, the long-term rebleeding rate was relatively low (4.04%). In H-graft portacaval shunting reported by Rosemurgy *et al*[15]. Collins *et al*[14],

late rehemorrhage was 5.4% and 8%, respectively. In small-stoma (10-12 mm) side-to-side portacaval shunting by Johansen *et al*[16], 4 patients (8%) had rebleeding. The most important reason for the successful prevention of rehemorrhage is to maintain a relatively hypertensive portal system (a higher FPP or portacaval pressure gradient). It was suggested that high portal pressures may reduce absorption of potential neurotoxins from the gut[17]. We aimed to obtain 2.64 kPa (26.94 cmH_2O) of FPP after shunting. The FPP before and after shunting showed that this requirement is essential. The mean FPP after shunting was slightly higher than target value (29.18 cm *vs* 26.94 cm) during the operation. The data by Sarfeh *et al*[18] showed that portal venous pressure would decrease after shunting. Although there is lack of direct information concerning long-term portal hemodynamics of shunting, our clinical observation suggested that higher FPP might have maintained in most cases.

As demonstrated by Bismuth *et al*[19], the small diameter side-to-side portacaval shunting was aimed to prevent hemorrhage by reducing variceal pressure while keeping considerable hepatic portal flow. However, the progressive enlargement of the stoma after shunting will eventually keep the total shunting of blood away from the liver[2], which will consequently increase the incidence of chronic encephalopathy. When compared with the results in a side-to-side portacaval anastomosis with small stomas, a low incidence of encephalopathy was observed in our study. There are two potential reasons to explain the difference. First, one important intervention was that continuous suture using nonabsorbent stitches was adopted in our study instead of using interrupted suture introduced by Capussotti *et al*[20] and Bismuth *et al*[19]. Although the underlying change of anastomosis after shunting was not fully understood, we could hypothesize that the stoma might keep its original size for a long time after operation. The second important factor was that FPP reached a higher level after shunting. Prevention of encephalopathy depends on the preservation of splanchnic venous pressure[17,21] and/or to preservation of nutrient hepatic blood flow[22]. However, it has been shown that long-term outcome after small-diameter H-graft portacaval shunt was not determined by direction or reversal of portal vein blood flow[23]. Johansen *et al*[16] suggested that portal vein pressure should not be reduced to normal range after portacaval shunting in order to prevent rehemorrhage and that maintenance of a higher portal vein pressure could keep a prograde portal flow.

The long-term survival rate was considerable in our cohort of patients. The 5-year and 10-year survival rates were 82% and 71% in patients with Child A and B variceal hemorrhage. After small-diameter H-graft portacaval shunting, a 7-year survival of 54% of Child A and B risk was reported by Collins *et al*[14], and 5/10-year survival of 67%/33% in Child A and 49%/16% in Child B reported by Rosemurgy *et al*[24]. Our long-term survival was higher than that of the two studies. Two reasons might exist: first, primary hepatic disease was different. Most of our patients suffered from hepatitis B compared with the patients with alcoholic cirrhosis in the Europe and United States. Second, some of the reported cases were emergent operations which might affect the long-term prognosis. In our study, all of the shunting was performed as selective operations. Progressive hepatic dysfunction was a major risk for late deaths. Eight of 13 patients died of hepatic dysfunction in this study. And there was no difference between Child A and Child B or between lower MELD scores in long-term survival. These results also suggested that there was a higher survival in patients with better liver function despite of pre-shunting or post-shunting. Although there are various prognostic factors such as age, occurrence of hepatocellular carcinoma, cardiovascular diseases, *etc*., liver function is still a determinant factor of survival on the whole.

As a nonsurgical intervention, transjugular intrahepatic portosystemic shunt (TIPS) has been used to treat the complications of portal hypertension. TIPS is accepted widely because of its high rate of decompressing the portal circulation, no requirement for anesthesia, very low procedure-related mortality and suitableness for severe cirrhotic patients. For control of esophageal and gastric variceal bleeding, TIPS has an excellent hemostatic effect (95%) with a low rebleeding rate (< 20%)[25]. However, encephalopathy and stent dysfunction are two major drawbacks[25]. Comparing with TIPS, small-diameter proximal splenorenal venous shunt can also acquire a high rate of bleeding control and low occurrence of encephalopathy. However, for patients with poor hepatic function, TIPS is more advantageous than partial portacaval venous shunt. As TIPS may not be the mainstream therapy in China, small-diameter proximal splenorenal venous shunting remains the first choice for the treatment of portal hypertension.

In conclusion, small-diameter proximal splenorenal venous shunting can be performed successfully in patients with better liver function. After shunting, the incidence of variceal rehemorrhage can be controlled effectively and the incidence of encephalopathy is reduced significantly as well.

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COMMENTS

Background

Total portacaval shunt provides an excellent effect of the treatment for hemorrhage due to esophageal and/or gastric varices but with a deleterious effect on liver function leading to a high encephalopathy rate. Partial portacaval shunt could effectively decompress portal vein while maintaining prograde portal flow to the liver to diminish postoperative encephalopathy.

Research frontiers

This retrospective investigation analyzed the long-term outcome of proximal splenorenal venous shunting with small stoma. Variceal rehemorrhage could be

prevented by this operation.

Innovations and breakthroughs

For patients with variceal hemorrhage, the suitable shunting diameter of anastomosis could be calculated according to the hemodynamic data of portal venous system by Duplex Doppler ultrasonography before the operation of proximal splenorenal venous shunt.

Applications

Small-diameter proximal splenorenal venous shunt could be used to control the occurrence of variceal rehemorrhage effectively while the incidence of encephalopathy can be reduced significantly.

Terminology

Small-diameter proximal splenorenal venous shunt: this is a surgical procedure in which the proximal splenic vein is attached to the left renal vein with small-diameter anastomosis; Esophageal variceal hemorrhage: bleeding from esophageal varices due to portal hypertension.

Peer review

This is an interesting paper with some restrictions. The period from 1996 to 2009 is very long and such studies have problems with changes in general management of these patients and the outcome. The authors did not mention the role of transjugular intrahepatic portasystemic shunting as an important part in the management of portal hypertension.

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CASE REPORT

Three cases of retroperitoneal schwannoma diagnosed by EUS-FNA

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Abstract

Schwannomas are peripheral nerve tumors that are typically solitary and benign. Their diagnosis is largely based on surgically resected specimens. Recently, a number of case reports have indicated that retroperitoneal schwannomas could be diagnosed with endoscopic ultrasound-guided fine-needle aspiration (EUS-FNA). We report the diagnosis of three cases of schwannoma using EUS-FNA. Subjects were two males and one female, ages 22, 40, and 46 years, respectively, all of whom were symptom-free. Imaging findings showed well-circumscribed round tumors. However, as the tumors could not be diagnosed using these findings alone, EUS-FNA was performed. Hematoxylin-eosin staining of the resulting tissue fragments revealed bland spindle cells with nuclear palisading. There was no disparity in nuclear sizes. Immunostaining revealed S-100 protein positivity and all cases were diagnosed as schwannomas. Ki-67 indexes were 3%-15%, 2%-3%, and 3%, respectively. No case showed any signs of malignancy. As most schwannomas are benign tumors and seldom become malignant, we observed these patients without therapy. All tumors demonstrated no enlargement and no change in characteristics. Schwannomas are almost always benign and can be observed following diagnosis by EUS-FNA.

Key words: Schwannoma; Endoscopic ultrasonography; Fine-needle aspiration; Retroperitoneal tumor; S100 proteins; Ki-67 index

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INTRODUCTION

Schwannomas are tumors that originate from peripheral nerve Schwann cells, and are typically solitary and benign. Schwannomas are difficult to diagnose using imaging

only, and as such, diagnoses are commonly confirmed with conventional surgical resection. In the present study, we diagnosed three schwannomas using endoscopic ultrasound-guided fine-needle aspiration (EUS-FNA) without the need for surgical resection, and provided follow-up of the patients.

CASE REPORT

Case 1

A 22-year-old male consulted our department for further examination of a 20 mm hypoechoic mass in the head of the pancreas that had been detected by abdominal ultrasonography (US). His abdomen was flat and soft without tenderness. US showed a well-encapsulated, smooth-surfaced, homogeneous and hypoechoic mass near the pancreas at the right side of the superior mesenteric artery (SMA) (Figure 1). Contrast-enhanced US with Sonazoid (Daiichi-Sankyo, Tokyo, Japan), a lipid stabilized suspension of perfluorobutane gas microbubbles, revealed progressively increased enhancement in the mass (Figure 2). Computed tomography (CT) showed a 20 mm diameter low-density mass located just dorsal to the head of the pancreas. Contrast-enhanced CT of the tumor revealed minimal early contrast enhancement followed by delayed enhancement (Figure 3). Magnetic resonance imaging (MRI) showed a round mass with high signal intensity on T2-weighted images and low signal intensity on T1-weighted images. The borders of the mass were clear (Figure 4). To obtain a pathological diagnosis, three passes of EUS-FNA of the mass were performed with a curvilinear echoendoscope (GF-UCT240-AL5; Olympus Medical Systems Co., Tokyo, Japan) under conscious sedation through the duodenal wall using a 22 gauge EchoTip® Ultra (Wilson-Cook Medical Inc., Tokyo, Japan). Pathological findings using a hematoxylin-eosin stain showed bland spindle cells with regular nuclear palisading. Immunochemical staining of the tumor demonstrated S-100 positivity. We diagnosed the tumor as a schwannoma, with a Ki-67 index of 3%-15% (Figure 5).

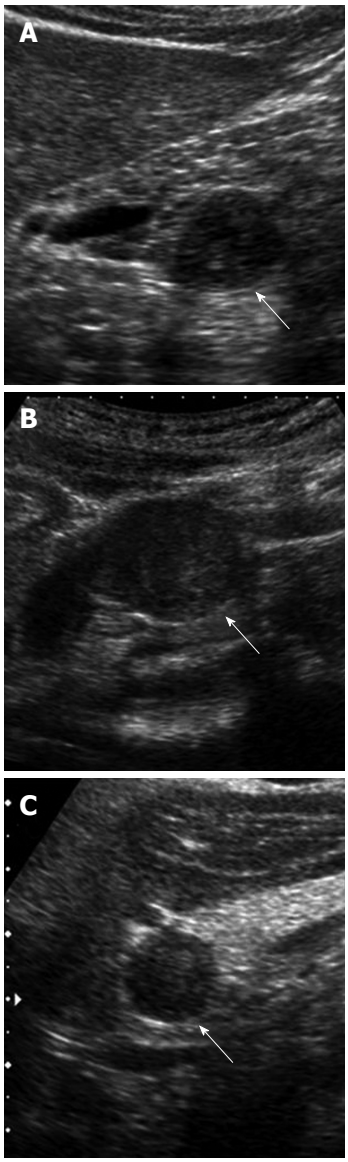

Figure 1 The borders of each of the tumors (arrows) are clearly defined. A: Case 1; B: Case 2; C: Case 3. Ultrasonography showed a round hypoechoic mass (arrows) with a slightly heterogeneous internal echo level.

Case 2

A 40-year-old female consulted an urologist and a retroperitoneal tumor was found incidentally on US. The abdomen was flat and soft without tenderness. When she consulted our hospital, she had no symptoms. US showed a well-encapsulated, smooth-surfaced, homogeneous and hypoechoic mass near the pancreas at the right side of the SMA (Figure 1). Contrast-enhanced US with Sonazoid revealed progressively increasing enhancement in the mass (Figure 2). CT showed a 27.4 mm diameter low-density mass located between the head of the pancreas and the SMA. Contrast-enhanced CT revealed deficient early contrast enhancement with slightly homogenous enhancement in the late phase. MRI showed a round mass with high signal intensity on T2-weighted images and low signal intensity on T1-weighted images. The borders of the mass were clear and the internal signal was heterogeneous. Two passes of EUS-FNA of the mass were performed through the duodenal bulb wall using a 22-gauge EchoTip Ultra. Pathologically the tumor consisted of bland spindle cells with nuclear palisading under hematoxylin-eosin staining. Immunochemical staining for S-100 was positive. The Ki-67 index of the tumor was 2%-3% (Figure 5).

Case 3

A 46-year-old male consulted a doctor in a nearby hospital for further examination of a 15 mm hypoechoic mass near the head of the pancreas that had been detected by US. The abdomen was flat and soft without tenderness and the patient was asymptomatic. US showed a well-encapsulated, smooth-surfaced, homogeneous and hypoechoic mass located dorsal to the uncinate process of the pancreas (Figure 1). US contrast-enhancement

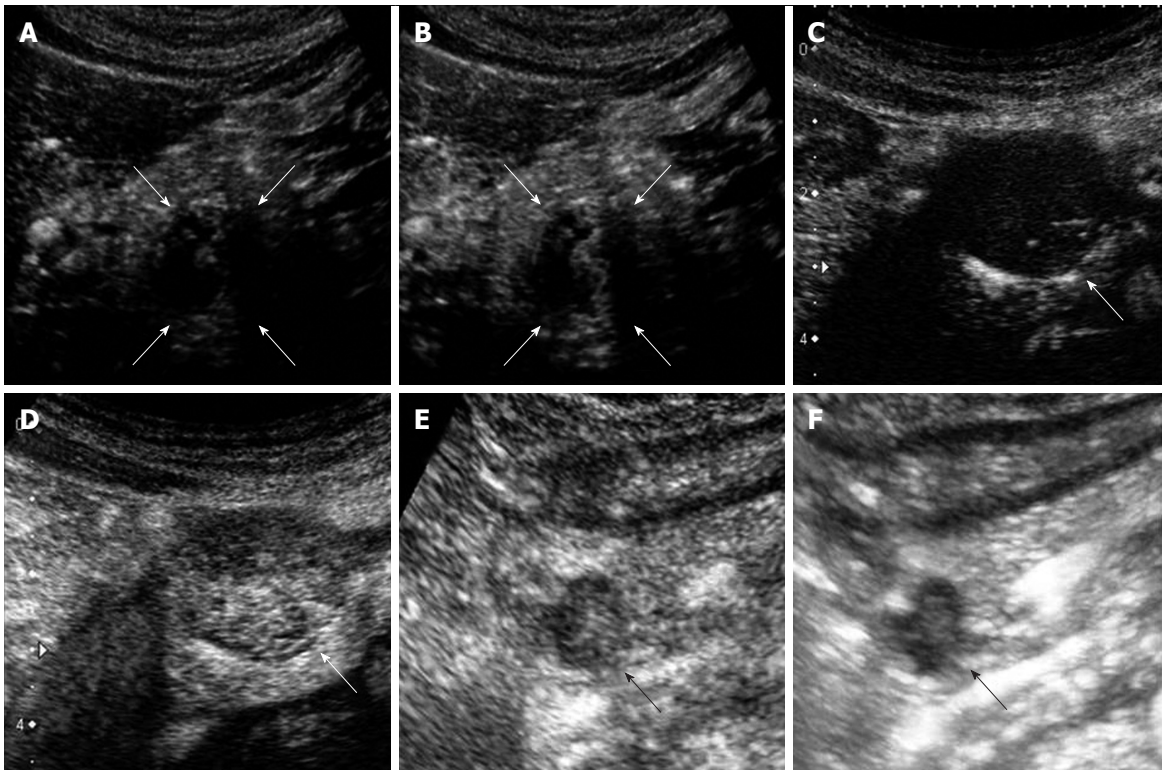

Figure 2 Sonazoid contrast-enhanced ultra sound showed no early enhancement and gradually increasing late phase enhancement. A: Vascular phase of Case 1; B: Post-vascular phase of Case 1; C: Vascular phase of Case 2; D: Post-vascular phase of Case 2; E: Vascular phase of Case 3; F: Post-vascular phase of Case 3. Tumors are indicated by arrows.

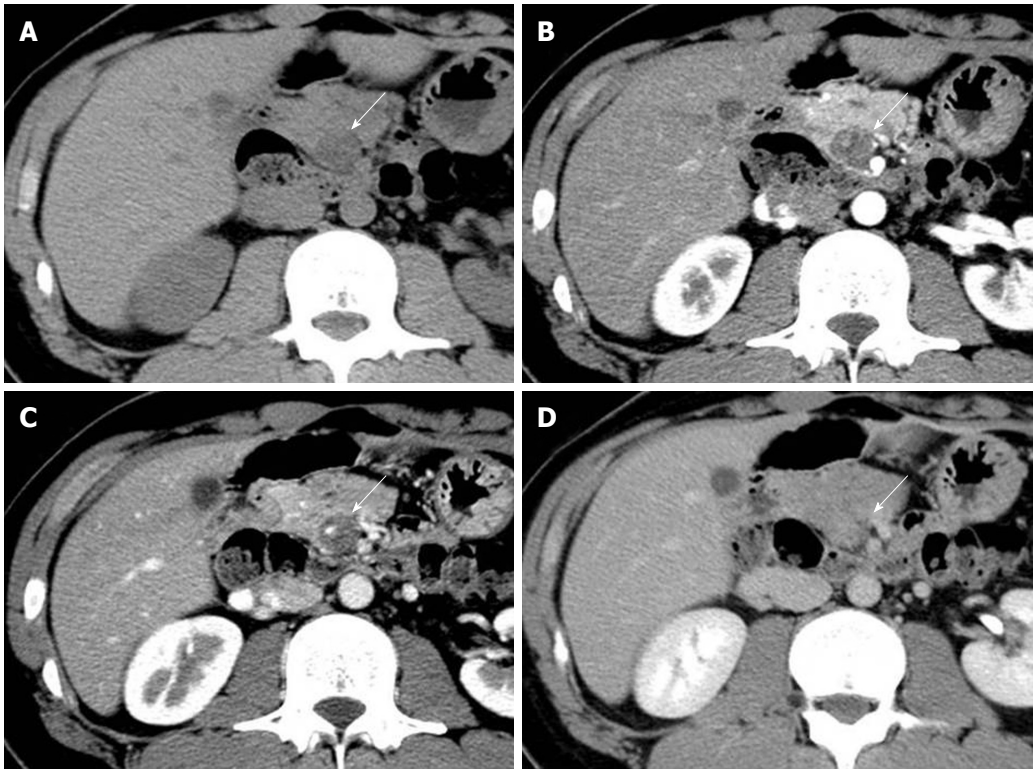

Figure 3 In Case 1, computed tomography indicated a round tumor (arrows) near the head of the pancreas. Gradually increasing enhancement of the tumor was shown by a dynamic computed tomography study. A: Plain; B: Early phase; C: Portal phase; D: Delayed phase.

with Sonazoid revealed slow contrast enhancement in the mass (Figure 2). CT showed a 15 mm diameter, smooth surfaced, well-bordered and round mass with a slightly higher density than normal pancreatic parenchyma. Con-

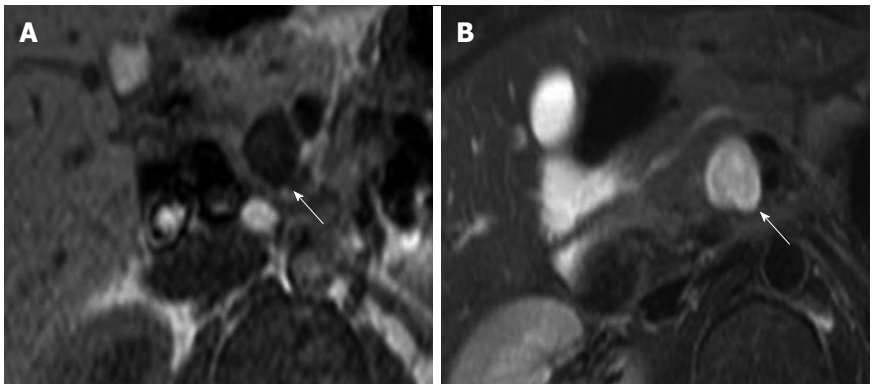

Figure 4 Magnetic resonance imaging of Case 1 showed a round mass (arrows) with low signal intensity in T1-weighted images and high signal intensity in T2-weighted images. The tumor signal was uniform. A: T1-weighted image; B: T2-weighted image.

Table 1 Patient characteristics

	Case 1	Case 2	Case 3
Age (yr), sex	22, M	40, F	46, M
Detection method	Screening	Screening	Screening
First assessment modality	US, CT	CT	US
CEA (ng/mL)	2.5	1.7	7.1
CA19-9 (U/mL)	0.0	14.0	10.0
Tumor diameter (mm)	20.0	27.4	15.0

CEA: Carcinoembryonic antigen; CA: Carbohydrate antigen; F: Female; M: Male; US: Ultrasonography; CT: Computed tomography.

Table 2 Lesion characteristics

	Case 1	Case 2	Case 3
Border	Clear	Clear	Clear
US	Hypoechoic	Hypoechoic	Hypoechoic
CT	Low density	Low density	Low density
MRI	T1 low, T2 high	T1 low, T2 high	T1 low, T2 high
Ki-67(%)	3-15	2-4	3
Follow-up duration (mo)	23	9	15

US: Ultrasonography; CT: Computed tomography; MRI: Magnetic resonance imaging.

trast-enhanced CT revealed that the tumor was slightly enhanced. MRI showed a round mass with high signal intensity on T2-weighted images and low signal intensity on T1-weighted images. The borders of the mass were clear and the internal signal was homogenous. Two passes of EUS-FNA of the tumor through the duodenal bulb wall were performed using a 22-gauge EchoTip Ultra. On pathological examination with hematoxylin-eosin staining, the tumor consisted of bland spindle cells with nuclear palisading. Immunochemical staining showed S-100 positivity, and the Ki-67 index was 3% (Figure 5). We diagnosed the tumor as a retroperitoneal schwannoma.

All three patients were asymptomatic and none of the tumors exhibited malignant signs (Tables 1 and 2). Therefore, after obtaining informed consent from the patients, we observed all three without therapy for 26, 12, and 18 mo of follow-up, respectively. No changes in tumor size or features were noted.

DISCUSSION

Schwannomas are tumors that originate from Schwann cells[1]. They are ordinarily seen in the head, neck, and extremities, and are only rarely found in the retroperitoneum; a retroperitoneal location accounted for 0.3%-3.2% of all primary schwannomas[2-6] and 0.3%-6.0% of all retroperitoneal tumors[7-12]. Schwannomas seldom cause symptoms and are often discovered incidentally. Most commonly, imaging studies show a well-defined round mass, sometimes with cystic changes[13]. Histologically, schwannomas consist of spindle cells in a hypercellular palisade arrangement area (Antoni type A) and myxomatous cells in a hypocellular organized area (Antoni type B). Schwannomas are characterized immunohistochemically by S-100 positivity[14,15]. As previously described, US findings of schwannomas generally show a well-defined hypoechoic mass with a slightly heterogeneous internal echo level. The majority of schwannomas have poor vascularity. Contrast-enhanced CT findings of these tumors often show a low-density and gradually enhanced mass. Our three cases demonstrated imaging findings similar to those described above, which are not specific to schwannomas. The MRI T2-weighted image signals of Antoni A areas were reported to exhibit a slightly high intensity, while those of Antoni B areas exhibited very high intensity[16]. According to a review of 199 schwannomas, 162 lesions (81%) showed biphasic macroscopic and microscopic patterns of central Antoni A and peripheral Antoni B cells, while 118 lesions (59%) also showed a biphasic pattern on MRI[17]. Gadolinium-enhanced T1-weighted images showed central high intensity and peripheral low intensity, whereas T2-weighted images showed peripheral high intensity and central low intensity. The specificity of these signs in schwannomas was 100% and the sensitivity was 59%[17]. However, when large in size, schwannomas are more likely to show secondary degenerative changes such as cystic degeneration, hemorrhage, necrosis, and calcification,

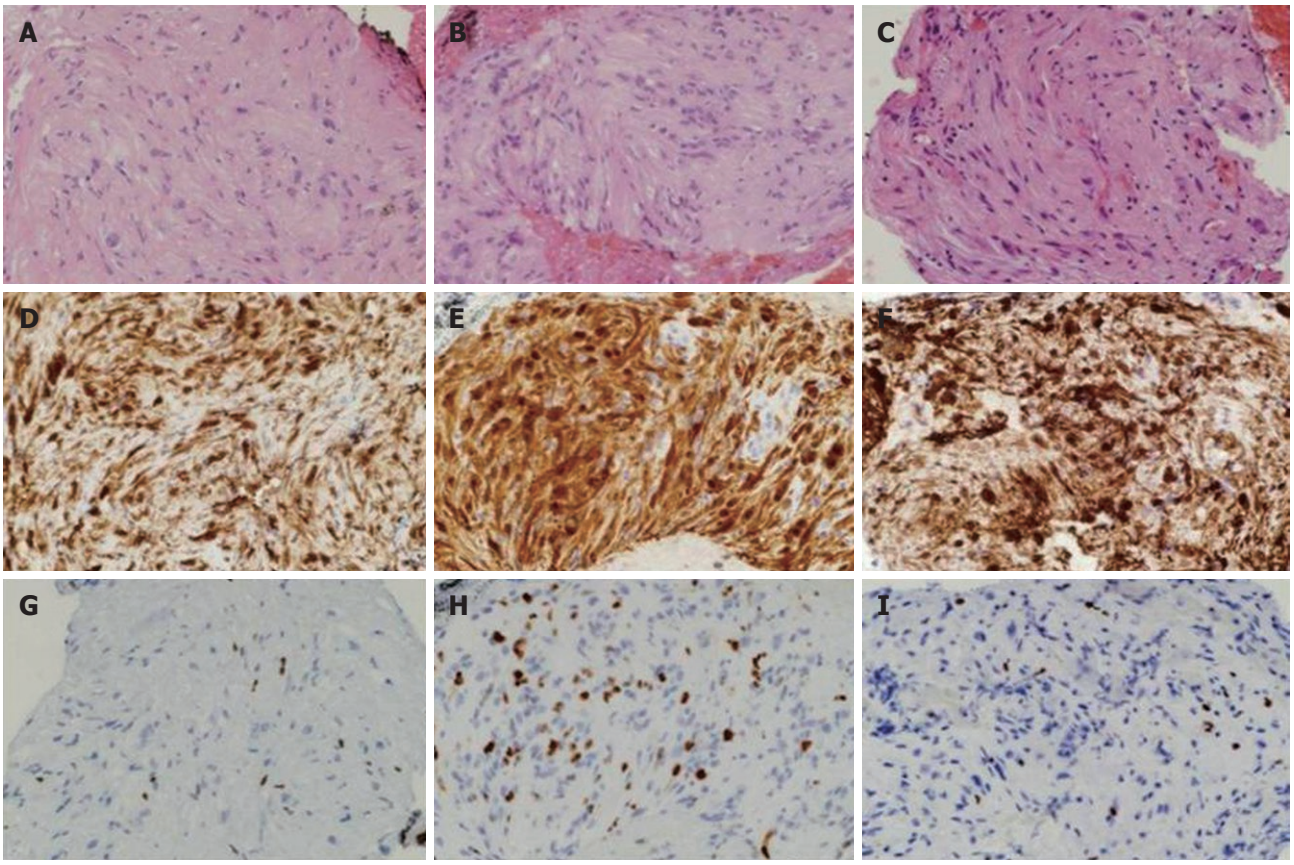

Figure 5 In all three cases the tissue fragments consisted of densely packed, bland spindle cells with nuclear palisading under hematoxylin-eosin staining (× 400). A: Case 1; B: Case 2; C: Case 3; D-F: S-100 reaction was also positive in all case. D: Case 1; E: Case 2; F: Case 3; G-I: Ki-67 indexes were 3%-15%, 2%-4%, and 3% for Cases 1, 2, and 3, respectively. G: Case 1; H: Case 2; I: Case 3.

Table 3 Reported cases of schwannoma diagnosed by endoscopic ultrasound-fine needle aspiration

Case No.	Age (yr)	Sex	Symptom	Tumor diameter (mm)	Position	US	Surgery
1[14]	79	F	Dry cough	30	SMP	Hypo	-
2[15]	59	M	None	40	Retro	Hypo	Done
3[16]	29	M	Epigastralgia	35	Retro	Hypo	Done
4[17]	37	M	None	16	Intra	Hypo	Done
5, 6, 7, 8[28]	54.5[1]	M:F = 3:1	None	23.7[1]	Retro	NA	-
Our case 1	22	M	None	20	Retro	Hypo	-
Our case 2	40	F	None	27.4	Retro	Hypo	-
Our case 3	46	M	None	15	Retro	Hypo	-

[1]Mean. Hypo: Hypoechoic; Intra: Intrapancreatic schwannoma; NA: Not available; Retro: Retroperitoneum; SMP: Superior mediastinum posterior; F: Female; M: Male; US: Ultrasonography.

and to reveal mixed patterns[13,18]. As such, it is difficult to confirm the diagnosis of a schwannoma using only the MRI findings discussed above. As a result, almost all retroperitoneal schwannomas are surgically resected and diagnosed from the resected specimen. CT- or US-guided FNAs for schwannoma diagnosis prior to surgery have been reported. However, the accuracy of both biopsy targeting and diagnosis were poor[18,19]. Li *et al*[20] reported that of 73 cases with total tumor resection and nine cases with exploratory laparotomy, 13 cases (15.9%) could be diagnosed as schwannomas preoperatively, and histological diagnosis by US-guided FNA was performed in only one case. In our three cases, we performed EUS-FNA to obtain a pathological diagnosis as we could not provide diagnosis by imaging alone. For the diagnosis of pancreatic tumors, the sensitivity of EUS-FNA was reported to be over 90%, with a specificity of approximately 100%[21,22]. A randomized comparison between EUS-FNA and CT- or US-guided FNA for malignant pancreatic tumors also revealed sensitivities of 84% and 62%, respectively. Thus, EUS-FNA was superior to CT- or US-guided FNA, especially for small masses difficult to detect with CT or US[23].

Using FNA of schwannomas, it is difficult to find a safe route from the skin to the lesion, especially in mediastinal, perirectal or retroperitoneal masses[21], as there are vessels

or other organs between the skin and the lesion. A recent report on the diagnosis of retroperitoneal tumors indicated a sensitivity of 50%, specificity of 100%, and a positive predictive value is 100%[24]. Previous case reports have described eight cases with schwannomas or neurilemmomas diagnosed preoperatively by EUS-FNA (retroperitoneum, seven cases; mediastinum, one case)[10,25-28]. In the present study the three cases with retroperitoneal schwannomas received surgery after diagnosis with EUS-FNA[10,26,27] (Table 3). Tumor recurrence or malignant transformation after complete resection is very rare for retroperitoneal schwannomas[15,29-31], and the features of malignant schwannomas include tumor diameter over 5 cm, ambiguous borders separating the tumor from the surrounding tissue, intratumor bleeding or necrosis, and a Ki-67 index of 5%-65%[32]. Therefore, asymptomatic retroperitoneal schwannomas with no indications of malignancy diagnosed by EUS-FNA[28] can be observed without the need for surgery.

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Is rectal cancer prone to metastasize to lymph nodes than colon cancer?

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Abstract

The biology of colorectal cancer differs according to its location within the large intestine. A report published in a previous issue of *World Journal of Gastroenterology* (November 2010) evaluated the importance of tumor location as a risk factor for lymph node metastasis in colorectal cancer, and showed that rectal cancer is prone to metastasize to lymph nodes as compared with colon cancer. However, in order to conclude that the tumor location is independently associated with the occurrence of lymph node metastasis, it is necessary to consider a selection bias or other patient- and tumor-related factors carefully.

Key words: Rectum; Colon; Lymph node; Metastasis; Cancer

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Akiyoshi T, Watanabe T, Ueno M, Muto T. Is rectal cancer prone to metastasize to lymph nodes than colon cancer? *World J Gastroenterol* 2011; 17(29): 3465-3466 Available from: URL: http://www.wjgnet.com/1007-9327/full/v17/i29/3465.htm DOI: http://dx.doi.org/10.3748/wjg.v17.i29.3465

TO THE EDITOR

We have read with great interest the article by Wang *et al*[1] in a recent issue of *World Journal of Gastroenterology* (November 2010). They retrospectively examined 2340 patients with colorectal cancer (stage Ⅰ-Ⅲ) who received radical resection between January 2000 and June 2008 at their institution. They showed that the proportion of lymph node positive cases (N+) was higher in the rectal cancer group compared with that in the colon cancer group (41.4% *vs* 35.5%, $P = 0.004$), despite the higher percentage of small or low-grade tumors in the rectal cancer group compared with the colon cancer group. The proportion of N+ cases in their study was close to the Japanese Society for Cancer of the Colon and Rectum (JSCCR) registry data from 1974 to 1993 that we reported previously[2]; the proportion of N+ cases among stage Ⅰ to Ⅲ was 41% (9271/22 686) in rectal cancer and 37% (8182/22 345) in colon cancer. Furthermore, they showed that the proportion of N+ cases stratified by T stage (T1, T2, and T3-4) was significantly higher in the rectal cancer group than in the colon cancer group ($P < 0.001$). They concluded that rectal cancer is prone to metastasize to lymph nodes as compared with colon cancer. However, there are some major issues about the conclusion and we would discuss them as follows.

The first point is about the selection bias. They excluded R1 or R2 resection, patients who received neoadjuvant chemoradiation, or patients with stage Ⅳ cancer.

However, excluding these patients might cause a significant selection bias when testing a hypothesis that rectal cancer is prone to metastasize to lymph nodes as compared with colon cancer.

The second point is about the significance of tumor location in the colon. Previous studies[3,4], including ours[5], have shown that patients with distal colon cancers exhibited significantly better survival than those with proximal cancers, suggesting that it might be better to analyze proximal colon and distal colon separately.

Finally, to show that tumor location is independently associated with a higher percentage of lymph node metastasis, multivariate analyses, including patient- (gender, age) and tumor-related factors (pathological grade, tumor size, tumor depth, or lymphovascular invasion[6,7]) other than tumor location, should be performed.

Although Wang *et al*'s article addresses important issues, further study is necessary to determine whether rectal cancer is truly prone to metastasize to lymph nodes as compared with colon cancer.

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Events Calendar 2011

January 14-15, 2011
AGA Clinical Congress of Gastroenterology and Hepatology: Best Practices in 2011 Miami, FL 33101, United States

January 20-22, 2011
Gastrointestinal Cancers Symposium 2011, San Francisco, CA 94143, United States

January 27-28, 2011
Falk Workshop, Liver and Immunology, Medical University, Franz-Josef-Strauss-Allee 11, 93053 Regensburg, Germany

January 28-29, 2011
9. Gastro Forum München, Munich, Germany

February 4-5, 2011
13th Duesseldorf International Endoscopy Symposium, Duesseldorf, Germany

February 13-27, 2011
Gastroenterology: New Zealand CME Cruise Conference, Sydney, NSW, Australia

February 17-20, 2011
APASL 2011-The 21st Conference of the Asian Pacific Association for the Study of the Liver
Bangkok, Thailand

February 22, 2011-March 04, 2011
Canadian Digestive Diseases Week 2011, Vancouver, BC, Canada

February 24-26, 2011
Inflammatory Bowel Diseases 2011-6th Congress of the European Crohn's and Colitis Organisation, Dublin, Ireland

February 24-26, 2011
2nd International Congress on Abdominal Obesity, Buenos Aires, Brazil

February 24-26, 2011
International Colorectal Disease Symposium 2011, Hong Kong, China

February 26-March 1, 2011
Canadian Digestive Diseases Week, Westin Bayshore, Vancouver, British Columbia, Canada

February 28-March 1, 2011
Childhood & Adolescent Obesity: A whole-system strategic approach, Abu Dhabi, United Arab Emirates

March 3-5, 2011
42nd Annual Topics in Internal Medicine, Gainesville, FL 32614, United States

March 7-11, 2011
Infectious Diseases: Adult Issues in the Outpatient and Inpatient Settings, Sarasota, FL 34234, United States

March 14-17, 2011
British Society of Gastroenterology Annual Meeting 2011, Birmingham, England, United Kingdom

March 17-19, 2011
41. Kongress der Deutschen Gesellschaft für Endoskopie und Bildgebende Verfahren e.V., Munich, Germany

March 17-20, 2011
Mayo Clinic Gastroenterology & Hepatology 2011, Jacksonville, FL 34234, United States

March 18, 2011
UC Davis Health Informatics: Change Management and Health Informatics, The Keys to Health Reform, Sacramento, CA 94143, United States

March 25-27, 2011
MedicReS IC 2011 Good Medical Research, Istanbul, Turkey

March 26-27, 2011
26th Annual New Treatments in Chronic Liver Disease, San Diego, CA 94143, United States

April 6-7, 2011
IBS-A Global Perspective, Pfister Hotel, 424 East Wisconsin Avenue, Milwaukee, WI 53202, United States

April 7-9, 2011
International and Interdisciplinary Conference Excellence in Female Surgery, Florence, Italy

April 15-16, 2011
Falk Symposium 177, Endoscopy Live Berlin 2011 Intestinal Disease Meeting, Stauffenbergstr. 26, 10785 Berlin, Germany

April 18-22, 2011
Pediatric Emergency Medicine: Detection, Diagnosis and Developing Treatment Plans, Sarasota, FL 34234, United States

April 20-23, 2011
9th International Gastric Cancer Congress, COEX, World Trade Center, Samseong-dong, Gangnam-gu, Seoul 135-731, South Korea

April 25-27, 2011
The Second International Conference of the Saudi Society of Pediatric Gastroenterology, Hepatology & Nutrition, Riyadh, Saudi Arabia

April 25-29, 2011
Neurology Updates for Primary Care, Sarasota, FL 34230-6947, United States

April 28-30, 2011
4th Central European Congress of Surgery, Budapest, Hungary

May 7-10, 2011
Digestive Disease Week, Chicago, IL 60446, United States

May 12-13, 2011
2nd National Conference Clinical Advances in Cystic Fibrosis, London, England, United Kingdom

May 19-22, 2011
1st World Congress on Controversies in the Management of Viral Hepatitis (C-Hep), Palau de Congressos de Catalunya, Av. Diagonal, 661-671 Barcelona 08028, Spain

May 21-24, 2011
22nd European Society of Gastrointestinal and Abdominal Radiology Annual Meeting and Postgraduate Course, Venise, Italy

May 25-28, 2011
4th Congress of the Gastroenterology Association of Bosnia and Herzegovina with international participation, Hotel Holiday Inn, Sarajevo, Bosnia and Herzegovina

June 11-12, 2011
The International Digestive Disease Forum 2011, Hong Kong, China

June 13-16, 2011
Surgery and Disillusion XXIV SPIGC, II ESYS, Napoli, Italy

June 14-16, 2011
International Scientific Conference on Probiotics and Prebiotics-IPC2011, Kosice, Slovakia

June 22-25, 2011
ESMO Conference: 13th World Congress on Gastrointestinal Cancer, Barcelona, Spain

June 29-2, 2011
XI Congreso Interamericano de Pediatria "Monterrey 2011", Monterrey, Mexico

September 2-3, 2011 Falk Symposium 178, Diverticular Disease, A Fresh Approach to a Neglected Disease, Gürzenich Cologne, Martinstr. 29-37, 50667 Cologne, Germany

September 10-11, 2011
New Advances in Inflammatory Bowel Disease, La Jolla, CA 92093, United States

September 10-14, 2011
ICE 2011-International Congress of Endoscopy, Los Angeles Convention Center, 1201 South Figueroa Street Los Angeles, CA 90015, United States

September 30-October 1, 2011
Falk Symposium 179, Revisiting IBD Management: Dogmas to be Challenged, Sheraton Brussels Hotel, Place Rogier 3, 1210 Brussels, Belgium

October 19-29, 2011
Cardiology & Gastroenterology | Tahiti 10 night CME Cruise, Papeete, French Polynesia

October 22-26, 2011
19th United European Gastroenterology Week, Stockholm, Sweden

October 28-November 2, 2011
ACG Annual Scientific Meeting & Postgraduate Course, Washington, DC 20001, United States

November 11-12, 2011
Falk Symposium 180, IBD 2011: Progress and Future for Lifelong Management, ANA Interconti Hotel, 1-12-33 Akasaka, Minato-ku, Tokyo 107-0052, Japan

December 1-4, 2011
2011 Advances in Inflammatory Bowel Diseases/Crohn's & Colitis Foundation's Clinical & Research Conference, Hollywood, FL 34234, United States

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Key words

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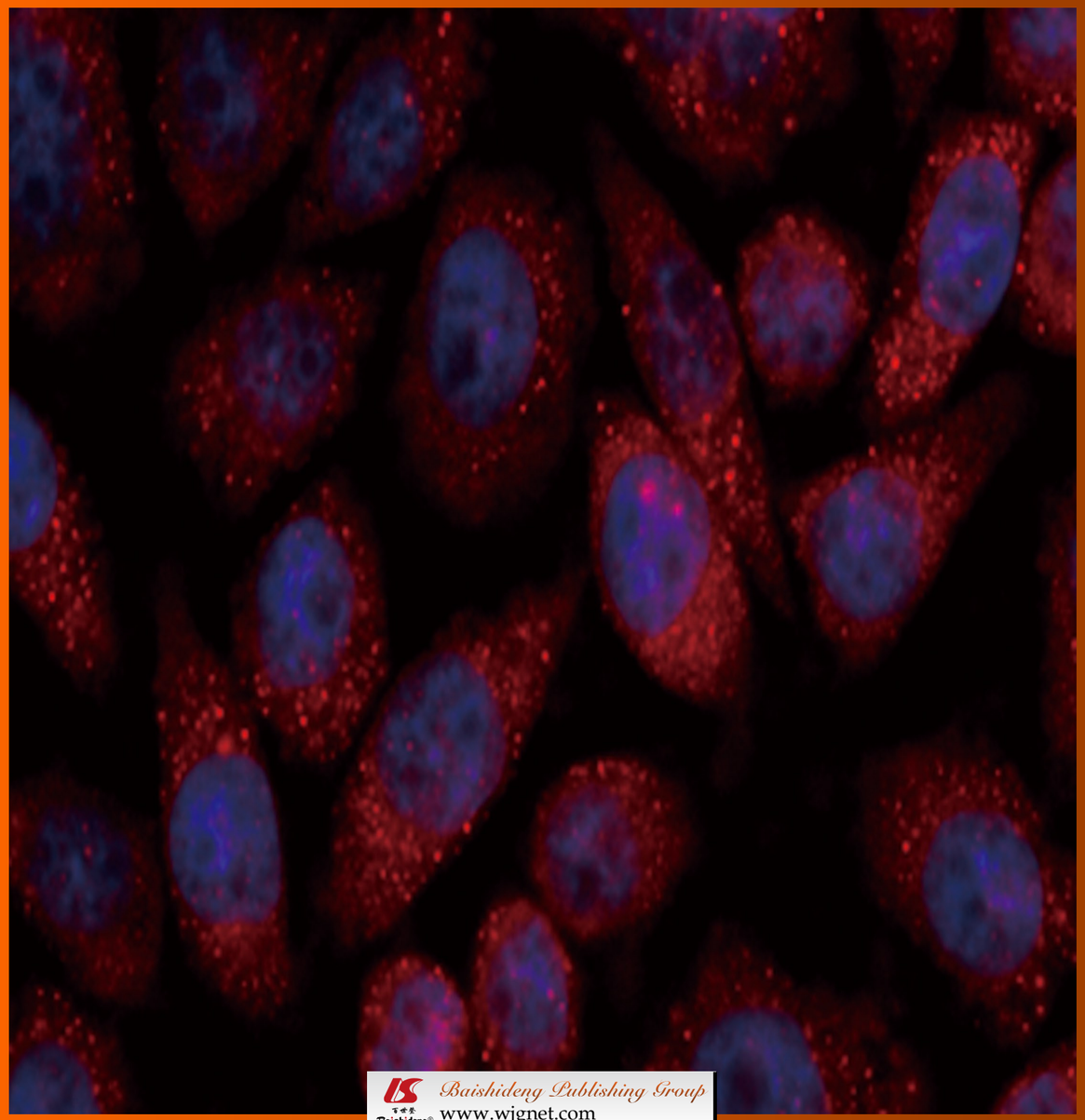

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EDITORIAL

Common misconceptions about 5-aminosalicylates and thiopurines in inflammatory bowel disease

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Abstract

Misconceptions are common in the care of patients with inflammatory bowel disease (IBD). In this paper, we state the most commonly found misconceptions in clinical practice and deal with the use of 5-aminosalicylates and thiopurines, to review the related scientific evidence, and make appropriate recommendations. Prevention of errors needs knowledge to avoid making such errors through ignorance. However, the amount of knowledge is increasing so quickly that one new danger is an overabundance of information. IBD is a model of a very complex disease and our goal with this review is to summarize the key evidence for the most common daily clinical problems. With regard to the use of 5-aminosalicylates, the best practice may to be consider abandoning the use of these drugs in patients with small bowel Crohn' s disease. The combined approach with oral plus topical 5-aminosalicylates should be the first-line therapy in patients with active ulcerative colitis; once-daily treatment should be offered as a first choice regimen due to its better compliance and higher efficacy. With regard to thiopurines, they seem to be as effective in ulcerative colitis as in Crohn' s disease. Underdosing of thiopurines is a form of undertreatment. Thiopurines should probably be continued indefinitely because their withdrawal is associated with a high risk of relapse. Mercaptopurine is a safe alternative in patients with digestive intolerance or hepatotoxicity due to azathioprine. Finally, thiopurine methyltransferase (TPMT) screening cannot substitute for regular monitoring because the majority of cases of myelotoxicity are not TPMT-related.

Key words: Crohn' s disease; Ulcerative colitis; Inflammatory bowel disease; Aminosalicylates; Steroids; Azathioprine; Mercaptopurine; Misconceptions

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INTRODUCTION

Daily clinical practice requires constant decision making, and each is open to possible errors[1-6]. Misconceptions are very common in clinical practice, but can be prevented[1-5]. More than 10 years ago, the Institute of Medicine issued its groundbreaking report, "To err is human: building a safer health system", which revealed that approximately 100 000 Americans die each year from preventable errors in hospitals[7]. The publication fundamentally changed the debate about health care quality in the United States and reconfigured how we think about the quality of care; attracted great interest

among payers and employers for improvement of care and patient safety; and produced substantial increases in research support[2]. In fact, safety issues have been a key factor in many human activities during the past few decades, and it is shocking how late the general culture of safety is reaching the health-care business. As recently summarized in a must-read book[8], "to get things right" can be a complex task but an indispensable one.

It has been pointed out that variation itself is a natural consequence of medicine being as much art as science, and thus some basal level of variation is to be expected[9]. However, in many instances, the current process of care exceeds the expected levels of natural variation, and at times may be extreme to the point of possibly indicating suboptimal overall care[9]. Medical advances have generated an increase in scientific literature and have made decision making more complex. From a scientific point of view, evidence-based medicine provides various highly useful tools for patient treatment, including clinical guidelines or consensus documents. However, frequent digressions from evidence-based recommendations and published guidelines exist, despite the wide dissemination of practice guidelines, which denotes poor quality of care[9-12].

When faced with the same set of facts, healthcare providers often make different diagnoses, employ different tests, and prescribe different therapies[9,13]. Wide practice variations might have several explanations, including the need for more evidence to determine the best course of action; the possibility that multiple approaches might be equally effective for a clinical scenario; or the need for existing evidence to be more effectively consolidated into guidelines and disseminated into practice[12]. Despite the wide dissemination of practice guidelines, clinical pathways and utilization review protocols, extreme variation continues to exist throughout all fields of medicine[9-11]. Within the field of gastroenterology, inflammatory bowel disease (IBD) is likely to generate diversions from clinical guidelines and extreme variations in the process of care[12,14-16]. There are at least three factors that establish IBD as a target for variation[9]: (1) the diagnosis of IBD is often uncertain, and this diagnostic uncertainty may lead to a potentially arbitrary sequence of diagnostic testing with various modalities; (2) the presentation of IBD is heterogeneous, and the multiple presentations of IBD mandate different diagnostic and therapeutic approaches; and (3) the treatments for IBD are themselves varied, and new treatments are always being developed and disseminated. It has been emphasized that demonstration of significant variations in the process of care in IBD indicates a need to disseminate better the available information in this area. Furthermore, identifying specific factors that predict extremes in resource utilization and clinical practice may allow for improved targeting of areas where doctor knowledge or education is inadequate[9].

Although experts and community providers are in general consensus about diagnostic decision making in Crohn' s disease, extreme variation exists both between and within groups for key therapeutic decisions in this disease[9]. When the standard of outpatient care provided has been evaluated, it has been demonstrated that the specialist IBD clinic provides better care than the non-specialist general gastroenterology clinics; even in the specialist clinic, however, the care of a relevant minority of patients does not fulfill certain criteria[17]. Some authors have performed a vignette survey to measure variations in decision making in areas of controversy dealing with ulcerative colitis, and have concluded that community gastroenterologists and ulcerative colitis experts vary dramatically in their approach to many areas of uncertainty, which suggests that current practice patterns are highly disparate and focus attention on specific areas of disconnect that should be further investigated[12]. Finally, a recent study has aimed to determine whether patients referred for a second opinion were receiving therapy in accordance with practice guidelines; it was concluded that patients with IBD often do not receive optimal medical therapy[18].

Our aim was to review several common misconceptions in the management of IBD. We focus on ambulatory patients who have predominantly mild or moderate disease treated with 5-aminosalicylates (5-ASAs) and thiopurines; the two most widely used drugs in IBD. Although decision making in the outpatient setting appears to be less difficult than in hospital situations, the reality of every day care makes human errors even more possible in outpatients. Thus, in the clinical setting, decisions need to be made immediately, with the pressure of limited time, and the understanding that an enormous variety of possible clinical situations exist. The approach taken in this paper is to state the most commonly found misconceptions in clinical practice, to review the related scientific evidence, and finally propose appropriate recommendations.

5-AMINOSALICYLATES

Aminosalicylates are the undisputed first-line option for treating and maintaining remission in ulcerative colitis[19-24]. Furthermore, they may have chemopreventive properties against colorectal cancer[25]. However, the role that these drugs may play in the management of Crohn' s disease has been controversial.

5-ASA drugs are as effective for the treatment of Crohn' s disease as for ulcerative colitis

Initially published trials have shown that oral aminosalicylates are effective treatment for active ileal, ileocolic, or colonic Crohn' s disease[26,27]. Sulfasalazine 3-6 g/d is effective in patients with colonic, but not in those with small bowel disease[28,29]. Asacol is effective in ileocolic or colonic disease[30] and Pentasa has been reported to be effective for ileitis, ileocolitis and colitis[31]. As a consequence, mesalazine has become a popular treatment for mild Crohn' s disease. However, more recently, a meta-analysis of the three placebo-controlled trials of Pentasa 4 g/d for active Crohn' s disease for 16 wk in a total of 615 patients, showed a mean reduction of the Crohn's disease activity index (CDAI) of 63 points, compared to 45 points for placebo (that is, a difference of only 18 points)[32].

Although this confirmed that a time-dependent de-

layed release formulation of mesalazine, Pentasa 4 g/d, is superior to placebo, the clinical significance of the reduction in CDAI is debatable because in individual trials, a 70- to 100-point decrease generally is required to establish clinical efficacy[32]. From these data, an alternative conclusion seems to be more plausible; namely, that Pentasa is ineffective for the treatment of symptomatic Crohn' s disease[33]. Thus, at this stage, mesalazine should be considered clinically no more effective than placebo for active ileal or colonic Crohn' s disease[33]. Accordingly, the European Crohn' s and Colitis Organization (ECCO) have concluded, "the benefit of mesalazine is limited"[26,27]. Therapeutic agents now exist that offer safe and highly effective alternatives to 5-ASA for the treatment of mild-to-moderate Crohn' s disease[34]. Specifically, in ileal or ileocolonic disease, budesonide provides the benefits of prednisone with less systemic side effects[35].

When faced with the same set of facts, healthcare providers often make different diagnoses, employ different tests, and prescribe disparate therapies. Esrailian *et al*[9] have constructed a survey with five vignettes to elicit provider beliefs regarding the appropriateness of therapies in Crohn' s disease. The authors measured agreement between community gastroenterologists and Crohn' s disease experts (the latter following, theoretically, more closely practice guidelines recommendations), and measured variation within each group. In the management of a patient with newly diagnosed Crohn' s disease, 75% of community providers endorsed the use of 5-ASA products, whereas less than half of experts (44%) employed 5-ASA therapies.

In summary, in the setting of modest efficacy and more potent alternatives, the best practice may be to consider abandoning the use of 5-ASA in patients with small bowel Crohn' s disease, until the appropriate patient population where these drugs may theoretically be effective is better delineated[33,34].

The combination of oral and topical 5-ASA treatment is not necessary, as each treatment on its own is similarly effective

Pharmacokinetic studies have demonstrated that, when given *per os*, the active moiety of mesalazine is delivered mainly to the distal ileum and proximal large bowel, thus ensuring a higher mucosal drug concentration in the right than in the left colon, with only negligible amounts of the drug reaching the rectal mucosa[36,37]. The increase in the oral dosage further increases the mucosal concentration in the proximal colonic segments, but does not significantly modify distal drug distribution[38]. Conversely, topical mesalazine administration assures considerable drug availability in the recto-sigmoid sites and, to a lower extent in the descending colon[39-41]. Therefore, it appears that, to increase mucosal mesalazine concentration in ulcerative colitis patients, along the entire length of their large bowel, besides oral dosage, topical treatment should be given[42].

As David Sachar has accurately emphasized, a form of undertreatment is overlooking the benefits of topical formulations[16]. The advantages of the combination of oral and topical aminosalicylates have been demonstrated for both inducing ulcerative colitis remission and for maintaining it. For treatment of an acute flare of the disease, on one hand, an already considered classic trial on patients with distal colitis has shown that combined therapy works more rapidly and effectively compared to oral or topical therapy alone[43]. Accordingly, the ECCO states that "left-sided active ulcerative colitis of mild-moderate severity should initially be treated with topical aminosalicylates combined with oral mesalazine. Mesalazine alone is also effective, but less effective than combination therapy"[44]. The beneficial effect of the combined regimen has also been confirmed in extensive colitis by Marteau *et al*[45]. Furthermore, patient-reported health-related quality of life in data collected from this study was investigated, and it was concluded that combined oral plus topical mesalazine treatment significantly improved this important parameter in patients with active ulcerative colitis[46].

On the other hand, there have been several randomized controlled trials comparing combination treatment, including oral mesalazine plus intermittent mesalazine enema, to oral mesalazine alone for maintaining remission[42,47-49], and success rates have been higher in patients receiving the combination regimen. Furthermore, combined oral and topical 5-ASA therapy also appear to have a favorable cost-effectiveness ratio in pharmacoeconomic analyses[47,48].

Although most authors have claimed that patients find long-term rectal treatment acceptable, a postal survey of British patients has shown that 80% preferred oral treatment alone[50]. Therefore, this form of combination treatment (with the aim of maintaining remission) could be appropriate and may be reserved for patients with a high probability of suffering relapse, because it has been demonstrated that the continuous use of topical mesalazine, associated with a high oral dosage, significantly improves the clinical course of ulcerative colitis in patients at high risk of relapse[42]. Thus, adding rectal therapy is a treatment option for patients who have relapsed on oral 5-ASA alone[44].

In summary, owing to the superiority of the combined approach - oral plus topical 5-ASA - it should be used as first-line treatment in patients with ulcerative colitis; mainly in those with predominant rectal syndrome[51].

Total 5-ASA dose should be divided at least twice daily, because a single daily dose is less effective

Oral 5-ASA is an established treatment for ulcerative colitis and the current standard of care for most patients requiring long-term maintenance treatment throughout their lives[52]. However, adherence rates - particularly in patients in remission - may be as low as 40% outside of the clinical trial setting[53]. It is now becoming relevant to find tools that improve patient adherence to treatment[54], as it has been found that multiple dosing is a predictor of non-compliance in IBD[55] and is related to a significantly increased risk of ulcerative colitis flare-ups[56].

Formulations to deliver 5-ASA to the disease activity

site, both orally and topically, have been often inconvenient and have classically required multiple daily dosing[57]. Such regimens can interfere with normal life and reduce the overall quality of life, with a negative impact on treatment adherence and poorer long-term outcomes[52]. Thus, ulcerative colitis patients cite treatment regimen complexity, tablet quantity and dose frequency as key negative influencers of adherence[52,57].

Pharmacokinetic studies in healthy volunteers have suggested that once-daily dosing may be an effective option in patients with ulcerative colitis. Hussain *et al*[58] have shown that serum, urinary, fecal, and rectal tissue concentrations are similar for once and three times daily mesalamine dosing regimens. Also, in a recent study, 4 g oral ethylcellulose-coated mesalamine given once daily was bioequivalent to a twice-daily regimen after single or repeated administration[59].

A new oral delayed-release formulation of mesalazine utilizing Multi Matrix System (MMX) technology was recently approved[60,61]. It is a high-dose (1.2 g/tablet), delayed-release form. Several studies with MMX have shown that mesalamine can be administered once-daily[62-64]. What is most important is that not only the new once-daily mesalazine formulations, but also older forms of 5-ASA may be administered in a single daily dose; apparently with adequate effects.

Response to 5-ASA is better correlated with tissue concentrations and best predicted by concentrations of the drug within the lumen of the colon. Some authors have used computer simulation to predict colonic 5-ASA levels after Asacol administration[65]. An Asacol dosage of 800 mg, three times daily, was compared to 2400 mg given once daily. The predicted maximum and average 5-ASA concentrations in the total colon and individual colonic segments differed by < 10% between dosing regimens. This model supports once-daily administration of 5-ASA as standard treatment for ulcerative colitis.

In a initial pilot clinical study, patients were randomized to receive either once daily or conventional (twice or three times daily) mesalazine for maintenance of remission in ulcerative colitis[66]. After 6 mo, patients in the once-daily arm appeared more satisfied with their regimen and consumed more medication than those in the conventional arm (90% *vs* 76%). More recently, preliminary results from a randomized trial have confirmed these encouraging results[67].

Data for the administration of a single daily dose of 5-ASA are available for both the induction and maintenance of remission of ulcerative colitis. On one hand, some authors have determined the therapeutic equivalence and safety of once-daily *vs* three times daily dosing of a total daily dose of 3 g Salofalk granules in patients with active ulcerative colitis[68]. On the other hand, other authors have confirmed this equivalence for patients with quiescent ulcerative colitis[69]. The results of the first long-term efficacy trial of maintenance therapy (with Pentasa as the 5-ASA) showed that 71% of patients receiving a single daily dose of 2 g mesalazine remained in remission, as compared to 59% of those taking 1 g twice daily; the differences being statistically significant[69]. Patients with ulcerative colitis given 5-ASA once daily had better remission rates, acceptability, and self-reported adherence to therapy compared with patients given 5-ASA twice daily. Another study was conducted to determine the efficacy and safety of once-daily dosing of delayed release mesalamine (Asacol) compared with twice-daily dosing for maintaining remission in ulcerative colitis patients, and demonstrated equivalent results with both regimens[70].

The totality of these data suggests that the success of once-daily dosing for all of these compounds may be due to the pharmacodynamic properties of 5-ASA, and may not depend on the specific characteristics of the formulation determining drug delivery[70]. In other words, given comparable efficacy between once-daily and divided dosing regimes for the treatment of ulcerative colitis with mesalazine MMX, and also with other 5-ASA formulations, the effect is likely to be generic rather than compound specific[44].

In summary, once-daily treatment should be offered as a first-choice regimen to ulcerative colitis patients. Indeed, the availability of treatments that can be taken once daily allows increased flexibility to tailor therapy according to patient preference and lifestyle, and may also have the potential to enhance compliance[69]. In fact, improved efficacy with once-daily dosing seems to be at least partly related to improved compliance[69]. These results and subsequent recommendations reinforce the principle that continued medication consumption, rather than actual drug regimen, is important in preventing disease relapse[67]. Also, that adherence, rather than medication regimen, appear to be important in disease outcome, mainly in the long term[67].

AZATHIOPRINE AND MERCAPTOPURINE

Thiopurine drugs azathioprine and mercaptopurine have been shown to be effective at inducing and maintaining remission in IBD[71,72]. These drugs are becoming increasingly popular, and their use is, at present, being considered at earlier phases of the disease than before.

Correct dose of azathioprine for Crohn's disease is 1-2 mg/kg, because higher doses are not more effective and are associated with increased adverse effects

The choice of azathioprine and mercaptopurine dose is generally based on the patient's weight, with the intention to achieve the highest therapeutic efficacy and, at the same time, to reduce the incidence of adverse effects[73-75]. Based on reported clinical trials, the most effective doses appear to be azathioprine 2.0-3.0 mg/kg and mercaptopurine 1.0-1.5 mg/kg, although there has not yet been a head-to-head comparison at various dose levels or a comparative trial evaluating the efficacy of mercaptopurine versus azathioprine in patients with IBD[76].

A meta-analysis has been performed to evaluate the

efficacy of these agents for the maintenance of remission of quiescent Crohn' s disease[77]. The pooled analysis for maintaining remission was stratified by the dose of azathioprine. When the maintenance therapy data were analyzed for the effect of azathioprine dose (1.0-2.5 mg/kg per day), the odds ratio (OR) for response increased from 1.20 (95% CI: 0.60-2.41) at 1.0 mg/kg per day to 3.01 (95% CI: 1.66-5.45) at 2.0 mg/kg per day, and to 4.13 (95% CI: 1.59-10.71) at 2.5 mg/kg per day. Thus, a common error is to step up the treatment strategy, giving up on thiopurine drugs (for example changing from these drugs to anti-tumor necrosis factor (TNF)α, before being absolutely sure that they have been administered at correct, maximal doses[16].

In summary, a form of undertreatment with thiopurines is underdosing[16]. The habit of automatically administering mercaptopurine or azathioprine at fixed doses of 50 mg/d should have been long abandoned, as higher doses of azathioprine (2.5 mg/kg per day) are more effective than lower doses (1.0 or 2.0 mg/kg per day) for treating Crohn' s disease.

Azathioprine and mercaptopurine are ineffective in ulcerative colitis (or, at best, much less effective than in Crohn' s disease)

Thiopurine drugs are the gold-standard treatment for steroid-dependent Crohn' s disease, because these drugs have been shown to be effective both at inducing and mainly, maintaining remission of the disease[71,72]. In addition, a clear steroid-sparing effect in active or quiescent Crohn' s disease has been observed with azathioprine/mercaptopurine therapy[71,72]. However, debate exists regarding whether thiopurine therapy is as effective in ulcerative colitis as it is in Crohn' s disease[78]. There have been surprisingly few randomized controlled trials, most of which were performed several decades ago and suffered from small sample sizes, used inadequate dosing of azathioprine, had ambiguous endpoints, and other methodological limitations[79].

Some meta-analyses have evaluated the efficacy of azathioprine/mercaptopurine in patients with ulcerative colitis[80-82]. The first one[80], which included studies up to the year 2003, identified only four clinical trials, and the pooled OR of the response to azathioprine therapy compared with placebo for the maintenance of remission was 2.26 (95% CI: 1.27-4.01). In the second meta-analysis[81], the literature search was performed up to the year 2006, and azathioprine was also shown to be superior for the maintenance of remission compared to placebo. Finally, the results of the most recent meta-analysis comparing azathioprine/mercaptopurine *vs* placebo or 5-ASA for the maintenance of remission in ulcerative colitis[82] has been published in 2009, and included six studies[83-88]. A therapeutic benefit of azathioprine, both overall (OR: 2.56; 95% CI: 1.51-4.34) and, particularly, when azathioprine was compared with placebo (OR: 2.59; 95% CI: 1.26-5.3), was demonstrated[82]. The number needed to treat (NNT) to prevent one recurrence with azathioprine/mercaptopurine, when compared with placebo, was only five (which compares favorably with the NNT of seven reported with azathioprine in Crohn' s disease[71]). These favorable results were confirmed when the experience from the non-controlled studies were reviewed: when these drugs were evaluated for the maintenance of remission of ulcerative colitis, the efficacy rate was as high as 76%[82].

A clinically meaningful steroid-sparing effect is achieved by thiopurine treatment, not only in Crohn' s disease patients but also in ulcerative colitis[89-92]. The number of cumulative hospitalizations significantly decreases during azathioprine treatment, both in Crohn' s disease and in ulcerative colitis patients[92,93]. Furthermore, the cumulative number of surgical interventions in patients treated with azathioprine/mercaptopurine has been reported to also be significantly lower after starting thiopurine treatment than before[92]. Finally, some authors have evaluated mortality by IBD medication, and have found that use of immunomodulators (mainly azathioprine and mercaptopurine) were associated with 50% decreased mortality in ulcerative colitis[94].

Few studies have directly compared thiopurine therapy efficacy between ulcerative colitis and Crohn' s disease. Kull *et al*[95] have compared the 6-mo efficacy of azathioprine in patients with both diseases, and found that clinical remission rates were slightly higher for ulcerative colitis than for Crohn' s disease (77% *vs* 70%); furthermore, complete corticosteroid weaning was obtained significantly more often in ulcerative colitis than in Crohn' s disease patients (59% *vs* 30%). Verhave *et al*[96] have concluded that patients with ulcerative colitis treated with azathioprine respond similarly to their Crohn' s disease counterparts. Moreover, they have determined that the beneficial effect occurs 1 mo sooner in ulcerative colitis patients than in Crohn' s disease patients. Fraser *et al*[97] have shown that azathioprine was more likely to achieve remission in patients with ulcerative colitis than Crohn' s disease (58% *vs* 45%), but was equally effective for the maintenance of remission. This study is also worth mentioning because of the long mean follow-up of patients, which provides valuable information to the clinician. In the study by Bastida *et al*[98], the beneficial effect of azathioprine was independent of the type of IBD. Finally, Gisbert *et al*[92] have found in a recent prospective study that azathioprine was similarly effective for Crohn' s disease and ulcerative colitis patients (49% *vs* 42%). Furthermore, azathioprine treatment resulted in a similar reduction in the number of hospitalizations and surgical procedures in both diseases[92].

In summary, it may be concluded that azathioprine and mercaptopurine seem to be at least as effective in ulcerative colitis as in Crohn' s disease patients.

Withdrawal of azathioprine should be recommended after several years if the patient is in remission

A form of undertreating with antimetabolites is suspending or discontinuing them too soon. Although azathioprine and mercaptopurine are effective for maintain-

ing remission in Crohn' s disease[99], no safe number of years has been determined after which these medications can be withdrawn without risk of relapse[16].

With the acceptance that Crohn' s disease is a chronic illness that needs long-term chronic therapy and the adoption of more aggressive goals of therapy (steroid-free remission, avoidance of surgery, and even mucosal healing), continuing an effective maintenance therapy is increasingly advised[100]. However, given the small but finite risk of significant adverse effects, coupled with the need for long-term therapy in patients who are often young and otherwise healthy, stopping immunomodulators in a patient in remission remains appealing[100,101].

The ECCO states that "for patients in remission on azathioprine as maintenance treatment, cessation may be considered after four years of "remission"[26,27]. It is also stated that "benefit and risks of continuing azathioprine should be discussed with individual patients"[27]. However, there has been no consensus about the duration of the treatment once remission has been obtained.

A retrospective study published in 1996 has suggested that withdrawal of azathioprine might be possible in patients who have been in complete remission without steroids for longer than 3.5 years, because the 2-year relapse rate seems similar whether the treatment is continued or stopped after this time[102]. This uncontrolled observation on a small subset of patients required confirmation by a prospective controlled trial. Therefore, Lemann *et al*[101] subsequently performed a multicenter, randomized, double-blind, noninferiority withdrawal trial. Patients who were in clinical remission on azathioprine for > 42 mo were randomized to continue azathioprine or to receive an equivalent placebo for 18 mo. Kaplan-Meier estimates of the relapse rate at 18 mo were 8% and 21%, respectively. Therefore, this study shows that azathioprine withdrawal is not equivalent to continued therapy with azathioprine for maintenance of remission in patients with Crohn' s disease who have been in remission on azathioprine for > 3.5 years. Consequently, the authors have concluded that azathioprine maintenance therapy should be continued beyond 3.5 years[101].

More recently, a cohort study of 66 patients in prolonged remission while being treated with azathioprine who stopped azathioprine, during or at the end of the aforementioned randomized controlled trial, underwent long-term follow-up evaluation[103]. The cumulative probabilities of relapse at 1, 3 and 5 years were 14%, 53%, and as high as 63%, respectively. In other words, two thirds of subjects still relapsed by 5 years when taken off azathioprine. This suggests that in many patients with Crohn' s disease, azathioprine withdrawal is not a feasible alternative, even after years of control, because it is associated with a high risk of relapse, whatever the duration of remission under this treatment[100].

In addition, two retrospective surveys have reported relapse rates after azathioprine or mercaptopurine withdrawal of 66%[97] and 85%[104], respectively, at 3 years. Another study[105] has reported the outcome of 29 patients in remission under continuous treatment with azathioprine for 2 years or more, randomized for continuation or withdrawal of azathioprine. At 1 year after randomization, the remission rate in each group was 85% and 47%, respectively ($P < 0.05$).

Discussion regarding the duration of an effective azathioprine treatment mainly concerns two points: (1) the magnitude of the relapse risk after stopping the drug; and (2) the toxicity of prolonged treatment[101]. As with all other agents, there will be some cost in relation to potential adverse events, including rare cases of infections and neoplasia that are probably related to the level of immunosuppression[106]. When the overall risks and benefits of prolonged maintenance therapy with azathioprine are balanced, it is likely that most clinicians and patients will accept the small, as yet unquantified, risk of a lymphoid malignancy, and the small risk of opportunistic infections, to prevent the ongoing morbidity and impact on quality of life that are related to the chronic symptomatic activity of Crohn' s disease[106].

In conclusion, even after a long duration of clinical remission under azathioprine, withdrawal of this drug is associated with a high risk of relapse. Therefore, as in transplanted patients, azathioprine maintenance therapy should probably be continued indefinitely in patients with Crohn' s disease once remission has been achieved[103,107].

In IBD patients who develop azathioprine digestive intolerance, thiopurine drugs should be definitively withdrawn

Azathioprine intolerance remains an important clinical problem in patients with IBD, which leads to withdrawal of therapy in up to 30% of patients[73]. In particular, its use is limited due to digestive intolerance in 10%-15% of patients[73]. This often mandates treatment with methotrexate, an alternative second-line immunosuppressive therapy in patients with Crohn's disease, or more recently, anti-TNF therapy. For patients with ulcerative colitis, colectomy may be precipitated in some individuals by azathioprine intolerance.

However, it has been suggested that the thiopurine drugs azathioprine and mercaptopurine could be interchangeable. Thus, an alternative strategy for azathioprine intolerance (mainly due to nausea or vomiting) is treatment with mercaptopurine (or *vice versa*). Several case series have addressed this question and have shown that mercaptopurine is tolerated in > 50% of azathioprine-intolerant patients (range: 47%-73%)[108-113].

In summary, treatment with mercaptopurine is a safe alternative in patients with IBD and previous digestive intolerance of azathioprine. Given the mild character of these symptoms, these patients may be cautiously switched to mercaptopurine (or *vice versa*) before being considered for other therapy or surgery[76].

Systematic blood controls may be avoided if thiopurine methyltransferase phenotype/genotype is normal

Azathioprine and mercaptopurine are inactive compounds that must be metabolized to 6-thioguanine nucleotides

(6-TGNs) to exert their cytotoxic and immunosuppressive properties. Thiopurine methyltransferase (TPMT) metabolizes mercaptopurine into inactive 6-methylmercaptopurine[114]. Therefore, reduction in TPMT activity predisposes to bone marrow suppression because of preferential metabolism of mercaptopurine to 6-TGN[115]. Quantification of TPMT activity has been considered a promising area, because it may identify unique metabolic profiles in patients at high risk of adverse reactions prior to drug exposure[115]. Thus, high concentrations of 6-TGN are detected in patients with low activity of TPMT, while low concentrations of these metabolites are found in patients with high TPMT activity, although not all studies have demonstrated this inverse correlation[116-120].

Several studies have reported a correlation between TPMT phenotype/genotype and the risk of myelotoxicity[115]. Homozygous patients for the low TPMT activity allele have an increased risk of suffering severe myelotoxicity due to excessive accumulation of 6-TGN[115]. It has been reported that the probability of having a complete TPMT deficiency or being homozygous for this enzyme is > 6 times higher among patients who have had a myelosuppression episode, when compared with those patients with good tolerance to thiopurine drugs[121]. Furthermore, other authors have even found an incidence of myelotoxicity of up to 100% in patients who are homozygous for the low activity allele[122]. However, some authors have reported that TPMT genotype/phenotype does not predict myelotoxicity in IBD patients treated with thiopurine drugs[123-132]. In this respect, a recent study has prospectively evaluated whether the choice of azathioprine or mercaptopurine dose based on TPMT activity prevents myelotoxicity in IBD patients. Among the four patients with myelotoxicity, one had intermediate basal TPMT levels, and three even had high levels, but no patient had low levels[133]. Finally, several studies have demonstrated that TPMT deficiency phenotype or genotype explains a variable proportion of myelotoxicity cases, but in no way explains all episodes of bone marrow suppression[116-122,125,128,129,134-142].

In summary, the majority of cases of leukopenia are not TPMT-related and therefore TPMT screening can never be viewed as a substitute for the current practice of regular monitoring of white blood cell counts. For this reason, it may be concluded that several factors (e.g., environmental and pharmacological) not related to TPMT activity may be responsible for azathioprine myelotoxicity, and systematic blood controls (complete blood count; mainly leukocyte count) should be done in these patients despite the function of this enzyme being normal.

Azathioprine should always be stopped and non-thiopurine therapy used instead if liver abnormalities are detected

Acute hepatocellular and cholestatic hepatitis have both been described during thiopurine therapy[143,144]. A small percentage of patients present with slight elevation of liver tests that do not have clinical implications, and abnormalities in liver tests return to normal during follow-up, which indicates that it is not always necessary to adjust immunomodulator dose. For example, abnormal liver tests resolved spontaneously while continuing on mercaptopurine in four out of five patients in the study by George *et al*[145], and in three out of four patients in the study by Markowitz *et al*[146].

When abnormalities in liver tests are more marked, but without associated jaundice, the dose of azathioprine/mercaptopurine may be reduced by 50%. It is probably not necessary to withdraw azathioprine or mercaptopurine completely, but frequent clinical and analytical controls should be strictly performed after reducing its dose. With this strategy, liver tests frequently normalize, and the initial azathioprine/mercaptopurine dose may be cautiously prescribed again[147,148].

A recent long-term follow-up study aimed to assess the incidence of azathioprine/mercaptopurine-induced liver injury in 786 patients with IBD (138 of whom received azathioprine/mercaptopurine)[149]. Among azathioprine/mercaptopurine-treated patients, the incidence of abnormal liver tests [liver tests between N (upper limit of the normal range) and 2 N] and hepatotoxicity (liver tests > 2 N) was, respectively, 7.1% and 2.6% per patient-year. In most patients, liver tests spontaneously normalized despite maintaining thiopurine treatment. These drugs were withdrawn due to hepatotoxicity (liver tests > 5 N, and lack of decrease despite 50% dose reduction) in only 3.6% of the patients, and all of them showed normalized liver tests.

If liver tests do not return to normal values with tapering of thiopurines, it has been recommended that therapy should be withdrawn. However, if azathioprine was initially prescribed, another possibility is to use mercaptopurine instead. Lopez-Sanroman *et al*[150] did so in 4/5 patients, and achieved complete resolution of liver test alterations in all patients. This finding is consistent with another smaller study in which seven out of eight patients with hepatotoxicity during azathioprine treatment tolerated mercaptopurine, and only one patient had hepatotoxicity again with mercaptopurine[151]. In this same way, in the study by Hindorf *et al*[111] 71% of patients with hepatotoxicity during azathioprine treatment subsequently tolerated mercaptopurine and only two of the patients had a recurrence of hepatotoxicity with mercaptopurine. Finally, Bermejo *et al*[152] have assessed tolerance to mercaptopurine in 31 patients with previous azathioprine-related liver injury; in 87% of patients, mercaptopurine was tolerated without further liver injury; and among these, 77% tolerated full mercaptopurine doses.

Nevertheless, it should be noted that in unusual cases, thiopurines may induce severe cholestatic jaundice that, in contrast to acute hepatocellular hepatitis that is generally associated with azathioprine/mercaptopurine, may not regress but even progress despite thiopurine withdrawal[153]. Therefore, these drugs should be completely withdrawn, and not only tapered, in patients who present with clinically significant jaundice during thiopu-

rine treatment[144].

In summary, most of the cases of thiopurine-induced hepatotoxicity in IBD patients are mild, and liver test abnormalities spontaneously returned to normal values despite maintenance of azathioprine/mercaptopurine; therapy withdrawal is necessary in < 5% of patients. However, when liver test abnormalities are more marked, the dose of azathioprine/mercaptopurine may be reduced by 50%. Finally, administration of mercaptopurine is a good alternative in patients with azathioprine-related liver injury before thiopurines are definitely withdrawn.

CONCLUSION

Misconceptions are common in medical practice in general and, in particular, in the health care of IBD patients. Many of these misconceptions are related to the use of 5-ASAs and thiopurines, the two most widely used drugs in IBD. A proportion of medical errors directly affects patient safety and causes accidental deaths, but the vast majority of them are effectiveness errors. However, we must not focus all our attention on prevention of safety errors while forgetting effectiveness ones. Prevention of errors needs knowledge to avoid errors being caused by ignorance. In fact, throughout history the main reason for medical errors has simply been ignorance[8]. However, at present, the amount of knowledge has increased so quickly that one new danger is overabundance of information. IBD is a model of a very complex problem, and our goal with this review is to summarize the key evidence for the most common daily clinical problems faced by physicians and patients.

With regard to the use of 5-ASAs, the best practice may to be consider abandoning the use of these drugs in patients with small bowel Crohn' s disease. The combined approach with oral plus topical 5-ASAs should be the first-line therapy in patients with active ulcerative colitis, because this is more effective than monotherapy; once-daily treatment should be offered as a first-choice regimen due to its better compliance and higher efficacy. With regard to thiopurine therapy, it seems to be as effective in ulcerative colitis as in Crohn' s disease. Underdosing with thiopurines is a form of undertreatment with these drugs. Thiopurine treatment should probably be continued indefinitely because its withdrawal is associated with a high risk of relapse. Mercaptopurine is a safe alternative in patients with digestive intolerance or hepatotoxicity due to azathioprine. Finally, TPMT screening cannot substitute for regular monitoring because the majority of cases of myelotoxicity are not TPMT-related.

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ORIGINAL ARTICLE

How albumin administration for cirrhosis impacts on hospital albumin consumption and expenditure

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Abstract

AIM: To assess the impact of guidelines for albumin prescription in an academic hospital, which is a referral center for liver diseases.

METHODS: Although randomized trials and guidelines support albumin administration for some complications of cirrhosis, the high cost of albumin greatly limits its use in clinical practice. In 2003, a multidisciplinary panel at Sant'Orsola-Malpighi University Hospital (Bologna, Italy) used a literature-based consensus method to list all the acute and chronic conditions for which albumin is indicated as first- or second-line treatment. Indications in hepatology included prevention of post-paracentesis circulatory dysfunction and renal failure induced by spontaneous bacterial peritonitis, and treatment of hepatorenal syndrome and refractory ascites. Although still debated, albumin administration in refractory ascites is accepted by the Italian health care system. We analyzed albumin prescription and related costs before and after implementation of the new guidelines.

RESULTS: While albumin consumption and costs doubled from 1998 to 2002, they dropped 20% after 2003, and remained stable for the following 6 years. Complications of cirrhosis, namely refractory ascites and paracentesis, represented the predominant indications, followed by major surgery, shock, enteric diseases, and plasmapheresis. Albumin consumption increased significantly after guideline implementation in the liver units, whereas it declined elsewhere in the hospital. Lastly, extra-protocol albumin prescription was estimated as < 10%.

CONCLUSION: Albumin administration in cirrhosis according to international guidelines does not increase total hospital albumin consumption if its use in settings without evidence of efficacy is avoided.

Key words: Human serum albumin; Cost analysis; Liver cirrhosis; Critical illness; Ascites

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INTRODUCTION

Serum albumin represents approximately 50% of the total

plasma protein content in healthy individuals, and generates about 70% of plasma oncotic pressure. The fairly elevated albumin concentration in extracellular fluids and its strong negative charge, which attract sodium, make albumin the main modulator of fluid distribution throughout body compartments. In addition, albumin carries many other biological properties, with implications for drug metabolism, free radical detoxification, inflammatory response, vascular integrity, and coagulation[1,2].

Human albumin is widely employed in clinical practice, but its administration is often inappropriate. This is largely due to a common belief in its efficacy, whereas many indications are still under debate or have been disproved by evidence-based medicine. Indeed, the high cost, the theoretical risk of viral disease transmission, and the availability of cheaper alternatives should be carefully weighed in a cost/effectiveness analysis of albumin prescription. Thus, it is not surprising that several clinical and economic studies have been performed to establish recommendations and guidelines for albumin prescription, but controversy persists[3-9].

At present, it is generally accepted that the administration of non-protein colloids and crystalloids represents the first-line treatment of resuscitation, while the use of albumin in critically ill patients should be reserved for specific conditions[10-15]. Albumin administration is not recommended to correct hypoalbuminemia *per se* (i.e. not associated with hypovolemia) or for nutritional intervention[6,7,9,16], but this is often disregarded in clinical practice. Albumin is also prescribed in certain conditions and diseases, such as kernicterus, plasmapheresis, and graft-*vs*-host disease[9], even though these indications are not supported by definite evidence.

Hepatology is a setting where the use of albumin is particularly common, since this treatment is currently employed to treat or prevent severe complications of cirrhosis. Indeed, randomized studies have shown that it is effective in preventing circulatory dysfunction after large-volume paracentesis[17] and renal failure induced by spontaneous bacterial peritonitis[18], and to treat hepatorenal syndrome in association with vasoconstrictors[19-21]. Although the recommendations on the use of albumin in cirrhosis have been endorsed by the International Ascites Club and other international scientific societies[22-26], albumin is not widely administered in clinical practice, even in specialized centers, mainly because of its high cost.

With the aim of rationalizing albumin prescription and reducing health care costs, clinical practice guidelines were devised in 2003, and subsequently implemented at the S Orsola-Malpighi University Hospital in Bologna, Italy, a third-level referral centre for many diseases, including liver cirrhosis and transplantation.

We here report the annual albumin consumption and costs comparing the 5 years before (January 1998-June 2003) with the 6 years after (July 2003-December 2008) implementation of the guidelines. We also analyze the indications for albumin, adherence to the protocol, and the distribution of albumin prescription among specialties in 2008.

MATERIALS AND METHODS

Protocol drafting

In the first half of 2003, clinical practice guidelines for the appropriate prescription of albumin were devised at the Sant'Orsola-Malpighi University Hospital of Bologna, Italy, using a systematic, literature-based consensus method. Briefly, a panel of experts from various disciplines (internal medicine, anesthesia, surgery, gastroenterology, nephrology, hematology, public health, and pharmacy) reviewed the available clinical literature and drew up draft guidelines which were submitted to a second panel of physicians from the same scientific areas, but not involved in the writing of the first draft. Then a consensus was reached by the two working groups and a final version was approved and distributed among the physicians employed at the hospital. Since July 2003, the in-hospital prescription of albumin has been regulated according to the recommendations reported in Table 1. Schematically, they list a series of acute and chronic clinical conditions for which albumin administration is indicated as a first- or second-line treatment or is not indicated at all. The level of scientific evidence and the strength of the recommendation are also reported according to the criteria summarized in Table 2. The guidelines were updated in 2007, but only minor changes were made regarding specific and limited indications.

Table 1 Levels of evidence and strength of recommendation

Levels of evidence	
1	Randomized clinical trials and/or meta-analyses
2	Single randomized clinical trail
3	Prospective observational studies
4	Retrospective studies
5	Cross-sectional surveys and descriptive studies
6	Opinion of experts in guidelines or consensus
Strength of recommendations	
A	Strong (levels of evidence 1 and 2)
B	Relatively strong (levels of evidence 3, 4 and 5)
C	Weak (level of evidence 6)

Data collection

Since the protocol was implemented, each order of albumin has been filled in by the prescribing physician using a specific form listing the amount requested, the diagnosis and the indication for albumin use, and the unit of the prescribing physician. All the data regarding in-hospital use of albumin are collected in a database at the hospital pharmacy service and a quarterly report on albumin prescription is sent by mail to all physicians.

Statistical analysis

Trends of albumin consumption before and after implementation of the clinical practice guidelines were analyzed using the Pearson correlation test. $P < 0.05$ were considered statistically significant. Data were analyzed using Graph PAD 4.0 software.

RESULTS

Consumption data

Albumin consumption and costs were monitored at the S Orsola-Malpighi University Hospital from 1998. The number of albumin vials (50 mL containing 10 g albumin,

Table 2 Practical recommendations for the prescription of albumin at the S Orsola-Malpighi University Hospital, Bologna, Italy

Acute diseases	First-line treatment	Second-line treatment
Hypovolemic shock [1, A]	Colloid/crystalloid solutions	Human albumin if: Sodium intake restriction Hypersensitivity to colloids or crystalloids Lack of response to combined use of colloids and crystalloids
Major surgery [6, C]		
(1) Cardiovascular surgery	Colloid/crystalloid solutions	Human albumin if: Lack of response to combined use of colloids and crystalloids
(2) Other surgery	As for hypovolemic shock	As for hypovolemic shock
Burns [6, C]	Colloid/crystalloid solutions	Human albumin plus crystalloid solutions if: Lack of response to colloid or crystalloid solutions alone Severe burns (> 50% body surface)
Chronic diseases	First-line treatment	Second-line treatment
Cirrhosis	Human albumin	
(1) Paracentesis [1, A]	8 g/L of removed ascites if paracentesis > 4 L	
(2) Spontaneous bacterial peritonitis [1, A]	1.5 g/kg at diagnosis and 1 g/kg on third day + antibiotic therapy	
(3) Hepatorenal syndrome [1, A]	1 g/kg at diagnosis followed by 20-40 g/d + vasoconstrictors	
(4) Ascites [1, A]	Diuretic treatment	Human albumin if: Ascites resistant to diuretics
Plasmapheresis [6, C]	Human albumin if plasma changes > 20 mL/kg per week	
Protein wasting enteropathy/ malnutrition	Enteral or parenteral nutrition	Human albumin only if: severe diarrhea (> 2 L/d) albuminemia < 2 g/dL clinical hypovolemia

Albumin is not recommended for cerebral ischemia (3, B), wound-healing (6, C), chronic pancreatitis (6, C), nephritic syndrome (6, C). Albumin is also provided for the following specific procedures: pulmonary endarterectomy, molecular adsorbent recirculating system (MARS), immunomagnetic selection system (CLINIMACS).

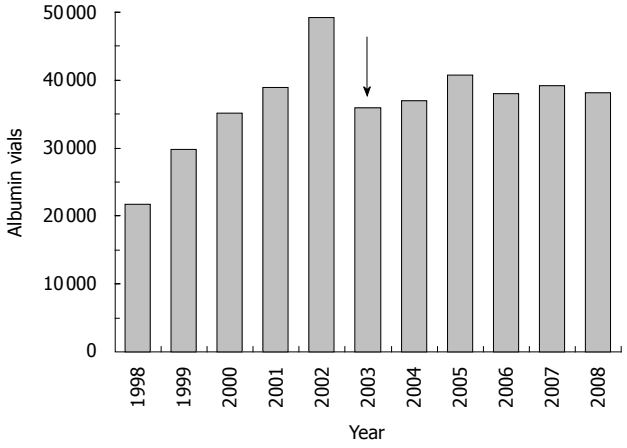

Figure 1 Annual consumption of albumin vials (50 mL, 20%) at the S Orsola-Malpighi University Hospital, Bologna, Italy. The black arrow indicates when the practical guidelines for in-hospital albumin prescription were implemented in July 2003.

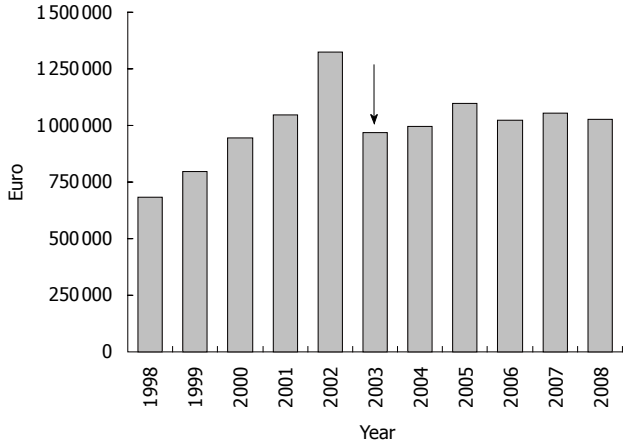

Figure 2 Global annual cost of albumin use at the S Orsola-Malpighi University Hospital, Bologna, Italy. The black arrow indicates when the practical guidelines for in-hospital albumin prescription were implemented in July 2003.

concentration 20%) and the related expenditure more than doubled from 1998 to 2002 (Figures 1, 2). The implementation of recommendations in July 2003 yielded a rapid 15%-20% reduction in albumin use (Figure 1), which was associated with a similar fall in albumin cost both expressed in absolute terms (Figure 2) or as percentage of the global pharmaceutical expenditure (Figure 3). Thereafter, albumin consumption and related costs remained substantially stable during the following 6 years (Figures 1-3). The data for the 2003 represent the sum of two periods with different prescription modalities (before and after guidelines implementation). The cost of each albumin vial paid for by our hospital remained stable throughout the study period.

Finally, as shown in Figure 4, the trend analysis of albumin consumption clearly indicates that its time-dependent increase was interrupted by the implementation of the recommendations, supporting their efficacy in regulating in-hospital albumin prescription.

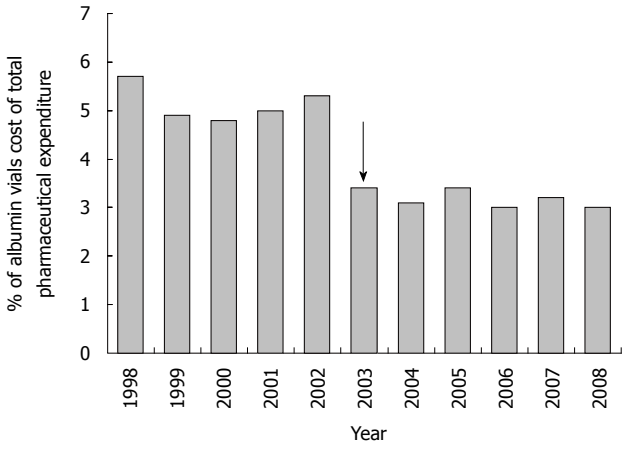

Figure 3 Annual cost of albumin use at the S Orsola-Malpighi University Hospital, Bologna, Italy, expressed as a percentage of the total pharmaceutical expenditure. The black arrow indicates when the practical guidelines for in-hospital albumin prescription were implemented in July 2003.

Prescription analysis

As the annual breakdown of albumin prescriptions among the major indications did not change significantly in the period 2003-2008, we present only the prescription analysis performed on the data for 2008 (Figure 4). Complications of cirrhosis represent the predominant indication for albumin use, accounting for 52% of vials utilized and 36% of patients treated. Major surgery was the second commonest indication for albumin use. Taking into account that liver transplantation and hepatic resection constitute approximately 30% of these surgical cases, it is evident that liver diseases represent the setting where the majority of albumin was prescribed. Other relatively common indications were shock not responsive to crystalloid/colloids in intensive care units (ICUs), bowel diseases associated with malnutrition, and plasmapheresis (Table 3).

The mean number of albumin vials per patient and the related cost were higher in patients with cirrhosis than in those presenting other indications, with the two expected exceptions being plasmapheresis and albumin dialysis with the molecular adsorbent recirculating system (MARS) (Table 3). Finally, the use of albumin outside protocol indications occurred only in about 10% of cases and mainly in patients with edematous and/or anasarcatic states (Table 3).

Ascites not responding to diuretic treatment represented the major indication for albumin prescription in patients with liver cirrhosis, and the high number of albumin vials per patient likely reflected the prolonged length of treatment. Repeated procedures also resulted in increased albumin consumption in patients after large-volume paracentesis to prevent post-paracentesis circulatory dysfunction (Table 4).

Although it appears that hypoalbuminemia was the major indication before guideline implementation (data not shown), we could not perform an accurate comparison between the two 5-year periods of prescription since the data were not systematically collected before 2003.

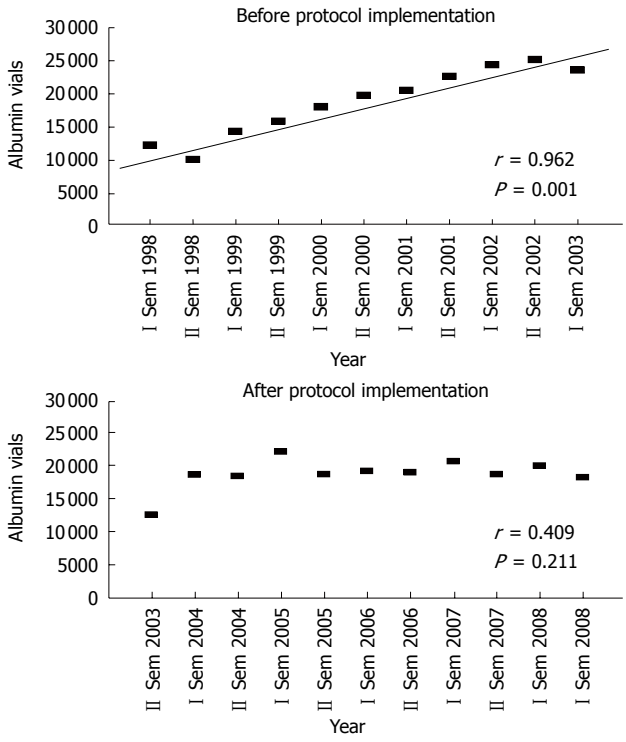

Figure 4 Top panel. Trend in albumin consumption at the S Orsola-Malpighi University Hospital, Bologna, Italy before implementation of the practical guidelines for albumin prescription. Albumin consumption is expressed per semester from January 1, 1998 to June 30, 2003. Data are analyzed using the Pearson correlation. Bottom panel. Trend in albumin consumption at the S. Orsola-Malpighi University Hospital, Bologna, Italy after implementation of the practical guidelines for albumin prescription. Albumin consumption is expressed per semester from July 1, 2003 to December 31, 2008. Data are analyzed using the Pearson correlation.

In an attempt to overcome this limitation of the study, we analyzed albumin consumption after grouping all the units into three main categories: hepatological medical and surgical units, i.e. units representing referral centers for liver diseases, non-hepatological medical and surgical units, and ICUs. While the proportion of albumin consumption remained stable over the years in the ICU, the increase observed in the "hepatological" units after guideline implementation was mirrored by a parallel decrease in the "non-hepatological" units (Figure 5).

DISCUSSION

Albumin utilization remains highly controversial in a variety of clinical settings in terms of indications, efficacy, and cost-benefit ratio. Over the last 10-15 years, specific conditions for which albumin administration is indicated have been defined, and liver disease possibly represents a field where this has been most clearly and convincingly demonstrated. However, besides inappropriate prescribing, the high cost of albumin infusion is the main issue leading health authorities and hospital administrators to restrict its use, and this has often prevented the widespread application of indications emerging from international guidelines in hepatology.

Table 3 Distribution of albumin consumption and cost among clinical indications in 2008

	Vials (number)(%)	Cost (euros)	Patients (number)(%)	Vials/patients (number)	Cost/patient (euros)
Cirrhosis	19 871 (52)	534.532	807 (36.3)	24.6	662
Major surgery	6196 (16.2)	166.982	495 (22.2)	12.5	337
Shock	5069 (13.3)	136.558	447 (20)	11.3	305
Enteric disease	2982 (7.8)	80.215	146 (6.5)	20.4	549
Plasmapheresis	2333 (6.1)	62.757	54 (2.4)	43.2	1162
Mars	196 (0.5)	5.272	6 (0.2)	32.6	878
Others[1]	201 (0.5)	5353.000	149 (6.7)	7.1	260
Extra-protocol	1240 (3.7)	33.418	119 (5.3)	10.5	281

[1]Others: Clinimacs system, neonatal jaundice, pediatric cardio-pulmonary bypass.

Table 4 Distribution of albumin consumption and cost among the clinical indications for cirrhosis in 2008

	Vials (number)(%)	Cost (euros)	Patients (number)(%)	Vials/patients (number)	Cost/patient (euros)
Ascites	12.540 (63.3)	337.976	453 (56.4)	27.7	746
Paracentesis	4.564 (23.1)	122.988	222 (27.6)	20.6	554
Hepatorenal syndrome	2.340 (11.8)	63.070	106 (13.2)	22.0	595
Spontaneous bacterial peritonitis	367.000 (1.8)	9.880	22 (2.7)	14.0	380

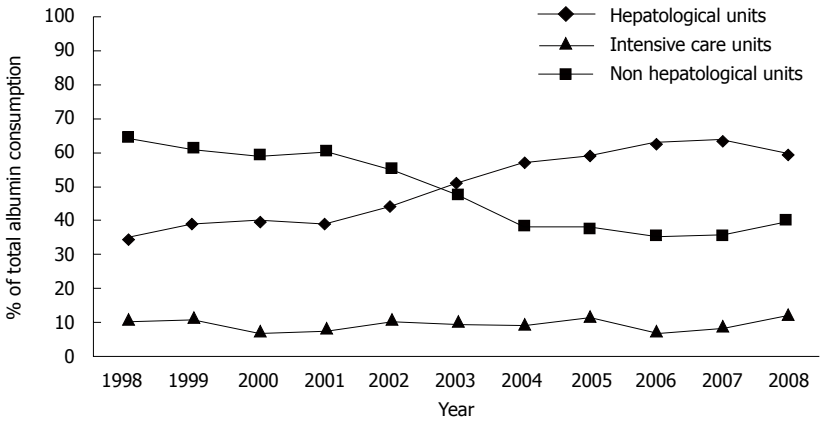

Figure 5 Distribution of the annual consumption of albumin vials (50 mL, 20%) among various Units of the S Orsola-Malpighi University Hospital, Bologna, Italy, grouped into three main categories: hepatological medical and surgical units, i.e. units representing a referral centre for liver diseases, non-hepatological medical and surgical units, and intensive care units. The black arrow indicates when the practical guidelines for in-hospital albumin prescription were implemented in July 2003.

The present study clearly shows that in a general hospital the recommendations for the use of albumin in patients with cirrhosis can be followed without increasing the global pharmaceutical costs, despite the large amounts of albumin required. This can be achieved by implementing rational albumin prescription guidelines, thereby avoiding its futile administration in settings where there is a lack of clinical evidence of efficacy. The clinical practice guidelines devised by our hospital to regulate albumin prescription used a literature-based consensus method and adopted all the recognized indications for albumin administration in hepatology, such as prevention of post-paracentesis circulatory dysfunction, prevention of renal impairment in patients with spontaneous bacterial peritonitis, and treatment of hepatorenal syndrome in association with vasoconstrictors. These guidelines managed to curb the incremental trend of albumin consumption and related costs recorded in previous years. In our view, these results are particularly important given the characteristics of our hospital, that acts both as an academic third-level and primary referral centre, hosts a program for liver transplantation, and where several medical and surgical units are devoted to the management of chronic liver diseases. Not surprisingly, the prevention or treatment of complications of cirrhosis was the commonest reason for prescribing albumin in our institution, and albumin use in this setting increased after our in-hospital clinical practice guidelines were implemented.

Refractory ascites was the most common specific indication for albumin infusion, and the high number of vials given to each patient likely reflects the need for sustained albumin administration in these cases. This finding merits some comment as the use of albumin to treat cirrhotic ascites is controversial with a lack of clear evidence in its

favor. Despite this debate, the Italian Drug Agency (AIFA) permits albumin reimbursement by the National Health Service in patients with refractory ascites based on the results of two Italian studies[27,28]. First, the Albumin Delphi Study, designed to gain a consensus among Italian physicians, showed that 80% of hepatologists agree that albumin treatment shortens the length of hospitalization, enhances the response to diuretics, lowers the relapse rate of ascites when given at home, and also improves patient general condition and well-being[26]. Second, a controlled clinical trial in 126 patients with decompensated cirrhosis reported that treatment with diuretics plus albumin favored the disappearance of ascites, shortened the hospital stay, and reduced the rate of ascites recurrence and readmission to hospital due to ascites compared with diuretics alone[27]. More recently, the same research group showed that long-term albumin administration increased patient survival and reduced the risk of ascites recurrence in patients with first-onset ascites followed for a median of 7 years[28]. No other controlled clinical trials have so far been performed to evaluate the effectiveness of prolonged albumin administration in the treatment of cirrhotic ascites. Thus, the lack of confirmatory multicenter randomized studies together with the high cost of albumin infusion explains why albumin is not usually included among the therapeutic options for difficult-to-treat ascites in countries other than Italy. For instance, the most recent international guidelines for the management of adult patients with ascites due to cirrhosis[25,29] do not even mention albumin as a possible therapeutic tool for either responsive or refractory ascites.

In the last 15 years, large randomized trials and meta-analyses have focused on the use of human albumin in the setting of critically ill patients, the clinical field where international guidelines for albumin prescription were first established. These studies showed that beside being cheaper, non-protein colloids and crystalloids are equally or even more effective than albumin for fluid resuscitation and blood volume expansion in patients with hypovolemic shock and critical illnesses, representing the first-line treatment in these cases. Thus, albumin should only be administered in the presence of contraindications to the use of colloids and/or crystalloids or specific conditions, such as the need for salt intake restriction[10-15]. Interestingly, the implementation of our in-hospital guidelines has not produced significant changes in albumin prescription by physicians working in ICUs, probably because they were already accustomed to established international guidelines, despite the ongoing dispute on the matter.

As global albumin consumption at our hospital declined by 15%-20%, the greatest saving occurred in non-hepatological medical and surgical units where hypoalbuminemia *per se* was probably the main reason for prescribing albumin before implementation of our in-hospital guidelines, despite the lack of scientific evidence supporting albumin administration to correct plasma albumin concentration and/or improve nutritional status[6,7,9,16].

Our hospital guidelines, like other local recommendations[3-9], also authorize albumin prescribing for specific procedures and clinical situations. These niche indications differ among guidelines and reflect the particular settings of highly specialized centers, such as university hospitals. Although these indications only affect a small number of patients, the albumin consumption per patient can be very high, as in the MARS procedure[30], or the treatment of orphan diseases, such as intestinal lymphangiectasia.

Several aspects of our study must be addressed for a correct evaluation of the results. The recommendations for albumin prescription in our hospital were devised using a systematic, literature-based consensus method, but not all of them were supported by solid scientific evidence derived from large randomized clinical trials, meta-analyses of trials or universally accepted guidelines. Despite this limitation, implementation of the protocol clearly interrupted the incremental trend in albumin consumption and related expenditure seen in previous years. Of course, most physicians must strictly adhere to the guidelines in order to control and, possibly, curb albumin consumption. Several *ad hoc* strategies were adopted: (1) involvement of experts from many disciplines in drafting the consensus document; (2) use of an albumin order form restating the recommendations at the time of prescription, a measure known to enhance compliance[31]; and (3) regular information sent to every physician in a quarterly report on in-hospital albumin prescription. Such a policy achieved a formal adherence to the protocol in approximately 85% of prescriptions. This figure, however, relied on the assumption that data reported on the order form correctly describe the patient's clinical status, because this concordance was not monitored on a regular basis. Finally, our study cannot provide any information on the impact of the recommendations on the patients' clinical outcomes and global hospital costs. This was beyond the scope of the present analysis and can only be determined by prospective studies designed to assess the cost/benefit ratio of albumin treatment for each specific disease.

In conclusion, this study indicates that prescribing albumin according to the current guidelines in hepatology does not increase total albumin consumption or costs in a hospital acting as both an academic third-level and primary referral center. This result can be achieved provided that albumin prescription is strictly regulated by practical recommendations designed to avoid ineffective albumin infusion in settings without scientific evidence of efficacy. The present data may promote the appropriateness of albumin prescription, particularly in clinical settings where the use of albumin is dogmatically limited rather than regulated on the basis of current scientific evidence. In this sense, our results may foster the acceptance of internationally endorsed indications for the use of albumin in hepatology by health authorities and hospital administrations.

COMMENTS

Background

Human albumin is widely employed in clinical practice, but its administration is often inappropriate. This is largely due to a common belief in its efficacy, whereas

many indications are still under debate or have been disproved by evidence-based medicine. In the field of hepatology, albumin is currently used to treat or prevent severe complications of cirrhosis. Although the recommendations on the use of albumin in cirrhosis have been endorsed by the International Ascites Club and other international scientific societies, albumin is not widely administered in clinical practice, even in specialized centers, mainly because of its high cost.

Research frontiers

As albumin utilization remains highly controversial in a variety of clinical settings in terms of indications, efficacy, and cost-benefit ratio, the implementation of practical recommendations and guidelines, based on solid scientific evidence, appears to be a necessary step to rationalize the use of this expensive hemoderivate.

Innovations and breakthroughs

The present study clearly showed that in an academic general hospital, the adherence to the international scientific guidelines for the use of albumin in patients with cirrhosis does not increase the global pharmaceutical costs, despite the large amounts of albumin required. This can be achieved by implementing rational albumin prescription guidelines, thereby avoiding its futile administration in settings that lack scientific evidence of efficacy.

Applications

The present data may promote the appropriateness of albumin prescription, particularly in clinical settings where the use of albumin is limited rather than regulated on the basis of current scientific evidence. The results may foster the acceptance of internationally endorsed indications for the use of albumin in hepatology by health authorities and hospital administrations. However, this study suffers an important limitation as the effects of the recommendations on the patients' clinical outcomes and global hospital costs could not be determined in the present investigation and only prospective studies specifically designed for this purpose will be able to provide the real cost-effectiveness of treatment for each specific disease.

Peer review

This study shed more light on the continued dilemma of consumption and costs of albumin administration particularly in patients with liver disease.

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ORIGINAL ARTICLE

Integrin-linked kinase in gastric cancer cell attachment, invasion and tumor growth

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Abstract

AIM: To investigate the effects of integrin-linked kinase (ILK) on gastric cancer cells both *in vitro* and *in vivo*.

METHODS: ILK small interfering RNA (siRNA) was transfected into human gastric cancer BGC-823 cells and ILK expression was monitored by real-time quantitative polymerase chain reaction, Western blotting analysis and immunocytochemistry. Cell attachment, proliferation, invasion, microfilament dynamics and the secretion of vascular endothelial growth factor (VEGF) were also measured. Gastric cancer cells treated with ILK siRNA were subcutaneously transplanted into nude mice and tumor growth was assessed.

RESULTS: Both ILK mRNA and protein levels were significantly down-regulated by ILK siRNA in human gastric cancer cells. This significantly inhibited cell attachment, proliferation and invasion. The knockdown of ILK also disturbed F-actin assembly and reduced VEGF secretion in conditioned medium by 40% ($P < 0.05$). Four weeks after injection of ILK siRNA-transfected gastric cancer cells into nude mice, tumor volume and weight were significantly reduced compared with that of tumors induced by cells treated with non-silencing siRNA or by untreated cells ($P < 0.05$).

CONCLUSION: Targeting ILK with siRNA suppresses the growth of gastric cancer cells both *in vitro* and *in vivo*. ILK plays an important role in gastric cancer progression.

Key words: Gastric cancer; Integrin-linked kinase; Small interfering RNA; Cell attachment; Cell proliferation; Cell invasion; Cell microfilament dynamics; Vascular endothelial growth factor; Nude mice

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INTRODUCTION

Gastric cancer is one of the most commonly diagnosed malignant tumors, and is also one of the most frequent causes of cancer mortality worldwide[1]. Its incidence is highest in Japan, China, Eastern Europe and Latin America. In 2002, 934 000 new cases were diagnosed, making it the fourth most common cancer, causing ap-

proximately 700 000 deaths[2]. The 5-year survival rate of gastric cancer is poor, being approximately 20%, even in patients treated with surgical resection, chemotherapy, radiotherapy and other approaches. However, in Japan, according to a systematic screening program, this figure reached 60%[3-5]. Therefore, an understanding of the molecular mechanisms involved in gastric cancer formation and progression, and the identification of specific targets for gene therapy should be helpful in developing more effective strategies. Integrin-linked kinase (ILK) is an ankyrin repeat-containing serine/threonine protein kinase[6] that interacts with the cytoplasmic domain of β1 and β3 integrins[7] and regulates integrin dependent functions. It mediates a diversity of cell functions by coupling integrins and growth factors to cascades of downstream signaling events. ILK is a downstream substrate of phosphoinositide 3-kinase, and is an important upstream kinase for the regulation of protein kinase B (PKB/Akt) and glycogen synthase kinase 3 (GSK-3)[8,9]. ILK is now recognized to play an important role in linking extracellular signaling to the regulation of survival, cell cycle progression, migration, and invasion. The expression and activity of ILK are increased in a range of tumors, and small-molecule inhibitors of ILK activity have been identified and shown to inhibit tumor growth, invasion and angiogenesis[10-12], although certain tumors have decreased or no ILK expression. In gastric cancer, there is no ILK expression in non-neoplastic gastric epithelia, while the number of cells expressing ILK increased to 69% in neoplastic gastric epithelia, which was associated with tumor cell invasion and nodal metastasis[13]. To further investigate the role of ILK in gastric cancer progression and to determine if ILK can be used as a therapeutic target, we specifically knocked down ILK expression using small interfering RNA (siRNA) in gastric cancer cell line, BGC-823. We also analyzed these cancer cells' spontaneous attachment, proliferation, invasion and cell morphology *in vitro* and their tumor growth *in vivo*.

MATERIALS AND METHODS

Cell culture and reagents

Human gastric cancer cell line, BGC-823, was obtained from the Institute of Geriatrics, Ministry of Health (Beijing, China), and was cultured in RPMI-1640 medium (Gibco BRL, Grand Island, United States) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Gibco) in a 5% CO_2 humidified atmosphere at 37 ℃. HiPerFect Transfection Reagent was purchased from Qiagen (Hilden, Germany). A First-Strand cDNA Synthesis kit and SYBR-green real-time polymerase chain reaction (PCR) Mastermix were purchased from Toyobo (Osaka, Japan). For Western blotting analysis, an anti-ILK antibody, purchased from Abcam (Cambridge, United Kingdom) was used at a dilution of 1:2000, and an HRP-goat anti-rabbit secondary antibody obtained from Rockland Immunochemicals (Gilbertsville, PA, United States) was used at a dilution of 1:3000. For immunocytochemistry, the ILK antibody was used at a dilution of 1:1000, and a goat anti-rabbit TRITC conjugated antibody was used at a dilution of 1:400. Anti-β-actin-HRP antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, United States), and SuperSignal West Pico Chemiluminescent Substrate was purchased from Pierce (Rockford, IL, United States). Matrigel was obtained from BD Biosciences (Bedford, MA, United States), while Alexa Fluor 488 phalloidin was purchased from Molecular Probes (Invitrogen, Carlsbad, CA, United States). All other reagents were purchased from Sigma (St. Louis, MO, United States).

siRNA and transfection assays

The ILK-specific (GenBank accession No. NM004517) siRNAs (ILK siRNA, forward: 5'-GAAUCUCAACCGUAUUCCATT-3'; reverse: 5'-UGGAAUACGGUUGAGAUUCTG-3') were chemically synthesized by Qiagen. BGC-823 cells were transfected with siRNA using HiPer-Fect Transfection Reagent according to the manufacturer's instructions. Briefly, the original stock of siRNA was suspended in siRNA suspension buffer provided by the manufacturer. The resulting suspension was aliquoted in a required amount for each experiment and stored at -20 ℃ until use. On the day of transfection, cells were seeded in plates at the recommended density according to the manufacturer's instructions. The siRNA was then gently introduced onto the cells by mixing with the required amount of HiPer-Fect Transfection Reagent as recommended by the manufacturer. In our study, the final concentration of siRNA was 10 nmol/L. Non-silencing siRNA (NS siRNA, forward: 5'-UUCUCCGAACGUGUCACGUTT-3'; reverse: 5'-ACGUGACACGUUCGGAGAATT-3')-treated cells were used to control any effects of the transfection reagent and the non-specific siRNA effects. The *in vitro* assays described here were performed 48 h after transfection. Chemically modified siRNAs used in animal models were synthesized by Qiagen according to the sequences described above.

Real-time quantitative RT-PCR

Isolation of total RNA was performed using Trizol solution according to the manufacturer's protocol. Reverse transcription was then performed using 100 ng RNA and the First-Strand cDNA Synthesis kit. Real-time quantitative PCR analysis was performed with the DNA Engine Opticon 2 System (Bio-Rad, Richmond, CA, United States) using the SYBR® green Real-time PCR Mastermix. We used the following primers: for ILK, forward 5'-TTTGCAGTGCTTCTGTGGGAA-3' and reverse 5'-CTACTTGTCCTGCATCTTCTC-3'; for GAPDH, forward 5'-GAAGGTGAAGGTCGGAGTC-3' and reverse 5'-GAAGATGGTGATGGGATTTC-3'. After initial denaturation at 95 ℃ for 3 min, reactions were cycled 40 times. Each cycle consisted of denaturation at 95 ℃ for 15 s, primer annealing at 60 ℃ for 15 s and primer extension at 72 ℃ for 45 s[14,15]. Results were collected and analyzed using MJ Opticon Monitor Analysis software (Bio-Rad). The quantity data of mRNA input was

controlled by measuring the reference gene, GAPDH. Experiments were performed in triplicate and repeated three times.

Western blotting analysis

Cells were washed with ice-cold phosphate buffered saline (PBS), and whole-cell extracts were prepared using cell lysis buffer [20 mmol/L Tris (pH 7.5), 0.1% Triton X, 0.5% deoxycholate, 1 mmol/L phenylmethylsulfonyl fluoride, 10 μg/mL aprotinin and 10 μg/mL leupeptin] and cleared by centrifugation at 12000 × *g* at 4 ℃. Total protein concentration was measured using the bicinchoninic acid assay with bovine serum albumin (BSA) as a standard. Equal amounts of protein were loaded and analyzed by immunoblotting. Enhanced chemiluminescence detection was performed in accordance with the manufacturer's instructions[16,17]. The ILK signal was quantified using BandScan software version 5.1 (Glyko, Novato, Calif., United States) and normalized to that of β-actin. Experiments were performed in triplicate and repeated 3 times.

Immunocytochemistry

Immunocytochemical assays were performed as previously described[18]. Briefly, cells were grown on fibronectin coated coverslips, washed in PBS, and fixed for 15 min in 4% paraformaldehyde. Cell monolayers were permeabilized in 0.1% Triton X-100, washed, and blocked in 10% normal goat serum. Cells were incubated with the anti-ILK antibody overnight at 4 ℃. Cells were then washed and incubated with fluorescently labeled secondary antibodies for 1 h at room temperature in the dark. Cells were washed and coverslips were mounted using Kaiser's glycerin gelatin (Merck, Darmstadt, Germany). Fluorescence signals were visualized and acquired using an epifluorescence microscope (Leica, Heidelberg, Germany) with appropriate excitation and emission filters under 40 × magnification. Pictures of observed fields were recorded digitally. Experiments were performed in triplicate and repeated three times.

Cell attachment assay

Plates of 96 wells were coated with 1.25 mg/mL fibronectin in 100 mL PBS overnight at 4 ℃. The plates were blocked with 2.5 mg/mL BSA for 2 h in DMEM at 37 ℃. Transfected cells were trypsinized and 1.5×10^4 cells were seeded in each well for 1 h at 37 ℃. Cells were then washed twice with PBS and the unattached cells were discarded. After the washing step, the number of attached cells was determined by the MTT assay in accordance with the manufacturer's instructions. Absorbance was measured using an enzyme-linked immunosorbent assay (ELISA) plate reader at 570 nm[19]. Experiments were performed in triplicate and repeated three times.

Cell proliferation assay

Cell proliferation was assessed using the MTT assay[20]. Gastric cancer BGC-823 cells were plated at 5×10^3 cells/well in 96-well plates in RPMI-1640 medium containing 10% FBS. After 24 h, the culture medium was replaced by fresh medium containing ILK siRNA or non-silencing siRNA. Six duplicate wells were set up for each group. Untreated cells served as control. After 4, 24, 48 or 72 h of incubation, 20 μL MTT (5 g/L, Sigma) was added to each well and incubation continued for 4 h. Cells were collected by centrifugation at 1000 × *g* for 5 min at room temperature. The reaction was stopped by the addition of 150 μL dimethyl sulfoxide. The absorbance of samples was measured at 570 nm. Each assay was performed in triplicate and repeated three times. Cell proliferation inhibition rate [proliferation inhibition rate = (1-A_{570} experiment group/A_{570} control group) × 100%] was plotted *vs* time.

Cell invasion assay

Polycarbonate membranes (8.0 μm pore size) of the upper compartment of 24-well Transwell culture chambers were coated with 18 μL of 5 mg/mL Matrigel (BD Biosciences) in serum-free medium. Cells (5×10^4) suspended in 250 μL of serum-free medium were applied on the upper compartment, and the lower compartment was filled with 750 μL of DMEM containing 10% fetal bovine serum. After incubation for 24 h, cells were fixed with 10% trichloroacetic acid at 4 ℃ for 1 h. Non-invaded cells on the upper surface of the filter were removed carefully with a cotton swab. Invading cells on the lower side of the filter were stained with 0.5% crystal violet for 2 h and the stained filters were photographed. The crystal violet dye retained on the filters was extracted with 30% acetic acid and cell invasion was measured by reading the absorbance at 590 nm[19]. Each assay was performed in triplicate and repeated three times.

Immunofluorescence analysis of microfilaments

Microfilament organization of RF/6A cells was assessed by a modification of an immunofluorescence protocol using rhodamine-phalloidin[21]. After transfection, cells were trypsinized and seeded onto coverslips for 6 h at 37 ℃ in 5% CO_2. Following this, the medium was aspirated, and adherent cells were fixed with 4% paraformaldehyde in PBS for 20 min. After they had been washed with PBS (pH 7.4) 3 times, cells were permeabilized with 0.1% Triton X-100 for 20 min and blocked with 1% BSA in PBS for 5 min. Cells were then incubated with rhodamine-phalloidin (200 U/mL) for 30 min and diamidinophenylindole (0.1 μg/mL) for 1 min in the dark. PBS was used as the base of all solutions and intervening rinses, and incubations were performed at room temperature. After mounting (Kaiser's glycerin gelatin; Merck, Darmstadt, Germany), slides were examined under an epifluorescence microscope (Leica, Heidelberg, Germany) with appropriate excitation and emission filters under a magnification × 40. Pictures of observed fields were recorded digitally. Experiments were performed in triplicate and repeated 3 times.

Enzyme-linked immunosorbent assay

siRNA-transfected cells were seeded in 6-well plates (3×10^5 cells/well) and incubated at 37 ℃. After 24 h, the cell culture supernatant was harvested, and cell counts were made after trypsinization. After collection, the medium was spun at 800 × *g* for 3 min at 4 ℃ to remove cellular debris[17]. The supernatants were frozen and stored at -80 ℃ until use. The levels of vascular endothelial growth factor (VEGF) were measured in culture medium samples with a VEGF ELISA kit according to the manufacturer's instructions. Experiments were performed in triplicate and repeated 3 times.

Tumor growth in nude mice

An equal number (1×10^7) of BGC-823 cells transfected with ILK siRNA, non-silencing siRNA, or untreated cells was harvested 48 h after transfection, washed twice with 1 × PBS, and resuspended in 0.2 mL of saline. Three groups (each group with 5 mice) of 4-6 wk old male BALB/c nude mice (Institute of Zoology, Chinese Academy of Sciences) were housed in a specific pathogen-free environment at the Animal Laboratory, then given subcutaneous injections with either untransfected cells, cells transfected with non-silencing siRNA, or cells transfected with ILK siRNA. The mice were monitored every 3 d for tumor formation. The date at which a palpable tumor first arose and the volume of the tumor ($V = L \times W^2 \times \pi/6$) were recorded[22]. At week 4 after injection of the cells, the mice were killed and the weights of tumors were recorded. The animal experiments performed on nude mice were approved by the Animal Ethics Committee of Peking University.

Statistical evaluation

Statistical analysis was performed using SPSS software (SPSS V 14.0; SPSS). All results were expressed as mean ± SD. To determine the significance of differences, ANOVA was performed. Differences with $P < 0.05$ were considered statistically significant.

RESULTS

SiRNA down-regulation of ILK expression in gastric cancer cells

Expression of ILK was significantly suppressed in gastric cancer BGC-823 cells transfected with ILK siRNA. The suppression of ILK occurred within 24 h after transfection and lasted a week. ILK siRNA caused a reduction of ILK mRNA of more than 85% after 48 h (88.2% ± 9.3% inhibition, $P < 0.01$) (Figure 1A), and a reduction of ILK protein of more than 85% after 48 h (86.8% ± 8.2% inhibition, $P < 0.01$) (Figure 1B) compared with non-silencing siRNA. Down-regulation of ILK expression in BGC-823 cells was further confirmed by immunocytochemistry (Figure 2): the fluorescence intensity representing the expression of ILK in untreated or non-silencing siRNA-treated cells was very strong, but that in ILK siRNA-transfected cells was barely detectable. Control cells also showed a higher degree of cell spreading when compared with the ILK-knockdown cells.

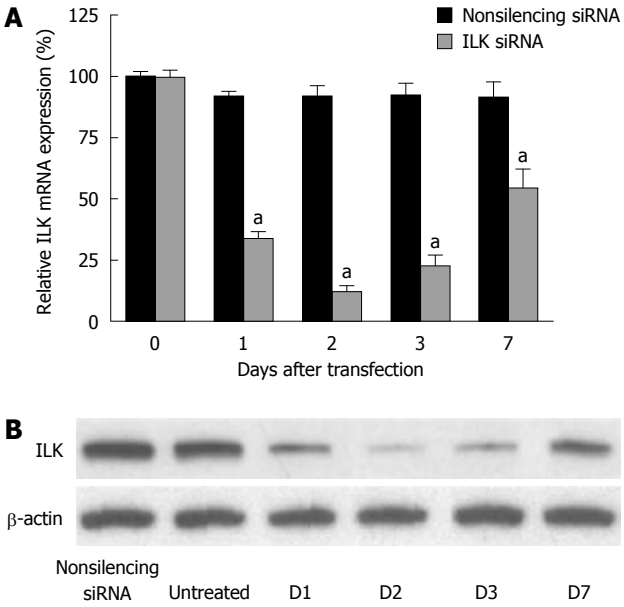

Figure 1 Effects of siRNA on integrin-linked kinase expression in gastric cancer cells. Integrin-linked kinase expression levels were analyzed by (A) real-time polymerase chain reaction and (B) Western blot analysis at different time points after incubation. Data are the mean ± SD. $^aP < 0.05$ *vs* control. Non-silencing siRNA is set to 100%.

ILK regulates cell attachment, proliferation and invasion in gastric cancer cells

We investigated the role of ILK in attachment of gastric cancer cells. In the cell attachment assay, we found that down-regulation of ILK lowered the ability of cells to attach to fibronectin compared with the non-silencing siRNA group ($P < 0.01$, Figure 3). The non-silencing siRNA and untreated groups had no significant difference in cell attachment ability ($P > 0.05$).

MTT assay was performed to observe whether down-regulation of ILK had an inhibitory effect on BGC-823 cell proliferation. We found that treatment of BGC-823 cells with ILK siRNA was associated with a time-dependent inhibition of cell proliferation, whereas no significant inhibitory effect was observed in cells treated with non-silencing siRNA (Figure 4).

Next, we investigated the role of ILK in the invasion of gastric cancer cells. We found that down-regulation of ILK reduced the ability of cells to invade through Matrigel-coated Boyden chambers to 10.7% ± 1.5% of that achieved by the non-silencing siRNA group ($P < 0.01$, Figure 5). The non-silencing siRNA and untreated groups had no significant difference in cell invasion ability ($P > 0.05$).

ILK regulates microfilament dynamics in gastric cancer cells

In the microfilament dynamics assay, ILK siRNA-transfected gastric cancer cells displayed different patterns of

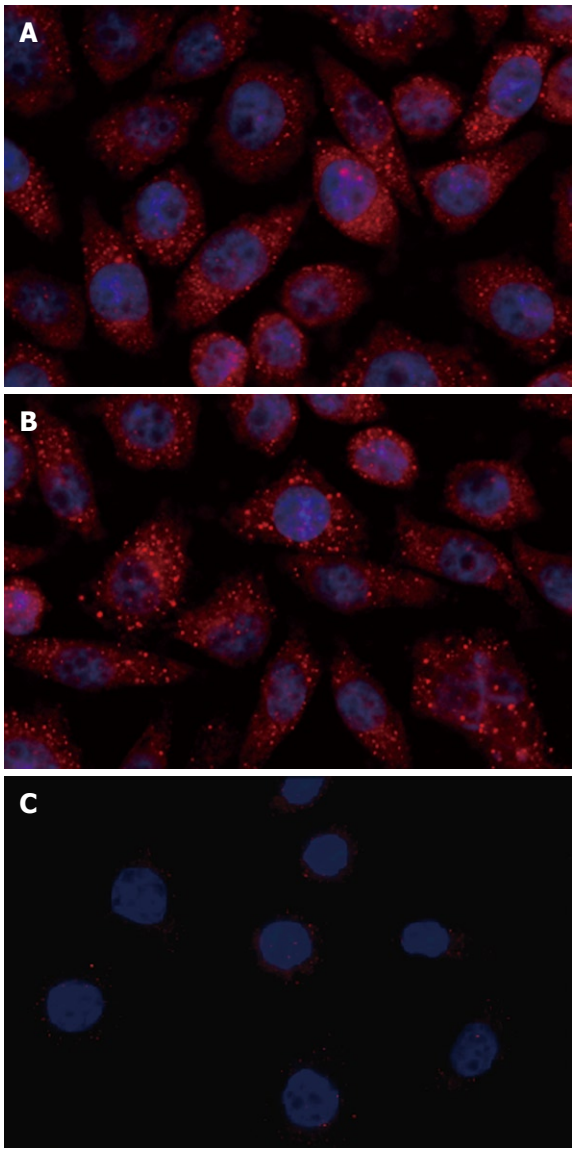

Figure 2 Immunocytochemical assays for integrin-linked kinase in gastric cancer cells. The fluorescence intensity represents the expression of integrin-linked kinase (ILK) in gastric cancer cells. A: Untransfected cells (Untreated); B: Non-silencing siRNA-transfected cells (Non-silencing siRNA); C: ILK siRNA-transfected cells (ILK siRNA). Experiments were repeated three times.

F-actin assembly and cell morphologies compared with the non-silencing siRNA group (Figure 6). F-actin assembly in the ILK siRNA group was significantly disturbed. There was less lamellipodia and filopodia formation in ILK-knockdown cells. However, cells in the non-silencing siRNA group displayed a well-organized actin skeleton with fibers extending throughout the cytoplasm into the cell membrane. Non-silencing siRNA-treated cells also showed a higher degree of cell spreading compared with the ILK siRNA-treated cells.

ILK regulates VEGF secretion from gastric cancer cells

VEGF plays an important role in tumor angiogenesis. To explore whether down-regulation of ILK can reduce VEGF activity in BGC-823 cells, we examined the levels of VEGF secreted into the culture medium by ELISA. We found that ILK knockdown led to a decrease in the level of VEGF secreted into the culture medium. As

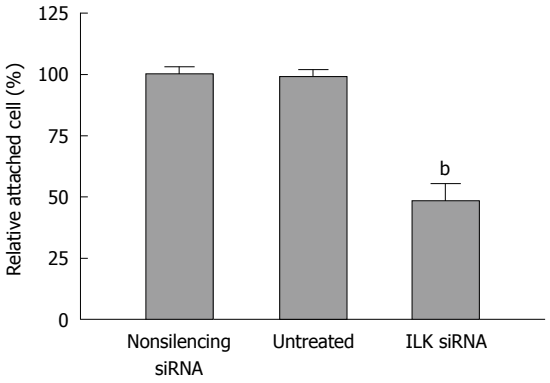

Figure 3 Effects of integrin-linked kinase on attachment of gastric cancer cells. Cell attachment was assessed after 1 h incubation and subsequent MTT test. Data are mean ± SD of three independent experiments ($^bP < 0.01$ *vs* control). Non-silencing siRNA is set to 100%. ILK: Integrin-linked kinase.

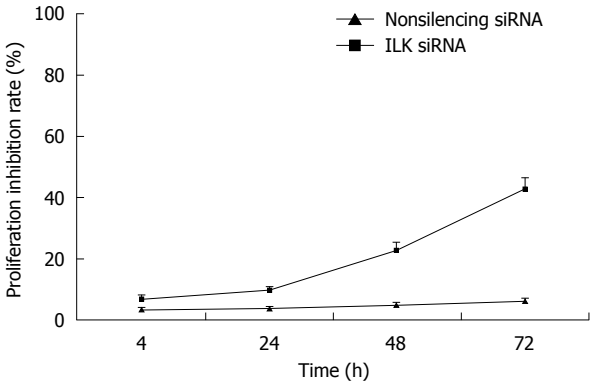

Figure 4 Effects of integrin-linked kinase on proliferation of gastric cancer cells. Gastric cancer BGC-823 cells were treated with integrin-linked kinase siRNA or non-silencing siRNA. Six duplicate wells were set up for each sample. After 4, 24, 48 or 72 h incubation, 20 μL MTT (5 g/L) was added to each well. Data are the mean ± SD of three independent experiments. Cell proliferation is plotted against time.

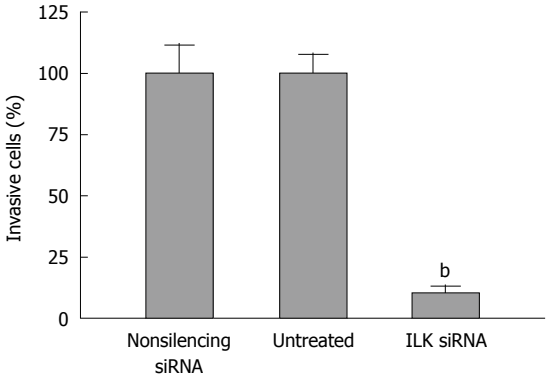

Figure 5 Effects of integrin-linked kinase on invasion of gastric cancer cells. Cell invasion was assessed 24 h after incubation in Matrigel coated transwell culture cambers. Data are the mean ± SD of three independent experiments ($^bP < 0.01$ *vs* control). Non-silencing siRNA is set to 100%. ILK: Integrin-linked kinase.

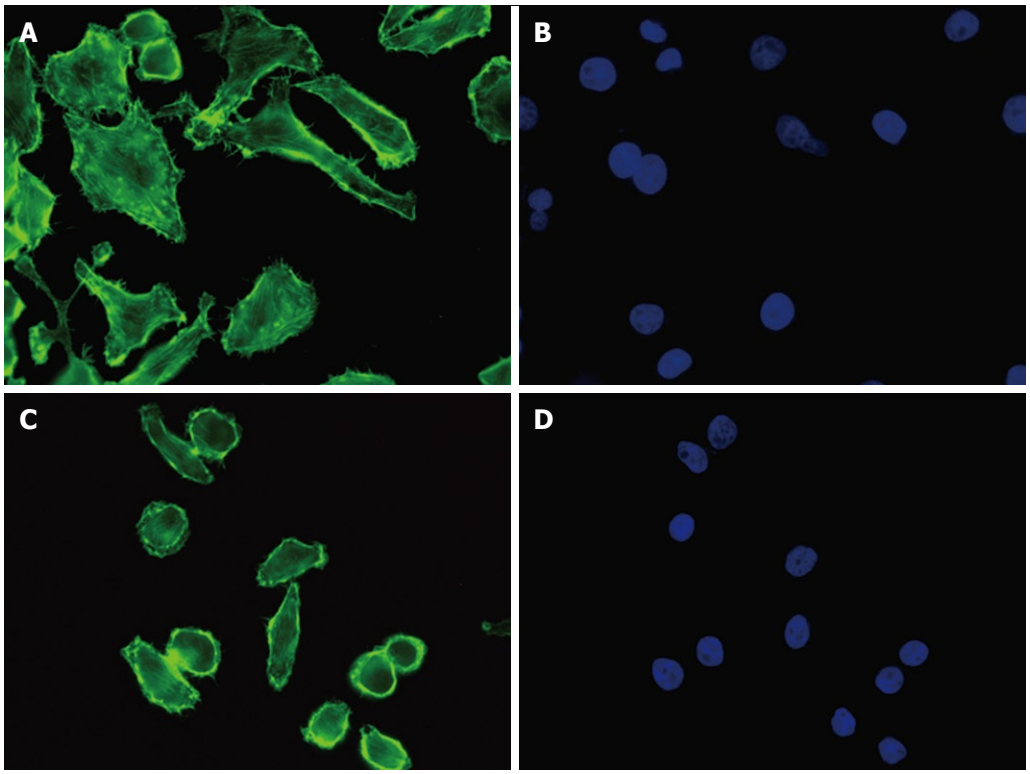

Figure 6 Effects of integrin-linked kinase on microfilament dynamics of gastric cancer cells. Microfilament organization was assessed by immunofluorescence analysis with rhodamine-phalloidin. A, B: Non-silencing siRNA-transfected cells displayed an elaborate network of precisely organized F-actin filaments and a high degree of cell spreading; C, D: The F-actin filament architecture became significantly disturbed with less lamellipodia and filopodia formation in integrin-linked kinase knockdown cells compared with control cells. Experiments were repeated three times.

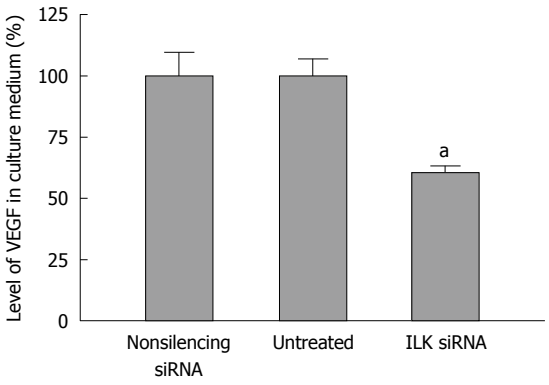

Figure 7 Effects of integrin-linked kinase on vascular endothelial growth factor secretion by gastric cancer cells. After transfection, the culture medium was harvested and cell counting was performed. Vascular endothelial growth factor (VEGF) released into the culture supernatant was measured by enzyme-linked immunosorbent assay. Data are the mean ± SD of three independent experiments ($^aP < 0.05$ *vs* control). Non-silencing siRNA cells are set to 100%. ILK: Integrin-linked kinase.

shown in Figure 7, the level of VEGF in the culture medium was decreased by 40% compared with non-silencing siRNA treated cells ($P < 0.05$). In contrast, there was no significant difference in the level of VEGF secretion between the non-silencing siRNA treated and untreated cells ($P > 0.05$).

ILK is important for gastric cancer growth in vivo

To further investigate the role of ILK in gastric cancer tumorigenesis, equal numbers (1×10^7) of BGC-823 cells transfected with ILK siRNA, non-silencing siRNA, or untreated cells were subcutaneously injected into nude mice. The growth of tumors was measured every 3 d. Four weeks after injection of the cells, mice were sacrificed and the weights of tumors were recorded. As shown in Figure 8, cells with down-regulation of ILK produced significantly smaller tumors in nude mice compared with untreated cells and cells treated with non-silencing siRNA (volume: 2.19 g ± 0.58 g *vs* 6.52 g ± 1.42 g, 6.68 g ± 1.40 g, $P < 0.05$; weight: 1.0 cm^3 ± 0.2 cm^3 *vs* 2.5 cm^3 ± 0.4 cm^3, 2.6 cm^3 ± 0.4 cm^3, $P < 0.05$, respectively) indicating that targeting ILK by siRNA may exert a strong anti-tumor effect on BGC-823 cells *in vivo*. All the tumors were analyzed by H&E staining and were verified to have similar cell morphologies that were consistent with gastric cancer.

DISCUSSION

Gastric cancer is a life-threatening disease with a high mortality worldwide, especially in Asia. Therefore, it is important to understand the molecular mechanisms of gastric cancer progression to discover useful targets to treat advanced gastric cancer. Previous findings showed that ILK has an increased expression and an association with tumor cell invasion and nodal metastasis in gastric cancer[13]. We, therefore, investigated whether ILK was important in different processes involved in gastric cancer progression, including cell attachment, growth, inva-

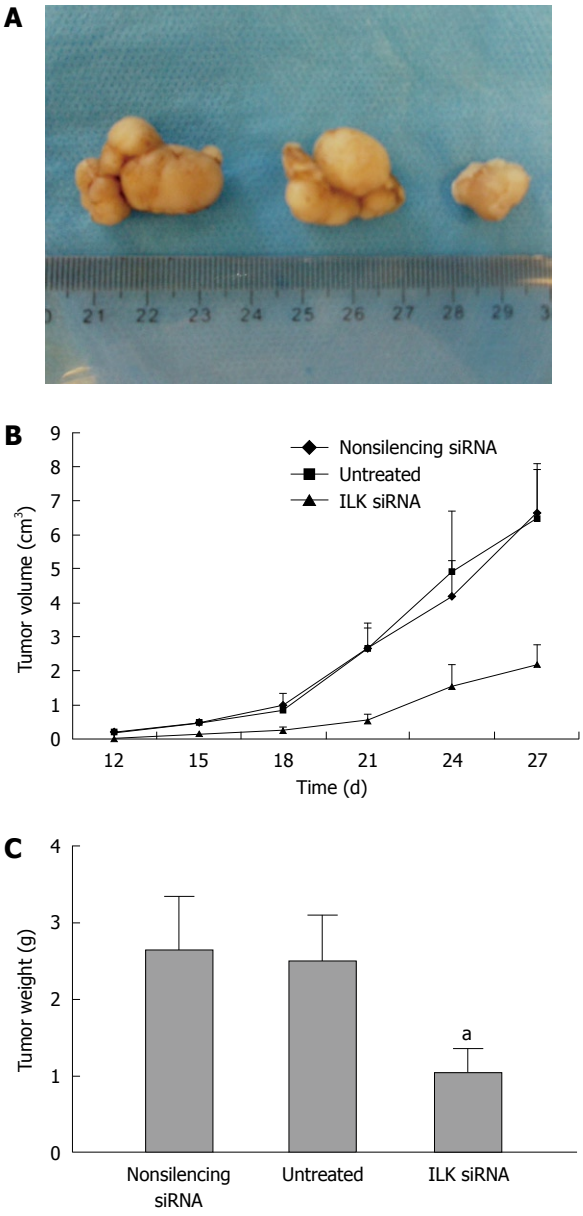

Figure 8 Integrin-linked kinase siRNA inhibits the growth of gastric cancer xenografts in nude mice. BGC-823 cells were treated with integrin-linked kinase (ILK) siRNA or non-silencing siRNA or left untreated. Equal numbers of cells (1×10^7) were injected 48 h later subcutaneously into the armpit of five nude mice in each group. Tumor formation was scored every 3 d. At week 4 after injection, the mice were killed and tumor weight recorded. A: images of tumors excised from the nude mice; B: estimated tumor volume of non-silencing siRNA, untreated or siRNA ILK tumors; C: estimated tumor weights of non-silencing siRNA, untreated or siRNA ILK tumors ($^aP < 0.05$ *vs* control).

sion, microfilament dynamics, angiogenesis and tumor growth *in vivo* by targeted siRNA knockdown of ILK.

Although RNA interference mediated by siRNAs is a powerful technology allowing the silencing of mammalian genes with high specificity and potency, non-specific effects both at the messenger RNA and protein levels can result from siRNA mediated mechanisms, and may represent one of the limitations of this technology[23]. Therefore, we used non-silencing siRNA-transfected cells to control these non-specific effects. We observed significant differences between the ILK-specific siRNA treated group and the non-silencing siRNA treated group in all assays. However, some studies indicated that the inhibition effect of synthetic siRNA may only last a short time. Therefore, following tumorigenesis, ILK expression may be released from RNAi inhibition in tumor tissues. Thus, to get a more stable inhibitory effect, we used a long-acting siRNA whose stability was improved by chemical modification. The expression of ILK was inhibited for about 7 d by this technique[24]. We observed that the suppression of ILK lasted a week and reached a peak 48 h after transfection. Thereafter, the expression of ILK gradually recovered, because of the remaining instability of the ILK siRNA.

We first showed that ILK is important for gastric cancer cells to attach to fibronectin-coated plates. Upon engagement with the extra cellular matrix (ECM), numerous signaling proteins are recruited to the adhesion sites, where cell-matrix contact is established[25]. Fibronectin is one of the major components in the ECM that connect cells through the extracellular domain of integrins. Here, we demonstrate that ILK plays a central role in the transduction of signals when gastric cancer cells engage in ECM-regulated cell attachment. The mechanism of how ILK regulates the dynamic rearrangement of cell-matrix adhesions and cell spreading is not well understood. Some scholars have suggested that ILK regulates cell-matrix adhesion dynamics through Rac-1[26]. In addition, inhibition of PI3K-dependent ILK activity in PTEN-null PC3 prostate cancer cells disrupted the localization of ILK/α-parvin/paxillin complex to focal adhesions, leading to decreased cell adhesion and migration[27]. However, others found that knock-down of human ILK by siRNA increased cell adhesion in diverse gastric carcinoma cell variants, including SNU16, integrin-α5-expressing SNU16, and integrin-α5-expressing SNU620 cells[28]. It appears that the correlation between ILK expression and cell adhesion is very sophisticated, and may depend on cell types and the signaling contexts. The epigenetic control of ILK expression and adhesion properties of gastric carcinoma cells appear to affect each other, through a bidirectional regulatory linkage[28].

Inhibition of ILK kinase activity by an ILK inhibitor is known to impede cell attachment and filamentous actin organization[27]. ILK over-expression induced the distribution of actin filaments onto the cell membrane to form cell motility structures[29]. We found that formation of the actin cytoskeleton and motility structures, which are important for the early events in cell spreading and migration, were severely affected in ILK knockdown gastric cancer cells. ILK may modulate cell spreading, migration and cytoskeletal organization by activating PAK-interactive exchange factor (PIX, also known as ARHGEF6), a guanine-nucleotide exchange factor for Rac1 and Cdc42[28], and by activating cofilin through an interaction with phosphorylated Scr[30]. This study suggests that targeting ILK seems to be linked with microfilament assembly, resulting in a decreased ability of the gastric cancer cells to attach, spread and migrate.

Tumor cell proliferation is another important event

in tumor progression. Knockdown of ILK using siRNA inhibited the proliferation of gastric cancer cells and impaired the growth of gastric cancer xenografts *in vivo*. ILK over-expression or constitutive activation leads to the stimulation of cell-cycle progression, and inhibition of ILK activity in some cancer cells results in inhibition of cyclin D1 expression and G1/S cell-cycle arrest[31-33]. ILK-mediated phosphorylation and consequent inhibition of the activity of GSK-3 may regulate several pathways, leading to stimulation if cyclin D1 expression. Inhibition of GSK-3 activity leads to activation of the AP1 transcription factor, cyclic-AMP-responsive-element-binding protein, and the β-catenin/TCF transcription factor[32,34,35], both of which can stimulate the expression of cyclin D1 and promote cell proliferation.

We also found that ILK is important for gastric cancer cell invasion; knockdown of ILK using siRNA inhibited gastric cancer cell invasion. Increased ILK expression was shown to stimulate the expression and activity of the matrix metallopeptidase 9 (MMP9), through activation of the AP1 transcription factor[36]. Inhibition of ILK activity in highly invasive human glioblastoma cells, resulted in substantial inhibition of invasion into matrigel, and pharmacological inhibition of MMP9 activity also inhibited invasion[36], demonstrating that ILK can promote invasion through up-regulation and activation of MMP9. In gastric cancer, the T allele of the 1562 C/T polymorphism in the MMP9 gene is associated with an invasive tumor phenotype[37] and elevated plasma MMP-9 correlates significantly with lymph node metastasis, lymphatic invasion, venous invasion and poor survival rates[38]. Therefore, it is reasonable to predict that down-regulating ILK and then inhibiting MMP9 hold promise for the treatment of gastric cancer.

Tumor angiogenesis is promoted by the expression and secretion of VEGF from tumor cells, which then binds to the VEGF receptor on the nearby endothelial cells, stimulating their survival, proliferation and migration, which are events required for the formation of new blood vessels. We observed that silencing ILK with siRNA significantly reduced VEGF secretion from gastric cancer cells. What is the mechanism by which ILK regulates VEGF? ILK has been reported to be essential for the regulation of hypoxia inducible factor (HIF)-1α expression, and for the consequent production of VEGF in a PKB/Akt- and mTOR/FRAP-dependent manner[10]. And HIF-1α is a major transcriptional activator of the VEGF gene[39]. A model has been proposed to explain this regulation[10]: phosphorylation of serine 473 of Akt/PKB by activated ILK results in the full activation of PKB/Akt, which promotes the phosphorylation of serine 2448 of mTOR/FRAP. This activates mTOR/FRAP, which raises the levels of HIF-1α protein translation. HIF-1α protein combines with HIF-1β to form an active transcription factor. This heterodimer binds to the VEGF promoter and activates VEGF transcription, translation, and secretion. VEGF binds to its receptor on the nearby endothelial cells and stimulates ILK activity. Furthermore, down-regulation of VEGF expression with siRNA not only impaired tube formation, but also inhibited the synthesis of multiple angiogenic proteins, such as angiogenin, interleukin (IL)-6, IL-8, transforming growth factor β1 and monocyte chemoattractant protein 1[40]. Collectively, these studies demonstrate a crucial role of ILK in the regulation of vascular morphogenesis, and indicate that ILK should be considered as a promising target for anti-angiogenic therapy.

In summary, our results indicate that knockdown of ILK with siRNA is able to inhibit not only gastric cancer cell attachment, proliferation, invasion and tumor angiogenesis *in vitro*, but also tumor growth *in vivo*. These findings also suggest that ILK could be a valid therapeutic target in gastric cancer.

COMMENTS

Background

Gastric cancer is one of the most commonly diagnosed malignant tumors and also is one of the most frequent causes of cancer mortality worldwide. Therefore, an understanding of the molecular mechanisms involved in gastric cancer formation and progression, and the identification of specific gene therapy targets are important for developing more effective approaches for gastric cancer treatment.

Research frontiers

Integrin-linked kinase (ILK), an ankyrin repeat-containing serine/threonine protein kinase, mediates a diversity of cell functions by coupling integrins and growth factors to cascades of downstream signaling events. The expression and activity of ILK are increased in a range of tumors, and small-molecule inhibitors of ILK activity have been identified, and shown to inhibit tumor growth, invasion and angiogenesis. ILK has become a hot topic in tumor research.

Innovations and breakthroughs

To investigate the role of ILK in gastric cancer, the authors specifically knocked down ILK expression using small interfering RNA (siRNA) in the gastric cancer cell line, BGC-823. The authors verified that knockdown of ILK significantly inhibited human gastric cancer cell attachment, proliferation, and invasion, and also disturbed F-actin assembly and reduced vascular endothelial growth factor secretion. Knockdown of ILK also suppressed the growth of gastric cancer cells *in vivo*. This research attempts to systematically understand the role of ILK in gastric cancer progression. The results could improve our understanding of gastric cancer progression.

Applications

The study provides the first evidence that ILK plays an important role in gastric cancer progression. The results indicate that ILK should be considered as a promising target for anti-gastric cancer treatment.

Peer review

The expression and activity of ILK are increased in a range of tumors, including gastric cancer. This study shows that targeting ILK with siRNA suppressed the growth of gastric cancer cells. The results indicate that ILK plays an important role in gastric cancer progression and that ILK could be a therapeutic target for gastric cancer.

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BRIEF ARTICLE

Gemcitabine in elderly patients with advanced pancreatic cancer

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Abstract

AIM: To assess feasibility, tolerability and efficacy of gemcitabine-based chemotherapy in patients ⩾ 75 years old with advanced pancreatic cancer.

METHODS: All consecutive patients ⩾ 75 years old with advanced pancreatic adenocarcinoma were included in this retrospective study. Necessary criteria to receive chemotherapy were: performance status 0-2, adequate biological parameters and no serious comorbidities. Other patients received best supportive care (BSC).

RESULTS: Thirty-eight patients (53% women, median age 78 years, range 75-84) with pancreatic cancer (metastatic: *n* = 20, locally advanced: *n* = 18) were studied. Among them, 30 (79%) were able to receive chemotherapy [median number: 9 infusions (1-45)]. Six patients (23%) had at least one episode of grade 3 neutropenia and one patient developed a grade 3 hemolytic-uremic syndrome. No toxic death occurred. Three patients (11%) had a partial tumor response, 13 (46%) had a stable disease and 12 (43%) had a tumor progression. Median survival was 9.1 mo (metastatic: 6.9 mo, locally advanced: 11.4 mo).

CONCLUSION: Tolerance and efficacy of gemcitabine-based chemotherapy is acceptable in elderly patients in good condition, with similar results to younger patients.

Key words: Elderly; Pancreas; Cancer; Gemcitabine

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INTRODUCTION

Although pancreatic cancer (PC) only accounts for 2% of all cancers, it is the fourth leading cause of cancer death in the United States (US)[1]. Prognosis is very poor, with an estimated incidence of 33 000 per year in the US and a similar death incidence rate[1]. Median survival in patients with advanced PC who receive the best support-

ive care (BSC) is only three to four months. Chemotherapy with gemcitabine has been considered the standard treatment of non-resectable PC since the study by Burris *et al*[2]; it slightly improves both survival and clinical response and is acceptably tolerated. Several drugs have been tested in combination with gemcitabine but with disappointing results. The only combination that showed a slight but significant increase in survival was erlotinib and gemcitabine in a study by Moore *et al*[3].

PC usually occurs in elderly patients. In the US, the incidence rate adjusted by age and for 100 000 is of 64.2 over 65 years old and of 3.7 under 65 years old[4]. In France 37.1% of PC cases occur in patients ≥ 75 years old[5]. Survival rates in this subgroup of patients seem to be shorter than in younger patients[4]. Physicians may hesitate to offer intravenous chemotherapy because of frequent comorbidities and short estimated survival; in addition, the motivation of elderly patients for this type of treatment should be carefully assessed. Nevertheless, it has clearly been shown that elderly patients are underrepresented in cancer trials[6,7]. The efficacy and tolerance of chemotherapy in elderly patients with colorectal cancer has been shown in previous studies[8-12]. Most phase Ⅲ studies of chemotherapy for PC include results of, but do not specifically analyze, the subset of patients ≥ 70-75 years old[2,13-15]. Results by Maréchal *et al*[16] in a pooled analysis of patients ≥ 70 years old who were included in seven prospective phase 2 or phase 3 studies testing various gemcitabine-based first line combinations, suggest that chemotherapy is feasible in the elderly as well as in younger patients with PC. Likewise, Locher *et al*[17] supported the use of gemcitabine in another study in elderly patients.

The aim of this retrospective monocentric study was to assess feasibility, tolerance and efficacy of gemcitabine-based palliative chemotherapy in patients ≥ 75 years old treated for PC.

MATERIALS AND METHODS

Selection of patients

All patients with digestive cancer in our hospital are discussed at the weekly multidisciplinary oncological committee meeting, even if they are only able to receive best supportive care on first intention. For the current study, all patients with pathologically-proven advanced adenocarcinoma of the exocrine pancreas who were ≥ 75 years old and listed in our database were considered. Patients with adenocarcinoma of the ampulla or the biliary tract were excluded. Overall, 40 patients were included for this retrospective analysis. Among them, 2 patients were excluded as they received gemcitabine in another institution (West Indies) and thus follow-up was not possible. Finally, 38 consecutive patients fulfilling these criteria and who were treated in our hospital between March 2000 and June 2006 were retrospectively studied. After clinical and imaging assessment, tumors were classified as locally advanced (stage Ⅲ) or metastatic (stage Ⅳ) according to the UICC classification (UICC).

Criteria required to propose chemotherapy were an Eastern Cooperative Oncology Group (ECOG) performance status of 0 to 2 and no serious comorbidities. Before starting chemotherapy, pain and biliary obstruction had to be controlled and adequate biological parameters (i.e., neutrophil count > 1500/mL, platelet count > 100 000/mL, serum creatinine < 1.5 × the upper limit of normal value (ULN), alkaline phosphatase < 5 × ULN, and bilirubin < 1.5 × ULN) were required. If one of these criteria was not fulfilled, BSC was decided.

Treatment

Chemotherapy included gemcitabine as a single agent according to the Burris regimen (gemcitabine 1000 mg/m² as a 30-min infusion weekly for 7 out of 8 wk and then for 3 out of 4 wk)[2] or combined with oxaliplatin according to the GemOx regimen (gemcitabine 1000 mg/m² as a 100-min infusion on day 1 and oxaliplatin 100 mg/m² as a 2-h infusion on day 2 every 2 wk)[18].

Patients who received at least one infusion of chemotherapy were placed in the "chemotherapy group". All the other patients received BSC.

Chemotherapy was stopped if there was an unacceptable/life-threatening adverse event, if performance status worsened (i.e., ECOG ≥ 3) and/or if tumor progression occurred according to imaging results. The type of chemotherapy, the number of infusions and the reason why chemotherapy was not administered or was stopped were analysed.

Safety and efficacy evaluation

Baseline assessment included medical history, physical examination with an evaluation of clinical symptoms, and biological analyses (blood cell count, serum creatinine, bilirubin, ASAT, ALAT, alkaline phosphatase). During the treatment period, blood tests, toxicity evaluation and a physical examination were performed before each infusion.

Toxicity was graded according to the National Cancer Institute Common Toxicity Criteria (NCI-CTC) version 2. Chemotherapy was delayed if the grade of toxicity ≥2; the dose of gemcitabine was reduced by 20% if the toxicity grade was ≥ 3.

Tumor response was assessed by computed tomography scan at three month intervals according to RECIST (Response Evaluation Criteria In Solid Tumors)[19]. Evaluation procedures were performed ahead of schedule if the patient's general condition worsened or severe toxicity occurred. Overall survival (OS) was calculated from the day of diagnosis of non-resectable PC to the date of death. This study was proposed after the agreement of our institution review board.

Statistical analysis

Qualitative data are expressed as numbers and percentages. Quantitative data are expressed as median (range).

Table 1 Characteristics of the 38 patients and their pancreatic cancers

	Metastatic	Locally advanced
Number of patients (%)	20 (53)	18 (47)
Median age (range)	78 (75-84)	78 (75-84)
Gender (M/F)	8/12	10/8
Site of metastases		
-liver	15	0
-other[1]	10	0

[1]Lung, peritoneum, lymph nodes.

Table 2 Characteristics of treatment in the 38 patients according to the stage of pancreatic cancer

	Metastatic	Locally advanced
Number of patients treated by gemcitabine-based chemotherapy (%)	15 (50%)	15 (50%)
Number of patients with BSC on first intention	5	3
-staff decision ECOG ⩾ 2	3	2
-others reasons	2 (Septicaemia, pulmonary embolism	1 (Duodenal stenosis and deep venous thrombosis)
Median number of infusions (range)[1]	*n* = 18 (1-45)	*n* = 7 (2-13)

[1]Data available for 28 patients.

Survival was determined by the Kaplan-Meier method.

RESULTS

General characteristics

Twenty women and 18 men were studied. Median age was 78 years old (75-84). Tumors were metastatic in 20 patients (including tumor relapse after surgical resection in five patients) and locally advanced in 18 patients. Median follow up was 7 mo (1-44) (Table 1). Thirty of the 38 patients (79%) received gemcitabine-based chemotherapy (single agent: $n = 28$, combined with oxaliplatin: $n = 2$) with a median of 9 infusions (1-45). Twenty-four patients (83%) completed at least 2 mo of chemotherapy (i.e., 7 infusions). The relative dose-intensity of gemcitabine was 83%. Chemotherapy was stopped due to tumor progression ($n = 21$), toxicity ($n = 1$) or fatigue ($n = 4$); it was replaced by chemoradiotherapy ($n = 2$) in patients with controlled disease. The eight remaining patients did not receive chemotherapy due to exclusion criteria ($n = 5$) or a life-threatening medical event that occurred after the decision to treat but before the beginning of the treatment ($n = 3$) (Table 2).

Safety evaluation

Tolerance data were available in 26 of 30 patients. Six patients (23%) had at least one episode of grade 3 hematological toxicity (neutropenia). One patient developed grade 3 hemolytic-uremic syndrome, so gemcitabine was discontinued. No grade 4 toxicity or toxic deaths occurred.

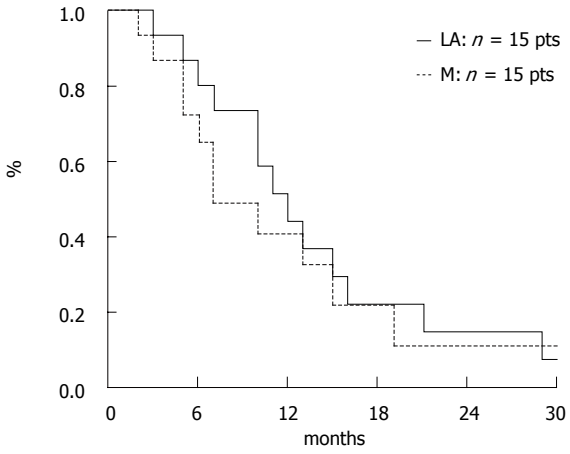

Figure 1 Overall survival of treated patients according to disease stage.

Tumor response rate

Response rate was available in 28 of 30 patients. During the first assessment (at 3 mo), 3 patients (11%) had partial tumor response (PR), disease was stable in 13 (46%) (SD) and 12 (43%) had tumor progression (PD). For 14/15 patients with metastatic PC, 1 PR (7%), 4 SD (29%) and 9 PD (64%) were observed.

Second line treatment included chemotherapy in four patients with progressive disease (GemOx after gemcitabine alone: $n = 3$, and Folfiri after GemOx: $n = 1$), and chemoradiotherapy was proposed in two 75-year old patients with locally advanced tumors who were in very good condition with controlled tumors after 3 mo of chemotherapy. The latter treatment involved irradiation of 50.4 Gy with a continuous infusion (200 mg/m^2) of 5-fluorouracile as a radiosensitizer based on the results of a previous study[20].

Overall survival

Median survival of all patients ($n = 38$) was 8.9 mo and the one-year survival rate was 33.2%. Median survival of the 8 patients who received BSC was 2.95 mo. In patients receiving chemotherapy, median survival was 9.1 mo; this was 6.9 mo in patients with metastatic cancer and 11.4 mo in patients with locally advanced cancer; the 1-year survival rate was 40.6% and 44%, respectively.

Overall survival in patients treated with gemcitabine-based chemotherapy according to disease stage is presented in Figure 1.

DISCUSSION

Although a direct comparison was not performed, this monocentric retrospective study suggests that the safety and efficacy of gemcitabine-based chemotherapy in elderly patients is similar to that in younger patients.

Most eligible patients (79%) received a median of 9 infusions of chemotherapy. Safety was acceptable with grade 3 neutropenia in 23% of patients (with no grade 4), and one case of grade 3 hemolytic-uremic syndrome requiring treatment discontinuation. There were no toxic deaths. These safety results are similar to those in randomised studies including younger patients which report neutropenia as the most frequent type of toxicity with gemcitabine (grade 3-4 toxicities from 9% to 27.6%)[2,3,13-15,18].

In our study, disease control was obtained in 57% of patients (PR: 11% and SD: 46%) who received chemotherapy, which compares favourably to other published randomised studies (41.2% to 52.8%)[2,3,13,21]. The objective response rates in these studies, which include patients with both locally advanced and metastatic cancers, was 7.1% to 17.3%[3,14,15,18]. The survival rate in our study was 9.1 mo in patients who received chemotherapy; this was 6.9 mo in patients in the metastatic subgroup and 11.4 mo in the locally advanced subgroup. The 1-year survival rate of patients with metastatic and locally advanced disease who received chemotherapy was 40.6% and 44%, respectively. In the randomised series with younger patients, median survival rates in the gemcitabine arm were 5.6 and 7.2 mo, respectively[2,3,13-15,18,21].

These results should be cautiously interpreted since methodological biases are inevitable in such a retrospective study. In addition, it was conducted in a tertiary care institution, thus our population should not entirely reflect the "true life" practice for elderly patients with PC. Likewise, our study does not allow distinguishing of the potential influence of performance status (i.e., 0-1 *vs* 2) on both treatment safety and efficacy.

One retrospective phase Ⅱ trial analysed the impact of age (< or ⩾ 65 years) on the efficacy and tolerance to gemcitabine in advanced non-small cell lung cancers. Hematological, non-hematological toxicities and dose reductions, or the mean number of cycles were similar in both age groups[22].

A recent study by Locher *et al*[17] reported 39 patients ⩾ 70 years old with PC treated by a fixed-dose rate of gemcitabine[23]. The authors showed a good efficacy of this treatment with a clinical benefit observed in 20%, a tumor response rate in 10% and a stabilization of the disease in 33% of patients. The median survival was 10 mo and the time to progression was 7 mo. Grade 3-4 neutropenia and alopecia occurred in respectively 38% and 18%. These side-effects were higher than in others trials probably due to the fixed dose rate of gemcitabine[2,3,13-15,18-23]. Maréchal *et al*[16] analyzed 42 patients > 70 years old pooled from seven prospective studies evaluating gemcitabine-based chemotherapy and compared them to 57 younger patients. Two thirds of the elderly patients received gemcitabine alone and one third received gemcitabine-based combinations (mainly gemcitabine-oxaliplatin). The median overall survival (220 d *vs* 240 d), time to progression (104 d *vs* 119 d), response rate (4.8% *vs* 8.9%) and clinical benefit (57.1% *vs* 59.6%) were similar in elderly and non-elderly patients. Tolerance to chemotherapy was acceptable in the elderly group despite a dose reduction or delay in therapy in 62%, a higher figure than that observed in our study. As in our study, neutropenia was the most common cause of grade 3-4 toxicity. Grade 3-4 neutropenia, anaemia and peripheral neuropathy occurred more often in the elderly group than in younger patients (30.9% *vs* 8.8%, 14.3% *vs* 8.8% and 4.8% *vs* 0%, respectively). Age was not an independent prognostic factor in multivariate analysis of the whole population. Multivariate analysis identified ASAT and Karnofsky index as independent prognostic factors in the elderly group[16].

A Japanese study specifically reported results in 25 patients ⩾ 70 years old receiving gemcitabine 800-1000 mg/m² compared to 43 patients receiving BSC. Patients receiving chemotherapy had a more favourable prognosis and acceptable tolerance[24]. Another retrospective study by Nakachi *et al*[25], presented in abstract form at the ASCO GI meeting in 2007, suggested that gemcitabine was effective and well tolerated in selected elderly patients. Thirty-seven patients ⩾ 75 years old were compared to 137 younger patients. Grade 3-4 neutropenia (18.9% *vs* 19 %) and tumor response rates (8.1% *vs* 4.3%) were similar. In contrast, median overall survival was better in the elderly group (8 mo *vs* 5.6 mo, $P = 0.009$).

Recently, the promising schema FOLFIRINOX (5-fluorouracil, irinotecan and oxaliplatin) was shown to be superior to gemcitabine in terms of tumor response and overall survival[26]. However, patients treated in this study were less than 75 years-old and in very good condition (PS 0-1). Moreover, significant toxicity was seen [45.7% of patients experienced a significant (grade 3-4) hematological toxicity with 5.4% of febrile neutropenia] that could be problematic in elderly patients[26]. Further studies are warranted in latter patients using such drugs.

In conclusion, gemcitabine chemotherapy seems to be effective and safe in elderly patients with PC in good condition. The risk/benefit ratio of this treatment should be discussed in a multidisciplinary context and these patients should actively participate in therapeutic decisions. Prospective studies of this specific subgroup of patients with PC are needed.

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COMMENTS

Background

Pancreatic cancer is a severe disease that is often treated using systemic chemotherapy as it is non-resectable in up to 80% of patients at the time of diagnosis. Significant rates of patients with this disease are older than 75 years. However, most phase Ⅲ studies of chemotherapy for pancreatic cancer include elderly patients, but they do not provide a specific analysis of patients ⩾ 70-75 years old. Thus, this specific population is strongly underrepresented in therapeutic trials for digestive cancers and thus guidelines for clinical practice are lacking. In this paper, the results suggest that elderly patients with pancreatic

cancer, when they are in acceptable condition, could receive gemcitabine-based chemotherapy which is safe and seems to be as efficient as in younger patients.

Research frontiers

Tumor response rates, toxicity and duration of tumor control were specifically analyzed in a homogeneous population of 38 elderly patients with pancreatic cancer treated in one center.

Innovations and breakthroughs

This is a homogeneous study of consecutive patients treated by an experienced team in digestive cancers, particularly pancreatic cancer. The authors have shown that toxicity of gemcitabine was manageable, and tumor control and overall survival were encouraging, as they appear to be similar to that of younger patients. The authors hope it will encourage physicians to evaluate and consider chemotherapy in such patients.

Applications

It is time to pave the way of chemotherapy in elderly patients with pancreatic cancer knowing that a significant subset of them may benefit of these treatments. In the future, patients should be better selected for the treatments using molecular markers (i.e., hENT-1 expression and gemcitabine).

Terminology

A locally advanced pancreatic cancer is a tumor involving the arterial axis (celiac trunk, mesenteric artery) and thus is non-resectable despite there being no detectable metastases. This form of cancer should be distinguished from metastatic tumors as the prognosis is different (slightly better, and some patients can return to surgical treatment in cases of good tumor response after chemotherapy), and thus separate analyses are needed.

Peer review

This is an article describing gemcitabine in elderly patients with advanced pancreatic cancer.

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BRIEF ARTICLE

Diagnostic efficacy of gadoxetic acid-enhanced MRI for hepatocellular carcinoma and dysplastic nodule

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Abstract

AIM: To evaluate the relationship between the signal intensity of hepatobiliary phase images on gadoxetic acid-enhanced magnetic resonance imaging (MRI) and histological grade.

METHODS: Fifty-nine patients with 82 hepatocellular lesions were evaluated retrospectively. Hepatobiliary phase images on gadoxetic acid-enhanced MRI were classified into 3 groups: low, iso or high. Angiography-assisted computed tomography (CT) findings were also classified into 3 groups: CT during arterial portography, and CT hepatic arteriography: A: iso, iso or low; B: slightly low, iso or low; and C: low, high. We correlated angiography-assisted CT, hepatobiliary phase findings during gadoxetic acid-enhanced MRI and histological grades. Furthermore, correlations between MRI findings and histological grade for each hemodynamic pattern were performed. Correlations among radiological and pathological findings were statistically evaluated using the chi-square test and Fisher' s exact test.

RESULTS: There was a significant correlation between histological grade and hemodynamic pattern ($P < 0.05$). There was a significant correlation between histological grade and signal intensity in the hepatobiliary phase ($P < 0.05$) in group A lesions. There was no significant correlation between histological grade and signal intensity in the hepatobiliary phase in group B or C lesions ($P > 0.05$).

CONCLUSION: Signal intensity in the hepatobiliary phase correlated with histological grade in the lesions that maintained portal blood flow, but did not correlate in lesions that showed decreased or defective portal blood flow.

Key words: Hepatocellular carcinoma; Gd-EOB-DTPA; Gadoxetic acid; Primovist; Early hepatocellular carcinoma

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INTRODUCTION

Early detection of hepatocellular carcinoma (HCC) is im-

portant in establishing an effective therapeutic strategy[1]. Early stage HCC does not have a hypervascular nature, and the lesions maintain portal blood flow[2,3]. Intranodular portal blood flow can only be evaluated by computed tomography (CT) during arterial portography (CTAP), and the absence or decrease of portal blood flow in the nodule can show that the lesion is malignant[4]. However, dysplastic nodules and some well-differentiated HCC lesions maintain portal blood flow, making differential diagnoses difficult[5]. Gadolinium-ethoxybenzyl diethylenetriaminepentaacetic acid (gadoxetic acid, Primovist®; Bayer-Schering, Osaka, Japan) is a liver-specific contrast medium which is taken into hepatocytes and excreted into bile; therefore a T1 shortening effect in liver parenchyma is obtained. The hepatobiliary phase begins 1.5 min after injection of the contrast medium and continues for 2 h, and the peak liver signal intensity is obtained 20 min after injection of contrast medium[6]. In the hepatobiliary phase, the tumor does not have normal functioning hepatocytes and is hypointense in most cases[7]. However, several investigators reported that some HCC can be iso or hyperintense regardless of overt HCC[8,9]. Gadoxetic acid-enhanced magnetic resonance imaging (MRI) yields a high tumor detection rate[10] and can detect lesions that maintain portal blood flow using angiography-assisted CT[11]. Therefore, gadoxetic acid-enhanced MRI can potentially distinguish the histological grade of hepatocellular lesions. We evaluated the relationships among angiography-assisted CT, hepatobiliary phase findings during gadoxetic acid-enhanced MRI and histological grades, and evaluated the diagnostic efficacy of gadoxetic acid for HCC and dysplastic nodule.

MATERIALS AND METHODS

Subjects

This retrospective study was approved by the Institutional Review Board, and the need for written informed consent was waived. Between January 2008 and June 2009, 460 patients received gadoxetic acid-enhanced MRI. Among them, patients satisfying all of the following criteria were enrolled; (1) gadoxetic acid-enhanced MRI had been performed; (2) angiography-assisted CT had been performed; (3) the duration between gadoxetic acid-enhanced and angiography-assisted CT was less than 60 d; and (4) their lesions had been pathologically confirmed. Patients in whom liver parenchymal enhancement was poor or absent due to severe portal hypertension or tumor invasion to the main portal vein were excluded. The subjects therefore consisted of 59 patients (37 men, 22 women), with 82 nodules. The mean age of the patients was 69 years (range 37-90 years). There were 8 patients with hepatitis B, 36 with hepatitis C, 4 alcoholic patients, 2 non-alcoholic patients with steatohepatitis, 1 with primary biliary cirrhosis and 8 cryptogenic patients. Six lesions were pathologically confirmed by operation. The other lesions were confirmed by ultrasound-guided biopsy (SSA-790A, Aplio XG; Toshiba Medical Systems Corp., Otawara, Japan). Biopsy specimens from the lesion and non-tumor area were obtained with a 20-gauge US-guided fine needle biopsy. Contrast medium (Sonazoid, Daiichi Sankyo, Tokyo, Japan) was used for obscure lesions on ultrasound. All lesions were detected on plain or contrast-enhanced ultrasound. The longest axis of the lesions was 5-66 mm (mean ± SD, 17.4 mm ± 9.5 mm). The longest dimension in 16 lesions was ≤ 10 mm, 21 were ≥10 mm, and 45 were > 15 mm. The long axis was measured on MRI.

Imaging

Angiography-assisted CT was performed with an angiography-combined 16 detector row CT system (Advantx ACT, GE Medical Systems, Milwaukee, WI). Immediately after injecting prostaglandin E2 (Liple®; Mitsubishi Tanabe Pharma, Osaka, Japan) through a catheter, 76 mL of contrast material (Iomeprol 350 mgI/mL; Eisai, Tokyo, Japan), which was diluted twice with physiological saline, was injected at a rate of 2 mL/s. CTAP was obtained 30 s after beginning the injection of contrast material through a catheter in the superior mesenteric artery. The parameters for CT acquisition were: table speed, 13.7 mm/0.5 s; collimation, 10 mm; and reconstruction, 5 mm. CT hepatic arteriography (CTHA) was obtained 6 s after the injection of contrast material through a catheter in the common hepatic or proper hepatic artery. In cases of hepatic artery bifurcation variation, the catheter was first inserted into the right and then the left hepatic artery, or *vice versa*. A total of 10-30 mL of contrast material (Iomeprol 350 mgI/mL) was injected at a rate of 0.8-1.5 mL/s. CTHA was obtained in 3 phases. Immediately after finishing the first phase, the second phase was obtained, and the third phase was obtained 2 min after beginning the injection of contrast material.

MR images were obtained using a 1.5 T superconductive MRI system (Avanto; Siemens, Erlangen, Germany). T1 weighted images (T1WI) included in-phase and opposed-phase images. The T1WI parameters (in-phase and opposed-phase) were: TR/TE, 120/4.76, 2.38 ms; flip angle, 75°; 1 averaging; matrix, 256 × 140; parallel acquisition technique (PAT) factor 2 with generalized autocalibration partially parallel acquisition (GRAPPA) algorithm; slice thickness, 6 mm; slice gap, 1.2 mm; and acquisition time, 13 s. The T2WI parameters were: TR/TE, 3600/99 ms; flip angle, 150°; echo train length, 29; matrix, 256 × 75(%); slice thickness, 6 mm; 1 averaging; PAT factor 2 with GRAPPA algorithm; and acquisition time, 14 s. T2WI was performed while subjects held their breath. 2 or 3 mL/s of gadoxetic acid (0.025 μmol/kg) was injected *via* the antecubital vein followed by 20 or 40 mL of physiological saline. The dynamic study included the arterial phase, portal phase, and 4 min after injecting the contrast material. A 3-dimensional (3-D) volumetric interpolated breath-hold examination (3D-VIBE) was used with the dynamic study. The 3D-VIBE parameters were : TR/TE, 4.28/1.78 ms; flip angle, 15°; matrix, 256 × 85 (%); PAT factor, 2; slice thickness, 3 mm; and acquisition time, 20 s. The monitoring scan technique (Care Bolus method) was used to obtain the optimal arterial phase. The hepato-

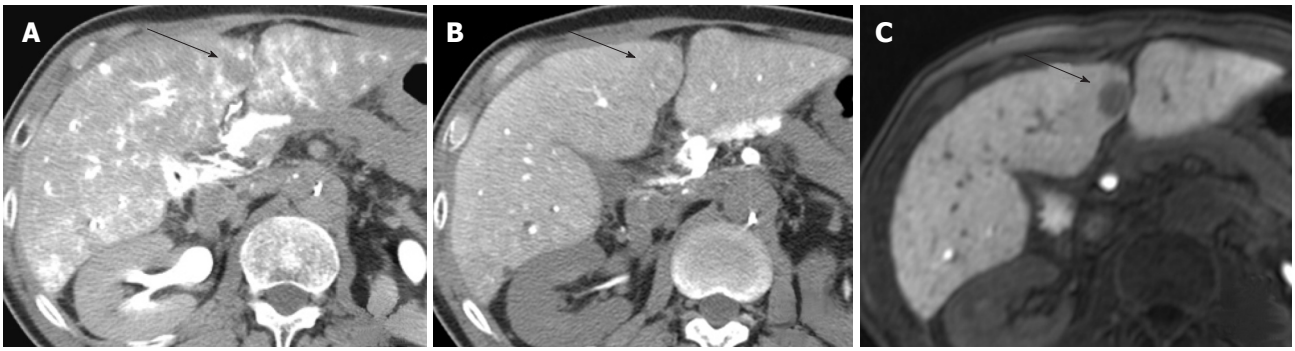

Figure 1 A 69-year-old man with moderately differentiated hepatocellular carcinoma. A: Computed tomography (CT) hepatic arteriography shows hypodensity; B: CT during arterial portography shows isodensity; C: Lesion clearly shows hypointensity in the hepatobiliary phase during gadoxetic acid-enhanced magnetic resonance imaging (arrow).

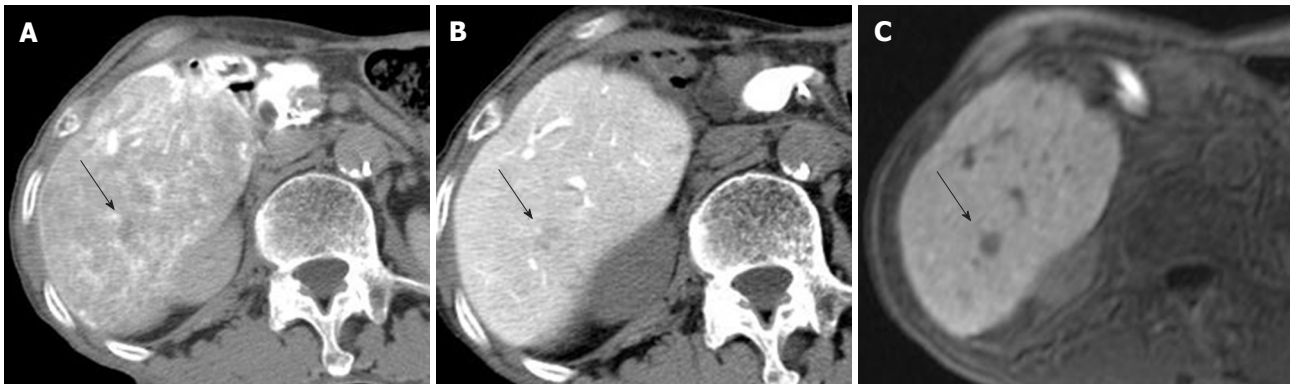

Figure 2 A 72-year-old man with well differentiated hepatocellular carcinoma. A: Computed tomography (CT) hepatic arteriography shows faint hypodensity; B: CT during arterial portography shows faint hypodensity; C: The lesion clearly shows hypointensity in the hepatobiliary phase on gadoxetic acid-enhanced magnetic resonance imaging (arrow).

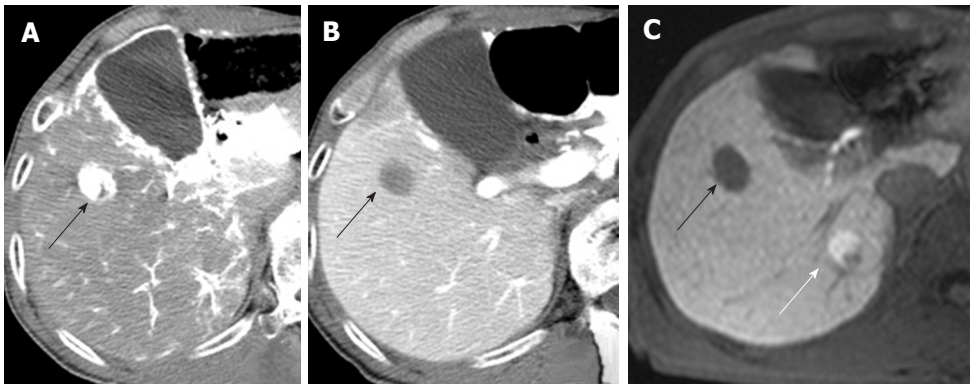

Figure 3 An 80-year-old man with poorly differentiated (black arrow) and well differentiated hepatocellular carcinoma (white arrow). A: Poorly differentiated hepatocellular carcinoma shows hypervascularity on computed tomography (CT) hepatic arteriography; B: Hypodensity on CT during arterial portography; C: Hypointensity in hepatobiliary phase on gadoxetic acid-enhanced magnetic resonance imaging (MRI). The lesion is not visible on CT hepatic arteriography (A) or on CT during arterial portography (B) and shows hyperintensity in the hepatobiliary phase on gadoxetic acid-enhanced MRI.

biliary phase was obtained with 3D-VIBE 20 min after injecting the contrast material.

Evaluation

A radiologist with 18 years of experience, whose specialty was interventional radiology, and a physician with 8 years of experience, whose specialty was liver imaging, evaluated the angiography-assisted CT by consensus. The findings of angiography-assisted CT were classified into 3 groups based on a previous report[12]: A, isodensity on CTAP and isodensity or low density on CTHA; B, slightly low density on CTAP and isodensity or low density on CTHA; and C, low density on CTAP and high density on CTHA (Figures 1-3). Partial hypodensity on CTAP and

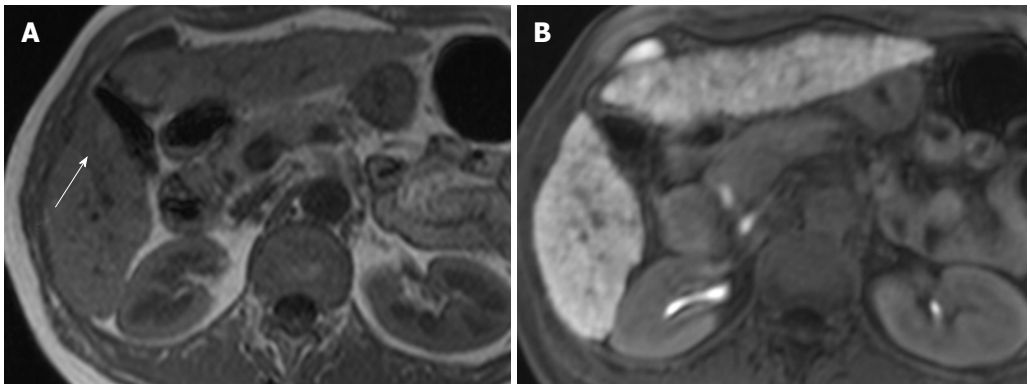

Figure 4 A 70-year-old woman with dysplastic nodule (arrow). A: The lesion shows hyperintensity on T1 weighted images; B: Isointensity in hepatobiliary phase on gadoxetic acid-enhanced magnetic resonance imaging.

Table 1 Correlation of histological grade and hemodynamic pattern

		Hemodynamic pattern			Total
		A	B	C	
Histological grade	DN	2	1	0	3
	Well	16[2]	8[2]	8[1]	32
	Mod.	7[1]	3	26[2]	36
	Poor	1	0	10[2]	11
Total		26	12	44	82

Hemodynamic patterns; A: Isodensity on computed tomography (CT) during arterial portography (CTAP) and isodensity or low density on CT hepatic arteriography (CTHA); B: Slightly low density on CTAP and isodensity or low density on CTHA; C: Low density on CTAP and high density on CTHA.DN: Dysplastic nodule; Well: Well differentiated hepatocellular carcinoma (HCC); Mod: Moderately differentiated HCC; Poor: Poorly differentiated HCC. [1]Significantly lower frequency; [2]Significantly higher frequency.

partial high density on CTHA were included in group C.

Two radiologists with 18 and 8 years of experience respectively, whose specialty was abdominal diagnostic radiology, evaluated MRI by consensus. Lesion signal intensity in the hepatobiliary phase during gadoxetic acid-enhanced MRI compared with the surrounding liver parenchyma was classified as either hypointensity, isointensity, or hyperintensity (Figures 3, 4). The same evaluation was performed for T1WI and T2WI.

We correlated angiography-assisted CT, hepatobiliary phase findings during gadoxetic acid-enhanced MRI and histological grades. Furthermore, correlations between MRI findings and histological grade for each hemodynamic pattern were performed. The signal intensities of T1WI and T2WI also correlated with the signal intensities of the hepatobiliary phase.

Correlations among radiological and pathological findings were statistically evaluated using the chi-square test and Fisher's exact test. $P < 0.05$ was considered statistically significant. Statistical analysis was performed using SPSS version 16.0 (SPSS Inc., Chicago, IL, United States) for Windows.

RESULTS

Correlation of histological grade and hemodynamic pattern

Twenty-six, 12 and 44 lesions were classified into hemodynamic pattern types A, B and C, respectively. Type A included 2 dysplastic nodules, 16 well, 7 moderately and 1 poorly differentiated HCC. Type B included 1 dysplastic nodule, 8 well and 3 moderately differentiated HCC. Type C included 8 well, 26 moderately and 10 poorly differentiated HCC.

There was a significant correlation between histological grade and hemodynamic pattern ($P < 0.05$). Well-differentiated HCC showed hemodynamic patterns of types A and B with significantly high frequency, and that of type C with significantly less frequency. Moderately differentiated HCC showed the hemodynamic pattern of type A significantly less frequently, and that of type C with significantly high frequency. Poorly differentiated HCC showed the hemodynamic pattern of type C with significantly high frequency (Table 1).

Correlations between histological grade and signal intensity in the hepatobiliary phase

There was a significant correlation between histological grade and signal intensity in the hepatobiliary phase ($P < 0.05$). Dysplastic nodules showed isointensity with significantly high frequency and hypointensity with significantly less frequency. Moderately differentiated HCC showed significantly less isointensity frequency (Table 2).

Correlation between histological grade and signal intensity in the hepatobiliary phase in each hemodynamic pattern

Type A lesions: There was a significant correlation between histological grade and signal intensity in the hepatobiliary phase ($P < 0.05$). Dysplastic nodules showed isointensity with significantly high frequency and lower hypointensity (Table 3).

Type B and C lesions: There was no significant correlation between histological grade and signal intensity in the hepatobiliary phase in either hemodynamic pat-

Table 2 Correlation between histological grade and signal intensity in the hepatobiliary phase

		Hepatobiliary phase			Total
		Hyper.	Iso.	Hypo.	
Histological grade	DN	0	2[2]	1[1]	3
	Well	1	3	28	32
	Mod.	1	0[1]	35	36
	Poor	0	0	11	11
Total		2	5	75	82

DN: Dysplastic nodule; Well: Well differentiated hepatocellular carcinoma (HCC); Mod: Moderately differentiated HCC; Poor: Poorly differentiated HCC. Hyper: Hyperintensity; Iso: Isointensity; Hypo: Hypointensity. [1]Significantly lower frequency; [2]Significantly higher frequency.

Table 3 Histological grade and signal intensity in the hepatobiliary phase in lesions which maintained portal blood flow

		Hepatobiliary phase			Total
		Hyperintensity	Isointensity	Hypointensity	
Histological grade	DN	0	2[b]	0[a]	2
	Well	1	0	15	16
	Mod.	0	0	7	7
	Poor	0	0	1	1
Total		1	2	23	26

DN: Dysplastic nodule; Well: Well differentiated hepatocellular carcinoma (HCC); Mod: Moderately differentiated HCC; Poor: Poorly differentiated HCC. [a]Significantly lower frequency, $P < 0.05$; [b]Significantly higher frequency, $P < 0.05$.

Table 4 Histological grade and signal intensity in the hepatobiliary phase in lesions with decreased portal blood flow

		Hepatobiliary phase		Total
		Isointensity	Hypointensity	
Histological grade	DN	0	1	1
	Well	1	7	8
	Mod.	0	3	3
	Poor	0	0	0
Total		1	11	12

DN: Dysplastic nodule; Well: Well differentiated hepatocellular carcinoma (HCC); Mod: Moderately differentiated HCC; Poor: Poorly differentiated HCC.

tern ($P > 0.05$) (Tables 4, 5).

Correlation between signal intensity on pre-contrast T1WI, T2WI and hepatobiliary phase

There was a significant correlation between the signal intensity of the T1-weighted in-phase image and that of the hepatobiliary phase ($P < 0.05$). The all isointense lesions in the hepatobiliary phase showed hyperintensity on T1-weighted in-phase imaging with significantly high frequency. The hyperintense lesions on T1-weighted in-phase imaging showed significantly less frequent hypointensity in the hepatobiliary phase. There were no significant correlations between the signal intensity of T1-weighted opposed-phase imaging and T2WI, or that of the hepatobiliary phase ($P > 0.05$) (Table 6).

Table 5 Histological grade and signal intensity in the hepatobiliary phase in lesions which lacked portal blood flow

		Hepatobiliary phase			Total
		Hyperintensity	Isointensity	Hypointensity	
Histological grade	DN	0	0	0	0
	Well	0	2	6	8
	Mod.	1	0	25	26
	Poor	0	0	10	10
Total		1	2	41	44

DN: Dysplastic nodule; Well: Well differentiated hepatocellular carcinoma (HCC); Mod: Moderately differentiated HCC; Poor: Poorly differentiated HCC.

Table 6 Correlation of signal intensity on pre-contrast T1WI, T2WI and hepatobiliary phase

		Hepatobiliary phase			Total
		Hyperintensity	Isointensity	Hypointensity	
T1WI In-phase	Hyperintensity	1	5[b]	21[a]	27
	Isointensity	0	0	27	27
	Hypointensity	1	0	27	28
T1WI Opposed phase	Hyperintensity	1	2	17	20
	Isointensity	0	3	23	26
	Hypointensity	1	0	35	36
T2WI	Hyperintensity	1	1	42	44
	Isointensity	0	4	27	31
	Hypointensity	1	0	6	7

[a]Significantly lower frequency, $P < 0.05$; [b]Significantly higher frequency, $P < 0.05$.

DISCUSSION

According to previous angiography-assisted CT studies, it is still unclear whether lesions that maintain portal blood flow are dysplastic nodules[13] or well-differentiated HCC[5,14]. In the present study, the lesions that maintained portal blood flow included dysplastic nodules and various types of differentiated HCC. Furthermore, well-differentiated HCC had a significantly high rate of maintenance of portal blood flow. A similar result was previously reported[14], indicating the presence of a high-grade malignant lesion within a small lesion that maintained portal blood flow.

Small HCC up to 1.5 cm in diameter and with an indistinct margin are called early HCC, and their malignant potential is relatively low[2,3,15,16]. The lesions are characterized by a high prevalence of maintained portal blood flow, infrequent intrahepatic metastasis or portal invasion, and generally consist of well-differentiated HCC. This entity is hard to detect and distinguish from dysplastic nodules because of the similarity of radiological findings[5,14,17,18], but it is clinically important to distinguish between these two entities. In the present study, isointense lesions in the hepatobiliary phase were dysplastic nodules or well differentiated HCC. Furthermore, among lesions maintaining portal blood flow, all isointense lesions were dysplastic nodules. However, hypointense lesions all appeared malignant. This finding may indicate that hypointense lesions, in which portal blood flow is maintained, are hepatocellular carcinoma.

Therefore, a combination of angiography-assisted CT and gadoxetic acid-enhanced MRI should improve the accuracy of the diagnosis of hepatocellular lesions.

In the present study, signal intensity in the hepatobiliary phase significantly correlated with histological grade. Dysplastic nodules showed isointensity with significantly high frequency. However, some well differentiated HCC also showed isointensity. In the present study, some hypervascular well differentiated HCC showed isointensity while portal blood flow-maintained well differentiated HCC, with less malignant potential, showed hypointensity. In general, distinguishing between dysplastic nodules and well differentiated HCC in which portal blood flow is maintained is difficult. Dysplastic nodules appeared as hypo- or iso-vascular[5,19,20]. Therefore the evaluation of tumor vascularity is important in distinguishing dysplastic nodules and hypervascular well differentiated HCC. The arterial phase is identifiable on gadoxetic acid, and we consider that elucidating the optimal arterial phase for imaging is essential.

Some HCC showed hyperintensity in the hepatobiliary phase. This finding has been reported previously and appeared in well or moderately differentiated HCC[8,11]. Narita *et al*[8] reported that uptake of gadoxetic acid in HCC was determined by expression of the organic anion transporter 1B3 (OATP1B3). Therefore the degree of expression of OATP1B3 may influence signal intensity in HCC. This is partly because isointense lesions appeared in the hepatobiliary phase. Therefore, we speculated that one of the reasons why isointense lesions in the hepatobiliary phase appeared was due to the expression of OATP1B3, and the pathological appearance was extremely similar to the surrounding liver parenchyma.

All isointense lesions in the hepatobiliary phase were detected on MRI and showed hyperintensity in T1-weighted in-phase images in the present study. Precontrast MRI sequencing has been reported to be able to detect dysplastic nodules and well differentiated HCC[21]. However, dysplastic nodules and well differentiated HCC frequently show hyperintensity[22,23] on T1WI, and the present study emphasized the significance of T1-weighted in-phase images to detect these lesions.

The present study has several limitations. Most nodules were diagnosed by biopsy, and therefore, the classification of the histological grade of differentiation was judged using only the biopsied part of the lesions. Second, it is difficult to differentiate dysplastic nodules from early HCC using biopsy[3] and there were few dysplastic nodules in the present study. Therefore further study is required.

In conclusion, signal intensity in the hepatobiliary phase on gadoxetic acid-enhanced MRI was correlated with histological grade in the lesions that maintained portal blood flow, but did not correlate in the lesions with decreased or no portal blood flow. These findings suggest that lesions in which portal blood flow is maintained, and which appear hypointense in the hepatobiliary phase on gadoxetic acid-enhanced MRI, are most likely to be HCC. These results indicate that gadoxetic acid-enhanced MRI can potentially be a powerful observation tool for HCC. These early-stage malignant lesions should be more easily detected, and enable appropriate work-up. This in turn should lead to improved clinical results of treatment.

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COMMENTS

Background

Dysplastic nodules and some well differentiated hepatocellular carcinoma (HCC) lesions maintain portal blood flow, making differential diagnoses difficult. Gadoxetic acid-enhanced magnetic resonance imaging (MRI) yields a high tumor detection rate and can detect lesions that maintain portal blood flow using angiography-assisted computed tomography (CT).

Research frontiers

According to previous angiography-assisted CT studies, it is still unclear whether lesions that maintain portal blood flow are dysplastic nodules or well differentiated HCC. In this study, the authors demonstrate relationships among angiography-assisted CT, hepatobiliary phase findings during gadoxetic acid-enhanced MRI and histological grades.

Innovation and breakthrough

According to the present study, gadoxetic acid-enhanced MRI should improve the accuracy of the diagnosis of hepatocellular lesions.

Applications

The lesions in which portal blood flow is maintained, and which appear hypointense in the hepatobiliary phase on gadoxetic acid-enhanced MRI, have a greater probability of being HCC. Gadoxetic acid-enhanced MRI can potentially be a powerful observation tool for HCC.

Terminology

Gadolinium-ethoxybenzyl diethylenetriaminepentaacetic acid (gadoxetic acid) is a liver-specific contrast medium for magnetic resonance imaging which is taken into hepatocytes and excreted into bile, producing a T1-shortening effect in liver parenchyma. In the hepatobiliary phase, the tumor does not have normal functioning hepatocytes and is hypointense in most cases.

Peer review

This is a comparison with the pathological and radiological findings of dysplastic liver nodules and HCC. They found a close correlation with the histological grade and the intensity of the radiological views.

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BRIEF ARTICLE

Prediction of nephrotoxicity induced by cisplatin combination chemotherapy in gastric cancer patients

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Abstract

AIM: To evaluate the treatment options for nephrotoxicity due to cisplatin combination chemotherapy.

METHODS: We retrospectively reviewed patients who had received cisplatin combination chemotherapy for gastric cancer between January 2002 and December 2008. We investigated patients who had shown acute renal failure (ARF), and examined their clinical characteristics, laboratory data, use of preventive measures, treatment cycles, the amount of cisplatin administered, recovery period, subsequent treatments, and renal status between the recovered and unrecovered groups.

RESULTS: Forty-one of the 552 patients had serum creatinine (SCR) levels greater than 1.5 mg/dL. We found that pre-ARF SCR, ARF SCR, and ARF glomerular filtration rates were significantly associated with renal status post-ARF between the two groups (P = 0.008, 0.026, 0.026, respectively). On the receiver operating characteristic curve of these values, a 1.75 mg/dL ARF SCR value had 87.5% sensitivity and 84.8% specificity (P = 0.011).

CONCLUSION: Cessation or reduction of chemotherapy should be considered for patients who have an elevation of SCR levels during cisplatin combination chemotherapy.

Key words: Acute renal failure; Cisplatin; Drug toxicities; Nephrotoxicity

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INTRODUCTION

Cisplatin is one of the most commonly used antineoplastic agents for the treatment of solid tumors[1,2]. It is generally used in combination with fluorouracil, docetaxel, paclitaxel, capecitabine or irinotecan for the treatment of gastric cancer[3]. However, cisplatin can induce severe side-effects such as bone-marrow suppression, gastrointestinal toxicity, nephrotoxicity, ototoxicity, and neuropathy. Of these, nephrotoxicity is the major side effect and main obstacle in the therapeutic use of cisplatin[1,4].

Many studies have attempted to determine the pathogenesis of nephrotoxicity caused by cisplatin in order to prevent and reduce patient symptoms. However, prevention cannot be achieved by the traditional manner of decreasing drug dosage, performing specific hydration procedures, and actively screening for renal abnormalities[5,6]. In fact, there are currently no unified recom-

mendations for the treatment of nephrotoxicity. In this study, patients who displayed nephrotoxicity induced by a cisplatin combination regimen for gastric cancer were retrospectively reviewed. The aim of this study was to determine the appropriate therapeutic steps when nephrotoxicity occurs due to cisplatin combination chemotherapy.

MATERIALS AND METHODS

Patient population

We retrospectively examined 552 patients who were diagnosed with gastric cancer, and who received cisplatin combination chemotherapy between January 2002 and December 2008 at the Kosin University Gospel Hospital. Of these patients, 41 who developed nephrotoxicity induced by cisplatin combination chemotherapy were chosen for further analysis; a serum creatinine (SCR) level of 1.5 mg/dL was used as the threshold for nephrotoxicity. Patients were excluded if they had renal disease, hydronephrosis, severe dehydration, SCR > 1.5 mg/dL before the administration of cisplatin, or lack of follow-up care.

Division of patients into recovered and unrecovered groups

Forty-one patients were reviewed in terms of gender, age, body surface area (BSA), combined chemotherapy drugs, stage of gastric cancer, hemoglobin levels, hematocrit, total protein, albumin, electrolytes, blood urea nitrogen, SCR, glomerular filtration rate (GFR), magnesium, phosphate and calcium levels, use of mannitol, furosemide and amifostine, amount of hydration, dose of cisplatin/cycle × BSA, cumulative dose of cisplatin/BSA, recovery period, and course of acute renal failure (ARF). Laboratory data were checked immediately before the administration of chemotherapy drugs; SCR levels greater than 1.5 mg/dL were used as pre-ARF laboratory data. Laboratory data were also collected at peak SCR values after SCR levels increased to greater than 1.5 mg/dL at the time of ARF. Patients were divided into two groups (recovered and unrecovered) according to their post-ARF renal status. The recovered patients were those whose SCRs decreased to less than 1.5 mg/dL after ARF; the unrecovered patients were those whose SCRs were maintained at levels greater than 1.5 mg/dL after ARF. The two groups were compared in terms of the above-mentioned characteristics, before and after collection of the ARF laboratory data, use of protective measures, dose of cisplatin, recovery period, and the course of recovery. With these results, the predictive values for post-ARF renal status were examined. Also, in each group, the relationship between treatment and subsequent renal status in response to treatment was examined. The treatments were then divided into the categories of stop, reduce and continue. The subsequent renal status in response to these treatments was divided into the normal, recovered and unrecovered groups. Normal patients were those whose SCRs did not increase to levels greater than 1.5 mg/dL; definition of the recovered and the unrecovered groups is the same as previously noted. Data collection ceased in June, 2009.

Table 1 Characteristics of 41 patients who developed nephrotoxicity induced by cisplatin combination chemotherapy

Gender	M	36
	F	5
Age	58.36 ± 10.54	
BSA[1]	1.677 ± 0.141	
Operation	None	3
	RSG c B I	15
	RSG c B II	5
	RSG c R-Y	7
	RTG c R-Y	5
	Pal[2]	6
Stage	I A	1
	I B	5
	II	5
	III A	9
	III B	2
	IV	19
Combination drug	5-FU[3]	18
	Docetaxel	10
	5-FU[3] + MMC[4]	5
	Paclitaxel	3
	TS-1	2
	Irinotecan	2
	Capecitabine	1

[1]Body surface area; [2]Palliative operation including gastrojejuno bypass, open and closure; [3]5-flourouracil; [4]Mitomycin.

Statistical analysis

Statistical analysis was performed using SPSS Statistics 17.0 for Windows. We collected the laboratory data, which were checked immediately before the SCR increased to > 1.5 mg/dL, for use as the pre-ARF laboratory data. Laboratory data were also checked at peak SCR values after levels increased to > 1.5 mg/dL at the time of ARF. The data on administration of the anticancer drug were reported in number and percentage with some overlap. Other data were reported as mean and standard deviation, and compared using the unpaired Student's *t* test. The predictive value of the post-ARF renal status was examined by receiver operating characteristic (ROC) analysis. The $\chi 2$ test was used to examine the relationship between treatment and subsequent renal status in response to treatment. *P* values less than 0.05 were considered statistically significant.

RESULTS

Patient characteristics

Five hundred and fifty-two patients were diagnosed with gastric cancer and received cisplatin combination chemotherapy between January 2002 and December 2008. The patients received several different cisplatin combination drugs, including 5-flourouracil (5-FU) in 193 patients (34.96%), docetaxel in 113 (20.47%), TS-1 in 86 (15.58%), paclitaxel in 71 (12.86%), capecitabine in 30 (5.43%), irinotecan in 29 (5.25%), mitomycin in 23 (4.17%), and

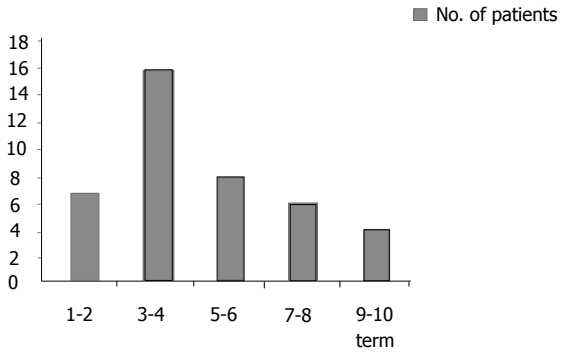

Figure 1 The term during which nephrotoxicity occurred due to cisplatin combination chemotherapy.

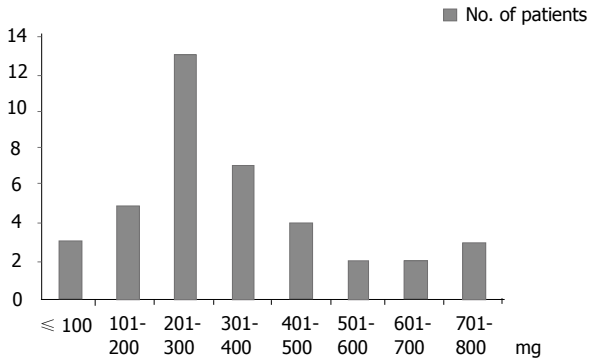

Figure 2 The cumulative dose of cisplatin/body surface area at which nephrotoxicity occurred due to cisplatin combination chemotherapy.

Table 2 Pre-acute renal failure (ARF) laboratory data[1] corresponding to renal status post-ARF[2]

			Renal status of post-ARF		*P* value[3]
Variable	Normal range	Unit	Recovered (*n* = 33)	Unrecovered (*n* = 8)	
Hb	14.0–16.7	g/dL	10.33 ± 1.28 (*n* = 33)	9.75 ± 0.95 (*n* = 8)	0.238
HT	14.7–50.7	%	29.88 ± 3.33 (*n* = 33)	28.56 ± 2.78 (*n* = 8)	0.307
Protein	6.3–8.3	g/dL	6.792 ± 0.69 (*n* = 26)	6.429 ± 0.39 (*n* = 7)	0.191
Albumin	3.5–5.0	g/dL	3.97 ± 0.54 (*n* = 26)	3.87 ± 0.38 (*n* = 7)	0.657
BUN	5–23	mg/dL	18.07 ± 5.35 (*n* = 33)	15.63 ± 4.03 (*n* = 8)	0.236
SCR	0.3-1.5	mg/dL	1.17 ± 0.20 (*n* = 33)	1.38 ± 0.13 (*n* = 8)	0.008
GFR	120-130	mL/min	68.03 ± 13.31 (*n* = 33)	59.13 ± 6.64 (*n* = 8)	0.076
Na	136–150	meg/L	139.24 ± 3.19 (*n* = 33)	140.13 ± 4.09 (*n* = 8)	0.510
Cl	98–110	meg/L	105.31 ± 4.42 (*n* = 24)	105.57 ± 3.65 (*n* = 7)	0.887
K	3.5–5.3	meg/L	4.49 ± 0.46 (*n* = 8)	4.66 ± 0.65 (*n* = 8)	0.284
P	3.0–4.5	mg/dL	3.97 ± 0.81 (*n* = 20)	3.93 ± 1.16 (*n* = 6)	0.940
Mg	1.6–2.6	mg/dL	2.08 ± 0.26 (*n* = 24)	2.03 ± 0.29 (*n* = 7)	0.635
Ca	8.0-10.0	mg/dL	9.15 ± 0.53 (*n* = 24)	8.86 ± 0.30 (*n* = 7)	0.170

[1]Pre-acute renal failure (ARF) Laboratory data were checked immediately before the administration of the chemotherapy drug that caused the serum creatinine (SCR) to increase to > 1.5 mg/dL. [2]We divided 41 patients into two groups; the recovered group included patients whose SCRs had decreased below 1.5 mg/dL after ARF, the unrecovered group included patients whose SCRs remained greater than 1.5 mg/dL. [3]Unpaired Student's *t*–test. BUN: Blood urea nitrogen; GFR: Glomerular filtration rate.

others in 4 patients (0.72%), with some overlap. In our investigation, 5-FU was the most frequently used anticancer drug in combination with cisplatin for gastric cancer chemotherapy. Table 1 lists the characteristics of the 41 patients who had an SCR > 1.5 mg/dL after receiving cisplatin combination chemotherapy for gastric cancer. There were 36 males and 5 females, with an average age of 58.36 years, and an average BSA of 1.677. 5-FU made up the largest proportion of the combined drug regimens (18 patients, 43.9%), and there were more stage Ⅳ patients than any other stage classification (19 patients, 46.3%).

The chemotherapy cycle during which nephrotoxicity occurred

Of 41 patients, nephrotoxicity occurred more frequently during the 3rd-4th cycle (16 patients), and 7 patients experienced nephrotoxicity during the 1st-2nd cycle. The most common cumulative dose of cisplatin/BSA at which nephrotoxicity occurred was 200-300 mg, while the second most common cumulative dose was 300-400 mg, and these were correlated with the greatest number of cycles and the dose of cisplatin/cycle × BSA (Figures 1, 2).

The recovery period for nephrotoxicity induced by cisplatin combination chemotherapy

The average length of recovery time among the patients was 15 d, and was less than 7 d for 27 patients, 8-14 d for 1 patient, 15-30 d for 2 patients, and more than 30 d for 3 patients. These results showed that approximately 70% of recovered patients reached this state within 2 wk.

Comparison between recovered and unrecovered patients

Patients were divided into two groups based on their post-ARF renal status: the recovered patients and the unrecovered patients. The average age of patients in the unrecovered group (51.88 ± 6.01) was lower than that of the recovered group (59.94 ± 10.86), with a *P* value ($P = 0.051$) near 0.05. In the analysis of the laboratory data (Tables 2, 3), the pre-ARF SCR, ARF SCR, and ARF GFR were significantly associated with the renal status post-ARF ($P = 0.008$, 0.026, 0.026, respectively). The ROC curve was constructed using these values (Figure 3). On the ROC curve, an ARF SCR value of 1.75 mg/dL

Table 3 Acute renal failure (ARF) laboratory data[1] corresponding to renal status post-ARF[2]

Variable	Normal range	Unit	Renal status post-ARF		*P* value[3]
			Recovered (*n* = 33)	Unrecovered (*n* = 8)	
Hb	14.0–16.7	g/dL	10.28 ± 1.62 (*n* = 31)	10.238 ± 1.30 (*n* = 8)	0.941
HT	14.7–50.7	%	29.52 ± 4.70 (*n* = 32)	29.93 ± 3.41 (*n* = 8)	0.820
Protein	6.3–8.3	g/dL	6.72 ± 0.78 (*n* = 24)	7.00 ± 0.95 (*n* = 3)	0.564
Albumin	3.5–5.0	g/dL	3.95 ± 0.61 (*n* = 24)	4.03 ± 0.65 (*n* = 3)	0.818
BUN	5–23	mg/dL	23.78 ± 13.60 (*n* = 33)	23.0 ± 4.87 (*n* = 8)	0.876
SCR	0.3-1.5	mg/dL	1.75 ± 0.48 (*n* = 33)	2.21 ± 0.61 (*n* = 8)	0.026
GFR	120-130	mL/min	43.30 ± 7.81 (*n* = 33)	36.0 ± 8.98 (*n* = 8)	0.026
Na	136–150	meg/L	136.60 ± 4.36 (*n* = 32)	137.63 ± 2.72 (*n* = 8)	0.529
Cl	98–110	meg/L	101.49 ± 6.59 (*n* = 24)	99.50 ± 2.65 (*n* = 4)	0.564
K	3.5–5.3	meg/L	4.22 ± 0.93 (*n* = 32)	4.46 ± 0.60 (*n* = 8)	0.491
P	3.0–4.5	mg/dL	4.13 ± 0.87 (*n* = 18)	3.93 ± 0.43 (*n* = 4)	0.659
Mg	1.6–2.6	mg/dL	1.83 ± 0.42 (*n* = 22)	1.833 ± 0.47 (*n* = 3)	0.995
Ca	8.0-10.0	mg/dL	9.06 ± 0.51 (*n* = 23)	9.40 ± 0.80 (*n* = 4)	0.269

[1]Laboratory data were checked at the time at which the value of serum creatinine (SCR) was highest after increasing the SCR > 1.5 mg/dL. [2]We divided 41 patients into two groups; recovered consisted of patients whose SCRs had decreased below 1.5 mg/dL after acute renal failure, unrecovered consisted of patients whose SCRs remained greater than 1.5 mg/dL. [3]Unpaired Student's *t*-test. BUN: Blood urea nitrogen; GFR: Glomerular filtration rate.

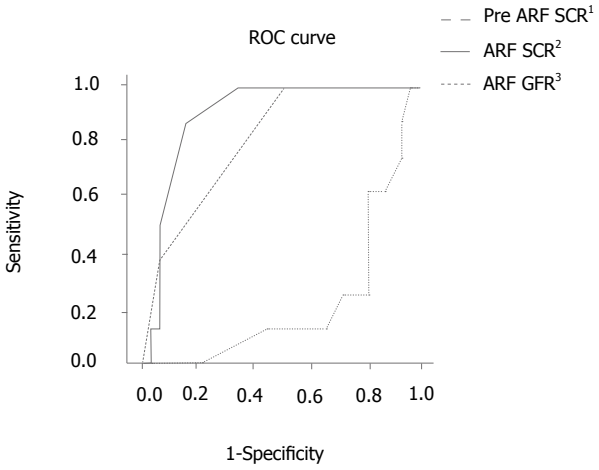

Figure 3 Receiver operating characteristic curves of pre acute renal failure serum creatinines and acute renal failure glomerular filtration rate. [1]Pre-acute renal failure serum creatinine (Pre-ARF SCRs) were checked immediately before the administration of the chemotherapy drug that caused the SCR to increase to > 1.5 mg/dL; [2]ARF SCRs were checked at the time at which the value of SCR was highest after increasing the SCR > 1.5 mg/dL; 3ARF glomerular filtration rate (GFRs) were checked at the time at which the value of SCR was highest after increasing the SCR > 1.5 mg/dL.

showed 87.5% sensitivity and 84.8% specificity. The use of amifostine, mannitol, and furosemide was not significantly different between the two groups ($P = 0.203$, $P = 0.587$, $P = 0.542$, respectively), as nearly all the patients who were followed-up, received a routine formula of hydration and diuretics. The time during which nephrotoxicity occurred and the cumulative dose of cisplatin in each group was assessed and compared (Table 4). The time during which nephrotoxicity occurred was greater in the unrecovered group than in the recovered group (6.63 cycles ± 2.62 cycles *vs* 4.24 cycles ± 2.09 cycles, respectively, $P = 0.009$), and the cumulative dose of cisplatin/BSA was also significantly greater in the unrecovered group compared to the recovered group (497.75 ± 222.61 *vs* 302.85 ± 152.73, respectively, $P = 0.005$).

Table 4 Comparison between the cycle during which nephrotoxicity occurred and the amount of accumulated cisplatin according to renal status post-acute renal failure (ARF)

	Renal status post-ARF		*P* value[3]
	Recovered[1] (*n* = 33)	Unrecovered[2] (*n* = 8)	
The cycle nephrotoxicity occurred	4.24 ± 2.09	6.63 ± 2.62	0.009
The cumulative dose of cisplatin/BSA, mg	302.85 ± 152.73	497.75 ± 222.61	0.005

[1]Recovered were patients whose serum creatinine (SCR)s had decreased below 1.5 mg/dL after acute renal failure. [2]Unrecovered were patients whose SCRs remained greater than 1.5 mg/dL. [3]Unpaired Student's *t*-test. ARF: Acute renal failure; BSA: Body surface area.

Relationship between treatment and renal status after ARF

In the recovered group, the relationship between treatment and renal status following ARF was examined. Table 5 shows that more recovered patients were present in the group that stopped therapy; their SCRs returned to normal. Meanwhile, there were more unrecovered patients in the group that continued treatment; their SCRs remained above 1.5 mg/dL. The relationship between treatment and renal status was significant ($P = 0.011$). Seven normal and recovered patients stopped treatment, including two patients who changed their chemotherapy regimens, two patients who ceased chemotherapy due to metastasis to other organs, two patients who ceased chemotherapy due to poor quality of life (weight loss, anorexia), and one patient who terminated cisplatin in their combination regimen.

The relationship between subsequent treatment and renal status was also examined in unrecovered patients, but it was not statistically significant (Table 6). Two patients stopped receiving cisplatin combination chemotherapy and were switched to another regimen. As a result, their SCRs returned to values less than 1.5 mg/dL after 150 and 181 d, respectively.

Table 5 Subsequent renal status corresponding to subsequent treatment in the recovered group

		Subsequent renal status				*P* value[4]
		Normal[1]	Recovered[2]	Unrecovered[3]	Total	
Subsequent treatment	Stop	5	2	0	7	
	Reduce	2	4	0	6	
	Continue	5	8	7	20	
	Total	12	14	7	33	
						0.011

[1]Normal were patients whose serum creatinine (SCR)s had not increased greater than 1.5 mg/dL after subsequent treatment. [2]Recovered were patients whose SCRs had decreased below 1.5 mg/dL after acute renal failure. [3]Unrecovered were patients whose SCRs remained greater than 1.5 mg/dL. [4]Linear by linear association.

Table 6 Subsequent renal status corresponding to subsequent treatment in the unrecovered group

		Subsequent renal status				*P* value[4]
		Normal[1]	Recovered[2]	Unrecovered[3]	Total	
Subsequent treatment	Stop	0	2	2	4	
	Reduce	0	0	3	3	
	Continue	0	0	1	1	
	Total	0	2	6	8	
						0.170

[1]Normal were patients whose serum creatinine (SCR)s had not increased greater than 1.5 mg/dL after subsequent treatment. [2]Recovered were patients whose SCRs had decreased below 1.5 mg/dL after acute renal failure. [3]Unrecovered were patients whose SCRs remained greater than 1.5 mg/dL. [4]Linear by linear association.

DISCUSSION

Nephrotoxicity induced by cisplatin

Cisplatin is the single most active antitumor agent in the treatment of solid tumors, including gastric cancer. Nevertheless, the use of cisplatin has been restricted because of its side effects, especially nephrotoxicity[1,2]. It has been reported that approximately 25% of patients who received a single dose of cisplatin developed reversible azotemia[7]. In addition, irreversible renal failure can occur when large doses are administered, or with repeated cycles of treatment[8]. In this study, the incidence of nephrotoxicity due to cisplatin combination chemotherapy was 7.43% (41/552). Since patients who had an SCR > 1.5 mg/dL as a measure of nephrotoxicity were selected, these results probably underestimated the incidence of nephrotoxicity. In this study, 5-FU was the most frequently used anticancer drug combined with cisplatin for gastric cancer chemotherapy; the 5-FU/cisplatin regimen is also the most traditional adjuvant chemotherapy for gastric cancer in South Korea.

Criteria for nephrotoxicity

Nephrotoxicity is evaluated by GFR and creatinine clearance values using the Modification of Diet in Renal Disease (MDRD) formula or the Cockcroft and Gault formula, as well as SCR values[9-11]. Only SCR was used for the selection of patients with nephrotoxicity, although the use of a single cutoff to define an elevated SCR is not appropriate[12,13]. The National Kidney Foundation (NKF) recommended that clinicians should not use serum creatinine concentration as the sole means of assessing the level of kidney function[14]. The Renal Insufficiency and Cancer Medications study group suggested that renal function should be evaluated in all cancer patients, including those with normal SCR levels, using either the Cockcroft-Gault formula or the MDRD formula[15]. In this context, the definition of nephrotoxicity in this study as > 1.5 mg/dL is a limitation. In 41 patients, the averages of the pre-nephrotoxic ARF SCR and GFR using MDRD were 1.21 mg/dL ± 0.20 mg/dL and 66.29 mg/dL ± 12.74 mL/min, respectively. Thus, their renal status prior to ARF was already stage 2 according to the clinical guidelines published by the Working Group of the NKF. However, in the case of ARF or acute renal injury (AKI), SCR can be used as a criterion for the definition of ARF or AKI[13,14]. RIFLE and AKIN defined an increase in SCR > 1.5 fold from baseline as a risk or stage 1[16,17]. In this methodology, the SCR can be used as one of the predictive values for renal status after a nephrotoxic event.

The purpose of this study was not to detect and evaluate renal toxicity due to cisplatin. Rather, this study was focused on choosing the appropriate next step after nephrotoxicity occurs due to cisplatin combination chemotherapy. Our data showed that the pre-ARF SCR, ARF SCR, and ARF GFR values were significantly associated with renal status post-ARF (P = 0.008, 0.026, 0.026, respectively). When the ROC curves of these values were assessed, an ARF SCR of 1.75 mg/dL showed 87.5% sensitivity and 84.8% specificity (Figure 3). This indicated that if a patient with nephrotoxicity experiences an SCR > 1.75 mg/dL, then that patient's renal status can progress to severe renal failure. Thus, an ARF SCR value of 1.75 mg/dL can be considered as a predictive measure for renal status post-ARF.

The mechanism of nephrotoxicity

Cisplatin accumulates in the kidneys, and the nephrotoxic effect of cisplatin is proportional to the amount of drug accumulated[3,5,18]. It is known that cisplatin accumulates in the mitochondrial DNA more than in the nucleus or other organelles[2,6]. In a rodent study, the mitochondrial DNA decreased by up to 63% 3–4 d after cisplatin injection[19,20]. Thus, repetitive cisplatin administration lowers the GFR in a dose-related manner[2,21]. In this respect, the dose-related toxicity of cisplatin correlated with the results from this study in terms of the number of cycles before nephrotoxicity occurred. Moreover, the cumulative dose of cisplatin/BSA was greater in the unrecovered group compared to the recovered group (Table 4), suggesting that the earlier nephrotoxicity occurs and the lower the cumulative dose of cisplatin combination chemotherapy, the more quickly the patient will recover (Table 4).

Figures 1 and 2 show that most of the nephrotoxicity occurred in the 3rd-4th cycles of treatment, and the

most common cumulative dose of cisplatin/BSA was 200-300 mg. However, in seven patients, nephrotoxicity occurred in the 1st-2nd cycle. Thus, it appears that the threshold of nephrotoxicity varies according to the individual. Furthermore, renal function should be evaluated, and chemotherapy must be carefully considered before administering cisplatin combination chemotherapy[15,22].

Subsequent chemotherapy and renal status

Upon analysis of the relationship between chemotherapy and renal status in the recovery group, it was found that continuing chemotherapy imparts an increased risk of severe renal failure, compared to ceasing treatment or decreasing the dosage of cisplatin combination chemotherapy (Table 5, $P = 0.011$). In the unrecovered group, all of the cases in which chemotherapy was not stopped remained unrecovered according to their renal status. There were only two patients who stopped receiving cisplatin combination chemotherapy and began another regimen. Their SCRs returned to values less than 1.5 mg/dL, although their recovery took a long time; 150 and 181 d, respectively. Therefore, if a nephrotoxic patient's SCR is > 1.5 mg/dL, chemotherapy should be stopped, the drug dosage should be reduced, or the regimen should be changed.

Other side effects of cisplatin

A common complication resulting from cisplatin treatment is electrolyte wasting, or hypomagnesemia[23,24]. The laboratory data of all the patients in this study were not checked routinely as this was not a prospective study. However, hyponatremia, hypokalemia, hypocalcemia, and hypomagnesemia were found in some patients (Tables 2, 3). Electrolyte imbalances are common in these types of patients, but are not severe[18]. Severe electrolyte imbalance can induce ototoxicity and neurotoxicity, or it can aggravate nephrotoxicity. Such conditions should be corrected by supplementation[18,25,26].

Protective measures

The most commonly used protective measure against renal toxicity is to establish solute diuresis[18,27]. Nearly all of the patients received a routine formula of hydration and diuretics which included hydrations of 1-2 L before and after administration of chemotherapy, diuretics after hydration, and sometimes amifostine. Despite the many recent physiopathological advances in the understanding of the mechanism of anticancer drug nephrotoxicity, especially that of cisplatin, prevention still relies on decreases in drug dosage, hydration measures, and active screening for renal abnormalities as part of the usual pre-therapeutic biological work-up in patients treated with anticancer drugs[6,18]. The European Society of Clinical Pharmacy Special Interest Group on Cancer Care suggested that hydration should be maintained for at least 3 d after the chemotherapy course, and by IV or oral route when feasible[6]. However, there are no specific recommendations or convincing data on the renal protective effect of cisplatin administration in fractionated doses[28].

Combined nephrotoxic drugs

In this study, the nephrotoxicity of combined anticancer drugs was not considered. Mitomycin is known to have renal toxicity. In fact, it has been reported that the onset of renal insufficiency induced by mitomycin administration occurs after an average time of 10-11 mo[29]. However, since the kidney is not a major route of mitomycin excretion, it is not suggested that the dose be adjusted in patients with renal insufficiency[29]. Paclitaxel and irinotecan are also known to cause potential nephrotoxicity, but the need for dosage adjustment has not been confirmed. A comparative prospective study of renal toxicity induced by combined drugs is needed[29,30]. This study has several limitations. Nevertheless, we believe that this issue is important and worthy of further prospective studies.

The author reviewed patients who were diagnosed with gastric cancer, who received cisplatin combination chemotherapy, and who displayed nephrotoxicity. The results show that the patients who experienced a SCR > 1.75 mg/dL after receiving cisplatin combination chemotherapy had a greater risk of chronic renal failure than did patients with a SCR < 1.75 mg/dL. Secondly, in subsequent chemotherapy regimens in patients who experienced SCR > 1.5 mg/dL, the patients who continued cisplatin combination chemotherapy had a greater tendency to experience severe chronic renal disease. Therefore, these results suggest that when a patient experiences a SCR > 1.5 mg/dL after receiving cisplatin combination chemotherapy, the chemotherapy should be stopped, reduced, or the regimen should be changed, and when a patient experiences a SCR > 1.75 mg/dL after receiving cisplatin combination chemotherapy, the chemotherapy should be stopped or changed. More prospective and comparative studies are needed on this subject.

COMMENTS

Background

Cisplatin is one of the most commonly used drugs in the chemotherapy of solid tumors. The major adverse effect of cisplatin is nephrotoxicity, with an incidence of up to 25%. Cisplatin accumulates in the kidneys, and the nephrotoxic effect of cisplatin is proportional to the accumulated drug dose. It is known that cisplatin accumulates in the mitochondrial DNA more than it does in the nucleus or other organelles. Thus, repeated cisplatin administration lowers the glomerular filtration rate (GFR) in a dose-related manner. The aim of this study was to determine the appropriate therapeutic steps when nephrotoxicity occurs due to cisplatin combination chemotherapy in gastric cancer.

Research frontiers

Nephrotoxicity is evaluated by the GFR and creatinine clearance (CrCl) using the Modification of Diet in Renal Disease formula or the Cockcroft and Gault formula, and not only by serum creatinine (SCR). However, in the case of acute renal failure (ARF) or acute renal injury (AKI), SCR can be used as a criterion for the definition of ARF or AKI. The authors suggest that the SCR can be used as one of the predictive values for renal status after a nephrotoxic event. The purpose of this study was not to detect and evaluate the renal toxicity of cisplatin. This study focused on choosing the next step after nephrotoxicity due to cisplatin combination chemotherapy.

Innovations and breakthroughs

Forty-one out of 552 patients, who received cisplatin combination chemotherapy, had SCR levels greater than 1.5 mg/dL. These patients were divided into two groups according to post-ARF renal status, the recovered patients and unrecovered patients. The two groups were compared in terms of the above-mentioned characteristics, before and after ARF laboratory data, use of protective measures, dose of cisplatin, recovery period, and the course of recovery. With these results, the predictive values for the post-ARF renal status were examined. The authors found that pre-AFR SCR, ARF SCR, and ARF GFR were significantly associated with renal status post-ARF in the two groups (P = 0.008, 0.026, 0.026, respectively). In the receiver operating characteristic curve of these values, a 1.75 mg/dL ARF SCR value showed 87.5% sensitivity and 84.8% specificity. This indicated that if a patient with nephrotoxicity experienced an SCR > 1.75 mg/dL, then the patient's renal status can progress to severe renal failure. Thus, an ARF SCR value of 1.75 mg/dL can be considered as a predictive value for renal status post-ARF. In addition, in each group, the relationship between subsequent treatment and renal status in response to treatment was examined. In the recovered group, the relationship between subsequent treatment and renal status following ARF was determined. The results showed that more recovered patients were present in the group who stopped therapy; their SCRs had returned to normal. Meanwhile, in patients who continued treatment, more unrecovered patients whose SCRs were maintained above 1.5 mg/dL were present. The relationship showed a significant difference (P = 0.011). Therefore, if a nephrotoxic patient's SCR is > 1.5 mg/dL, chemotherapy should be stopped, the drug dosage should be reduced, or the regimen should be changed.

Applications

In cisplatin combination chemotherapy in gastric cancer patients, when a patient has experienced a SCR level greater than 1.5 mg/dL, cessation or reduction of chemotherapy should be considered. Furthermore, when a patient experiences a SCR greater than 1.75 mg/dL, chemotherapy should be stopped or changed.

Terminology

The recovered patients consisted of those whose SCRs had decreased to less than 1.5 mg/dL after ARF. The unrecovered patients consisted of those whose SCRs were maintained at greater than 1.5 mg/dL. Subsequent treatment is the next chemotherapy regimen after cisplatin-induced nephrotoxicity, which was divided into stop, reduce and continue. Subsequent renal status is the renal status (recovered or unrecovered) corresponding to subsequent treatment.

Peer review

Despite the many recent physiopathological advances in the understanding of the mechanism of anticancer drug nephrotoxicity, especially that of cisplatin, prevention still relies on a drug dosage decrease, hydration measures, and active screening for renal abnormalities as part of the usual pre-therapeutic biological work-up in patients treated with anticancer drugs. In addition, there are no specific recommendations or convincing data about the renal protective effect of the administration of cisplatin and the subsequent step of nephrotoxicity.

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BRIEF ARTICLE

Stomach cancer screening and preventive behaviors in relatives of gastric cancer patients

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Abstract

AIM: To investigate gastric cancer screening and preventive behaviors among the relatives of patients with gastric cancer [i.e., gastric cancer relatives (GCRs)].

METHODS: We examined the Korean National Health and Nutrition Examination Survey 2005 (KNHANES Ⅲ) database and compared the gastric cancer screening and preventive behaviors of GCRs (n = 261) with those of non-GCRs (n = 454) and controls without a family history of cancer (n = 2842).

RESULTS: The GCRs were more likely to undergo gastric cancer screening compared with the control group (39.2% *vs* 32.3%, adjusted odds ratio: 1.43, CI: 1.05-1.95), although the absolute screening rate was low. Dietary patterns and smoking rates did not differ significantly between the groups, and a high proportion of GCRs reported inappropriate dietary habits (i.e., approximately 95% consumed excessive sodium, 30% were deficient in vitamin C, and 85% were deficient in dietary fiber).

CONCLUSION: The gastric cancer screening and preventive behaviors of GCRs have yet to be improved. To increase awareness among GCRs, systematic family education programs should be implemented.

Key words: Family history of cancer; Cancer relatives; Gastric cancer screening; Preventive behaviors; Cancer prevention

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INTRODUCTION

Gastric cancer is the most common cancer, and the third leading cause of death from cancer, in Korea[1]. It is also the fourth most prevalent cancer in the world[2], although recent trends show stabilization of incidence rates and a continued decrease in cancer death rates[3].

Prevention of gastric cancer can be broadly divided

into primary and secondary prevention. Primary prevention is essentially behavioral modification, which seeks to control the etiological agents of gastric cancer[4]. Several modifiable risk factors contribute to the development of gastric cancer. Infection with *Helicobacter pylori* (*H. pylori*) is a well-established risk factor[5], and the potential of preventing gastric cancer by eradicating *H. pylori* infection has been emphasized in the recent studies[6,7]. Salt intake levels of at least 10 g/d (4000 mg Na) significantly increase the risk of gastric cancer[8]. Fresh fruits and vegetables contain sufficient amounts of vitamin C and dietary fiber, which strongly reduce the risk of gastric cancer[4,9]. A previous study found that subjects in the bottom third of the distribution of vitamin C intake had a 2.5-fold higher risk of developing gastric cancer[10]. Additionally, there is a significant dose-dependent relationship between smoking and gastric cancer[11].

Secondary prevention relies on early detection, which can be achieved through regular cancer screenings[12]. This form of prevention is a priority in Korea, which has one of the highest incidence rates of stomach cancer in the world. The Korean National Cancer Screening Program (KNCSP) recommends that individuals aged 40 years or older undergo biennial gastric cancer screening (Table 1). Although the effect of mass screening remains controversial, it may help by identifying cancer at an early stage[13,14]. According to a study in Korea, the proportion of early gastric cancer (EGC) was 96% in a repeated screening group and 71% in an infrequent screening group, among patients with newly diagnosed gastric cancer[15]. The 5-year survival rate of EGC is greater than 90%[16].

A positive family history of gastric cancer is one of the most important factors, increasing the risk of developing the disease by three-fold[17,18]. There is evidence that there may be a synergistic interaction between family history and *H. pylori* infection in the development of gastric cancer[18]. In addition, patients with a family history tend to have larger or more deeply infiltrated tumors[15]. As many risk factors of gastric cancer are modifiable, it is meaningful to investigate gastric cancer screening and preventive behaviors among high risk groups, such as the relatives of patients with gastric cancer [i.e. gastric cancer relatives (GCRs)] such that early detection and prevention can be achieved. The main purpose of this study is to investigate the current status of gastric cancer screening and preventive behaviors in GCRs.

MATERIALS AND METHODS

Study design

We performed a cross-sectional study of Koreans (n = 3557) who were at least 40 years old, with the aim of investigating the gastric cancer screening rates and preventive behaviors of GCRs compared with those of the general population. To differentiate the impact of family history of gastric cancer from that of other cancers, we studied subjects with a family history of cancer other than gastric cancer [i.e., non-GCRs (NGCRs)] and subjects without a family history of any cancer (controls).

Data source

We analyzed data from the 2005 Korea National Health and Nutrition Examination Survey (KNHANES Ⅲ), which was conducted by the Korea Centers for Disease Control to evaluate the health and nutrition status of the Korean population. The KNHANES Ⅲ categorized the nation into 600 regions at the first stage, selecting 20 households from each region at the second stage. Data collected from the samples were adjusted to represent the entire population of Korea. The questionnaire consisted of four parts: a health interview survey, a health behavior survey, a health examination survey, and a nutrition survey. Information about family histories of cancer were obtained from the health examination survey, cancer screening behaviors and smoking behaviors were assessed using the health behavior survey, and 1 d food intake (i.e. for the last 24 h prior to the survey) was evaluated using the nutrition survey.

Study subjects

The completion rate of the health examination survey in KNHANES Ⅲ was 70.2%. Of the 7597 subjects who responded to the health examination survey, we excluded respondents under the age of 40 years (n = 4008), former and current patients with stomach cancer (n = 23), and those who did not complete questions about their family history (n = 9). Data from the remaining 3557 respondents were analyzed (Figure 1). The following questions from the health examination survey supplement were used to categorize the subjects into three groups: (1) "Has your father, mother, brother or sister ever been clinically diagnosed with any form of cancer?" (responses included "yes" or "no") and (2) "If you responded 'yes', write the type of the cancer." These questions were asked three times to identify exactly which family member, if any, had a history of cancer. According to the answers, respondents were categorized into the following three groups: (1) GCRs; (2) NGCRs; and (3) controls. We defined "cancer family history" as subjects whose parents or siblings had a history of cancer.

We compared the screening patterns for other common cancers (i.e., breast, cervical, and colon cancers) with those of gastric cancer. We excluded subjects with a history of breast, cervical, or colon cancer, respectively. Only females were included in the analysis of breast and cervical cancer. Only subjects 50 years and older were included in the analysis of colon cancer.

Variables

Factors known or thought to affect gastric cancer screening behavior were used as covariates, including socioeconomic factors (e.g., sex, age, education level, marital status, and income), health-related behaviors (e.g., smoking and alcohol consumption), and psychological factors (e.g.,

Table 1 The national cancer screening program in Korea

Cancer	Target population	Frequency	Test or procedure	Co-payment[1] (US $)
Stomach	40 and over (adults)	Every 2 yr	Endoscopy or Upper Gastrointestinal Series	7
Colorectal	50 and over (adults)	Every 1 yr[2]	Fecal Occult Blood Test[3]	0.5
Breast	40 and over (women)	Every 2 yr	Mammography and Clinical breast exam	3.5
Cervix	30 and over (women)	Every 2 yr	Pap smear	0

[1]Co-payments only applied to people with a higher income (i.e. upper 50%), and account for 20% of the total price. No co-payment is applied to the low-income population (i.e., lower 50%). There is no co-payment for cervical cancer screening regardless of income level; [2]Colorectal screening is provided every 2 years to most of the target population, with the exception of low-income people or manual laborers; [3]Colonoscopy or barium enema are performed if the fecal occult blood test is positive.

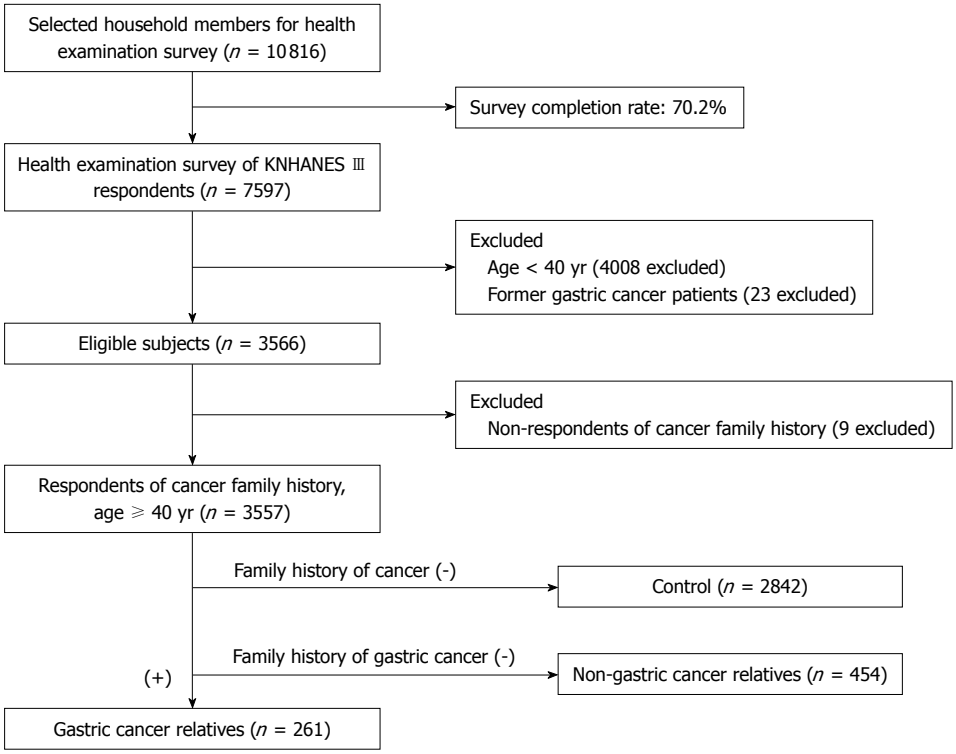

Figure 1 Selection of gastric cancer relatives and controls.

self-reported health status)[19].

Gastric cancer screening behaviors were assessed *via* the question, "When was the last time you were screened for gastric cancer (i.e., gastroscopy or upper gastrointestinal series)?" Responses included, "Less than 1 year ago", "1 year to 2 years ago", "More than 2 years ago" and "Never". In accordance with the KNCSP guidelines, we distinguished screened and unscreened subjects based on whether they had undergone gastric cancer screening within the previous 2 years, and whether they had received a mammography or ultrasonography for breast cancer, a pap smear for cervical cancer, or a colonoscopy or barium enema for colon cancer within the past 2, 2, or 5 years, respectively (Table 1).

The 1 d intakes of sodium, vitamin C, and dietary fiber were calculated using the subjects' responses to the interviewer-administered 24-h dietary recall, a tool that has been used in American surveys because of the accurate and complete self-reported information that it provides[20]. In our analyses, sodium, vitamin C, and dietary fiber were dichotomized according to current dietary recommendations, with a maximum recommended sodium intake of 2000 mg[21], a minimum recommended vitamin C intake of 60 mg[22], and a minimum recommended dietary fiber intake of 20 g[23].

Statistical analysis

The STATA program (version 10.0) was used to analyze the data. The chi-squared test was used to analyze the general characteristics, cancer screening, and preventive behaviors of each group. Adjusted means and adjusted rates of each group were analyzed *via* analysis of covariance, after adjustment for age, sex, education, marital status, smoking status, alcohol consumption, income, and self-reported health status. Crude odds ratios were analyzed *via* simple logistic regression, while adjusted odds ratios (aORs) were analyzed *via* multiple logistic regression, after adjustment for factors that affect gastric cancer screen-

Table 2 Characteristics of gastric cancer relatives and controls *n* (%)

	Controls (*n* = 2842)	Non-gastric cancer relatives (*n* = 454)	Gastric cancer relatives (*n* = 261)	*P*[1]
Sex				
Male	1258 (44.3)	190 (41.9)	105 (40.2)	0.321
Female	1584 (55.7)	264 (58.2)	156 (59.8)	
Age (yr)				
40-49	1024 (36.0)	211 (46.5)	104 (39.9)	< 0.001
50-59	710 (25.0)	118 (26.0)	85 (32.6)	
60-69	665 (23.4)	85 (18.7)	51 (19.5)	
≥ 70	443 (15.6)	40 (8.8)	21 (8.1)	
Education				
Elementary	1136 (40.0)	116 (25.6)	79 (30.3)	< 0.001
Middle to high school	1271 (44.7)	246 (54.2)	146 (55.9)	
University and higher	435 (15.3)	92 (20.3)	36 (13.8)	
Marital status				
Married	2188 (77.0)	387 (85.4)	211 (80.8)	< 0.001
Single	653 (23.0)	66 (14.6)	50 (19.2)	
Smoking status				
Non-smoker	1588 (57.3)	260 (59.8)	152 (59.6)	0.657
Ex-smoker	582 (21.0)	86 (19.8)	45 (17.7)	
Current smoker	602 (21.7)	89 (20.5)	58 (22.8)	
Alcohol drinking				
None	1524 (55.0)	241 (55.4)	145 (56.9)	0.841
More than once a month	1248 (45.0)	194 (44.6)	110 (43.1)	
Income (US $/mo)[2]				
< 1000	801 (28.2)	87 (19.2)	48 (18.4)	< 0.001
1000-5000	1825 (64.2)	315 (69.4)	192 (73.6)	
≥ 5000	216 (7.6)	52 (11.5)	21 (8.1)	
Self-reported health status				
Good	873 (30.8)	149 (33.0)	82 (31.7)	0.502
Intermediate	1017 (35.9)	171 (37.8)	96 (37.1)	
Bad	946 (33.4)	132 (29.2)	81 (31.3)	
Stress				
Low	1822 (65.7)	294 (67.6)	168 (65.9)	0.703
Moderate	770 (27.8)	120 (27.6)	73 (28.6)	
High	180 (6.5)	21 (4.8)	14 (5.5)	

[1]*P* values were calculated by using a χ^2 test; [2]1 US $ = 1000 won.

ing behaviors, as mentioned above. Association analysis weights were used to minimize selection bias.

RESULTS

Characteristics of subjects

The socioeconomic environment, health behaviors, and psychological factors of the subjects are shown in Table 2. Of the 3557 subjects in the study population, 715 had a family history of cancer and 261 had a family history of gastric cancer. The factors listed in Table 2 were used as variables in subsequent multivariate logistic regression analyses.

Gastric cancer screening behavior

Our analysis of gastric cancer screening rates revealed that GCRs were significantly more likely than the control group to undergo gastric cancer screening (39.2% *vs* 32.3%, aOR: 1.43, CI: 1.05-1.95). The gastric cancer screening rate of NGCRs was not significantly different from that of the control group (37.2% *vs* 32.3%, aOR: 1.08, CI: 0.83-1.41) (Table 3).

The rate of gastric cancer screening was higher among younger than older GCRs (42.4% *vs* 31.0%), and higher among younger GCRs than among controls (aOR 1.53 *vs* 1.08) Similarly, GCRs with a high income were screened more often than were GCRs with middle or low incomes (68.4% *vs* 41.8% and 17.0%, respectively), or controls (aOR: 2.70 *vs* 1.56 and 0.70, respectively). Gastric cancer screening did not vary according to education level (Table 4).

Other cancer screening behaviors

The prevalence rates of cancer screening were slightly higher in GCRs and NGCRs compared with control subjects, although these differences were not consistently significant. Female NGCRs were more likely to undergo breast cancer screening (40.8% *vs* 29.6%, aOR: 1.42, CI: 1.02-2.00) and cervical cancer screening (53.9% *vs* 39.9%, aOR: 1.51, CI: 1.04-2.20) when compared with controls. Female GCRs were slightly more likely to undergo breast cancer screening compared with the control group (40.9% *vs* 29.6%, aOR: 1.40, CI: 0.95-2.08), although this difference was insignificant. The groups did not differ with regards to colon cancer screening.

Gastric cancer preventive behaviors

Gastric cancer preventive behaviors were similar among the three groups (Table 5). Sodium consumption was elevated in all three groups. The proportion of individuals with excessive sodium intake (i.e. more than 2000 mg per day) was more than 90% in all three groups, even in GCRs (94.6%). There was a tendency toward higher intake of vitamin C in GCRs and NGCRs compared with the control group [mean ± SE (mg): 110.0 ± 6.2 and 114.1 ± 4.9 *vs* 98.5 ± 1.6, respectively], but this difference was not statistically significant after adjustment. Approximately 30% of the subjects in each groups consumed less than 60 mg vitamin C per day. The average consumption of dietary fiber was not significantly different among the groups. The proportion of individuals with a deficient intake of dietary fiber (< 20 g/d) was approximately 85% in all three groups. The current smoking rate was similar in the three groups.

DISCUSSION

To our knowledge, this is the first study of gastric cancer screening and preventive behaviors among GCRs. The strengths of this study are the use of a nationally representative sample and the inclusion of three comparison groups to more clearly reveal relationships. Our findings suggest that GCRs undergo gastric cancer screening more often than others, although the gastric cancer screening rate among GCRs was still relatively low (39.2%). The rates of breast cancer, cervical cancer, and colon cancer screening were not significantly higher in GCRs than in the

Table 3 Prevalence of cancer screening *n* (%)

	Controls (*n* = 2842)	Non-gastric cancer relatives (*n* = 454)	Gastric cancer relatives (*n* = 261)
Stomach cancer screening (within 2 yr)			
Crude rate	894 (32.3)	162 (37.2)	100 (39.2)
Adjusted rate (%, 95% CI)[2]	32.2 (30.5, 34.0)	35.2 (30.8, 39.8)	38.1 (32.3, 44.2)
Crude OR (95% CI)	1 (referent)	1.16 (0.89, 1.50)	1.47 (1.08, 2.00)[a]
Adjusted OR (95% CI)[1]	1 (referent)	1.08 (0.83, 1.41)	1.43 (1.05, 1.95)[a]
Breast cancer screening (within 2 yr)			
Crude rate	456 (29.6)	102 (40.8)	61 (40.9)
Adjusted rate (%, 95% CI)[2]	28.9 (26.7, 31.4)	36.5 (30.7, 42.8)	38.5 (31.0, 46.7)
Crude OR (95% CI)	1 (referent)	1.68 (1.21, 2.33)[a]	1.53 (1.05, 2.23)[a]
Adjusted OR (95% CI)[1]	1 (referent)	1.42 (1.02, 2.00)[a]	1.40 (0.95, 2.08)
Cervical cancer screening (within 2 yr)			
Crude rate	596 (39.9)	133 (53.9)	71 (47.7)
Adjusted rate (%, 95% CI)[2]	39.1 (36.5, 41.8)	47.3 (40.7, 54.1)	43.2 (35.1, 51.7)
Crude OR (95% CI)	1 (referent)	1.90 (1.35, 2.68)[a]	1.33 (0.90, 1.97)
Adjusted OR (95% CI)[1]	1 (referent)	1.51 (1.04, 2.20)[a]	1.14 (0.76, 1.71)
Colon cancer screening (within 5 yr)			
Crude rate	309 (17.5)	55 (23.6)	33 (21.4)
Adjusted rate (%, 95% CI)[2]	17.2 (15.4, 19.0)	22.6 (17.7, 28.5)	20.2 (14.6, 27.3)
Crude OR (95% CI)	1 (referent)	1.17 (0.78, 1.74)	1.48 (0.85, 2.56)
Adjusted OR (95% CI)[1]	1 (referent)	1.10 (0.73, 1.67)	1.41 (0.83, 2.41)

[a]$P < 0.05$. [1]Calculated *via* multiple logistic regression and adjusted for age, sex, education, marital status, smoking, alcohol consumption, income, and self-reported health status; [2]Calculated *via* analysis of covariance adjusted for age, sex, education, marital status, smoking, alcohol consumption, income, and self-reported health status. OR: Odds ratio.

Table 4 Gastric cancer screening prevalence by education level, age and income subgroups

	Controls (*n* = 2842)	Non-gastric cancer relatives (*n* = 454)	Gastric cancer relatives (*n* = 261)
Education level			
Elementary			
n (%)	322 (29.2)	37 (32.5)	27 (35.1)
aOR (95% CI)[1]	1 (referent)	0.93 (0.57, 1.51)	1.59 (0.85, 2.96)
Middle and higher			
n (%)	572 (34.3)	125 (38.9)	73 (41)
aOR (95% CI)[1]	1 (referent)	1.13 (0.82, 1.55)	1.36 (0.97, 1.92)
Age group (yr)			
40-59			
n (%)	585 (34.5)	118 (37.7)	78 (42.4)
aOR (95% CI)[1]	1 (referent)	1.11 (0.82, 1.49)	1.53 (1.07, 2.17)[a]
≥ 60			
n (%)	309 (28.8)	44 (36.1)	22 (31)
aOR (95% CI)[1]	1 (referent)	0.99 (0.57, 1.69)	1.08 (0.59, 1.98)
Income (US $/mo)			
< 1000			
n (%)	214 (27.1)	22 (26.2)	8 (17)
aOR (95% CI)[1]	1 (referent)	0.86 (0.47, 1.57)	0.70 (0.28, 1.77)
1000-5000			
n (%)	580 (32.8)	113 (37.7)	79 (41.8)
aOR (95% CI)[1]	1 (referent)	1.16 (0.84, 1.60)	1.56 (1.10, 2.21)[a]
≥ 5000			
n (%)	100 (47.4)	27 (53.0)	13 (68.4)
aOR (95% CI)[1]	1 (referent)	1.02 (0.46, 2.26)	2.70 (0.82, 8.88)

[a]$P < 0.05$. [1]Adjusted odds ratios (aOR) were calculated *via* multiple logistic regression.

control group. The dietary patterns and smoking behaviors of GCRs were similar to those of the other two groups.

The finding that GCRs undergo more frequent gastric cancer screening is consistent with previous reports for other cancers. Female relatives of patients with breast cancer are more likely to undergo mammogram screening than are females without a family history of breast cancer[24]. Similarly, men with a family history of prostate cancer are more likely to undergo prostate cancer screening. These findings suggest that a family history of cancer creates a greater sense of vulnerability and is an important factor in the decision to undergo screening[25]. Nonetheless, the screening rates for cancers other than gastric cancer were not different from those of the controls, suggesting that gastric cancer screening behaviors in GCRs is incidental and opportunistic, and not necessarily the result of a formal, systematic training on the importance of cancer screening in general. In addition, it is supposed that GCRs are motivated to undergo gastric cancer screening because of worries about possible cancer development rather than recognition of the benefits of screening. This hypothesis is also explained by the fact that individuals' awareness of the benefits of screening, which is thought to be the result of educational campaigns, was no higher in GCRs than in the control group (63.9% *vs* 64.4%, Table 6). The diagnosis of cancer in a first-degree relative may spur a person into action, as suggested by the Health Belief Model[26], which might explain the increased rate of gastric cancer screening in GCRs.

More importantly, the absolute screening rate in GCRs was only 39.2%, indicating that more than half of the GCRs had not yet undergone regular gastric screening. Endoscopic mass screening for gastric cancer is effective in identifying cancer at an early stage and is cost-effective, especially in moderate- to high-risk popu-

Table 5 Gastric cancer-preventive behaviors

	Controls (n = 2842)		Non-gastric cancer relatives (n = 454)		Gastric cancer relatives (n = 261)	
	Crude	Adjusted	Crude	Adjusted	Crude	Adjusted
Na (mg)						
Mean intake (SE)[2]	5582 (66)	5602 (64)	5574 (166)	5522 (162)	5516 (213)	5483 (212)
P value			0.86	0.99	0.76	0.50
High sodium intake (> 2000 mg)						
Rate, *n* (%)[2]	2625 (92.4)	93.8	429 (94.5)	94.9	247 (94.6)	95.1
Odds ratio[1]	1 (referent)	1 (referent)	1.67 (1.04, 2.67)[a]	1.56 (0.95, 2.57)	1.34 (0.71, 2.54)	1.17 (0.61, 2.26)
Vitamin C (mg)						
Mean intake (SE)[2]	98.5 (1.6)	100.0 (1.6)	114.1 (4.9)	109.7 (4.1)	110.0 (6.2)	107.2 (5.4)
P value			0.03[a]	0.15	0.07	0.19
Low vitamin C intake (< 60 mg)						
Rate, *n* (%)[2]	922 (32.4)	30.7	129 (28.4)	30.5	78 (29.9)	30.8
Odds ratio[1]	1 (referent)	1 (referent)	0.79 (0.59, 1.06)	0.91 (0.67, 1.23)	0.79 (0.55, 1.13)	0.89 (0.62, 1.28)
Dietary fiber (g)						
Mean intake (SE)[2]	8.0 (0.1)	8.0 (0.1)	8.4 (0.2)	8.3 (0.2)	8.3 (0.3)	8.3 (0.3)
P-value			0.20	0.30	0.08	0.14
Low fiber intake (< 20 g)						
Rate, *n* (%)[2]	2447 (86.1)	87	383 (84.4)	86.9	225 (86.2)	87.4
Odds ratio[1]	1 (referent)	1 (referent)	0.86 (0.44, 1.66)	0.86 (0.44, 1.68)	1.17 (0.50, 2.72)	1.20 (0.50, 2.85)
Current smoking status						
Rate, *n* (%)[2]	602 (21.7)	12.9	89 (20.5)	13.2	58 (22.8)	15.4
Odds ratio[1]	1 (referent)	1 (referent)	0.95 (0.69, 1.29)	1.04 (0.73, 1.47)	1.10 (0.78, 1.55)	1.18 (0.76, 1.83)

[a]$P < 0.05$. [1]Adjusted odds ratios were calculated *via* multiple logistic regression and adjusted for age, sex, education, marital status, smoking, alcohol consumption, income, and self-reported health status; [2]Adjusted means and adjusted rates were calculated *via* analysis of covariance adjusted for age, sex, education, marital status, smoking, alcohol consumption, income, and self-reported health status.

Table 6 Perception of the benefits of screening *n* (%)

	Controls (n = 2842)	Non-gastric cancer relatives (n = 454)	Gastric cancer relatives (n = 261)
Beneficial	1783 (64.4)	292 (67.1)	163 (63.9)
Not beneficial	292 (10.5)	47 (10.8)	36 (14.1)
Have never received	696 (25.1)	96 (22.1)	56 (22.0)

lations[14,27]. In Korea, gastric cancer screening is provided as a part of the national cancer screening program, with virtually no economic barrier (Table 1). Therefore, the gastric cancer screening rate should theoretically be high, even in the general population, and GCRs should undergo at least biennial screening, barring a contraindication. Proper educational programs are needed to emphasize the benefits of screening to GCRs, especially those who are older and earn a lower income.

Although GCRs underwent gastric cancer screening more often than other people, their dietary habits and smoking behaviors were not significantly different from those of the control group. Many members of the GCR group had inappropriate dietary habits, with 94.6% consuming excessive sodium, 29.9% deficient in vitamin C, and 86.2% deficient in dietary fiber. This finding was consistent with a previous study of breast cancer relatives, which found that female relatives were more likely to undertake medical actions but not lifestyle preventive behaviors[28]. However, another study suggested that relatives were motivated to change their consumption of fruits, vegetables, and fat once they understood that their behavior could increase their risk of cancer[29]. It is possible that a large proportion of the subjects did not completely understand the extent to which unhealthy behaviors increase the risk of gastric cancer. Healthy lifestyle changes are most successful when individuals believe that the changes will reduce their risk of adverse conditions[30]. For example, perceived vulnerability was a primary motivator for efforts to quit smoking among family members of lung cancer patients[31]. These findings suggest that GCRs should be made aware of the elevated risk of gastric cancer due to unhealthy behaviors. However, a survey has shown that the general Korean public did not clearly understand the risk factors for gastric cancer[32]. Therefore, family education programs should be developed to ensure that GCRs are aware of the risk factors for gastric cancer and the importance of regular screening and preventive behaviors. As the cancer diagnosis and treatment provide a teachable moment for family members as well as the patients themselves[26,31], hospital-based education programs involving both patients and family members could be considered as a potential method to deliver educational messages about gastric cancer screening and other preventive behaviors to them. In a similar example, a family-based health education and counseling intervention program was effective in changing health behaviors of children with a family history of cardiovascular diseases[33]. Another promising method of intervention is clinical treatment that is combined with computerized family-history tools, such as Family Healthware[34], which provides tailored preventive health messages focused on health behaviors and screening, not only for patients, but also for their doctors to of-

fer appropriate recommendations.

This study had several limitations. First, we were unable to assess the prevalence of *H. pylori* existence in the subjects because of the retrospective nature of the study. *H. pylori* eradication is recommended for patients who are first degree relatives of patients with gastric cancer[6,7,35]. Second, the survey did not assess whether the subjects were aware of the causes of gastric cancer or the recommended biennial gastric cancer screening. Third, the statistical significance may have been limited by the relatively small number of GCRs. Fourth, as the design of this study is cross-sectional, we have no information regarding the gastric screening adherence at follow-up. Thus, further research is needed to determine how many subjects actually continue to undergo a 2-year screening procedure. Fifth, only 70.2% of the selected household members responded to the health examination survey. Therefore, it cannot be excluded that the other 29.8% of the household members who did not participate in the survey were less interested in health. As a result, preventive behaviors could be even worse than the findings of this study. Finally, the survey was based on self-reported data, which can potentially increase the risk of inaccuracy. However, the validity of self-reported cancer screening histories and interviewer-administered 24-h dietary recall have been shown to be accurate and reliable[20,36], although few studies have examined the validity of self-reported upper endoscopy history, which is still used in national surveys.

In conclusion, GCRs were found to be more likely to undergo gastric cancer screening compared with the control group. However, this behavior may be incidental, opportunistic, and motivated by concern rather than a true recognition of the benefits of screening by systematic education. The overall gastric cancer screening rate was relatively low in GCRs. The GCRs did not differ from controls with regards to the 1 d intake of sodium, vitamin C, and dietary fiber and a high proportion of GCRs reported inappropriate dietary habits. In addition, the smoking rate was similar in GCRs and controls. To promote awareness about gastric cancer screening and prevention in GCRs, family education programs should be developed and implemented in a systematic manner.

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COMMENTS

Background

These days, increasing emphasis is placed on early detection and prevention of cancers, as it is difficult to cure them when they develop. Gastric cancer is one of the cancers that are modifiable through lifestyle preventive behaviors and regular cancer screening. Currently, only a few Asian countries, including Korea, Japan, and Matsu Island in Taiwan, are conducting population-based screening for gastric cancer.

Research frontiers

Regular screening and health behaviors of high-risk groups have been always emphasized. However, it has not been unequivocally addressed as to how regularly or strictly gastric cancer relatives (GCRs), one of the high-risk groups for gastric cancer, are practicing these measures. In this study, the authors demonstrated that GCRs had much room for improvement in their cancer screening and preventive behaviors.

Innovations and breakthroughs

There have been previous reports that highlighted the low gastric cancer screening rate in the general public. This is the first study to use a nationally representative sample and report that GCRs were more likely to undergo gastric cancer screening, even though their lifestyle preventive behaviors did not show significant differences compared to controls. Furthermore, this study suggests that GCRs were not fully aware of the importance of screening and the potential impacts of risk factors for gastric cancer.

Applications

This study highlights the necessity of targeted intervention for GCRs and also proposes a future strategy through systematic family education programs.

Peer review

This is an interesting, well-written study.

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BRIEF ARTICLE

Virological response to adefovir monotherapy and the risk of adefovir resistance

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Abstract

AIM: To evaluate virological response to adefovir (ADV) monotherapy and emergence of ADV-resistant mutations in lamivudine (LAM)-resistant chronic hepatitis B patients.

METHODS: Seventy-seven patients with documented LAM resistance who were treated with 10 mg/d ADV for > 96 wk were analyzed for ADV resistance.

RESULTS: At week 48 and 96, eight (10%) and 14 (18%) of 77 LAM-resistant patients developed the ADV-resistant strain (rtA181V/T and/or rtN236T mutations), respectively. Hepatitis B virus (HBV) DNA levels during therapy were significantly higher in patients who developed ADV resistance than in those who did not. Incidence of ADV resistance at week 96 was 11%, 8% and 6% among patients with complete virological response (HBV DNA level < 60 IU/mL); 0%, 5% and 19% among patients with partial virological response (HBV DNA level ⩾ 60 to 2000 IU/mL); and 32%, 34% and 33% among patients with inadequate virological response (HBV DNA levels > 2000 IU/mL) at week 12, week 24 and week 48, respectively. HBV DNA levels > 2000 IU/mL at week 24 showed best performance characteristics in predicting ADV resistance.

CONCLUSION: Development of ADV resistance mutations was associated with HBV DNA levels, which could identify patients with LAM resistance who are likely to respond to ADV monotherapy.

Key words: Hepatitis B virus; Viral DNA; Adefovir; Lamivudine; Drug resistance

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INTRODUCTION

Lamivudine (LAM) has been the most popular antiviral agent for the treatment of chronic hepatitis B for many years, but its efficacy is hampered by the high incidence of drug resistance[1]. Adefovir dipivoxil (ADV) is a nucleotide analog that exhibits activity against wild-type and LAM-resistant hepatitis B virus (HBV)[2-4]. Early

studies have demonstrated potent viral suppression of LAM-resistant HBV by either switching to or adding ADV to LAM[5]. However, ADV-resistant strains have been reported after either switching to or adding ADV in patients with LAM resistance, and several recent clinical studies have found that combined LAM with ADV is associated with improvements in virological response and lower rates of ADV resistance than sequential ADV monotherapy[6-8]. Thus, recent guidelines suggest adding ADV to LAM as a better approach than sequential monotherapy for patients with LAM resistance[9-12].

Although ADV add-on therapy represents a new paradigm that is highly effective at restoring viral suppression and preventing the emergence of resistance in patients with LAM resistance[13], the higher cost of add-on therapy may allow ADV monotherapy to retain its role in selected patients, particularly in developing countries[14]. In clinical practice, some patients with LAM resistance do respond to ADV monotherapy[15]. Identification of patients who may be sufficiently treated with ADV monotherapy alone may reduce medical costs in areas where resources are limited.

HBV DNA levels on treatment may help select patients who are likely to respond to ADV monotherapy. Maintenance of undetectable HBV DNA levels during treatment with nucleoside/nucleotide analogs has been suggested as a desirable endpoint[10]. Recently proposed "on-treatment strategy" for patients receiving oral nucleoside/nucleotide therapy is also based on HBV DNA levels[16]. On-treatment monitoring strategies are based on the nature of virological response during treatment, and HBV DNA levels during treatment may be a valuable predictor of treatment response to ADV monotherapy.

The aim of this study was to establish whether HBV DNA levels during treatment are associated with the emergence of genotypic ADV resistance. We studied HBV DNA levels at weeks 12, 24 and 48 after the start of ADV monotherapy among LAM-resistant patients who had received ADV monotherapy for > 96 wk and assessed genotypic resistance to ADV at weeks 48 and 96.

MATERIALS AND METHODS

Patients

Data were collected retrospectively from 85 LAM-resistant chronic hepatitis B patients who started ADV monotherapy between March 2004 and May 2006, and maintained ADV monotherapy for at least 96 wk at the Samsung Medical Center, Seoul, Korea. All 85 patients were treated with LAM for chronic HBV infection and experienced virological breakthrough (VB), which was defined as an increase in the level of HBV DNA of at least 1 $\log_{10}$ IU/mL from the lowest point during treatment. Serum samples were collected every 3 mo during treatment and kept at -80℃ until mutation analyses were performed. Eight patients were excluded from analyses for the following reasons: (1) serum samples were not available for six patients; and (2) an ADV-resistant strain (rtA181V/T) was present at baseline in two patients. Finally, a total of 77 patients were included in this study. This study was approved by the Institutional Review Board at Samsung Medical Center.

Blood tests

Routine biochemical tests were performed by standard procedures every 12 wk during therapy. Hepatitis B surface antigen, hepatitis B e antigen (HBeAg), and hepatitis B e antibody were tested by electrochemiluminescence immunoassay (Elecsys; Roche Diagnostics, Indianapolis, IN, USA). LAM resistance was tested with direct sequencing (ABI 3130 Genetic Analyzer; Applied Biosystems, Foster City, CA, United States).

HBV DNA assay

HBV DNA was quantified using the COBAS TaqMan HBV test (detection limit of 12 IU/mL, Roche Molecular Systems, Branchburg, NJ, USA) at the onset of ADV treatment (baseline), and at weeks 12, 24 and 48 using stored serum samples. Virological response was defined as complete virological response (CVR, HBV DNA level < 60 IU/mL); partial virological response (PVR, HBV DNA levels ⩾ 60 to 2000 IU/mL); and inadequate virological response (IVR, HBV DNA levels > 2000 IU/mL)[13]. VB was defined as an increase in the level of HBV DNA of at least 1 $\log_{10}$ IU/mL from the lowest point during treatment[13].

Detection of ADV resistance

Genotype resistance to ADV was determined at baseline and at weeks 48 and 96 by direct sequencing after amplification of polymerase chain reaction (PCR) products. To detect mutations, PCR amplification was performed using the following primers: external primers were RTF (5'-tat gtt gcc cgt ttg tcc tc-3', position 460-479) and RTR (5'-tga cat act ttc caa tca ata gg-3', position 970-992); internal primers were RTNF (5'-aaa acc ttc gga cgg aaa ct-3', position 574-593) and RTNR (5'-tgc ggt aaa gta ccc caa ct-3', position 895-914). The PCR-amplified DNA was purified by using a QIAquick PCR purification kit (QIAGEN, Hilden, Germany). Purified DNA was treated with an ABI Prism BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems). The primers used for direct sequencing were RTNS and RTNR. DNA sequencing was performed in both directions by an Applied Biosystems 3130 Genetic Analyzer (Applied Biosystems). In this study, ADV resistance was defined as the presence of mutations that confer resistance to ADV, which were rtN236T and/or rtA181V/T[17,18].

Statistical analysis

Differences between patient groups were tested using *t* tests or Mann-Whitney *U* tests, as appropriate, for numeric variables, and χ^2 tests or Fisher's exact tests, as appropriate, for categorical variables. For statistical analysis, HBV DNA levels < 12 IU/mL were considered to be 12 IU/mL. Sensitivity, specificity, positive predictive value, and negative predictive value of HBV DNA levels

Table 1 Baseline characteristics of patients

Variables	
No. of patients	77
Age (yr, mean ± SD)	49.3 ± 11.7
Female: Male (*n*, % male)	18: 59 (77%)
HBeAg positive (*n*, % positive)	21: 56 (73%)
ALT (U/L, median, range)	119 (25–926)
AST (U/L, median, range)	77 (30–483)
Albumin (g/dL, median, range)	4.0 (2.6–4.6)
Total bilirubin (mg/dL, median, range)	1.0 (0.3–4.0)
Prothrombin time (INR, median, range)	1.1 (0.9–1.7)
Baseline creatinine level (mg/dL, mean ± SD)	0.90 ± 0.13
Creatinine level at weeks 96 (mg/dL, mean ± SD)	0.94 ± 0.16
LAM resistance mutation profile (*n*, %)	
rtM204 I ± rtL180M	48 (62%)
rtM204V ± rtL180M	29 (38%)

ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; INR: International normalized ratio; LAM: Lamivudine.

Table 2 Patterns of ADV-resistant mutations at weeks 48 and 96 after ADV monotherapy in LAM-resistant patients

Variables	Week 48	Week 96
rtA181T	5	5
rtA181V	2	5
rtA181V + rtN236T	1	2
rtA181T + rtN236T	0	2
Total *n* (%)	8 (10)	14 (18)

LAM: Lamivudine; ADV: Adefovir dipivoxil; rtA181V/T and/or rtN236T: ADV-resistant strain.

Table 3 HBV DNA levels during ADV monotherapy according to emergence of ADV-resistant mutations in LAM-resistant patients

HBV DNA level	ADV resistance at week 96 (*n* = 14)	No ADV resistance at week 96 (*n* = 63)	*P* value
Baseline (log10 IU/mL, mean ± SD)	7.1 ± 0.7	6.8 ± 1.0	0.245
Month 3 (log10 IU/mL, mean ± SD)	4.2 ± 1.6	3.1 ± 1.6	0.027
Month 6 (log10 IU/mL, mean ± SD)	4.1 ± 1.4	2.7 ± 1.6	0.002
Month 12 (log10 IU/mL, mean ± SD)	3.9 ± 1.3	2.4 ± 1.5	0.002

LAM: Lamivudine; ADV: Adefovir dipivoxil; HBV: Hepatitis B virus.

were calculated for the prediction of genotypic resistance to ADV at week 96. Receiver operator curve (ROC) analysis was performed to compare the performance of HBV DNA levels at each time points. Statistical analysis was conducted using PASW Statistics 17.0 (SPSS, Inc., Chicago, IL, United States) and $P < 0.05$ was considered significant.

RESULTS

Genotypic ADV resistance at weeks 48 and 96

Baseline characteristics of enrolled patients are shown in Table 1. At week 48, 8 (10%) of 77 LAM-resistant patients had developed the rtA181V/T and/or rtN236T mutations. At week 96, 14 (18%) of 77 LAM-resistant patients had developed rtA181V/T and/or rtN236T mutations (Table 2).

Table 4 Incidence of ADV resistance according to HBV DNA levels

HBV DNA level	Patient number	ADV resistance at week 48 *n* (%)	ADV resistance at week 96 *n* (%)
Week 12[a]			
< 60 IU/mL	18	2 (11)	2 (11)
≥ 60 to < 2000 IU/mL	21	0 (0)	0 (0)
> 2000 IU/mL	38	6 (16)	12 (32)
Week 24[b]			
< 60 IU/mL	26	2 (8)	2 (8)
≥ 60 to < 2000 IU/mL	19	1 (5)	1 (5)
> 2000 IU/mL	32	5 (16)	11 (34)
Week 48[c]			
< 60 IU/mL	34	2 (6)	2 (6)
≥ 60 to < 2000 IU/mL	16	0 (0)	3 (19)
> 2000 IU/mL	27	6 (22)	9 (33)

[a]*P* value; week 48 = 0.162, week 96 = 0.007. [b]*P* value; week 48 = 0.431, week 96 = 0.008. [c]*P* value; week 48 = 0.036, week 96 = 0.022. ADV: Adefovir dipivoxil; HBV: Hepatitis B virus.

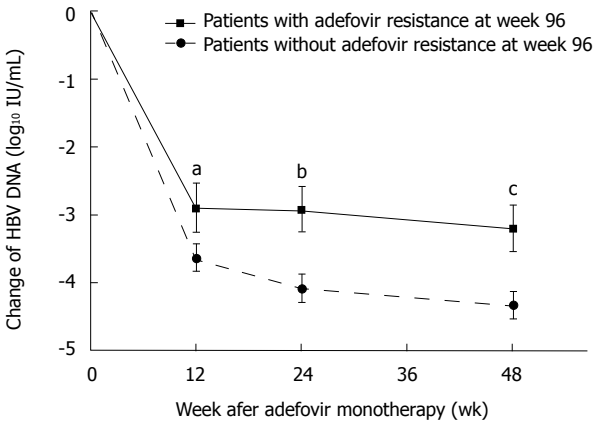

Figure 1 Hepatitis B virus DNA levels after adefovir monotherapy. There were significant differences in the degree of Hepatitis B virus (HBV) DNA reduction between patients who developed adefovir resistance and those who did not (*P* value; [a]week 12 = 0.027, [b]week 24 = 0.002, [c]week 48 = 0.002).

HBV DNA levels on treatment and emergence of ADV resistance

HBV DNA levels during treatment were significantly lower in patients who did not develop ADV resistance than in those who did, while pretreatment HBV DNA levels were not significantly different (Table 3). The degree of reduction of HBV DNA level was also significantly greater among patients who developed ADV resistance (Figure 1). There was a significant difference in the incidence of ADV resistance at week 96 according to HBV DNA levels at week 12 ($P = 0.007$), at week 24 ($P = 0.008$), and at week 48 ($P = 0.022$) (Table 4). Only 8% and 6% of patients with CVR at weeks 24 and 48 developed ADV resistance at week 96, whereas, > 30%

Table 5 Virological response in predicting ADV resistance at week 96

Variables	Adefovir resistance *n* (%)	Sensitivity (%, 95% CI)	Specificity (%, 95% CI)	Positive predictive value (%, 95% CI)	Negative predictive value (%, 95% CI)
HBV DNA level ≥ 60 IU/mL at Week 12 (n = 59)	12 (20)	85 (56–97)	25 (15–38)	20 (11–33)	89 (63-98)
HBV DNA level > 2,000 IU/mL at Week 12 (n = 38)	12 (32)	85 (56–97)	58 (45–70)	31 (18–48)	84 (81–99)
HBV DNA level ≥ 60 IU/mL at Week 24 (n = 51)	12 (24)	85 (56–97)	38 (26-51)	23 (13–38)	92 (73–98)
HBV DNA level > 2000 IU/mL at Week 24 (n = 32)	11 (34)	78 (48–94)	66 (53–77)	34 (19–53)	93 (80–98)
HBV DNA level ≥ 60 IU/mL at Week 48 (n = 43)	12 (28)	85 (56–97)	51 (38–63)	28 (16–44)	72 (56–84)
HBV DNA level > 2000 IU/mL at Week 48 (n = 27)	9 (33)	64 (35–86)	71 (58–81)	33 (17–53)	90 (77–96)

ADV: Adefovir dipivoxil; HBV: Hepatitis B virus.

of patients with IVR at weeks 12, 24 and 48 developed ADV resistance at week 96.

The HBV DNA levels at weeks 12, 24 and 48 were tested for sensitivity, specificity, positive predictive value and negative predictive value for the prediction of ADV resistance at week 96 (Table 5). ROC curve analysis showed that the area under the curve in predicting ADV resistance was lowest at HBV DNA level ≥ 60 IU/mL at week 12 (area = 0.556, P = 0.51) and highest at HBV DNA level > 2000 IU/mL at week 24 (area = 0.726, P = 0.008).

DISCUSSION

In this study, we found that on-treatment serum HBV DNA levels were associated with genotypic ADV resistance. Patients who developed ADV resistance showed higher HBV DNA levels at weeks 12, 24 and 48 after the start of ADV monotherapy. The incidence of ADV resistance was lowest among patients with CVR, and highest among patients with IVR (Table 4). These data suggest that risk of ADV resistance is low among patients who achieve CVR at weeks 12, 24 and 48 after ADV monotherapy, and they may continue ADV monotherapy. IVR at weeks 12, 24 and 48 was associated with development of ADV resistance, and ADV monotherapy should not be continued. Patients with PVR at weeks 12 and 24 showed low incidence of ADV resistance, and may continue ADV monotherapy with careful follow-up, but patients with PVR at weeks 48 may not continue ADV monotherapy because of significant risk of ADV resistance at week 96. The best predictor of ADV resistance was IVR at week 24 (Table 5).

The findings of this study are in line with the recently proposed "on-treatment strategy" for patients receiving oral nucleoside/nucleotide therapy[13]. Keeffe *et al*[13] have suggested that management strategies should be changed for patients with IVR response at week 24. Shin *et al*[19] also have described the importance of HBV DNA levels on treatment among patients with LAM resistance who received ADV monotherapy. They have reported that patients who had HBV DNA levels < 200 IU/mL at week 48 were unlikely to develop VB and genotype mutations. Chen *et al*[20] have reported that ADV resistance was associated with higher HBV DNA levels and lower HBV DNA reduction during the first 6 mo of ADV treatment, compared to patients who did not develop ADV resistance. Gallego *et al*[21] also have shown that initial virological response (reduction ≥ 4 $\log_{10}$ IU/mL) in HBV DNA at 6 mo is an important factor for predicting treatment outcome. These studies, as well as the present study, suggest that HBV DNA level during treatment is a valuable parameter for making early decisions regarding the continuation of ADV monotherapy or switching to another therapy in patients who show LAM resistance[19].

For patients with LAM resistance, adding ADV is a better approach than switching to ADV, as has been demonstrated by several studies[6-12]. However, because of the higher cost of add-on therapy, in areas with limited resources, ADV monotherapy may still be considered. If so, these data suggest that ADV monotherapy may be tried for up to 24 wk, depending on virological response. Patients who show favorable virological response may continue ADV monotherapy.

The cumulative probability of ADV resistance in our series was 10% and 18% at weeks 48 and 96, respectively. However, in this study, only patients who maintained ADV monotherapy for at least 96 wk were enrolled, which indicates that patients who were good responders to ADV were preferentially selected for the study. Thus, the incidence of ADV resistance in this study does not reflect true genotypic resistance rates among LAM-resistant patients who received ADV monotherapy. We included patients only for those who had received at least 96 wk of ADV monotherapy, because the aim of this study was to determine which patients could continue ADV monotherapy.

In conclusion, the results of this study demonstrate the importance of HBV DNA levels during treatment as an indicator of future ADV resistance. The development of ADV-resistant mutations was closely associated with HBV DNA levels during therapy. The risk of developing ADV-resistant mutations in patients who experi-

enced IVR at week 24 was high. These findings suggest that ADV monotherapy is a viable alternative for LAM-resistant patients with good on-treatment virological response, in areas with limited resources.

COMMENTS

Background

A major concern with adefovir (ADV) treatment in lamivudine (LAM)-resistant patients is the selection of ADV-resistant mutations.

Research frontiers

Recent studies suggest that combination therapy with ADV and LAM is better than ADV monotherapy in preventing development of ADV resistance among LAM-experienced patients. However cost is of concern, in areas with limited resources.

Innovations and breakthroughs

Recent reports have highlighted the importance of hepatitis B virus (HBV) DNA levels during antiviral therapy. On-treatment monitoring strategies are based on the nature of virological response during treatment. This study showed that HBV DNA levels during treatment were also useful in predicting ADV resistance in LAM-resistant patients, thus helps to identify patients that might respond to ADV monotherapy.

Applications

This study suggests that ADV monotherapy could be a viable alternative for LAM-resistant patients with good on-treatment virological response to ADV. ADV monotherapy may still be alternative, cost-effective approach especially in areas with limited resources.

Terminology

Genotypic resistance refers to the detection of mutations that have been shown in *in vitro* studies to confer resistance to the drug that is being administered. Antiviral-resistant mutations can be detected at months and sometimes years before biochemical breakthrough. Thus, early detection and intervention can prevent hepatitis flares and hepatic decompensation.

Peer review

This is a retrospective study that evaluated the virological response to ADV monotherapy. This was a good study.

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BRIEF ARTICLE

Histological origin of pseudomyxoma peritonei in Chinese women: Clinicopathology and immunohistochemistry

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Abstract

AIM: To investigate the histological origin of pseudomyxoma peritonei (PMP) in Chinese women.

METHODS: The clinical and pathological data were reviewed for 35 women with PMP, and specimens of the peritoneal, appendiceal and ovarian lesions of each patient were examined using the PV-6000 immunohistochemistry method. Antibodies included cytokeratin (CK)7, CK20, mucin (MUC)-1, MUC-2, carbohydrate antigen (CA)-125, estrogen receptor (ER), and progesterone receptor (PR).

RESULTS: Abundant colloidal mucinous tumors were observed in the peritoneum in all 35 cases. Thirty-one patients had a history of appendectomy, 28 of whom had mucinous lesions. There was one patient with appendicitis, one whose appendix showed no apparent pathological changes, and one with unknown surgical pathology. Ovarian mucinous tumors were found in 24 patients. The tumors were bilateral in 13 patients, on the right-side in nine, and on the left side in two. Twenty patients had combined appendiceal and ovarian lesions; 16 of whom had undergone initial surgery for appendiceal lesions. Four patients had undergone initial surgery for ovarian lesions, and relapse occurred in these patients at 1, 11, 32 and 85 mo after initial surgery. Appendiceal mucinous tumors were found in each of these four patients. Thirty-three of the 35 patients showed peritoneal lesions that were positive for CK20 and MUC-2, but negative for CK7, MUC-1, CA125, ER and PR. The expression patterns in the appendix and the ovary were similar to those of the peritoneal lesions. In one of the remaining two cases, CK20, CK7 and MUC-2 were positive, and MUC-1, CA125, ER and PR were negative. The ovaries were not resected. The appendix of one patient was removed at another hospital, and no specimen was evaluated. In the other case, the appendix appeared to be normal during surgery, and was not resected. Peritoneal and ovarian lesions were negative for CK20, MUC-2, CK7, MUC-1, CA125, ER and PR.

CONCLUSION: Most PMP originated from the appendix. Among women with PMP, the ovarian tumors were implanted rather than primary. For patients with PMP, appendectomy should be performed routinely. The ovaries, especially the right ovaries should be explored.

Key words: Pseudomyxoma peritonei; Peritoneum; Tumor origin; Ovary; Appendix; Immunohistochemistry

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Guo AT, Song X, Wei LX, Zhao P. Histological origin of pseudomyxoma peritonei in Chinese women: Clinicopathology and immunohistochemistry. *World J Gastroenterol* 2011; 17(30): 3531-3537 Available from: URL: http://www.wjgnet.com/1007-9327/full/v17/i30/3531.htm DOI: http://dx.doi.org/10.3748/wjg.v17.i30.3531

INTRODUCTION

Pseudomyxoma peritonei (PMP) is a rare disease that was

first described in association with a mucinous tumor of the ovary in 1884[1]. It is characterized by dissemination of voluminous jelly-like mucus on the peritoneal surface. Benign or malignant epithelial cells may be found in the mucinous deposit. Its etiology and pathological origin are not yet fully understood to date. It is reported in the literature that PMP often originates from the appendix[2-10]; however, a third to a half of PMP cases are accompanied by ovarian mucinous tumors in women. It has been controversial over the years whether the ovarian lesions in patients with PMP are primary or metastatic or implanted from the appendix. Most studies have indicated that PMP associated with ruptured mucinous lesions originates from the appendix. However, some authors consider that PMP originates from the ovaries[1,11-14].

Cytokeratin (CK)7 is a specific marker of primary ovarian epithelial tumor, which is rarely expressed in epithelial cells of the gastrointestinal tract[4,6]. CK20 is mainly expressed in the epithelium of the gastrointestinal tract[4,6]. Mucin (MUC)2 is a specific marker of goblet cells in the gastrointestinal tract[4,6]. MUC1 is often expressed in epithelial tumors of the breast and female reproductive system[6]. Carbohydrate antigen (CA)125 is a membrane surface glycoprotein that is associated with ovarian cancer cells, which is expressed in the epithelium in most cases of ovarian cancer[6]. In female patients, different levels of estrogen receptor (ER) and progesterone receptor (PR) expression are present in most primary ovarian epithelial tumors.

We reviewed 35 cases of PMP diagnosed at out hospital since the establishment of our hospital, and collected the clinical and pathological data of all the female patients. We carried out immunohistochemical studies to explore the causes and pathological origin of PMP in women, and to provide guidance for clinical diagnosis and treatment of PMP. To the best of our knowledge, this is the first large sample study on the origin of PMP in Chinese women.

MATERIALS AND METHODS

Clinical data

Our hospital treated 83 patients with PMP from 1962 to January 2010, including 38 women (45.8%) and 45 men (54.2%). Three of the 38 women with PMP did not undergo tumor resection or cytoreductive surgery, and underwent pathological examination only via puncture or biopsy, and were therefore, excluded from analysis. The clinical data for the remaining 35 female patients were reviewed, including age of onset, main symptoms and physical signs, imaging findings, intraoperative findings, surgical approach, pathological diagnosis, postoperative adjuvant therapy, recurrence, number of surgical procedures, and survival. The survival rate of patients was analyzed using the Kaplan-Meier method.

Methods

Pathological sections were obtained from all patients to the best of our ability, and all pathological sections obtained were reviewed. For each patient, tissue blocks of lesions of the peritoneum, appendix, and ovary were selected for immunohistochemical staining. Three-micrometer sections of the paraffin-embedded tissue were deparaffinized, rehydrated in a graded series of alcohol and microwave-treated for 10 min in a citrate buffer (pH 6.0). Endogenous peroxidase activity was blocked using 0.3% hydrogen peroxide. The tissues were processed in an automatic immunohistochemical staining machine using the standard protocols (Lab Vision Autostainer; Lab Vision Co., Fremont, CA, USA) with DAKO Real™ EnVision™ Detection System (K5007, DAKO). We used the the following primary antibodies: CK7 (clone OV.TL-12/30, Dako; dilution 1:15 000), CK20 (clone Ks20.8, Dako; dilution 1:2000), MUC-1 (clone Ma 695, Novocastra; dilution 1:200) , MUC-2 (clone ccp58, Novocastra; dilution 1:100), CA125 (clone OC125, Dako; dilution 1:500), ER (clone 6F11, Novocastra; dilution 1:100), and PR (clone 16, Novocastra; dilution 1:200). All antibodies were incubated for 1 h at room temperature. The sections were visualized with 3-3'-diaminobenzidine and tissues were counterstained with Mayer's hematoxylin. We used colon mucinous carcinoma tissue as a positive control for MUC2 and CK20, ovarian mucinous carcinoma tissue as positive control for MUC1, CA125 and CK7, and endometrial carcinoma tissue as a positive control for ER and PR. The same tissues without labeling by primary antibody were used as negative controls. Reactions were interpreted as positive, based on the presence of cytoplasmic staining for MUC2, CK7 and CK20 or cytoplasmic and membranous staining for MUC1 and CA125. For descriptive purposes, the staining was scored semi-quantitatively based on the percentage of positive cells: 1, negative; 2, < 10%; 3, 10-50%; and 4, > 50%. For comparative purposes, scores of 2-4 were considered to be positive.

RESULTS

Clinical characteristics

The 35 female patients were 24-76 years of age, with a mean age of 52.5 years. Most patients complained of abdominal distension and abdominal pain, and physical examination showed abdominal distension with ascites.

During surgery, a large volume of jelly-like mucous substances was seen in the abdomen. Multiple mucous lesions could be observed on the surface of the peritoneum and visceral organs. Thirty-one patients (88.6%) underwent appendectomy. A space-occupying lesion of the gallbladder was seen in one patient, whose appendix was not resected because intraoperative exploration of the appendix did not show any obvious abnormalities. The remaining three patients did not have a history of appendectomy, and the appendix was not explored during surgery.

Twenty-seven patients underwent ovarian resection, and mucinous ovarian tumors were observed in 24. In the remaining eight patients, no obvious abnormalities of the ovaries were observed during surgery, and the ovaries were not resected.

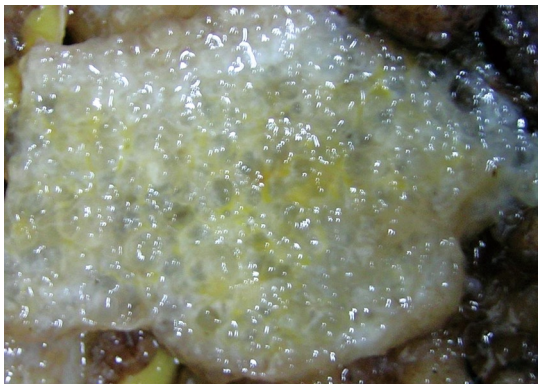

Figure 1 Upon sectioning, the peritoneal lesions were full of a jelly-like mucous substance.

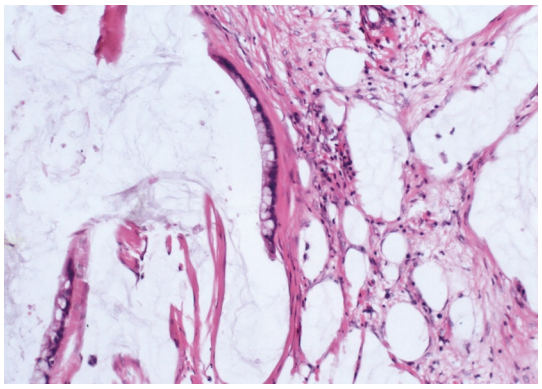

Figure 2 Microscopically, some mucous glandular structures were floating in a large number of mucous lakes (HE stain, × 200).

Lesions of the appendix and ovary were present in 20 patients (57.1%); 16 of whom underwent initial surgery for appendiceal lesions and four for ovarian lesions. None of these four patients underwent appendectomy, and the appendix was not explored during surgery. However, mucinous tumors recurred 1, 11, 32 and 85 mo after initial surgery, respectively, and appendiceal mucinous tumors were found in them. The other four patients with ovarian mucinous tumors did not undergo appendectomy, and the appendix was not explored during surgery in three patients, and a space-occupying lesion of the gallbladder was present in the remaining patient. The ovary was negative for tumor in 10 patients with appendiceal mucinous tumors.

Twenty-five patients (71.43%) underwent at least two surgical procedures, and all 35 patients received varying degrees of abdominal and systemic chemotherapy after surgery. Eleven patients died during follow-up (survival was 3-312 mo); five patients were lost to follow-up after 2-12 mo; and 19 patients survived with tumors (3-129 mo).

Pathological characteristics

In all the patients, the intra-abdominal masses consisted of multiple nodules or grape-like masses; most of which had a smooth and shiny surface. Upon sectioning, the nodules were full of a jelly-like mucous substance (Figure 1). Microscopically, some mucous glandular structures were floating in a large number of mucous lakes, whose epithelium showed varying degrees of differentiation; mostly highly differentiated (Figure 2). Part of the epithelium was poorly differentiated. Mucinous tumors or tumor-like lesions were observed in 28 of 31 patients who underwent appendectomy (Figure 3A). Among the three patients who underwent surgery at other hospitals, the surgical pathology result was a chronic inflammatory mass of the appendix in one patient, no significant changes of the appendix in one patient, and unclear in the remaining patient. Ovarian mucinous tumors were found in 24 of the 27 patients who underwent resection of the ovary (Figure 3B). The tumors were bilateral in 13 cases, on the right side in nine, and on the left side in two (Table 1). The morphological changes in lesions of the appendix and ovary were similar to those of the peritoneal lesions.

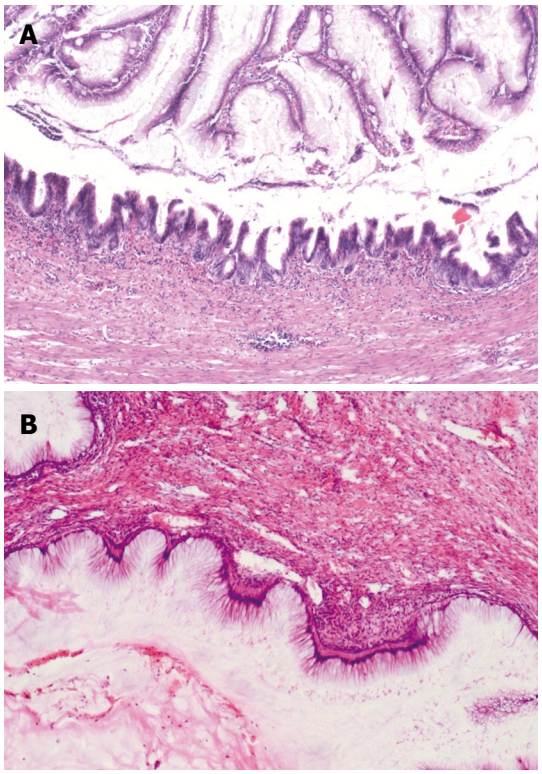

Figure 3 Mucinous tumors were observed in the appendix (A) (HE stain, × 100) and ovary (B) (HE stain, × 100).

Immunohistochemistry results

CK20 and MUC-2 were positive in 33 of the 35 patients with peritoneal lesions (94.3%) (Figure 4A), but CK7, MUC-1 and CA125 were negative. The staining results of the appendix and/or ovaries (Figure 4B and C) were consistent with those of peritoneal lesions. In one patient, peritoneal tumors expressed CK20, CK7 and MUC-2, but not MUC-1 and CA125. That patient underwent appendectomy at another hospital, and no specimen was examined. No ovarian tumors were seen during surgery; consequently, the ovaries were not resected. The peritoneal and ovarian lesions were negative for CK20, MUC-2, CK7,

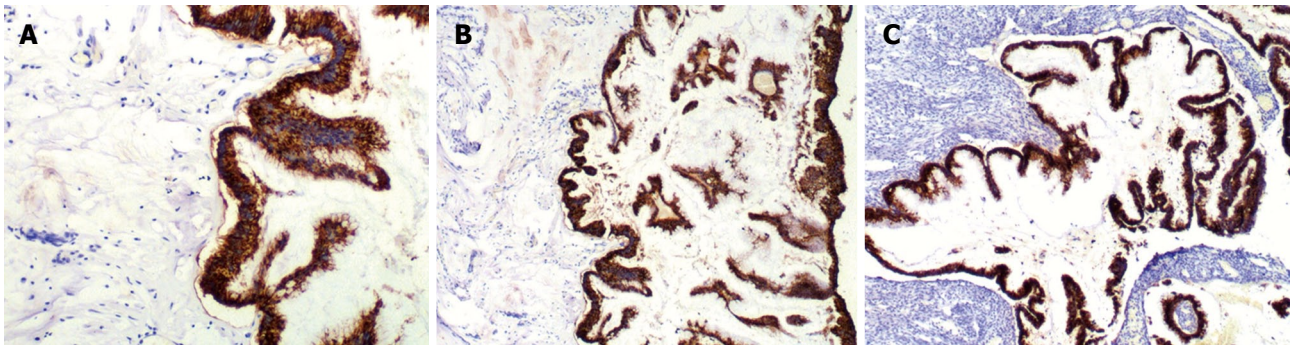

Figure 4 Mucin-2 was positive in peritoneal (A) (× 200), appendiceal (B) (× 100) and ovarian (C) (× 100) lesions.

Table 1 Pathological findings in 35 women with pseudomyxoma peritonei

		Ovary (*n*)					Other organs	Total (*n*)
		Bilateral	Right-side	Left-side	NSC	UR		
Appendix (*n*)	Mucocele	1	0	0	0	0	No	1
	MA	3	5	1	0	3	No	12
	MAC	4	3	1	3	4	No	15
	Appendicitis	1	0	0	0	0	No	1
	UP	1	0	0	0	0	No	1
	NSC	0	0	0	0	1	No	1
	UR	1	0	0	0	0	Gallbladder	1
	UDS	2	1	0	0	0	No	3
Total (*n*)		13	9	2	3	8		35

MA: Mucinous adenoma; MAC: Mucinous adenocarcinoma; UP: Unknown pathology; NSC: No significant changes; UR: Unresected; UDS: Unexplored during surgery.

MUC-1 and CA125 in one patient with a space-occupying lesion of the gallbladder. Additionally, ER and PR status was examined in the peritoneal, appendiceal, and ovarian lesions of the 24 patients with ovarian mucinous lesions, and the results were negative.

Survival

The survival of patients ranged between 2 and 312 mo. The average survival of patients was 47 mo, as calculated using the Kaplan-Meier survival curve. The 3-, 5- and 10-year survival was 54.3%, 23.8% and 13.6%, respectively (Figure 5).

DISCUSSION

PMP is a rare disease with a large volume of extensively implanted gelatinous mucous substance on the surface of the peritoneum or omentum majus[1,2]. The jelly-like substance is called pseudo-mucin, whose chemical properties are different from those of the mucous proteins. PMP was first discovered by Werth[1] in 1884, and its incidence is approximately 2/10 000 in all the patients who are undergoing laparotomy. This disease, with a mean age of 60 years (range: 30-88 years) and a male:female ratio 1:3.4, is characterized by unrelenting pain of gradual onset, abdominal distension, and mucous ascites in literatures. Ultimately, the tumor may occupy the majority of the abdominal cavity and "jelly belly" syndrome may occur. Definitive clinical diagnosis is very difficult to make, and almost all cases are diagnosed with assistance of laparotomy. Detection of jelly-like ascites through abdominal puncture has high diagnostic value for this disease. Our patients with PMP were 24-76 years of age, (mean age, 52.5 years), and the female:male ratio was 1:1.2.

The causes of PMP have remained controversial for many years. It has been reported that the primary tumor of PMP is present in many organs, of which the most common are the appendix and ovaries, followed by the Fallopian tube, pancreas, and intestine. The primary foci are hard to detect in some cases[2,3]. Most patients with PMP either suffer from appendiceal mucinous diseases (including cystadenoma and cystadenocarcinoma) or have a history of recent appendectomy[4-10]. In women, PMP may be accompanied by ovarian mucinous tumors, which often occur bilaterally. If the tumor occurs unilaterally, it more often affects the right ovary[9,10]. Clinically, about a third to a half of women with PMP have concurrent ovarian and appendiceal mucinous tumors.

Our hospital has treated 83 cases of PMP (45 men and 38 women) since the establishment of our hospital. The number of male patients was greater than female patients, which is different from that reported in the literature. Thirty-one of the 35 female patients in our study underwent appendectomy, and appendiceal mucinous tu-

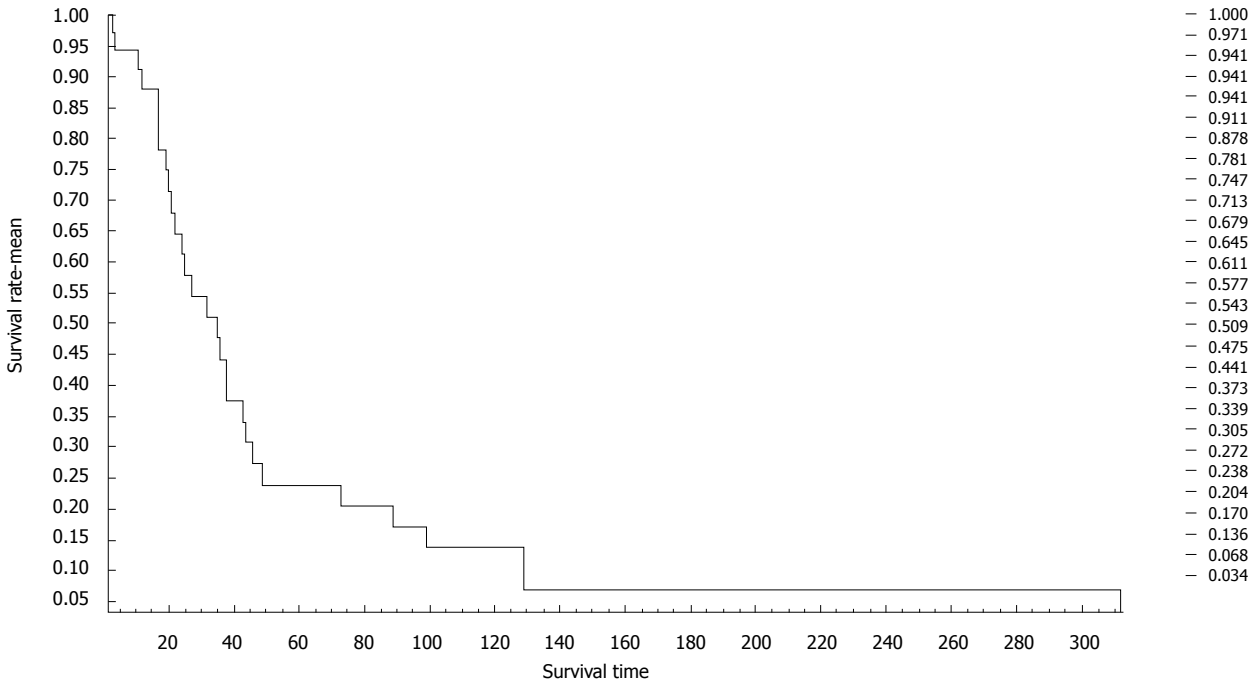

Figure 5 Survival of 35 women with pseudomyxoma peritonei.

mors were seen in 28 of these. Three patients underwent surgery at other hospitals, and the pathological results of two were obtained. The pathological results of the remaining patient were unknown. For the two patients with pathological results, the pathological diagnosis was chronic inflammatory mass of the appendix in one patient, and no significant change in the appendix in the other. The remaining four patients did not have a history of appendectomy, and in three of them, the appendix was not explored during cytoreductive surgery for peritoneal tumor.

Twenty-four of the 35 patients had ovarian mucinous lesions, which were bilateral in 13 cases, on the right side in nine, and on the left side in two. Concurrent appendiceal and ovarian lesions occurred in 20 cases. It is controversial whether the ovarian lesions in these patients were primary or secondary to the appendiceal lesions.

In recent years, the techniques of immunohistochemistry and molecular biology have become more sophisticated, and have greatly enhanced our ability to identify the origin of this disease. In 1991, Young *et al*[5] analyzed 22 cases of ovarian mucinous tumors and PMP-induced appendiceal mucinous tumors. Their results showed that PMP and ovarian lesions both originated from the appendix. Subsequently, Ferreira[6] and Ronnett[7] have made the same observation. Using immunohistochemical methods, Dong *et al*[10] in China have analyzed CK7, CK20 and CA125 expression in peritoneal, ovarian and appendiceal tumors in women with PMP. These investigators drew the same conclusion. Szych *et al*[8] have analyzed the *k-ras* mutations and chromosome 18q, 17p, 5q and 6q alleles in patients with PMP. Their results support the conclusion that the ovarian lesions originate from the appendix in patients with PMP.

It has been reported in the literature that CK7 is a specific marker of primary ovarian epithelial tumors, which is rarely expressed in gastrointestinal epithelial cells[4,6]; however, our experience has shown that CK7 is not specific enough, and sometimes CK7 is positive in typical gastrointestinal adenocarcinoma. CK20 is mainly expressed in the epithelial cells of the gastrointestinal tract[4,6], but it can also be positive in intestinal-type ovarian mucinous tumors. MUC2 is a highly specific marker of intestinal goblet cells[4,6]. MUC1 is often expressed in epithelial tumors of the breast and female reproductive systems[6], but it is negative in epithelial cells of the gastrointestinal tract. CA125 is a surface membrane glycoprotein that is associated with ovarian cancer cells, which is positive in epithelial cells of most ovarian cancers[6]. In women, different levels of ER and PR expression are present in most of the primary ovarian epithelial tumors. Application of the aforementioned antibodies in the detection of tissue antigens is helpful in differentiating whether the tumor originates in the appendix or the ovaries.

Thirty-three of the 35 patients in our study had CK20- and MUC-2-positive peritoneal lesions, but CK7, MUC-1, CA125, ER and PR were negative. The expression pattern of the appendix and the ovary was similar to that of the peritoneal lesions. In one of the remaining two cases, CK20, CK7 and MUC-2 were positive, and MUC-1, CA125, ER and PR were negative. The ovaries were not resected because there were no abnormal intraoperative findings. This patient's appendix was removed at another hospital, and no specimen was examined. In the other case, the appendix appeared to be normal during surgery and was not resected. Peritoneal and ovarian lesions were negative for CK20, MUC-2, CK7, MUC-1, CA125, ER and PR. The above results suggest that PMP

and ovarian lesions were implanted and metastatic appendiceal tumors in 34 of the 35 cases.

It has been reported in the literature that the appendix can be normal in patients with PMP and ovarian mucinous tumors[11]. Lee *et al*[11] have studied 196 patients with borderline ovarian mucinous tumors; of whom, only 11 had PMP. These investigators stated that the apparent absence of appendiceal lesions could have been explained by a variety of circumstances, but that the appendix was not truly normal. First, the appendix may have been left unresected because the surgeon might have observed a normal-appearing appendix during surgery. Second, even if the appendix was resected, the sample collection might not have been sufficient and complete. Finally, even if a sufficient sample was collected, tiny foci of ruptured wall might have been missed due to failure to cut serial sections. In cases with apparent absence of appendiceal lesions, we believe that lesions may have been missed.

In our study, concurrent appendiceal and ovarian lesions occurred in 20 patients, of which, 16 underwent initial surgery for appendiceal lesions, and four for ovarian tumors. Abdominal recurrence occurred at 1-85 mo after surgery, and lesions of appendiceal mucinous tumors were found in all four patients. For one patient who underwent surgery that involved the right ovary, the surgeon explored the appendix during surgery, but the appendix was not resected because no obvious abnormalities were observed by the naked eye. Mucinous tumors were found throughout the abdominal cavity in that patient at 39 mo after surgery, and the resected appendix was confirmed to contain mucinous cystadenoma.

PMP caused by pancreatic mucinous tumor occasionally has been reported in the literature[15]. In our study, bilateral ovarian mucinous tumors were seen in one patient, accompanied by a space-occupying lesion of the gallbladder. The gallbladder was not resected due to the difficulty of the surgery. Immunohistochemical studies of the peritoneal and ovarian lesions showed that CK20, MUC-2, CK7, MUC-1, CA125, ER and PR were negative, which suggested that the ovarian tumor might be metastatic. In the literature, mucinous tumors of the gallbladder have not been reported to cause PMP, and this needs further study.

Treatment and prognosis of PMP

Treatment for PMP is complete resection of the tumors, supplemented by intraperitoneal and systemic chemotherapy. The disease often relapses, and recurrence occurs in 60%-76% of patients after surgery. Multiple surgical resections are often required, and sometimes > 20 operations are performed. Extent and invasiveness of PMP are the important causes of post-surgical recurrence. Although this disease may progress slowly, it is often fatal. The reported median survival was 5.9 years, and the 3-, 5- and 10-year survival was 81.1%-83%, 50.0%-81%, and 18.2%-32%, respectively. Common causes of death are systemic infection secondary to wound infection, bowel obstruction, hernia, and pleural pseudomyxoma caused by tumor passing through the diaphragm. Twenty-five of the 35 patients in our study underwent two or more operations, and 11 patients died. The 5- and 10-year survival was 23.8% and 13.6%, respectively, which was lower than the survival reported in the literature. Therefore, we suggest close follow-up of patients with a diagnosis of PMP; especially those patients whose appendix has not been resected or explored during the initial surgery.

In summary, we believe that PMP often originates in the appendix, and that mucinous ovarian lesions are implanted or metastatic appendiceal tumors. Therefore, appendectomy should be performed routinely for patients in whom PMP is considered during laparotomy. The pathologist should carefully examine the gross specimen of the appendix, and collect as many tissue blocks as possible. Serial sections should be made for suspicious tissue blocks in order to search for small lesions. Additionally, because the incidence of ovarian involvement with implanted tumor is high in women with PMP, adnexa should be explored bilaterally during surgery; especially the right-side adnexa. Patients with PMP should be followed up closely; especially those whose appendix has not been resected or explored during initial surgery.

COMMENTS

Background

Pseudomyxoma peritonei (PMP) is a rare disease that is characterized by dissemination of voluminous jelly-like mucus on the peritoneal surface. Its etiology and pathological origin are not yet fully understood. It has been reported that PMP often originates from the appendix; however, a third to a half of PMP cases are accompanied by ovarian mucinous tumors in women.

Research frontiers

It has been controversial over the years whether the ovarian lesions in patients with PMP are primary or metastatic or implanted from the appendix. Most studies have indicated that PMP associated with ruptured mucinous lesions originates from the appendix. However, some authors consider that PMP originates from the ovaries. In this study, the authors demonstrated that most PMP in Chinese women originated from the appendix, and the ovarian tumors were implanted but not primary.

Innovations and breakthroughs

Recent reports have highlighted the origin of PMP in women. Unfortunately, few reports have observed the origin of PMP in Chinese women, and most of them were case reports or small studies. To the best of our knowledge, this study is the first large sample study on the origin of PMP in Chinese women.

Applications

By understanding the appendiceal origin of PMP in women, appendectomy should be performed routinely for patients in whom PMP is considered during laparotomy. The pathologist should carefully examine the gross specimen of the appendix, and collect as many tissue blocks as possible. Serial sections should be made for suspicious tissue blocks in order to search for small lesions. Additionally, because the incidence of ovarian involvement with implanted tumor is high in women with PMP, the adnexae should be explored bilaterally during surgery, especially the right-side adnexa.

Terminology

PMP is a disease with a large volume of extensively implanted gelatinous mucous substance on the surface of the peritoneum. The term comes from the jelly-like substance, whose chemical properties are different from those of the mucous proteins, and called pseudo-mucin.

Peer review

This study considers the investigation of the histological origin of PMP in Chinese women, using immunohistochemical methods for detection of several mucin and tumor markers. The authors found that most PMP originated from

the appendix, and among women, the PMP was predominately originated from implanted ovarian tumors. The study was set up correctly. The aim of the study was fulfilled. The figures give a good overview of the results. The methods used and the results are not described sufficiently well.

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BRIEF ARTICLE

Propofol *vs* traditional sedative agents for endoscopic retrograde cholangiopancreatography: A meta-analysis

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Abstract

AIM: To investigate the efficacy and safety of propofol sedation for endoscopic retrograde cholangiopancreatography (ERCP).

METHODS: Databases including PubMed, Embase, and the Cochrane Central Register of Controlled Trials updated as of October 2010 were searched. Main outcome measures were ERCP procedure duration, recovery time, incidence of hypotension and hypoxia.

RESULTS: Six trials with a total of 663 patients were included. The pooled mean difference in ERCP procedure duration between the propofol and traditional sedative agents was -8.05 (95% CI: -16.74 to 0.63), with no significant difference between the groups. The pooled mean difference in the recovery time was -18.69 (95% CI: -25.44 to -11.93), which showed a significant reduction with use of propofol sedation. Compared with traditional sedative agents, the pooled OR with propofol sedation for ERCP causing hypotension or hypoxia was 1.69 (95% CI: 0.82-3.50) and 0.90 (95% CI: 0.55-1.49), respectively, which indicated no significant difference between the groups.

CONCLUSION: Propofol sedation during ERCP leads to shorter recovery time without an increase of cardiopulmonary side effects. Propofol sedation can provide adequate sedation during ERCP.

Key words: Endoscopic retrograde cholangiopancreatography; Propofol; Sedative agents; Meta-analysis; Outcomes

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INTRODUCTION

Endoscopic retrograde cholangiopancreatography (ERCP), the most complex gastrointestinal procedure since its introduction in 1968[1], is a highly effective tool to diagnose or treat a variety of biliopancreatic diseases. It is generally recognized that ERCP is a lengthy and potentially uncomfortable procedure that should be performed under

at least conscious sedation[2]. Over the past two decades, propofol, a short-acting agent with rapid metabolism *in vivo* has been used frequently worldwide as a sedative agent for standard endoscopic procedures[3]. However, propofol may lead to deep sedation or even dangerous adverse events that require cardiopulmonary support[4]. Previous studies and several meta-analyses[5,6] have demonstrated that, compared with the traditional sedative agents, propofol sedation is associated with a lower risk of complications in gastrointestinal endoscopy. To date, several studies have compared the effectiveness of propofol with conventional sedation during ERCP. However, the results of individual studies have been inconclusive. Thus, we propose that pooling all available studies together systematically may provide a better understanding of the procedure. Here, we performed a meta-analysis to assess the safety and efficacy of propofol sedation for ERCP, including all randomized controlled trials (RCTs).

MATERIALS AND METHODS

Searching strategy

Related articles in all languages were identified and selected by searching multiple electronic databases including PubMed, Embase, and the Cochrane Central Register of Controlled Trials updated to October 2010, and all bibliographies were identified in the reference lists to identify eligible studies. Due to the relatively small number of articles in this field, we did not use an automated RCT filter in the searching strategy. Key words including ERCP, propofol and diprivan, were used to identify as many articles as possible. Internet search engines, Google Scholar and Yahoo, were also searched with relevant keywords. Major proceedings of international meetings were handsearched.

Inclusion and exclusion criteria

The primary objective of this meta-analysis was to determine the safety and efficacy of propofol sedation for ERCP by comparing with traditional sedative agents such as meperidine, midazolam, scopolamine, and/or pentazocine. Only RCTs in adult patients aged > 18 years who underwent ERCP, published as full articles or meeting abstracts in peer-reviewed journals were considered. Studies were included if they provided the sedation-related outcomes: patient monitoring and complications (i.e., hypoxia or hypotension), procedure-related outcomes (i.e., ERCP duration, sedation and recovery time). All the studies that used propofol plus other agents simultaneously in the same group were excluded. We also excluded studies that could not provide actual frequencies of the complications rather than percentages of complications or percentage decline in complications.

Data extraction and validity assessment

Two authors (Bo LL and Bai Y) selected the studies, extracted the data, and assessed study quality using a predesigned form. This process resulted in high inter-observer agreement (K = 0.86). Disagreement was resolved by consensus or discussion with the third author (Deng XM). Extracted information includes study design, interventions, outcomes, and adverse effects. When necessary, authors were contacted for data not reported or not fully clarified in the original article.

Included studies were assessed for methodological quality on a scale validated by Jadad *et al*[7] and scored from 0 to 5: randomization (0-2 points), blinding (0-2 points), and full accounting of all patients (0-1 point); a higher score indicating better quality. All the included studies had a score of at least 1 because randomization was a requirement for inclusion.

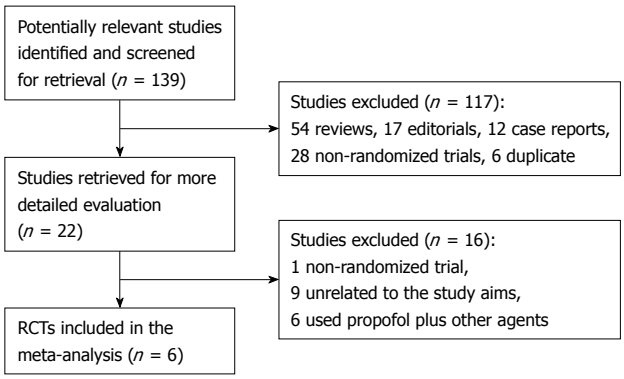

Figure 1 Flow diagram of included and excluded trials.

Statistical analysis

All statistical analysis was performed using Review Manager (RevMan version 5.0), the Cochrane Collaboration's software for preparing and maintaining Cochrane systematic reviews. Meta-analysis was performed using fixed-effect or random-effect methods, depending on the absence or presence of significant heterogeneity. We used the χ^2 test to assess heterogeneity between trials and the I^2 statistic to assess the extent of inconsistency. $P < 0.10$ was defined as significant heterogeneity. Results were expressed as OR or mean difference with 95% CI. $P < 0.05$ was considered statistically significant. Potential publication bias was examined by funnel plot.

RESULTS

Selected RCTs

Figure 1 shows the process of study selection. Our initial searching strategy yielded 139 citations in Embase, PubMed, and Cochrane library (updated to October 12, 2010), of which 117 were excluded on the basis of the title or abstract. Of the remaining 22 articles, we excluded one study that was not randomized, nine unrelated to the study aims, and six having used some other agents plus propofol in the same group or in the control.

Finally, six RCTs[8-13], with a total of 663 subjects, 331 who received propofol, and 332 who received traditional agents for sedation, fulfilled our inclusion criteria.

Table 1 Characteristics of studies included in the meta-analysis

Included studies	Country	Administrator	Procedure	Sedation	Sample size	Hypoxia (SaO_2 < 90%)	Hypotension (SBP < 90 mmHg)	Procedure duration (min)	Recovery time (min)	Jadad score
Chen *et al*[11], 2005	China	ICU physician	ERCP	Propofol	35	2	7	49.22 ± 24.51	5.20 ± 1.94	2
				Meperidine + scopolamine	35	3	0	69.59 ± 25.16	63.94 ± 78.02	
Jung *et al*[10], 2000	Germany	Anesthesiologist	ERCP	Propofol	40		1			2
				Midazolam	40		0			
Kongkam *et al*[13], 2008	Thailand	ACLS trained gastroenterologist	ERCP	Propofol	67	15	6	39.79 ± 32.49	17.24 ± 5.99	5
				Meperidine + midazolam	67	21	6	41.82 ± 21.85	34.25 ± 16.06	
Krugliak *et al*[12], 2000	Israel	Anesthesiologist	ERCP	Propofol	15				13.1 ± 5.8	5
				Midazolam	17				58.4 ± 29.4	
Riphaus *et al*[8], 2005	Germany	ICU physician	ERCP	Propofol	75	8	6		22 ± 7	5
				Meperidine + midazolam	75	7	4		31 ± 8	
Wehrmann *et al*[9], 1999	Germany	Physician unspecified	ERCP	Propofol	99	11	7	27 ± 16	19 ± 8	4
				Midazolam + pentazocine	98	8	2	32 ± 14	29 ± 8	

SBP: Systolic blood pressure; ERCP: Endoscopic retrograde cholangiopancreatography.

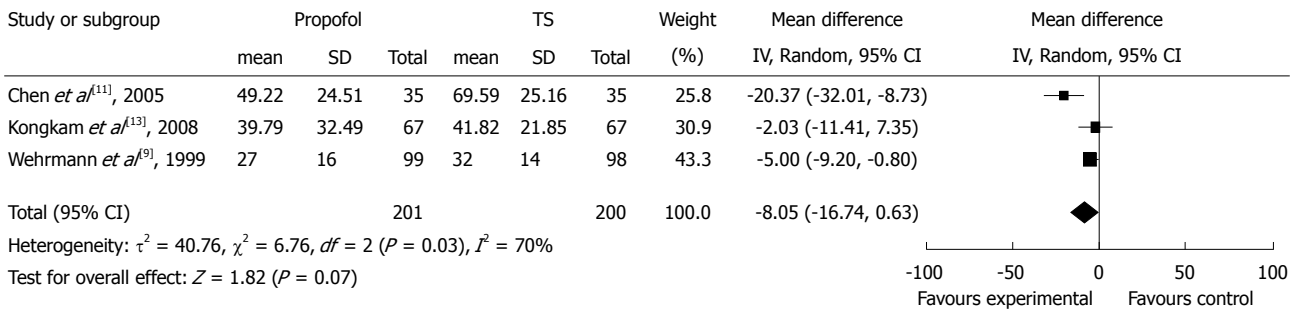

Figure 2 Forest plot of meta-analysis of propofol *vs* traditional sedative agents in endoscopic retrograde cholangiopancreatography procedure duration. IV: Inverse variance; TS: Traditional sedation.

Among them, three trials were reported from Germany[8-10] (Riphaus, 2005 #30), one from China[11], one from Israel[12], and one from Thailand[13]. All eligible articles were reported in the form of full-text articles.

Characteristics of the selected studies

The characteristics of the six included studies are summarized in Table 1. The median number of enrolled patients was 107 (range, 32-197). The indication for ERCP in these trials was generally biliary diseases. All of them were randomized controlled single-center trials. Four of them[8,10,12,13] reported the method of randomization with a Jadad score of $\geqslant$ 3, which suggested a good study design or high quality of report.

Meta-analysis results

Procedure time: The duration of ERCP procedure between propofol and control groups was measured in three studies. Although all of them showed a trend towards duration reduction in the propofol group, the pooled mean difference between the propofol and control groups was -8.05 (95% CI: -16.74 to 0.63), which suggested a statistically non-significant difference between the two groups. The χ^2 and I^2 were 6.76 ($P < 0.10$) and 70%, which indicated heterogeneity among the studies (Figure 2).

Recovery time: Five studies with 583 patients reported recovery time. All of them found a shorter mean recovery time using propofol with pooled weighted mean difference (WMD) of -18.69 (95% CI: -25.44 to -11.93), which indicated a statistically significant difference between the two groups. The χ^2 and I^2 were 46.9 ($P < 0.10$) and 91%, which suggested heterogeneity among the studies. Sensitivity analysis omitting two studies[11,12] with a high risk of bias did not alter the findings, pooled WMD -11.61 (95% CI: -15.45 to -7.78) (Figure 3).

Complications: The complications of hypotension and hypoxia were recorded in most of the studies. However, amnesia was recorded in only three studies. Systolic blood pressure < 75% of baseline and heart rate < 75% of baseline were recorded in only two studies. Due to the limited number of studies, and different criteria for amnesia recognition, only hypotension and hypoxia were

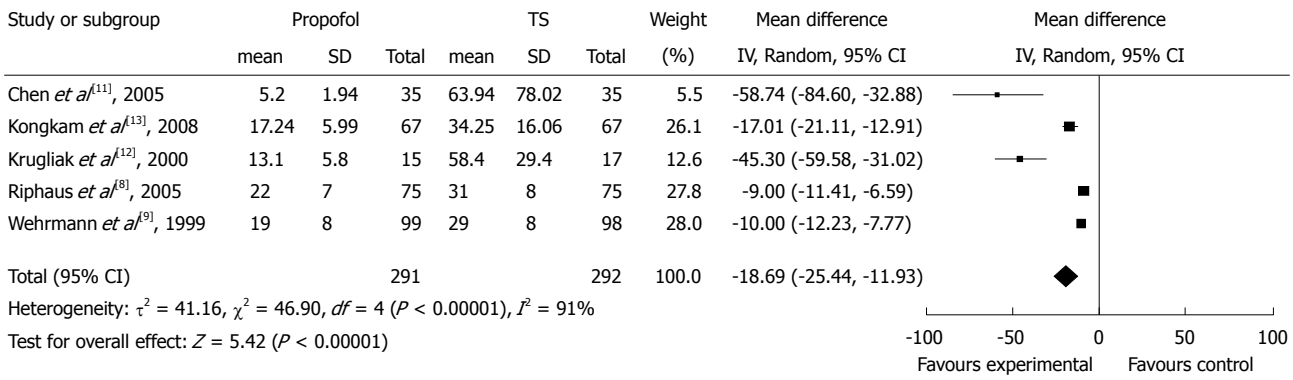

Figure 3 Forest plot of meta-analysis of propofol *vs* traditional sedative agents in endoscopic retrograde cholangiopancreatography recovery time. IV: Inverse variance; TS: Traditional sedation.

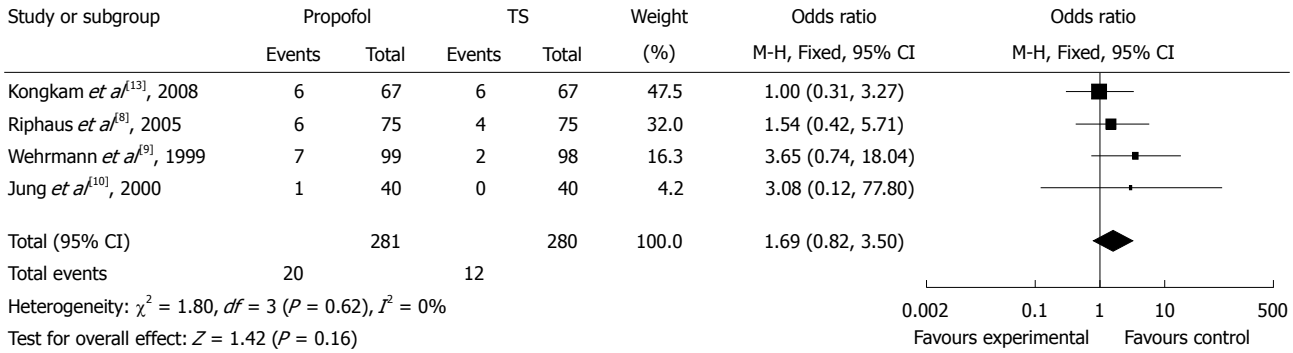

Figure 4 Forest plot of meta-analysis of propofol *vs* traditional sedative agents in occurrence of hypotension during endoscopic retrograde cholangiopancreatography. M-H: Mantel-Haenszel; TS: Traditional sedation.

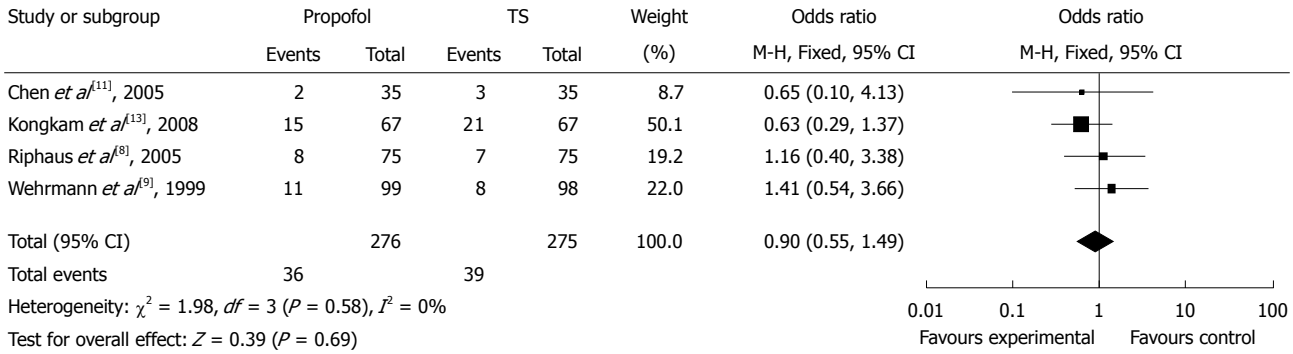

Figure 5 Forest plot of meta-analysis of propofol *vs* traditional sedative agents in occurrence of hypoxia during endoscopic retrograde cholangiopancreatography. M-H: Mantel-Haenszel; TS: Traditional sedation.

eligible for inclusion in the present meta-analysis.

Of the four studies, the OR of hypotension in three studies was in favor of traditional agents, with one showing no difference. The meta-analysis demonstrated that hypotension occurred in 4.29% of controls (12/280) *vs* 7.12% (20/281) of the propofol group. Compared with traditional agents for sedation, the pooled OR of developing hypotension using propofol was 1.69 (95% CI: 0.82-3.50), which indicated no statistically significant difference between the two groups (Figure 4).

In evaluating the OR between propofol and traditional sedation agents causing hypoxia, two studies favored propofol, whereas two studies favored traditional sedation agents. The meta-analysis demonstrated that hypoxia occurred in 14.19% of controls (39/275) *vs* 13.04% (36/276) of the propofol group. Overall, the pooled OR of developing hypoxia using propofol was 0.90 (95% CI: 0.55-1.49), which suggested no statistically significant difference between the two groups (Figure 5).

Publication bias: Funnel plot analysis was conducted using the occurrence of hypotension as the index. The graphical funnel plot of the five studies appeared to be asymmetrical (Figure 6).

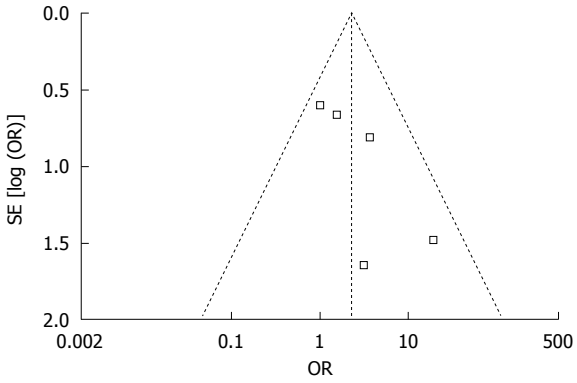

Figure 6 Funnel plot of trials of propofol sedation during endoscopic retrograde cholangiopancreatography. OR: Odds ratio.

DISCUSSION

By summarizing the current best evidence, this meta-analysis conclusively revealed that there are clear benefits of propofol sedation during ERCP regarding the recovery time, without an increase in hypotension and hypoxia occurrence.

Propofol is widely used to induce and maintain anesthesia. It is also used to induce moderate to deep sedation for other procedures, and its advantages include rapid onset, rapid recovery time, and absence of nausea or vomiting[14]. During the past decade, with the growing interest in sedation for gastrointestinal endoscopy worldwide, the use of propofol sedation during endoscopy has been increased[15]. In a previous meta-analysis[6], propofol sedation for colonoscopy was associated with significantly fewer adverse effects. Our present meta-analysis of propofol in ERCP indicated that propofol was not inferior to traditional sedation agents.

Our present meta-analysis showed that the recovery time with propofol sedation was significantly reduced when compared with that with traditional sedation. We also confirmed that the incidence of hypotension and hypoxia during ERCP with propofol sedation was comparable to traditional sedation. It has been reported that propofol for sedation during colonoscopy for generally healthy individuals can lead to a faster recovery time without an increase in side effects[6]. Our results in ERCP also found a significant reduction in recovery time. Qadeer *et al*[6] also concluded that propofol is not inferior to other agents when used for ERCP/endoscopic ultrasound sedation (EUS) in terms of complications of hypoxia and hypotension. However, their meta-analysis of propofol sedation in ERCP included only three studies; since then, three new RCTs have been published[8,11,13]. Estimation based on the three trials, involving only 304 patients, was underpowered to detect the risk of hypoxia or hypotension. Several differences should also be highly noted. First, our present meta-analysis focused specifically on ERCP, whereas the previous meta-analysis focused on colonoscopy. Second, the procedure duration and recovery time were compared in the present meta-analysis, whereas the previous analysis only estimated the risk of hypoxia and hypotension caused by propofol sedation during ERCP/EUS.

Guidelines and a position statement[16] published jointly by four American gastroenterology and hepatology societies regarding non-anesthesiologist administration of propofol for gastrointestinal endoscopy state that, non-anesthesiologist administration of propofol is more cost-effective than standard sedation with benzodiazepines and opioids. Propofol has the potential to induce general anesthesia, and there is no pharmacological antagonist to reverse its effect. Although propofol sedation appears to be a promising strategy during ERCP, its side effects should never be underestimated. With respect to its potential side effects, the administrator should be aware of the risk of hypotension and respiratory depression[4]. Further studies with standardized end-points are also needed to compare propofol administration by anesthesiologists to that by non-anesthesiologists.

The objectives of a meta-analysis include increasing power to detect an overall therapeutic effect by estimating the degree of benefit associated with a particular study treatment[17]. In the case of propofol sedation during ERCP, the current meta-analysis pooled all available data from published RCTs, which substantially reduced the type II error. However, the present meta-analysis also has several limitations that need to be taken into account in interpreting the results.

First, this meta-analysis is a study-level but not an individual patient-level meta-analysis. It is known that study-level analysis can lead to biased assessments, and use of aggregated summary values has some limitations for explaining the heterogeneity[18]. Second, the administrator of propofol sedation was not the same in all the included studies: two studies by anesthesiologists[10,12], two by ICU physicians[8,11], one by ACLS trained gastroenterologists[13], and one by an unspecified physician[9]. This may be considered as a source of heterogeneity. However, due to the limited number of included studies and differently recorded data, subgroup analysis was not carried out. Third, we originally intended to analyze other complications (e.g., arrhythmias, antegrade amnesia, and apnea), assessment of the procedure by the patients (i.e. satisfaction, pain or discomfort), and assessment of the procedure by physicians (i.e., satisfaction with sedation and patient cooperation). However, due to the limited number of studies that reported relevant outcomes, and the different methods in reporting outcomes, it was not appropriate to combine them together for the present meta-analysis. It should be emphasized that future studies should take into a comprehensive consideration of uniform outcome reporting.

A high incidence of hypotension was noticed among all original studies except one[13]. Although the present meta-analysis found no significant statistical difference between two sedative agents, there was a trend toward a higher incidence of hypotension with propofol sedation. This result may have been caused by the relatively small

numbers included in each study, leading to a high possibility of type Ⅱ error, which could weaken the conclusions. Further studies with a large number of patients are warranted to clarify the safety of propofol sedation during ERCP.

In conclusion, propofol sedation during ERCP can lead to a shorter recovery time without an increase of cardiopulmonary side effects. Propofol sedation seems to be an effective method for providing adequate sedation during ERCP.

ACKNOWLEDGMENTS

The authors thank all the authors of the primary studies, without which this study would not have been possible.

COMMENTS

Background

Endoscopic retrograde cholangiopancreatography (ERCP), a highly effective tool to diagnose or treat a variety of biliopancreatic diseases, is considered as the most complex gastrointestinal procedure. It is generally recognized that ERCP is a lengthy and potentially uncomfortable procedure that should be performed under at least conscious sedation.

Research frontiers

Propofol, a short-acting agent with rapid metabolism *in vivo*, has been used frequently worldwide as a sedative agent for standard endoscopic procedures. However, propofol may lead to deep sedation or even dangerous adverse events that require cardiopulmonary support.

Innovations and breakthroughs

The current meta-analysis summarized all available studies to support the propofol sedation for ERCP. The authors found from this meta-analysis that propofol can lead to a shorter recovery time without an increase of cardiopulmonary side effects. Propofol sedation can provide adequate sedation during ERCP.

Applications

This study generated the best evidence to support the clinical use of propofol for ERCP sedation.

Terminology

Propofol: a short-acting, intravenously administered hypnotic agent. It is used in the induction and maintenance of general anesthesia, sedation for mechanically ventilated adults, and procedural sedation. ERCP: a technique that combines the use of endoscopy and fluoroscopy to diagnose and treat certain problems of the biliary or pancreatic ductal systems.

Peer review

The authors made a meta-analysis comparing the effect and adverse effects of propofol for ERCP sedation. Nowadays, propofol is more frequently used especially to sedate patients undergoing ERCP. Clearly, there is a need to update on the propofol effect and safety. The great effort provided by the authors is appreciated.

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BRIEF ARTICLE

Contrast-enhanced multiple-phase imaging features in hepatic epithelioid hemangioendothelioma

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Abstract

AIM: To investigate and review the contrast-enhanced multiple-phase computed tomography (CEMP CT) and magnetic resonance imaging (MRI) findings in patients with pathologically confirmed hepatic epithelioid hemangioendothelioma (HEHE).

METHODS: Findings from imaging examinations in 8 patients (5 women and 3 men) with pathologically confirmed HEHE were retrospectively reviewed (CT images obtained from 7 patients and MR images obtained from 6 patients). The age of presentation varied from 27 years to 60 years (average age 39.8 years).

RESULTS: There were two types of HEHE: multifocal type (n = 7) and diffuse type (n = 1). In the multifocal-type cases, there were 74 lesions on CT and 28 lesions on MRI with 7 lesions found with diffusion weighted imaging; 18 (24.3%) of 74 lesions on plain CT and 26 (92.9%) of 28 lesions on pre-contrast MRI showed the target sign. On CEMP CT, 28 (37.8%) of 74 lesions appeared with the target sign and a progressive-enhancement rim and 9 (12.2%) of 74 lesions displayed progressive enhancement, maintaining a state of persistent enhancement. On CEMP MRI, 27 (96.4%) of 28 lesions appeared with the target sign with a progressive-enhancement rim and 28 (100%) of 28 lesions displayed progressive-enhancement, maintaining a state of persistent enhancement. In the diffuse-type cases, an enlarged liver was observed with a large nodule appearing with persistent enhancement on CEMP CT and MRI.

CONCLUSION: The most important imaging features of HEHE are the target sign and/or progressive enhancement with persistent enhancement on CEMP CT and MRI. MRI is advantageous over CT in displaying these imaging features.

Key words: Liver; Neoplasm; Epithelioid hemangioendothelioma; Computed tomography; Magnetic resonance imaging

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INTRODUCTION

Hepatic epithelioid hemangioendothelioma (HEHE) is

a rare vascular tumor in adults with a variable but often long clinical course, which is intermediate between hemangioma and angiosarcoma in clinical biological behavior[1]. This tumor is histologically characterized by an epithelial appearance and endothelial nature in tumor cells[1]. Clinical, imaging and pathologic diagnosis of HEHE is difficult[2-4], however, its correct diagnosis is very important because long-term survival (5-10 years) of HEHE is possible[5]. Treatment modalities include hepatic resection, orthotopic liver transplantation (even in cases with known metastases), radiotherapy, chemotherapy and use of interferon alpha-2[3,6].

No more than 200 cases of HEHE have been reported since its first description[1-19] and most of them were of sporadic cases and small case series[1,2,8,10,12-15]. The contrast-enhanced multiple-phase computed tomography (CEMP CT) and magnetic resonance imaging (MRI) findings of HEHE have not been well addressed. In this paper, we highlight the predominant imaging features of this type of tumor, focusing on the target sign and/or progressive enhancement with persistent enhancement on CEMP CT and MRI, which have not been extensively described previously in the English-language literature, to the best of our knowledge.

MATERIALS AND METHODS

Subjects

CT (n = 7), MRI (n = 6), clinical (n = 8) and pathological (n = 8) features of 8 cases of HEHE were retrospectively reviewed at our institution from 2004 to 2009. This study was approved by the Institutional Research and Ethics Board of our institution. Among the 8 cases, there were 3 males and 5 females, with ages ranging from 27 to 57 years (mean, 39.8 years). The duration of symptoms ranged from 10 d to 2 years.

The clinical signs and symptoms included epigastric pain (n = 2), discomfort (n = 3), weight loss (n = 3), weakness (n = 2), hepatomegaly (n = 5), splenomegaly (n = 4) and ascites (n = 1). Two patients without any complaints were incidentally found by a routine physical examination. One of 8 cases was accompanied by lung epithelioid hemangioendothelioma. Laboratory tests showed abnormal liver function in two cases, with mild elevation of serum bilirubin, alkaline phosphatase and aspartate aminotransferase levels. HBsAg was positive in two patients. Tumor marker levels, including α-fetoprotein, carcinoembryonic antigen (CEA) and cancer antigen 19-9, were negative in all patients except for an increased level of CEA in one patient.

Radiological examination

CT imaging was performed in seven patients using Siemens Somatom Sensation 16-row CT scanners with 5-mm axial sections from the dome of the diaphragm to the last plane of the liver. All patients were examined in a fasting state with plain scanning at first, and then non-ionic contrast medium (Omnipaque 300 g/L, GE Healthcare, USA) 80 mL per bolus injection was given *via* antecubital vein for enhanced scanning. Images were obtained separately at the arterial phase (25-35 s after injection), portal venous phase (65-75 s after injection) and equilibrium phase (100-110 s after injection).

MR scanning was performed using a 1.5 T or 3.0 T magnet (Signa, GE Healthcare, USA) with an eight-channel torso-array coil. Axial T1-weighted images (T1WI) and T2-weighted images (T2WI) were obtained from all six patients, and additional contrast-enhanced T1WI (Omniscan, GE Healthcare, USA, 0.1 mmol/kg body weight) images were obtained from four patients. Dynamic breath-hold T1WI acquisitions were obtained at 15-27 s, 40-52 s, 70-82 s and 130-142 s after contrast enhancement. The imaging parameters for T1WI and T2WI were as follows: repetition time/echo time (TR/TE) of 205/3.2 ms and 6000/102.5 ms. The matrix was 256 × 256, the standard field-of-view was 400 mm and slice thickness was 4.0 mm with no interslice gap. Additional diffusion weighted single-shot echo-planar imaging was performed in two patients using the following parameters: TR/TE = 1300/52.5 ms, 7-8 mm thickness, water selective excitation for fat suppression, matrix size = 128 × 128, field of view = 36 cm × 36 cm, number of excitations = 6.0, slice thickness/gap = 5 mm/1.0 mm, 20 axial slices, scan time = 2 min 24 s, b value = 0 and 600 s/mm^2, under breath-hold.

Image analysis

All CT and MR images were reviewed separately by two radiologists who were blinded to the identity of the patient and clinical outcome. Discordance between the two was resolved by consensus.

Pathological examination

Histologic specimens of HEHE were obtained by percutaneous needle biopsy in five patients and by exploratory laparotomy and nodule biopsy in three patients. HEHE was diagnosed on the basis of light microscopic examinations of histologic specimens. HE staining and immunohistochemical staining for at least one endothelial marker, i.e., factor Ⅷ-related antigen, CD34, or CD31, were performed on all tumors to confirm the endothelial origin[2,8]. All HEHE specimen analyses were confirmed by an experienced pathologist for diagnostic accuracy.

RESULTS

Imaging findings

There were two types of HEHE in the 8 cases of our study: multifocal type (n = 7) and diffuse type (n = 1). In the 7 multifocal type cases, a total of 74 lesions were found with CT, 28 lesions with MRI and 7 with diffusion weighted imaging (DWI). Eighteen (24.3%) of 74 lesions on plain CT showed a low density with peripheral isodensity (Figure 1A), which looked like a "target" with an inner low density/intensity and a peripheral hyperdensity/intensity or isodensity/intensity (target sign).

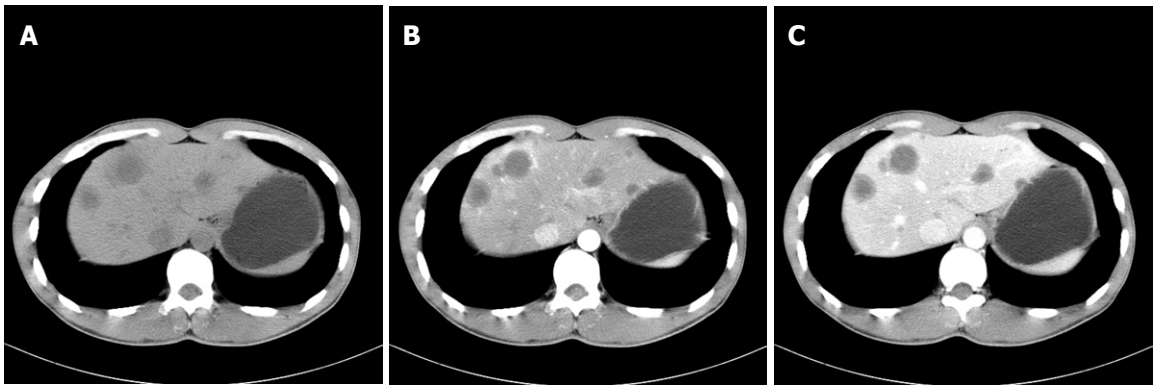

Figure 1 Multifocal hepatic epithelioid hemangioendothelioma in a 27-year-old male. (A) Unenhanced axial computed tomography scan of liver shows multiple discrete masses, which are round with a low density and peripheral iso-density. (B-C) Partial lesions show peripheral ring-like enhancement in the arterial phase (B) with even stronger enhancement in the portal venous phase (C).

On CEMP CT images, 28 (37.8%) of 74 lesions showed peripheral ring-like enhancement in the arterial phase with even stronger enhancement in the portal venous and equilibrium phases, appearing as a target sign with a progressive-enhancement rim (Figures 1B, C). Nine (12.2%) of 74 lesions displayed progressive enhancement, with 8 (10.8%) lesions showing progressive enhancement in the center and 1 (1.4%) lesion showing lamellar progressive enhancement on CEMP CT, maintaining the state of persistent enhancement.

Twenty-six (92.9%) of 28 lesions showed hypointensity relative to normal liver parenchyma with peripheral faint hyperintensity on T1WI (Figure 2A) and hyperintensity with peripheral hypointensity and an area of evident hyperintensity in the center on T2WI (Figure 2B), appearing as the target sign. Six (85.7%) of 7 lesions showed hyperintensity with a peripheral hypointense rim on DWI (Figure 2C) and 1 (14.3%) showed lamellar hyperintensity on DWI.

On CEMP MRI, 27 (96.4%) of 28 lesions displayed peripheral ring-like enhancement in the arterial phase, and even stronger enhancement in the portal venous and equilibrium phases, appearing as a target sign with a progressive-enhancement rim; 28 (100%) of 28 lesions displayed progressive-enhancement in the arterial, portal venous and equilibrium phases and became isointense to liver parenchyma in the delayed phase, with 27 (96.4%) lesions showing progressive enhancement in the center of the target (Figures 2D-G, Figures 3A-E) and 1 (3.6%) lesion showing lamellar progressive enhancement on CEMP MRI (Figures 3F-J), maintaining the state of persistent enhancement.

One diffuse case manifested an enlarged liver with a large nodule appearing slightly hyperdense relative to normal liver parenchyma on plain CT (Figure 4A), isointensity on T1WI (Figure 4E) and hypointensity on T2WI (Figure 4F), with slight enhancement in the arterial phase (Figure 4B), evident enhancement in the portal venous phase (Figures 4C and G) and isodense/intense in the equilibrium phase (Figures 4D and H), which manifested persistent enhancement on CEMP CT and MRI, associated with splenomegaly and ascites (Figure 4F). All the imaging features are summarized in Table 1.

Pathologic results

All tumors were consistent with a diagnosis of HEHE on pathological review. Grossly, the tumors were solid, firm and hyperemic in the outer portions. Histologically, the tumors were composed of dendritic and epithelioid cells. Signet ring–like structures appeared in the tumor cells with intracytoplasmic lumina, occasionally containing red blood cells (Figure 5). The tumors consisted of large amounts of mucinous and dense stroma in the center and rich cellular zones in the periphery. Immunohistochemically, tumors were positive for factor Ⅷ -related antigen in 3 patients (Figure 6A), CD34 in 5 patients (Figure 6B) and CD31 in 4 patients (Figure 6C). Tumors were negative for epithelial markers (cytokeratin and CEA).

DISCUSSION

HEHE is a rare tumor of vascular origin, first defined as a specific entity by Weiss and Enzinger in 1982[5]. Because of the prolonged course and nonspecific clinical manifestations, the age of the patients varies greatly at the time of HEHE detection by biopsy or imaging studies. The incidence of this neoplasm is higher in females than in males (a female to male ratio of 3:2), with a peak incidence occurring between 30 and 40 years of age[2]. Most patients survive 5-10 years after diagnosis[4]. This study covered 5 females and 3 males and their average age was 39.8 years, which was comparable with other reports in the literature.

Clinical manifestation is variable, with most showing nonspecific symptoms such as right upper quadrant pain and weight loss. Physical examination findings are uncommon but may include hepatomegaly, a palpable mass, or jaundice. Some patients present with hemoperitoneum[20] and Budd-Chiari syndrome due to hepatic vein invasion[9]; others present with incidental findings[2]. Liver function tests reveal mild abnormalities in most patients. Tumor marker levels are negative apart from el-

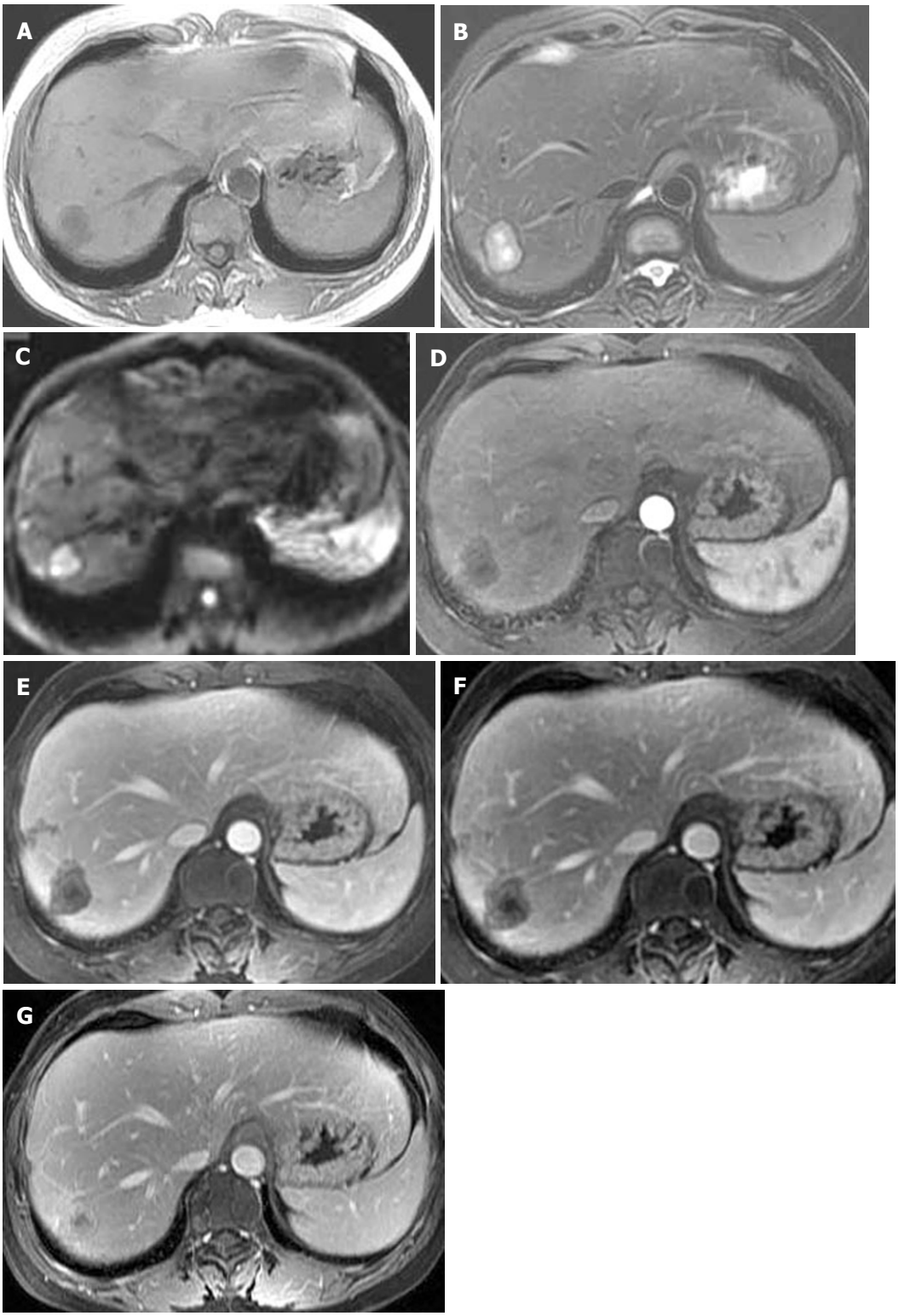

Figure 2 Multifocal hepatic epithelioid hemangioendothelioma in a 48-year-old female. Precontrast axial magnetic resonance imaging scan of the liver shows multiple lesions of low signal intensity with peripheral faint hyperintensity on T1WI (A), high signal intensity with peripheral hypointensity and an area of evident hyperintensity in the center of one lesion on T2WI (B), and hyperintensity with peripheral hypointensity on diffusion weighted imaging (C). Lesions show peripheral ring-like enhancement in the arterial phase (D), and heterogeneously progressive reinforcement in the portal venous phase (E), equilibrium phase (F) and it approaches isointensity to liver parenchyma in the delayed phase (G). There is an association with an area of unenhanced necrosis in the center, which looks like a conspicuous "target" with an inner low intensity, in-between high intensity and outer lower intensity layers.

evated CEA levels in a small number of patients, which is in conformity with our series. No risk factors or specific causes of HEHE were identified. Two patients in our study were HBsAg positive, which is similar to the rate reported in a few studies[10]. However, more cases are needed to clarify the relationship between HBV infection and the occurrence of HEHE.

Pathologically, there are three types of growth patterns in the gross appearance of HEHE: multiple nodules, diffuse nodules and a single mass[3,8,11]. Histologically, the tumors are composed of dendritic and epithelioid cells. Immunohistochemically, tumors are positive for factor Ⅷ-related antigen, CD34 or CD31, demonstrating the endothelial origin of these tumors[2,8]. Two types of HEHE were found in our cases, i.e., multifocal and diffuse, and the immunohistochemical results of our series were consistent with other observations. HEHE may have been present in some patients with pulmonary

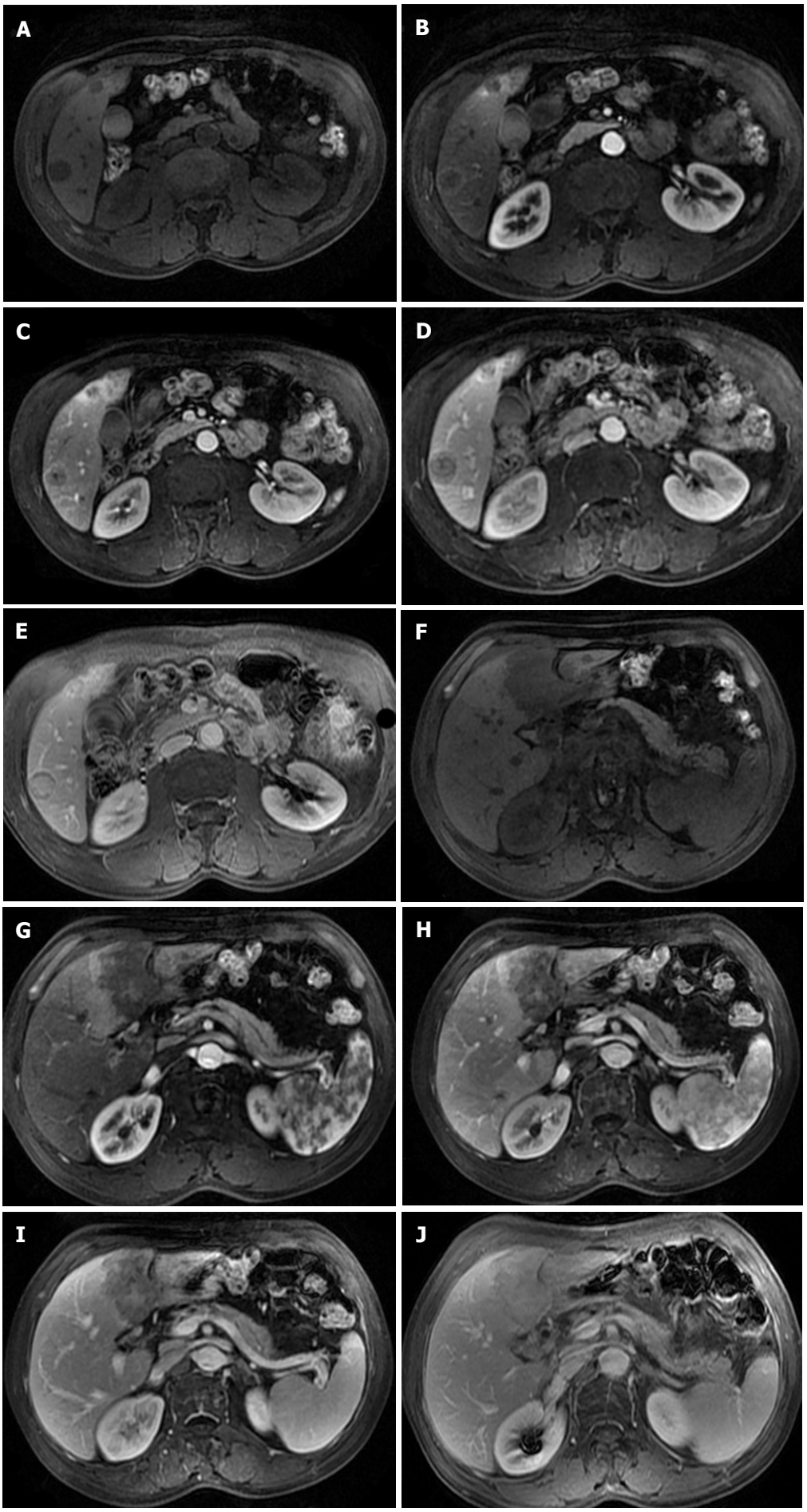

Figure 3 Multifocal hepatic epithelioid hemangioendothelioma in a 60-year-old man. Contrast-enhanced multiple-phase magnetic resonance imaging shows a progressive-enhancement target sign in the lesions in segment Ⅵ of the liver (A-E) and lamellar lesions with progressive enhancement in the left lobe of the liver (F-J).

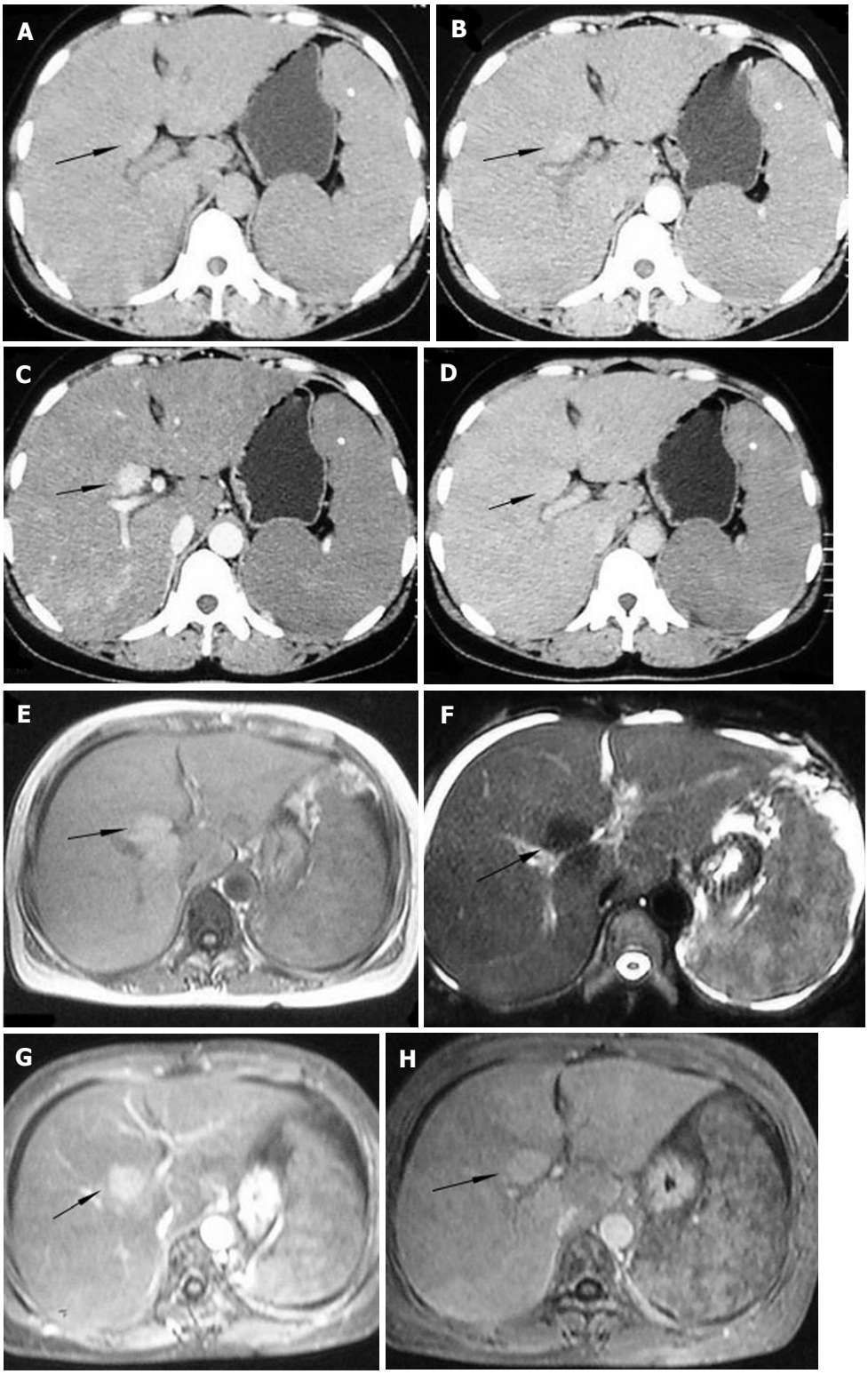

Figure 4 Diffuse hepatic epithelioid hemangioendothelioma in a 48-year-old woman. Plain computed tomography and magnetic resonance imaging manifests an obviously enlarged liver with a large nodule (black arrow) appearing slightly hyperdense relative to normal liver parenchyma (A), isointense on T1WI (E) and hypointense on T2WI (F), with slight enhancement in the arterial phase (B), evident enhancement in the portal venous phase (C and G) and iso-density/intensity in the equilibrium phase (D and H), associated with splenomegaly and ascites (F).

EHE or soft tissue EHE, but was considered metastatic[5,21-23]. In our series, there was one case of HEHE associated with pulmonary EHE.

A few radiological findings in HEHE have been described[1,2,8,10,12-15], however, CEMP CT and MRI findings have not been well addressed. According to the literature on multifocal-pattern HEHE[1,2,8,12,13], plain CT usually shows multiple discrete low-attenuation lesions and extensive confluent masses. Contrast-enhanced CT findings include marginal enhancement during the arterial phase[12], becoming isodense to liver parenchyma on contrast-enhanced scans[8], and a halo or target pattern of enhancement with larger lesions[1,13]. Lin *et al*[10] found that about 38 (48.1%) of 79 lesions showed the "halo" sign on contrast-enhanced CT. We had similar findings with CT, but 24.3% of the lesions showed the target sign on plain CT, 37.8% lesions showed the target sign with a progressive-enhancement rim and 12.2% le-

Table 1 Radiological findings of hepatic epithelioid hemangioendothelioma (HEHE)

Cases	Sex	Age (yr)	Total lesions (*n*)	Plain CT findings of HEHE	Pre-enhanced MRI of HEHE	CEMP imaging findings of HEHE		Radiological findings of extra-hepatic lesions
						Lesions of target sign with peripheral progressive enhancement (*n*)	Lesions of central or lamellar progressive enhancement (*n*)	
Case 1 multifocal-type (Figure 1)	Male	27	42	All discrete, peripheral, low-attenuation lesions with 1 coalescence and 13 target sign	No MRI obtained	42	0	No extra-hepatic lesions
Case 2 multifocal-type	Female	30	14	All discrete, peripheral, low-attenuation lesions with 1 coalescence and 5 target sign	All lesions showing hypointense on T1WI and hyperintense on T2WI with 14 target sign	3 (CT), 14 (MRI)	3 (CT), 14 (MRI)	No extra-hepatic lesions
Case 3 multifocal-type	Female	28	2	All discrete, peripheral, low-attenuation lesions	No MRI obtained	2 (CT), 2 (MRI)	2 (CT), 2 (MRI)	No extra-hepatic lesions
Case 4 multifocal-type	Female	53	4	All discrete, peripheral, low-attenuation lesions	No MRI obtained	4 (CT)	0	No extra-hepatic lesions
Case 5 multifocal-type (Figure 2)	Female	48	10	All discrete, peripheral, low-attenuation lesions with 1 coalescence	All lesions showing hypointense on T1WI and hyperintense on T2WI with 10 target sign, and 5 hyperintense with peripheral hypointense on DWI	10 (CT), 10 (MRI)	4 (CT), 10 (MRI)	No extra-hepatic lesions
Case 6 multifocal-type	Female	56	2	All discrete, peripheral, low-attenuation lesions, with compensatory hypertrophy in the left lobe of liver	No MRI obtained	0	2 (CT)	Pleural effusion in both sides
Case 7 multifocal-type (Figure 3)	Male	60	2	No CT obtained	Two lesions showing hypointense on T1WI and hyperintense on T2WI, and 1 hyperintense with peripheral hypointense on DWI	1 (MRI)	2 (MRI)	No extra-hepatic lesions
Case 8 diffuse – type (Figure 4)	Female	48	Diffuse	Diffuse hepatomegaly with a slightly hyperdense nodule	Diffuse hepatomegaly with a nodule appearing isointense on T1WI and hypointense on T2WI	Diffuse hepatomegaly with a nodule appearing persistent enhancement		Splenomegaly and ascites

HEHE: Hepatic epithelioid hemangioendothelioma; CEMP: Contrast-enhanced multiple-phases; *n*: Number; CT: Computed tomography; MRI: Magnetic resonance imaging; DWI: Diffusion weighted imaging.

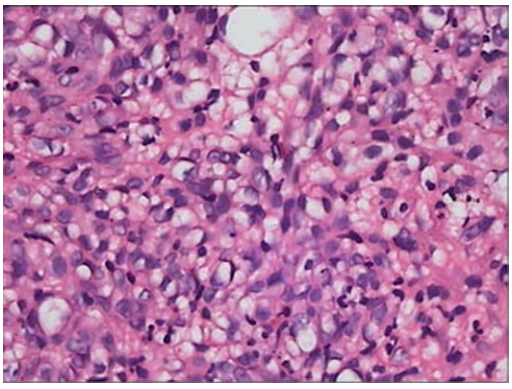

Figure 5 Photomicrography shows epithelioid cells with intracytoplasmic lumina, occasionally containing red blood cells, appearing as signet ring–like structures (hematoxylin and eosin, HE × 200).

sions displayed progressive enhancement on CEMP CT, maintaining the state of persistent enhancement. The CT findings in our cases were not compatible with other reports in the literature.

According to the literature on multifocal pattern HEHE[1,2,8,12,13], precontrast MR imaging revealed hypointense lesions relative to normal liver parenchyma on unenhanced T1-weighted images, heterogeneously increased signal intensity on T2-weighted images and hyperintensity with peripheral hypointensity on DWI[7,8]. Some lesions have a peripheral halo or a target-type enhancement pattern on enhanced MR imaging, with occasional observation of a thin peripheral hypointense rim[2,8,12]. Lin *et al*[10] found that 9 (23.1%) of 39 lesions presented the characteristic "halo" sign on contrast-enhanced MRI. In our series, we had similar findings with precontrast MRI and DWI, but 92.9% of the lesions showed the target sign on precontrast MRI and 14.3% of the lesions showed lamellar hyperintensity on DWI, which is in contrast to the previous literature. In our series, 96.4% of the lesions appeared with the target sign and a progressive-enhancement rim and 100% of the lesions displayed progressive-enhancement, maintaining the state of persistent enhancement. The MRI findings in our cases were not compatible with the previous lit-

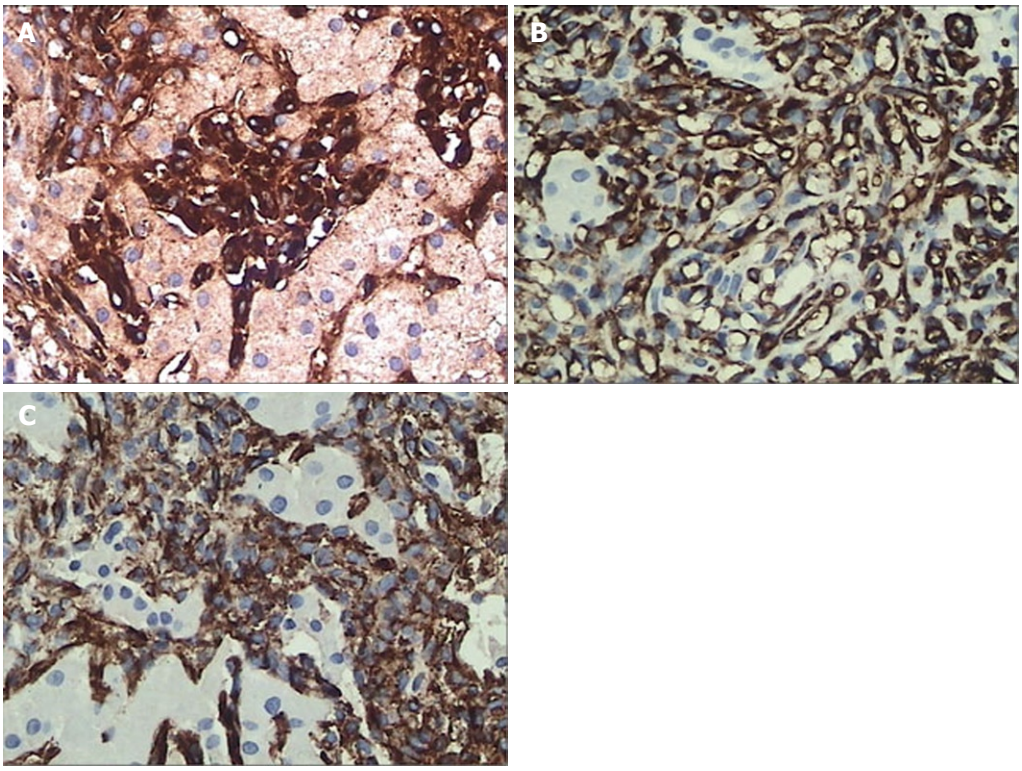

Figure 6 Immunohistochemical staining shows that the tumor cells are positive for factor Ⅷ-related antigen (A), CD34 (B) and CD31 (C) (× 250).

erature. The MRI features of progressive reinforcement on CEMP MRI have not been reported previously according to our literature search.

It could be considered that the distinctive image appearance of the tumor is correlated with the pathologic characteristics in many ways. Histologically, the tumors consisted of large amounts of mucinous and dense stroma in the center and rich cellular zones in the periphery. These findings might account for the central low density and peripheral isodensity on plain CT images, hypointensity with peripheral faint hyperintensity on T1WI and hyperintensity with peripheral hypointensity on T2WI and DWI. The actively proliferating, increased cellular periphery of the nodules may account for the peripheral progressive-enhancement target sign on CEMP CT. The tumor also produced a fibrous myxoid stroma that was most dense in the center of the nodules, which may attribute to the heterogeneously progressive reinforcement on CEMP MRI. Tumor infiltration and occlusion of hepatic sinusoids and small vessels caused a narrow avascular zone between the tumor nodules and liver parenchyma. This may be the reason for the halo appearance on CT or MRI.

According to the reported studies on diffuse-pattern HEHE, a multifocal nodular pattern of infiltration is usually considered as the early stage of a diffuse pattern[1,8,13]. Local lesions may increase in size and coalesce, thus forming the diffuse pattern. The diffuse lesions contain many lowly attenuating, round or irregular spots, which may be associated with calcified foci and dilated bile ducts in the lesions. The lesions were slightly enhanced on dynamic CT scans and became iso-attenuated to non-tumorous liver on subsequent scans, but spots of lower attenuation remained inside or showed marked contrast enhancement during and after intra-arterial contrast material injection and disappeared within 1 min after the contrast material injection. In our series, the diffuse case was also associated with splenomegaly and ascites, but had different imaging findings manifesting an obviously enlarged liver with a large nodule. The manifestation of diffuse-pattern HEHE appearing as an obviously enlarged liver was only reported by Lorber *et al*[11]. Necropsy showed that the liver was grossly enlarged without cirrhosis, and contained a very discrete red area (0.2-2.5 cm in diameter). However, according to our search, the MRI findings of diffuse-pattern HEHE with a large nodule and the imaging feature of a large nodule manifesting persistent enhancement on CEMP CT and MRI have not been reported previously.

Because the histologic specimens of this diffuse-pattern HEHE were obtained by percutaneous needle biopsy of many sites in the liver, but not with exploratory laparotomy, it is hard to analyze the correlation between the imaging findings of the large nodule in a diffuse case with the pathologic findings. We suppose that the large nodule in the diffuse case might consist of large amounts of tumor cells, manifesting persistent enhancement on CEMP CT and MRI, and the nodule appearing slightly hyper-dense on plain CT, isointense on T1WI and hypointense on T2WI might correlate with hemorrhage in the nodule.

According to the isolated case report literature of single-pattern HEHE, it only accounted for about 18%[3]. Jeong *et al*[14] and Hsieh *et al*[15] found that the single lesion is usually ovoid, with a low density and calcification in segment 7 of the liver in the pre-contrast phase of

liver dynamic CT. The mass exhibited central enhancement in the arterial phase, heterogeneous peripheral enhancement in the portal phase, and then peripheral enhancement was washed out in the delayed phase, or with central enhancement in the delayed phase on CT. According to these reports, the single nodular pattern of HEHE typically preferentially involved the right lobe of the liver, and the pattern of contrast enhancement was different and more studies are needed in the future. To our regret, there was no single-pattern HEHE in our cases.

For differential diagnosis, the most important imaging features of a target sign and progressive enhancement could differentiate HEHE from intrahepatic multiple metastatic tumors, cavernous hemangioma and primary hepatic angiosarcoma.

In conclusion, MRI is more advantageous over CT in displaying the imaging features of a target sign and progressive enhancement. Although the incidence of HEHE is low and the diagnosis can only be confirmed by pathological examination, it should be considered in the differential diagnosis list of intrahepatic nodules appearing with a target sign and/or progressive enhancement with persistent enhancement on CEMP CT and MRI, which demonstrates a vasoformative nature, especially in multiple lesions in middle-aged women.

COMMENTS

Background

Hepatic epithelioid hemangioendothelioma (HEHE) is a rare vascular tumor. Clinical assessment, imaging and pathologic diagnosis of HEHE is difficult, however, its correct diagnosis is very important because long-term survival of HEHE is possible.

Research frontiers

No more than 200 cases of HEHE have been reported since its first description. The contrast-enhanced multiple-phase computed tomography (CEMP CT) and magnetic resonance imaging (MRI) findings of HEHE have not been well addressed. In this study, the authors highlight the predominant imaging features of this tumor, which have not been described previously in the English-language literature.

Innovations and breakthroughs

Most of the radiologic studies on HEHE were sporadic case reports and small case series reports. In this study, the authors evaluated and described target sign and/or progressive enhancement with persistent enhancement in CEMP CT and MRI of HEHE. MRI is advantageous over CT in displaying these imaging features. Furthermore, the authors described a diffuse-type HEHE, manifesting diffuse hepatomegaly with a slightly hyperdense nodule appearing with persistent enhancement on CEMP CT and MRI.

Applications

By demonstrating imaging features of HEHE on CEMP CT and MRI, this study may represent a future strategy for correct diagnosis of HEHE and therapeutic intervention in the treatment of HEHE.

Terminology

A target sign is an image that looks like a "target" with inner density/intensity and peripheral hyper-density/intensity or iso-density/intensity. On CEMP CT and MRI, it appears as peripheral ring-like and progressive enhancement. In some typical cases, it may show hyperintensity with peripheral hypointensity and an area of evident hyperintensity in the center on T2WI or an area of unenhanced necrosis in the center.

Peer review

The authors investigated and described CEMP CT and MRI findings in patients with pathologically confirmed HEHE. It revealed the most important imaging features of HEHE may be a target sign and/or progressive enhancement with persistent enhancement on CEMP CT and MRI. MRI is advantageous over CT in displaying these imaging features. The results are interesting and may represent the imaging features in HEHE.

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CASE REPORT

Simultaneous bile duct and portal venous branch ligation in two-stage hepatectomy

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Iida H, Yasui C, Aihara T, Ikuta S, Yoshie H, Yamanaka N. Simultaneous bile duct and portal venous branch ligation in two-stage hepatectomy. *World J Gastroenterol* 2011; 17(30): 3554-3559 Available from: URL: http://www.wjgnet.com/1007-9327/full/v17/i30/3554.htm DOI: http://dx.doi.org/10.3748/wjg.v17.i30.3554

Abstract

Hepatectomy is an effective surgical treatment for multiple bilobar liver metastases from colon cancer; however, one of the primary obstacles to completing surgical resection for these cases is an insufficient volume of the future remnant liver, which may cause postoperative liver failure. To induce atrophy of the unilateral lobe and hypertrophy of the future remnant liver, procedures to occlude the portal vein have been conventionally used prior to major hepatectomy. We report a case of a 50-year-old woman in whom two-stage hepatectomy was performed in combination with intraoperative ligation of the portal vein and the bile duct of the right hepatic lobe. This procedure was designed to promote the atrophic effect on the right hepatic lobe more effectively than the conventional technique, and to the best of our knowledge, it was used for the first time in the present case. Despite successful induction of liver volume shift as well as the following procedure, the patient died of subsequent liver failure after developing recurrent tumors. We discuss the first case in which simultaneous ligation of the portal vein and the biliary system was successfully applied as part of the first step of two-stage hepatectomy.

INTRODUCTION

Hepatectomy has been established as an effective treatment method for liver metastases from colon cancer, but there are some limits to this procedure when indicated in cases of synchronous multiple bilobar liver metastases. One of the reasons that limit resectability for these cases is an insufficient volume of the future remnant liver, which poses a risk of postoperative liver failure. To address this concern and improve the resectability, ligation or embolization of the portal vein is used in clinical settings. In addition, according to several animal experiments, ligation of the bile duct effectively induces atrophy of the ipsilateral liver.

For the present case of synchronous multiple bilobar liver metastases from cecal cancer, we first performed a right hemicolectomy and a segment 3 resection combined with microwave coagulation therapy (MCT) of the metastatic tumors situated in the left hepatic lobe, together with simultaneous ligation of the right portal vein and right-sided bile duct. Second, we performed an extended right hepatic lobectomy. Here, we discuss the effectiveness of intraoperative simultaneous ligation of the unilateral portal

Table 1 Laboratory findings on admission

Laboratory finding	Values
Leukocytes	8300/μL
Hemoglobin	9.7 g/dL
Hematocrit	30.70%
Platelets	408 000/μL
Alkaline phosphatase	595 IU/L
Lactate dehydrogenase	1295 IU/L
Aspartate aminotransferase	49 IU/L
Alanine aminotransferase	47 IU/L
Total-bilirubin	0.4 mg/dL
Direct-bilirubin	0.1 mg/dL
CEA	22 120 ng/dL
CA 19-9	16 354 U/mL

CEA: Carcinoembryonic antigen; CA: Carbohydrate antigen.

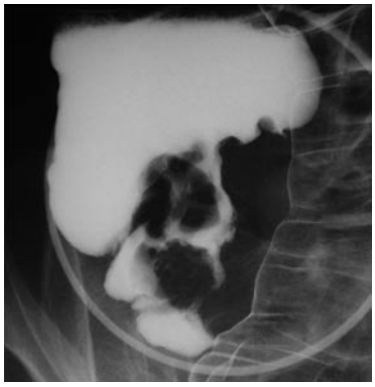

Figure 1 Barium enema findings. A filling defect caused by a tumor in the cecum was observed.

vein and bile duct applied to two-stage hepatectomy, as a therapeutic strategy for multiple bilobar liver metastases.

CASE REPORT

A 50-year-old woman began to have back pain and nausea around December 2004 and was referred to our hospital. Her liver was palpable at three finger widths below the right costal arch. The palpebral conjunctiva was anemic but bulbar conjunctiva was not icteric. Blood tests suggested that she had anemia (hemoglobin and hematocrit levels were 9.7 g/dL and 30.7%, respectively) and liver dysfunction (lactate dehydrogenase, aspartate aminotransferase, alanine aminotransferase, and alkaline phosphatase were 1295 IU/L, 49 IU/L, 47 IU/L and 595 IU/L, respectively). The serum levels of carcinoembryonic antigen (CEA) and carbohydrate antigen (CA) 19-9 were 22 120 ng/mL and 16 354 U/mL, respectively (Table 1). Barium enema showed a filling defect in the cecum. The results of biopsy indicated moderately differentiated adenocarcinoma (Figure 1). Contrast-enhanced computer tomography (CT) of the abdomen revealed many metastatic nodules with 2-6 cm diameter in the right hepatic lobe and three nodules with 3-3.5 cm diameter in the left hepatic lobe. A preoperative volume rate of the right hepatic lobe was 56.3% (Figure 2). There were no other metastases. Before implementation of two-stage hepatectomy, preoperative chemotherapy (TS-1: tegafur, gimeracil, and oteracil) was initiated at a starting dose of 80 mg/d. Four weeks later, the liver metastases shrank markedly and the serum levels of CEA and CA 19-9 decreased to 12 680 ng/dL and 8640 U/mL, respectively.

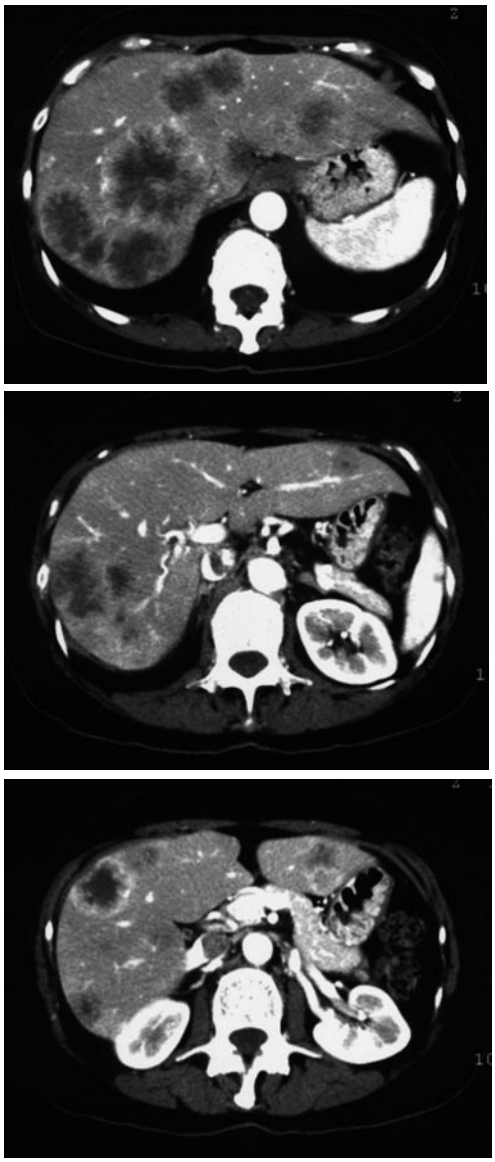

Figure 2 Preoperative findings from contrast-enhanced computer tomography of the abdomen. Multiple metastatic nodules with 2-6 cm diameter were observed in the right hepatic lobe and three nodules measuring 3-3.5 cm diameter in the left hepatic lobe. A preoperative volume rate of the right hepatic lobe was 56.3%.

Written informed consent was obtained from the patient and her family prior to the new surgical procedure. In March 2005, a right hemicolectomy was performed. At the same time, the metastatic tumor in segment 3 was resected, and the metastatic tumors in segments 2 and 4 were coagulated using MCT (90 W, 60 s). Furthermore, the right bile duct and right branch of the portal vein were ligated simultaneously (Figure 3). Intraoperative cholangiography was performed after ligation to confirm complete occlusion of the right bile duct and no constriction of the contralateral bile duct. Contrast-

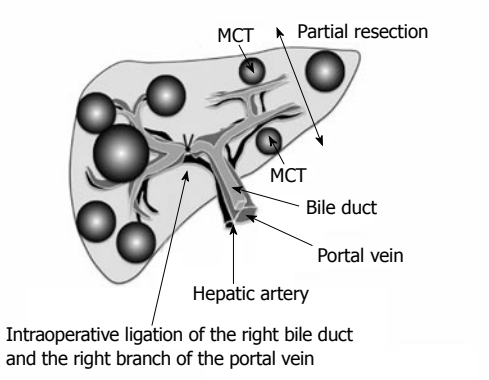

Figure 3 Initial operation scheme. Right hemicolectomy was performed. At the same time, the metastatic tumor in segment 3 was resected and the metastatic tumors in segments 2 and 4 were coagulated using MCT (90 W, 60 s). Furthermore, the right bile duct and right branch of the portal vein were ligated simultaneously. MCT: Microwave coagulation therapy.

enhanced CT of the abdomen performed at 1 wk after surgery showed coagulated areas in the left hepatic lobe and dilation of the right bile duct. Ligation of the right branch of the portal vein resulted in a complementary increase in the right hepatic arterial blood flow. The volume rate of the right hepatic lobe was 55.0%, which was similar to the preoperative rate (Figure 4). However, abdominal CT performed at 1 mo after surgery showed that the volume rate of the right hepatic lobe decreased to 44.3%, along with compensatory hypertrophy of the left hepatic lobe. This liver volume shift was approximately 12% from the right to the left lobe. No viable tumors were observed in the left hepatic lobe (Figure 5).

An extended right hepatic lobectomy was performed in May 2005. There were 10 nodules with 1-4 cm diameter in the right hepatic lobe. The resection was performed using a suture of the right bile duct and the right branch of the portal vein. The weight of the resected specimen was 555 g (Figure 6). There was no complication in the postoperative course. However, in October 2005, two recurrent nodules with 3.2 cm and 1.8 cm diameter were found in the remaining liver. She did not accept repeat resection, therefore, percutaneous radiofrequency ablation was performed.

In November 2005, the biweekly administration of oxaliplatin (80 mg), levofolinate calcium (125 mg), and 5-fluorouracil (1250 mg) was started, leading to some improvement for a while; specifically, a decrease in the serum levels of CEA and CA 19-9 to 36.3 ng/dL and 96 U/mL, respectively. Nevertheless, further recurrence was observed in the remaining liver. At 1 year and 8 mo after the initial operation, the patient developed liver failure and died (Figure 7). There was no other distant metastasis.

DISCUSSION

Liver metastasis occurs in 40%-70% of patients with colon cancer, and 15%-30% of these patients develop multiple liver metastases in both lobes[1]. However, in comparison to other cancers, we can expect long-term survival by using a combined therapy of resection and chemotherapy. In particular, hepatectomy is widely used as the most effective surgical treatment, and the 5-year survival rate after a curative resection is 24%-44%[2]. Even for multiple liver metastases in both lobes previously considered unresectable, complete resection is feasible in some cases by adopting preoperative portal vein embolization in addition to chemotherapy. In 2004, Jaeck *et al*[3] reported a method called two-stage hepatectomy; applicable to cases of multiple bilobar liver metastases. In the first stage of the operation, they conducted a partial hepatic resection or ablation for the unilateral lobe, and at the same time embolized the portal vein in order to induce hypertrophy of the future remnant lobe. In the

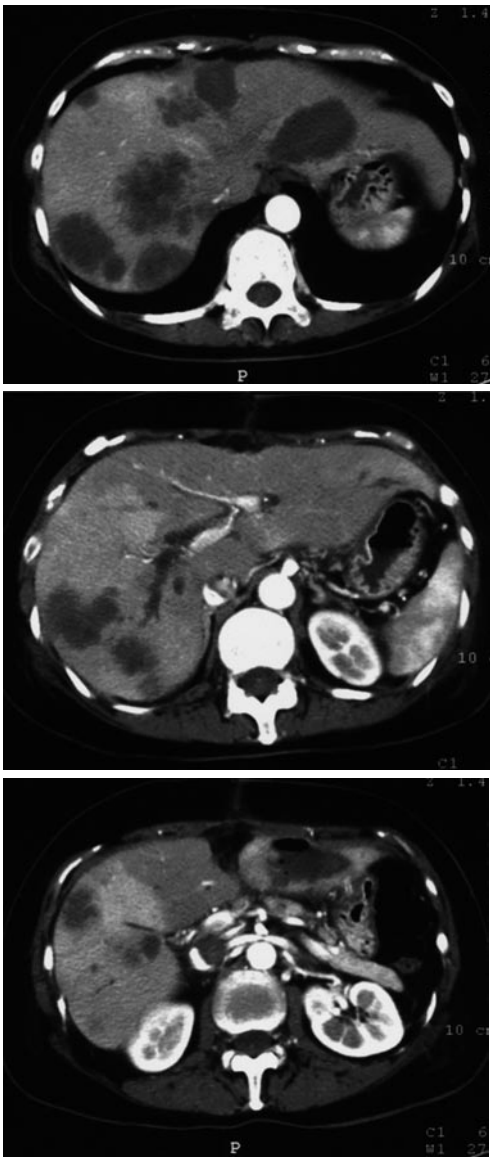

Figure 4 Findings from contrast-enhanced computer tomography of the abdomen at 1 wk after surgery. Two coagulated areas in the left hepatic lobe and dilation of the right bile duct were observed. Ligation of the right branch of the portal vein resulted in a complementary increase in the right hepatic arterial blood flow. The volume rate of the right hepatic lobe was 55.0%, which was similar to the preoperative rate.

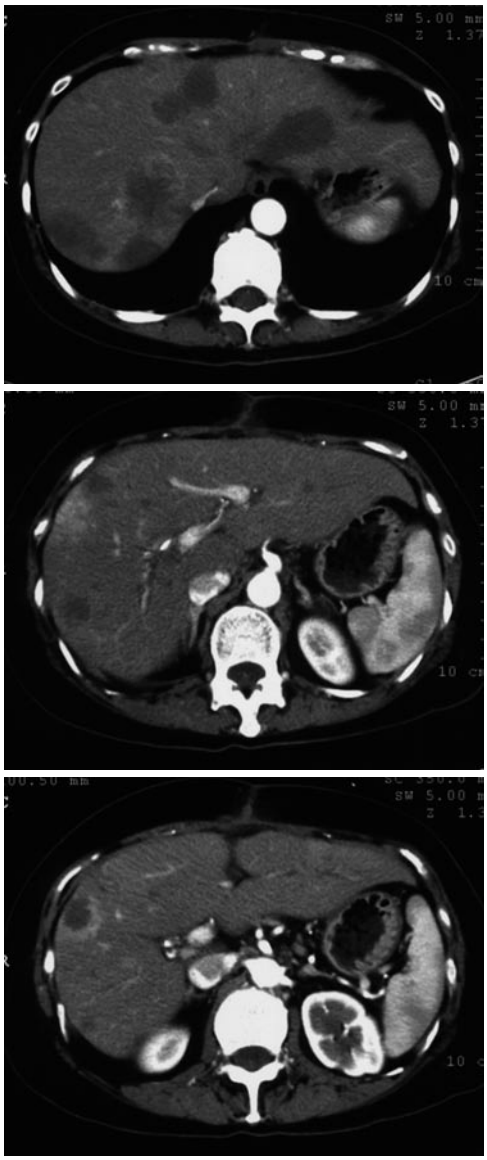

Figure 5 Findings from contrast-enhanced computer tomography of the abdomen at 1 mo after surgery. The volume rate of the right hepatic lobe decreased to 44.3% and the volume of left hepatic lobe underwent compensatory hypertrophy. This liver volume shift was approximately 12% from the right to the left lobe. There were no viable tumors in the left hepatic lobe.

second stage of the operation, hepatic lobectomy was performed in the atrophic lobe. They reported that the 1-year survival rate was 70% and the 3-year survival rate was 54.4%[3]. Moreover, for two-stage hepatectomy conducted by Togo *et al*[4], the 1-year survival rate was 90% and the 3-year survival rate was 45%. The treatment strategy includes combined therapy of systemic chemotherapy, arterial infusion therapy and ablation therapy. Such a multidisciplinary therapy allows resection of marginal or so-called unresectable tumors and contributes to improvement in the prognosis.

Ligation of the portal vein of the unilateral lobe is well-known to induce atrophy of the unilateral hepatic lobe and hypertrophy of the future remnant liver. Honjo *et al*[5] clinically applied this technique to a case of liver metastasis in 1961. Such a method was adopted for cases

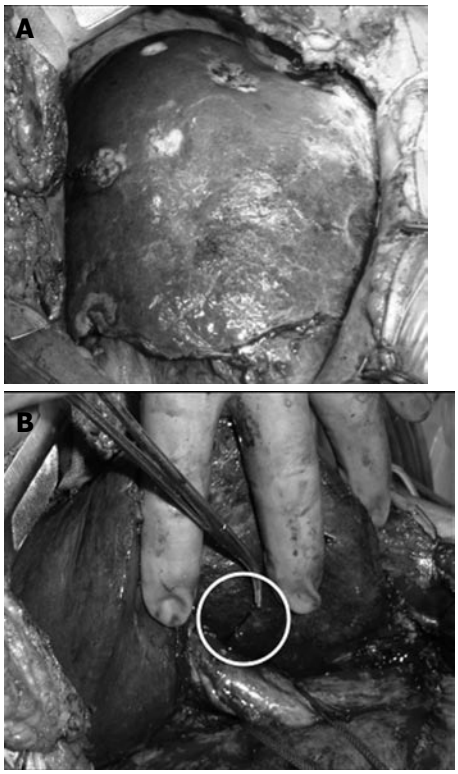

Figure 6 Intraoperative findings from the secondary operation. A: There were 10 nodules with 1-4 cm diameter in the right hepatic lobe; B: Resection was performed using the suture of the right bile duct and the right branch of the portal vein.

of major resection initially considered to have a high risk of postoperative liver failure based on preoperative prognosis[6-9]. Reportedly, at 3-4 mo after occlusion of the right portal vein, 9.5%-14.5% of the liver volume was transferred from the right to the left lobe[10]. In addition, according to a literature review of animal experiments, in 1920, Rous *et al*[11] reported that atrophy of the ligated lobe and hypertrophy of the future remnant lobe were induced in mice after bile duct ligation. Subsequently, Tanaka *et al*[12] have reported that the atrophic rate was enhanced after portal vein embolization in a rabbit that had undergone bile duct ligation. It was also pointed out that occlusion of the bile duct and portal vein could reduce biligenesis and occurrence of complications[12].

We applied these findings to the initial surgery in the present case and used simultaneous ligation of the bile duct and portal vein, with an aim of promoting an atrophic effect in the right hepatic lobe. As a result, 12.0% of the liver volume was transferred from the right to the left hepatic lobe at 1 mo after ligation, and the amount of this volume shift could reverse the primary unresectablity. The possible explanation that we consider for the benefit of our simultaneous ligation strategy is that it can overcome several factors that may diminish the atrophic effect of the conventional technique. The bile duct not being ligated may allow the portal blood flow to remain, due to backflow from the hepatic vein and the use of an arterioportal shunt. In our clinical experience of four cases in

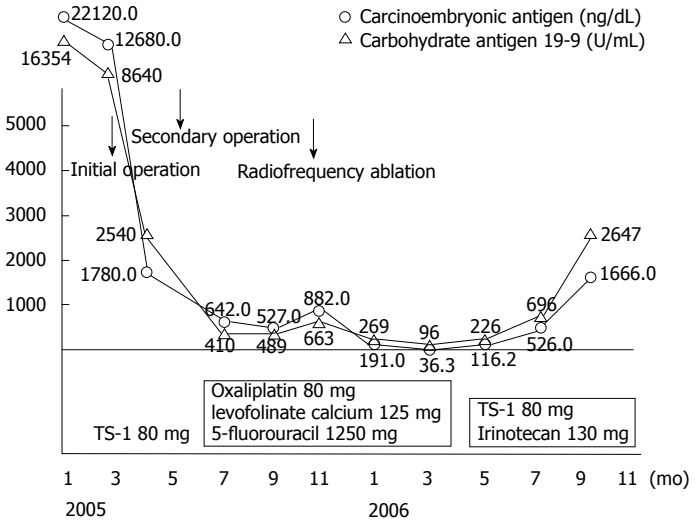

Figure 7 Postoprative course. The serum levels of carcinoembryonic antigen and carbohydrate antigen 19-9 decreased to 36.3 ng/dL and 96 U/mL, respectively. But further recurrence was observed in the remaining liver, and at 1 year and 8 mo after the initial operation, the patient developed liver failure and died. TS-1: Tegafur, gimeracil, and oteracil.

Table 2 Atrophy achieved in our previous four PVL cases

Case No.	Age	Sex	Primary disease
Case 1	55 years	Female	Multiple liver metastasis
Case 2	61 years	Male	Multiple liver metastasis
Case 3	61 years	Male	Hepatocellular carcinoma
Case 4	58 years	Male	Hepatocellular carcinoma

PVL: Portal vein ligation; RHL: Right hepatic lobe.

Table 3 Atrophy achieved in our previous four PVL cases

Case No.	Preoperative volume rate of RHL	The volume rate of RHL 1 mo after PVL
Case 1	74.80%	68.50%
Case 2	68.30%	62.50%
Case 3	58.60%	55.50%
Case 4	58.90%	52.40%

PVL: Portal vein ligation; RHL: Right hepatic lobe.

which the right portal vein alone was ligated, the volume shift at 1 mo after the procedure was only 5.4% ± 1.6% (Tables 2, 3), which suggests that ligation of the bile duct made a substantial contribution to inducing atrophy of the ipsilateral liver in the present case.

Regarding the limitations of our simultaneous ligation strategy, it should be noted that this technique should be performed with careful consideration of the risks of obstructive cholangitis and involution of the contralateral bile duct. Intraoperative cholangiography may help avoid these occurrences. With respect to the risks of the aforementioned complications, the safety of our procedure remains to be confirmed in a future study.

To the best of our knowledge, there has not been any reported case in which simultaneous ligation of the portal vein and bile duct has been used in two-stage hepatectomy. There was no postoperative biliary tract infection in our patient. Unfortunately, the patient developed further recurrent tumors after hepatectomy and died of subsequent liver failure. Yet, it can be concluded that the simultaneous ligation strategy could be used to accelerate atrophy of the hemiliver to be resected, and thereby provide us with a further possibility of addressing the primary unresectability of metastatic bilobar liver tumors.

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CASE REPORT

Endoscopic naso-pancreatic drainage for the treatment of pancreatic fistula occurring after LDLT

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Abstract

Pancreatic fistula is a quite rare complication in patients who undergo living donor liver transplantation (LDLT). However, in the cases that show pancreatic fistula, the limited volume of the graft and the resultant inadequate liver function may complicate the management of the fistula. As a result, the pancreatic fistula may result in the death of the patient. We present 2 cases in which endoscopic treatment was effective against pancreatic fistulas that developed after LDLT. In case 1, a 61-year-old woman underwent LDLT for primary biliary cirrhosis. Because of a portal venous thrombus caused by a splenorenal shunt, the patient underwent portal vein reconstruction, and a splenorenal shunt was ligated on postoperative day (POD) 7. The main pancreatic duct was injured during the manipulation to achieve hemostasis, thereby necessitating open drainage. However, discharge of pancreatic fluid continued even after POD 300. Endoscopic naso-pancreatic drainage (ENPD) was performed, and this procedure resulted in a remarkable decrease in drain output. The refractory pancreatic fistula healed on day 40 after ENPD. In case 2, a 58-year-old man underwent LDLT for cirrhosis caused by the hepatitis C virus. When the portal vein was exposed during thrombectomy, the pancreatic head was injured, which led to the formation of a pancreatic fistula. Conservative therapy was ineffective; therefore, ENPD was performed. The pancreatic fistula healed on day 38 after ENPD. The findings in these 2 cases show that endoscopic drainage of the main pancreatic duct is a less invasive and effective treatment for pancreatic fistulas that develop after LDLT.

Key words: Pancreatic fistula; Endoscopic treatment; Living donor liver transplantation; Complications

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Nagatsu A, Taniguchi M, Shimamura T, Suzuki T, Yamashita K, Kawakami H, Abo D, Kamiyama T, Furukawa H, Todo S. Endoscopic naso-pancreatic drainage for the treatment of pancreatic fistula occurring after LDLT. *World J Gastroenterol* 2011; 17(30): 3560-3564 Available from: URL: http://www.wjgnet.com/1007-9327/full/v17/i30/3560.htm DOI: http://dx.doi.org/10.3748/wjg.v17.i30.3560

INTRODUCTION

The incidence of complications associated with living donor liver transplantation (LDLT) is known to be greater than that associated with deceased donor liver transplantation (DDLT)[1-4]. In the patients who undergo LDLT, the incidence of complications, including mild complications, during the perioperative period can be as high as 82.8%. In particular, the rate of development of biliary complications after LDLT is twice that after DDLT[1]. Moreover, patients who undergo LDLT often have inadequate liver function because of the limited volume of the graft. Therefore, the management of complications is very difficult, and the mortality rate in critical cases is high.

Compared to other abdominal surgery, pancreatic fistula is a quite rare complication after LDLT[1-5], but it is theoretically possible because LDLT involves surgery in the area surrounding the portal vein, the pancreas, and the spleen. Pancreatic fistula causes hemorrhage, abscess, etc., and may result in the death of the patient[6-8]. Only 1 previous study has reported 2 cases of leakage of pancreatic fluid after liver transplantation. In those cases, leakage of a mixture of pancreatic fluid and bile was observed at the anastomosis site of the bile duct after DDLT[9]. However, there is no clear consensus on the management of pancreatic fistula after liver transplantation. Generally, conservative therapy is the first line of treatment, and surgery is performed only when the patient does not respond to conservative therapy[6-8]. Recently, however, endoscopic treatment has attracted more attention because it is less invasive than surgical treatment. We present 2 cases in which endoscopic treatment was effective against refractory pancreatic fistulas that developed after LDLT.

CASE REPORT

Case 1

A 61-year-old woman underwent LDLT for primary biliary cirrhosis; the graft was obtained from the left liver lobe of her son. Abdominal computed tomography (CT) performed on postoperative day (POD) 7 revealed a portal vein thrombus; therefore, urgent exploratory laparotomy was performed. The thrombus was believed to have been induced by reduced portal blood flow, which was caused by splenorenal steal by an artificial shunt. After removing the thrombus, portal vein reconstruction was performed by using the right external iliac vein, and this procedure was followed by splenectomy. To increase the portal blood flow, a splenorenal shunt was ligated. The main pancreatic duct on the dorsal side of the pancreas was injured at the time of hemostatic manipulation; however, this injury was not identified immediately. CT performed on POD 13 revealed a hematoma at the lower edge of the pancreas. CT on POD 21 revealed that the hematoma under the pancreas had decreased in size (Figure 1). The amylase level of the drainage fluid was 22 690 IU/L; therefore, the hematoma at the inferior edge of the pancreas was considered to have ruptured because of a pancreatic leak. Another CT examination revealed fluid collection in the mesentery on the ventral side of the upper pole of the left kidney; therefore, open drainage was performed. To this end, a drain was placed at the tail of the pancreas and administration of octreotide was started. However, the leakage of pancreatic fluid from the drain did not stop. After several days, the patient's general status stabilized, but surgical treatment for pancreatic fistula was still unsafe because of inadequate liver function. Therefore, the patient was discharged on POD 128 with the drain in place and was followed up. The patient was in a stable state at discharge. However, on POD 318, she was readmitted to our department because of fever. Examinations revealed that the drain at the tail of the pancreas had deviated and that the patient had developed liver necrosis, supposedly because of contact with the drain. On POD 320, we repositioned the drain by using a fluoroscope. A contrast test performed at that time revealed that the main pancreatic duct was completely disrupted (Figure 2). The patient was diagnosed with refractory pancreatic fistula, and an endoscopic naso-pancreatic drainage (ENPD) tube was inserted to the proximal side of the leakage on POD 331 (Figure 3); this procedure resulted in a remarkable decrease in drain output (Figures 4 and 5). The ENPD tube was removed on POD 368, and the drains at the tail of the pancreas were removed on POD 371. The patient was discharged on POD 375 without abnormal fluid collection around the pancreas (Figure 6). The patient is well without the recurrence of pancreatic fistula up to this time.

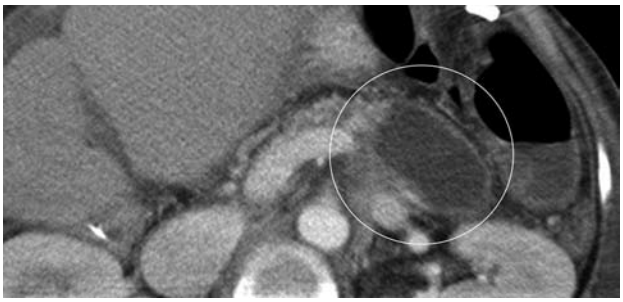

Figure 1 Computed tomography performed on postoperative day 13 showing fluid collection (circle) at the lower edge of the pancreas. The hematoma was considered to be ruptured.

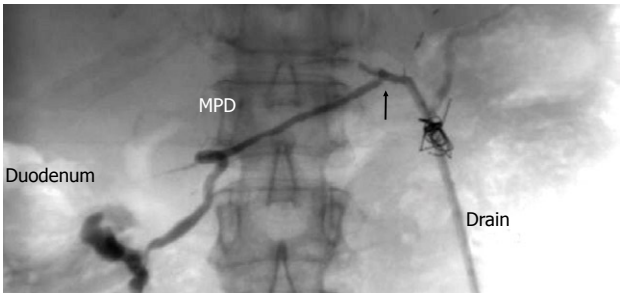

Figure 2 Pancreatographic examination of the drain at the tail of the pancreas (Drain) in case 1 reveals the disrupted (arrow) main pancreatic duct with flow of contrast into the duodenum. MPD: Main pancreatic duct.

Case 2

A 58-year-old man underwent LDLT for cirrhosis C; the graft was obtained from the right liver lobe of his daughter. Because the portal vein was occluded by a thrombus,

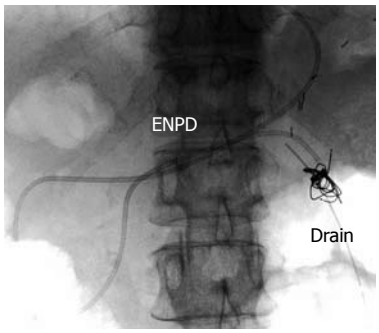

Figure 3 A radiograph showing postprocedure endoscopic naso-pancreatic drainage in case 1. Excellent drainage of the pancreatic duct is noted. ENPD: Endoscopic naso-pancreatic drainage.

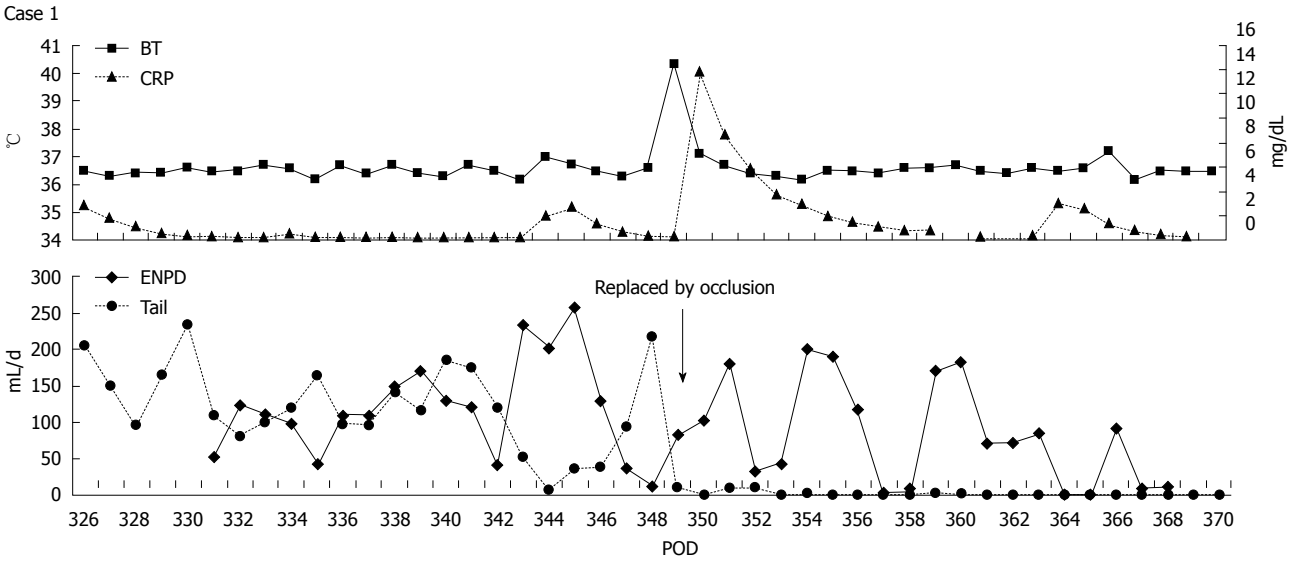

Figure 4 Upper chart shows the body temperature and serum C-reactive protein level. Lower one shows daily output of the endoscopic naso-pancreatic drainage tube and the drain at the tail of the pancreas (Tail) in case 1. The patient had an episode of fever caused by the occlusion of the endoscopic naso-pancreatic drainage (ENPD) tube. After the tube was replaced, the pancreatic fistula healed completely. BT: Body temperature; CRP: C-reactive protein; POD: Postoperative day.

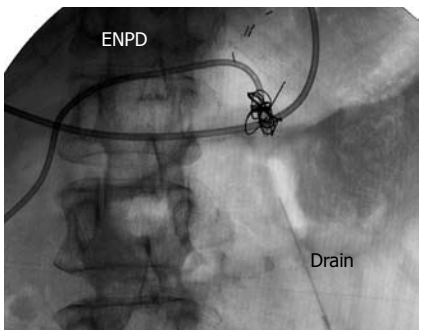

Figure 5 Contrast examination from the drain at the tail of the pancreas (Drain) in case 1 on postoperative day 363 reveals the closure of fistula. ENPD: Endoscopic naso-pancreatic drainage.

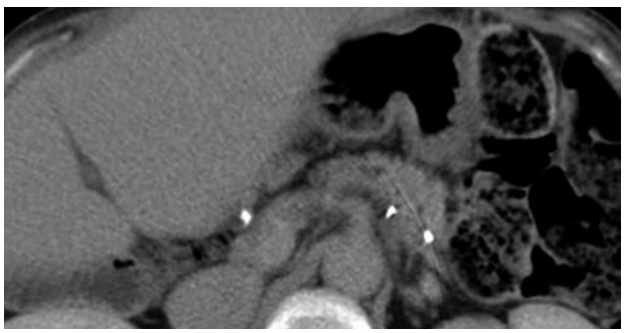

Figure 6 Computed tomography performed 2 d after removal of the drain in case 1 showing no fluid collection around the pancreas.

the portal and splenic veins were stripped off from the surrounding tissue and were exposed in order to remove the thrombus. However, the upper edge of the pancreatic head was injured during this process. The amylase level measured at the upper edge of the pancreatic drain was high on POD 1; therefore, the patient received octreotide on POD 2. On POD 5, the patient showed high fever and acute peritonitis. Therefore, an emergency exploratory laparotomy was performed. Because fluid collection was observed around the pancreas, drains were placed at both the right and left edges of the pancreas. On POD 21, the total output from the drain was 460 mL/d, and the amylase level of the drainage fluid was 166 700 IU/L. The patient was

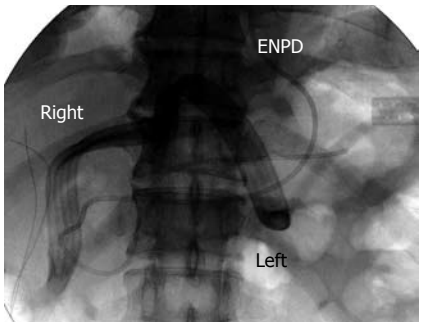

Figure 7 A radiograph showing postprocedure endoscopic naso-pancreatic drainage in case 2. The image shows drains placed at both the right and left edges of the pancreas (Right and Left) and endoscopic naso-pancreatic drainage (ENPD).

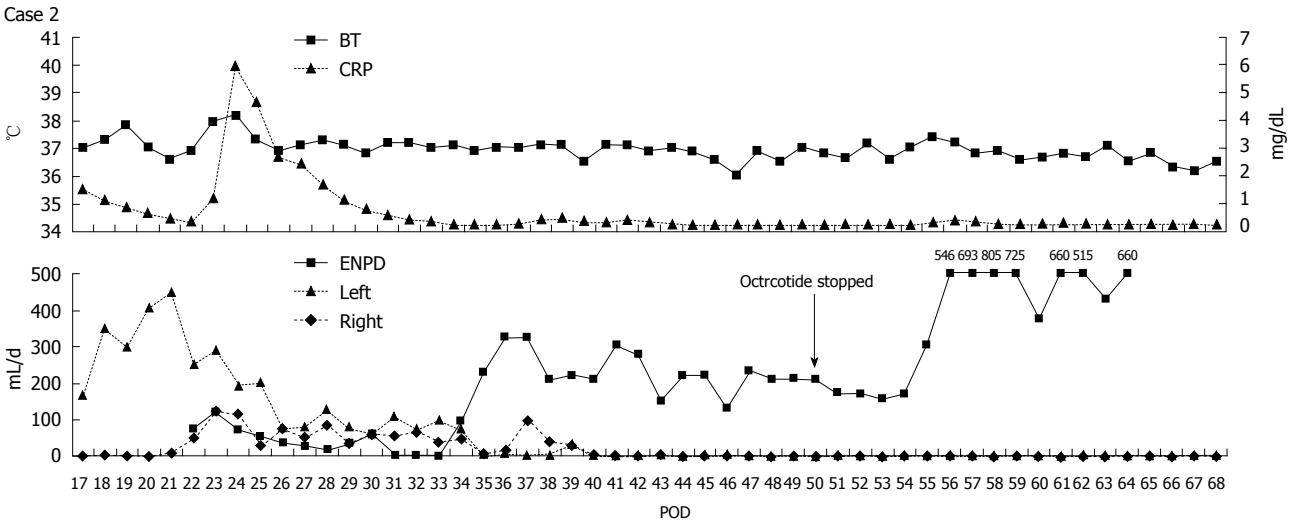

Figure 8 Upper chart shows the body temperature and serum C-reactive protein level. Lower one shows daily output of endoscopic naso-pancreatic drainage (ENPD) tube and the drains at both the edges of the pancreas (Right and Left) in case 2. The pancreatic fistula healed completely on post-ENPD day 38. BT: Body temperature; CRP: C-reactive protein; POD: Postoperative day.

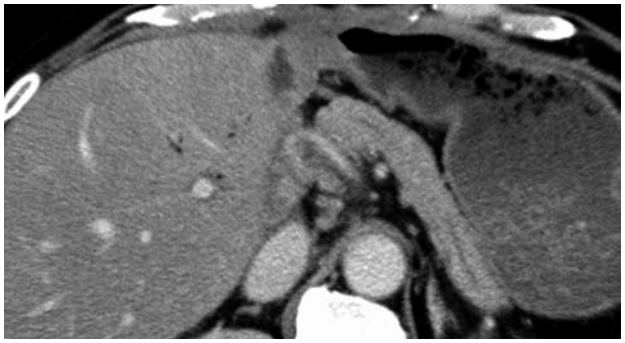

Figure 9 Computed tomography performed 14 d after removal of the drains in case 2 showing no fluid collection around the pancreas.

diagnosed with high-output pancreatic fistula, and ENPD was performed on POD 22 (Figure 7). The drain output decreased very rapidly (Figure 8); therefore, the patient was allowed to consume solid foods on POD 49, and octreotide administration was stopped on POD 50. The ENPD tube was removed on POD 65, and the drains placed at the right and left edges of the pancreas were removed on POD 68 and POD 70, respectively. The patient was discharged on POD 100 without abnormal fluid collection around the pancreas (Figure 9). The patient is well and receiving regular out-patient treatment and is showing no recurrence of pancreatic fistula.

DISCUSSION

Pancreatic fistula is primarily treated by conservative therapy, which includes rapid total infusion or enteral nutrition along with administration of octreotide. The recovery rate after conservative therapy ranges from 44% to 85%[6-8]; thus, a number of cases are not resolved by conservative treatment. Surgical treatment has been performed in such cases. However, surgical treatment is highly invasive and may lead to various complications. Further, surgical treatment is associated with high mortality rates, with the mortality rate being as high as 23%-67% in the cases showing early peritonitis after the operation[9]. Endoscopic drainage of the main pancreatic duct *via* the ampulla of Vater, which was first reported in 1991[10], has drawn considerable attention. Boerma *et al*[11] (2006) reported an excellent recovery rate (87%) after endoscopic treatment of 15 cases of pancreatic fistula. In addition, other studies

have reported recovery rates of about 58%-100% in the cases of pancreatic fistulas that do not respond to conservative therapy and involve endoscopic treatment[12-20]. To date, only 1 death caused by acute pancreatitis has been reported. However, since this death may also have been caused by inadequate drainage, a direct relationship between the death and endoscopic treatment could not be confirmed[13]. Unlike LDLT, endoscopic treatment for pancreatic fistula allows greater accessibility to the ampulla of Vater. Further, endoscopic treatment is less invasive than surgical treatment; therefore, it can easily replace conservative therapy if sufficient drainage is achieved. Thus, patients who undergo endoscopic treatment for pancreatic fistula can be expected to make an early recovery. Irrespective of their merits and demerits, both ENPD and endoscopic pancreatic stenting (EPS) have been referred to in the reports. ENPD causes a sense of discomfort in the pharynx; however, this technique enables easy diagnosis of occlusion and dropout because it allows monitoring of the pancreatic fluid. In contrast, in EPS the diagnosis of occlusion and dropout is difficult; however, this technique causes no sense of discomfort in the pharynx. We selected ENPD to enable safe monitoring of 2 channels of drainage: the endoscopic retrograde pancreatic drain as well as the intraperitoneal drain. In case 1, the drain tube had to be replaced because of the fever caused by occlusion; therefore, the choice of ENPD was considered to be reasonable. The patient in case 1 could have recovered earlier if the endoscopic treatment for pancreatic fistula had been initiated earlier. In each case, the patient recovered within approximately 40 d after ENPD. Further, the treatment had no influence on the patients' general status. Endoscopic treatment is considered to be safe for treating pancreatic fistulas that develop after LDLT. New endoscopic techniques, such as ultrasonography (US)-guided drainage, have also been used to treat refractory cases that do not respond to drainage via the ampulla of Vater; however, only few reports have described these techniques. These new techniques may also be less invasive than surgical treatment[21,22].

In conclusion, we described 2 cases of pancreatic fistula after LDLT that were not responsive to conservative therapy. In each case, the patient recovered within approximately 40 d after ENPD. Thus, endoscopic treatment for pancreatic fistula after LDLT should be adopted because of its high recovery rate and low invasiveness.

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Events Calendar 2011

January 14-15, 2011
AGA Clinical Congress of Gastroenterology and Hepatology: Best Practices in 2011 Miami, FL 33101, United States

January 20-22, 2011
Gastrointestinal Cancers Symposium 2011, San Francisco, CA 94143, United States

January 27-28, 2011
Falk Workshop, Liver and Immunology, Medical University, Franz-Josef-Strauss-Allee 11, 93053 Regensburg, Germany

January 28-29, 2011
9. Gastro Forum München, Munich, Germany

February 4-5, 2011
13th Duesseldorf International Endoscopy Symposium, Duesseldorf, Germany

February 13-27, 2011
Gastroenterology: New Zealand CME Cruise Conference, Sydney, NSW, Australia

February 17-20, 2011
APASL 2011-The 21st Conference of the Asian Pacific Association for the Study of the Liver
Bangkok, Thailand

February 22, 2011-March 04, 2011
Canadian Digestive Diseases Week 2011, Vancouver, BC, Canada

February 24-26, 2011
Inflammatory Bowel Diseases 2011-6th Congress of the European Crohn's and Colitis Organisation, Dublin, Ireland

February 24-26, 2011
2nd International Congress on Abdominal Obesity, Buenos Aires, Brazil

February 24-26, 2011
International Colorectal Disease Symposium 2011, Hong Kong, China

February 26-March 1, 2011
Canadian Digestive Diseases Week, Westin Bayshore, Vancouver, British Columbia, Canada

February 28-March 1, 2011
Childhood & Adolescent Obesity: A whole-system strategic approach, Abu Dhabi, United Arab Emirates

March 3-5, 2011
42nd Annual Topics in Internal Medicine, Gainesville, FL 32614, United States

March 7-11, 2011
Infectious Diseases: Adult Issues in the Outpatient and Inpatient Settings, Sarasota, FL 34234, United States

March 14-17, 2011
British Society of Gastroenterology Annual Meeting 2011, Birmingham, England, United Kingdom

March 17-19, 2011
41. Kongress der Deutschen Gesellschaft für Endoskopie und Bildgebende Verfahren e.V., Munich, Germany

March 17-20, 2011
Mayo Clinic Gastroenterology & Hepatology 2011, Jacksonville, FL 34234, United States

March 18, 2011
UC Davis Health Informatics: Change Management and Health Informatics, The Keys to Health Reform, Sacramento, CA 94143, United States

March 25-27, 2011
MedicReS IC 2011 Good Medical Research, Istanbul, Turkey

March 26-27, 2011
26th Annual New Treatments in Chronic Liver Disease, San Diego, CA 94143, United States

April 6-7, 2011
IBS-A Global Perspective, Pfister Hotel, 424 East Wisconsin Avenue, Milwaukee, WI 53202, United States

April 7-9, 2011
International and Interdisciplinary Conference Excellence in Female Surgery, Florence, Italy

April 15-16, 2011
Falk Symposium 177, Endoscopy Live Berlin 2011 Intestinal Disease Meeting, Stauffenbergstr. 26, 10785 Berlin, Germany

April 18-22, 2011
Pediatric Emergency Medicine: Detection, Diagnosis and Developing Treatment Plans, Sarasota, FL 34234, United States

April 20-23, 2011
9th International Gastric Cancer Congress, COEX, World Trade Center, Samseong-dong, Gangnam-gu, Seoul 135-731, South Korea

April 25-27, 2011
The Second International Conference of the Saudi Society of Pediatric Gastroenterology, Hepatology & Nutrition, Riyadh, Saudi Arabia

April 25-29, 2011
Neurology Updates for Primary Care, Sarasota, FL 34230-6947, United States

April 28-30, 2011
4th Central European Congress of Surgery, Budapest, Hungary

May 7-10, 2011
Digestive Disease Week, Chicago, IL 60446, United States

May 12-13, 2011
2nd National Conference Clinical Advances in Cystic Fibrosis, London, England, United Kingdom

May 19-22, 2011
1st World Congress on Controversies in the Management of Viral Hepatitis (C-Hep), Palau de Congressos de Catalunya, Av. Diagonal, 661-671 Barcelona 08028, Spain

May 21-24, 2011
22nd European Society of Gastrointestinal and Abdominal Radiology Annual Meeting and Postgraduate Course, Venise, Italy

May 25-28, 2011
4th Congress of the Gastroenterology Association of Bosnia and Herzegovina with international participation, Hotel Holiday Inn, Sarajevo, Bosnia and Herzegovina

June 11-12, 2011
The International Digestive Disease Forum 2011, Hong Kong, China

June 13-16, 2011
Surgery and Disillusion XXIV SPIGC, II ESYS, Napoli, Italy

June 14-16, 2011
International Scientific Conference on Probiotics and Prebiotics-IPC2011, Kosice, Slovakia

June 22-25, 2011
ESMO Conference: 13th World Congress on Gastrointestinal Cancer, Barcelona, Spain

June 29-2, 2011
XI Congreso Interamericano de Pediatria "Monterrey 2011", Monterrey, Mexico

September 2-3, 2011 Falk Symposium 178, Diverticular Disease, A Fresh Approach to a Neglected Disease, Gürzenich Cologne, Martinstr. 29-37, 50667 Cologne, Germany

September 10-11, 2011
New Advances in Inflammatory Bowel Disease, La Jolla, CA 92093, United States

September 10-14, 2011
ICE 2011-International Congress of Endoscopy, Los Angeles Convention Center, 1201 South Figueroa Street Los Angeles, CA 90015, United States

September 30-October 1, 2011
Falk Symposium 179, Revisiting IBD Management: Dogmas to be Challenged, Sheraton Brussels Hotel, Place Rogier 3, 1210 Brussels, Belgium

October 19-29, 2011
Cardiology & Gastroenterology | Tahiti 10 night CME Cruise, Papeete, French Polynesia

October 22-26, 2011
19th United European Gastroenterology Week, Stockholm, Sweden

October 28-November 2, 2011
ACG Annual Scientific Meeting & Postgraduate Course, Washington, DC 20001, United States

November 11-12, 2011
Falk Symposium 180, IBD 2011: Progress and Future for Lifelong Management, ANA Interconti Hotel, 1-12-33 Akasaka, Minato-ku, Tokyo 107-0052, Japan

December 1-4, 2011
2011 Advances in Inflammatory Bowel Diseases/Crohn's & Colitis Foundation's Clinical & Research Conference, Hollywood, FL 34234, United States

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GENERAL INFORMATION

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The columns in the issues of *WJG* will include: (1) Editorial: To introduce and comment on major advances and developments in the field; (2) Frontier: To review representative achievements, comment on the state of current research, and propose directions for future research; (3) Topic Highlight: This column consists of three formats, including (A) 10 invited review articles on a hot topic, (B) a commentary on common issues of this hot topic, and (C) a commentary on the 10 individual articles; (4) Observation: To update the development of old and new questions, highlight unsolved problems, and provide strategies on how to solve the questions; (5) Guidelines for Basic Research: To provide guidelines for basic research; (6) Guidelines for Clinical Practice: To provide guidelines for clinical diagnosis and treatment; (7) Review: To review systemically progress and unresolved problems in the field, comment on the state of current research, and make suggestions for future work; (8) Original Article: To report innovative and original findings in gastroenterology; (9) Brief Article: To briefly report the novel and innovative findings in gastroenterology and hepatology; (10) Case Report: To report a rare or typical case; (11) Letters to the Editor: To discuss and make reply to the contributions published in *WJG*, or to introduce and comment on a controversial issue of general interest; (12) Book Reviews: To introduce and comment on quality monographs of gastroenterology and hepatology; and (13) Guidelines: To introduce consensuses and guidelines reached by international and national academic authorities worldwide on basic research and clinical practice gastroenterology and hepatology.

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Please list 5-10 key words, selected mainly from *Index Medicus*, which reflect the content of the study.

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Figures should be numbered as 1, 2, 3, *etc.*, and mentioned clearly in the main text. Provide a brief title for each figure on a separate page. Detailed legends should not be provided under the figures. This part should be added into the text where the figures are applicable. Figures should be either Photoshop or Illustrator files (in tiff, eps, jpeg formats) at high-resolution. Examples can be found at: http://www.wjgnet.com/1007-9327/13/4520.pdf; http://www.wjgnet.com/1007-9327/13/4554.pdf; http://www.wjgnet.com/1007-9327/13/4891.pdf; http://www.wjgnet.com/1007-9327/13/4986.pdf; http://www.wjgnet.com/1007-9327/13/4498.pdf. Keeping all elements compiled is necessary in line-art image. Scale bars should be

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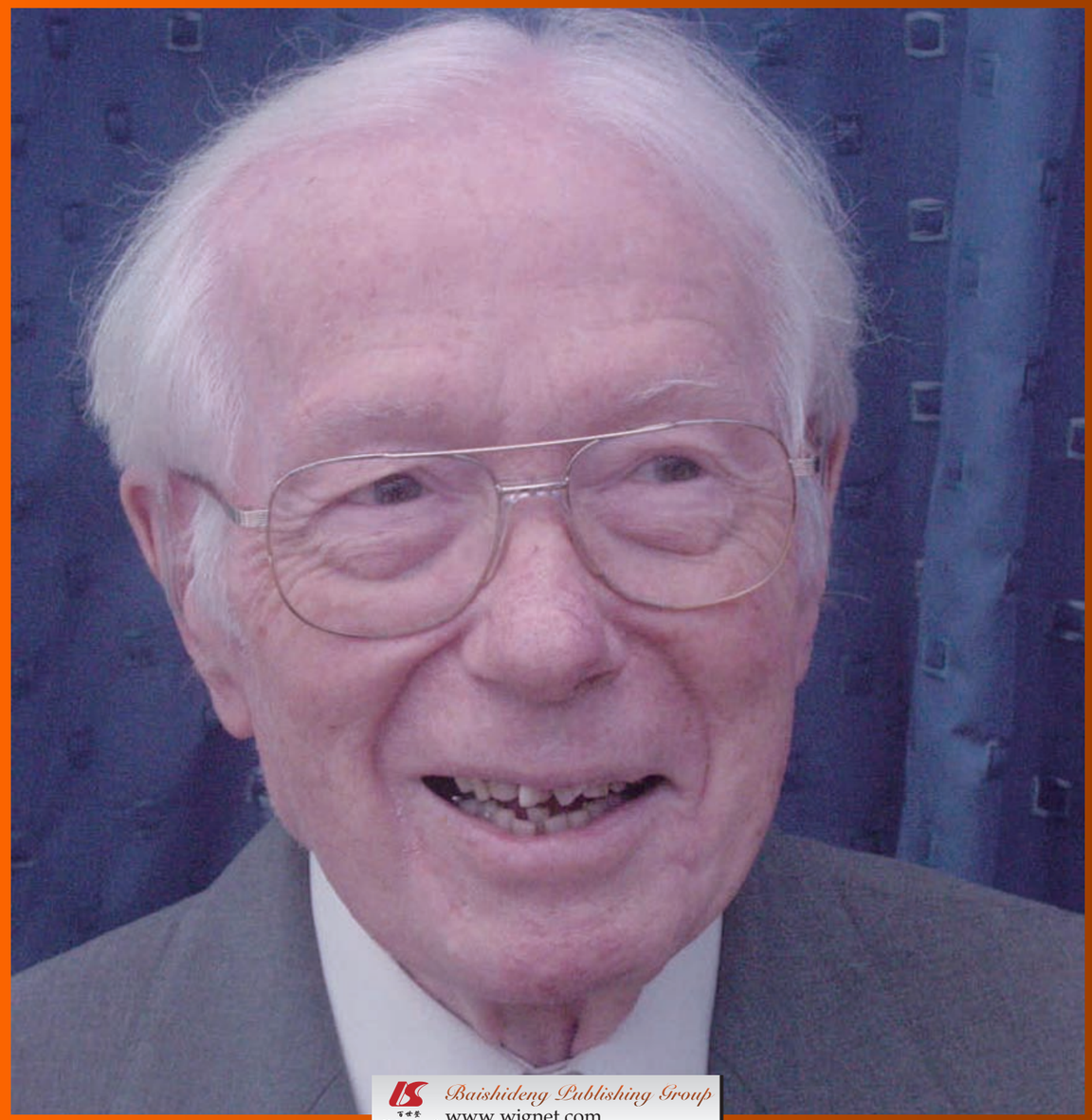

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EDITORIAL

A tribute to Dr. Frank I Tovey on his 90th birthday

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Figure 1 Frank I Tovey, OBE, ChM, FRCS (Eng), Honorary Senior Research Associate, Department of Surgery and Interventional Science, University College London, London W1W 7EJ, United Kingdom.

Abstract

This paper pays a tribute to Dr. Frank I Tovey on his 90th birthday which happens on September 1, 2011, and briefly describes the major findings in his research career and contributions as follows. The geographical prevalence of duodenal ulceration is related to staple diets. Unrefined wheat and maize, soya, certain pulses and millets are associated with a low prevalence while refined wheat, maize and rice, yams, cassava and green banana with a high prevalence. Predominant foodstuffs from low prevalence areas are ulceroprotective in rat peptic ulcer models. The protective activity lies in the lipid fraction present in these foodstuffs. The lipid fraction also promotes ulcer healing, is active both orally and intramuscularly and is ulceroprotective against non-steroidal anti-inflammatory drugs (NSAIDs). The phospholipids and phytosterols present in the lipid have been identified to be responsible for this protective activity. The combination of phospholipids and phytosterols may be of value in the prevention and treatment of duodenal ulceration and protection against the ulcerogenic effect of NSAIDs.

Key words: Duodenal ulceration; Staple diets; Protective factors; Phospholipids; Phytosterols; Non-steroidal anti-inflammatory drugs; *Helicobacter pylori*

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September 1st, 2011 marks the 90th birthday of Dr. Frank I Tovey, ChM, FRCS (Eng) (Figure 1).

Dr. Tovey worked as a surgeon at the Methodist Hospital in Zhaotong, Yunnan, southwest China from 1948 to 1949 and at the Holdsworth Memorial Hospital, Mysore, South India from 1951 to 1967. He was appointed OBE in 1966 for services to surgery and leprosy in India. From 1968 to 1986, he was a Consultant Surgeon in the United Kingdom at Basingstoke District Hospital and Honorary Research Fellow in the Department of Surgery, University College Hospital, London. His present appointment is Honorary Senior Research Associate, Department of Surgery and Interventional Science at University College London.

His main research interests include reconstructive surgery in leprosy, the nutritional effects of surgery for pep-

tic ulceration both in the United Kingdom and in developing countries, the relationship between the prevalence of duodenal ulceration and staple diets worldwide, and the absence of any relationship between the prevalence of duodenal ulceration and the prevalence of *Helicobacter pylori* (*H. pylori*) infection. These interests have led to a number of publications.

His interest in duodenal ulceration arose when working in Mysore in India where duodenal ulceration was a major problem particularly in men and requiring surgery. He found that partial gastrectomy was inappropriate for people living on one large meal a day because they were not able to eat enough. This led to trials of the long-term nutritional effect of different types of vagotomy and drainage procedures, which continued after his return to the United Kingdom[1-3]. He also noted that the majority of duodenal ulcer patients came from the wetlands where rice was the staple diet and very rarely from the dry areas where millets or pulses were the staple food. This suggested a relationship between staple diets and the prevalence of duodenal ulceration and led to researches which extended over 55 years. Information was gathered from all over India and confirmed a higher prevalence in rice-eating areas particularly in the South and a lower prevalence in the unrefined wheat or millet-eating drier areas in the North. In association with Denis Burkitt and the Medical Research Council, information was obtained from many countries including Africa, China and Malaysia. This was at a time when surgery was the accepted procedure for duodenal ulceration and information about the incidence of surgical procedures thus reflected its prevalence. The evidence showed a consistent pattern. A higher prevalence was found in areas where the staple diet was principally milled rice, refined wheat or maize, yams, cassava, sweet potato or green bananas, and a lower prevalence in areas where the staple diet was based on unrefined wheat or maize, soya, certain millets or certain pulses. These diets and individual foods were investigated using several rat peptic ulcer models, and the results confirmed the ulceroprotective activity of the foods predominating in the diet in the lower duodenal ulcer prevalence areas. The experiments showed that the protective activity lay in the lipid component of these foods. The lipid fraction was protective when given orally or intramuscularly, and it also promoted ulcer healing. The activity was found to lie in the phospholipid and sterol fractions of the lipid, and their nature has been subsequently identified. This combination of phospholipids and phytosterols, may prove to be of value in giving protection against not only duodenal ulceration but also the ulcerogenic effect of non-steroidal anti-inflammatory drugs[4-17].

The geographical study of the prevalence of duodenal ulceration also showed no relationship with the prevalence of *H. pylori* infection[18-22].

Dr. Tovey served as a member of the *World Journal of Gastroenterology* (*WJG*) Editorial Board for 11 years, during which he reviewed 55 articles and published 8 articles in *WJG*[3,13,19-24], making a great contribution to the improvement of the academic quality of *WJG*.

On behalf of all the *WJG* Editorial Board members and all *WJG* editorial staff, I would like to wish Dr. Frank I Tovey a very happy birthday!

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Management of Crohn's disease in smokers: Is an alternative approach necessary?

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Abstract

Inflammatory bowel disease (IBD) is a chronic condition with a pathogenic background that involves both genetic and environmental factors. Although important progress has been made regarding the former in the last decade, scarce knowledge is available for the latter. In this sense, smoking remains the most important environmental factor in IBD. Active smoking increases the risk of developing Crohn's disease (CD). Moreover, CD patients who start or continue smoking after disease diagnosis are at risk for poorer outcomes such as higher therapeutic requirements and disease-related complications, as compared to those patients who quit smoking or who never smoked. However, the harmful effect of active smoking is not uniform in all patients or in all clinical scenarios. Interventions designed to facilitate smoking cessation may impact the course of the disease. In this article, the available evidence of the deleterious effects of smoking on CD is reviewed in detail, and alternative therapeutic approaches to CD in smokers are proposed.

Key words: Tobacco; Crohn's disease; Therapy; Smoking cessation; Recurrence; Complications

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INTRODUCTION

Crohn's disease (CD) is a chronic idiopathic inflammatory bowel disease of unknown etiology. The pathogenesis of CD is multifactorial, and several factors have been implicated in its development including genetic and environmental ones[1]. Differences in incidence rates across age, time, and geographic areas suggest that environmental factors are implicated in inflammatory bowel disease (IBD), but only cigarette smoking and appendectomy have consistently been identified as risk factors, the former being the one with the greatest impact on both CD development and disease behavior. The disease is more frequent in active smokers than in non-smokers[2,3], and smoking may alter the natural course of CD since smokers are more likely to develop complications and relapses, and to need surgery[4].

There is evidence that interventions designed to facilitate smoking cessation may improve the course of the disease[5]. This article reviews the available data on smoking and its effects among patients with an established diagnosis of CD.

CLINICAL COURSE AND PROGNOSIS OF CD IN SMOKERS

It is well known that CD is more common in smokers[3]. The relationship between smoking and worsening of the clinical course of the disease has also been established, though the underlying mechanisms are complex and are still subject to research. Of note is the fact that in the case of ulcerative colitis (UC), the effects of smoking are the opposite of those seen in CD. The potential mechanisms explaining such opposing behavior include the different effects of smoking upon cellular and humoral immune function in both diseases, their cytokine profiles, and bowel wall motility and permeability[6].

Among the elements contained in tobacco smoke, it seems clear that nicotine has the greatest impact upon the clinical course of CD[7]. However, it has been recently established that tobacco glycoprotein may be responsible for promoting a Th1 cell response[8], while nicotine maintains its relevance as the cause of anti-inflammatory action in UC[9]. Smokers show an increased production of reactive oxygen species and a lessened antioxidant capacity[10]; this, in turn, could act synergically with oxidative stress in CD[11]. Moreover, environmental factors may interact with genetic factors. In this sense, recent research has demonstrated that smoking may influence the gene expression profile of the colonic mucosa in CD patients[12].

Smoking has been associated with a poorer prognosis of CD and a worse quality of life[13]. Holdstock *et al*[14] reported for the first time that CD patients who were active smokers had an increased number of disease relapses and more severe pain; this was also associated with an increased probability of hospital admission and intestinal resection among patients with ileal involvement. This increased relapse rate was later estimated to be two-fold higher among smokers in a Canadian prospective study involving 152 CD patients[15].

Some epidemiological factors such as gender or disease location may influence the impact of smoking habits on CD outcomes. Most data on the impact of smoking on CD natural history come from the studies performed in the Hôpital Saint Antoine in Paris[16-18]. One of their first studies included 400 consecutive CD patients who were specifically interviewed to assess the effects of smoking upon the long-term course of the disease[16]. The need for corticosteroids and immunomodulators was greater among smokers, but no differences were found in terms of intestinal resection requirements except in those patients who started smoking after CD diagnosis. In addition, the deleterious effect of smoking was found to be dose-dependent, and more marked among women. In a later study, the same authors underscored the existence of a poorer prognosis among women[18].

Exclusive colon involvement possibly does not imply a poorer disease course in smokers as compared to non-smokers. In another study published by the same French group that involved 622 CD patients, the risk of relapse was found to be significantly greater among patients with inactive disease and without colon involvement[17]. A multicenter survey involving 457 CD patients from 19 European countries evaluated several clinical parameters at the time of disease diagnosis. Prescription of corticosteroids or immunomodulators - as a surrogate marker of a less favorable disease course - proved greater among smokers within the first year from disease diagnosis[19]. The proportion of individuals with ileal involvement was likewise significantly greater among smokers, in agreement with the findings in our own setting[20]. In fact, these higher surgical requirements among smokers could be related to a more frequent ileal involvement[21].

The negative effects of smoking seem to be dose-dependent, with the risk of a poor disease prognosis being particularly high among heavy smokers. Lindberg *et al*[22], in a series of 231 CD patients, reported that heavy smokers (over 10 cigarettes/d) presented an increased risk of surgery at 5 and 10 years from diagnosis as compared to patients who never smoked (OR 1.14 and 1.24, respectively). The risk for further operations was even higher, with an OR at 10 years of 1.79. In another study, the proportion of time with intestinal inflammatory activity during follow-up was reported to be 37% among non-smokers, 46% among patients who smoked less than 10 cigarettes/d, and 48% among heavy smokers[23]. Surprisingly, a recent study has reported no unfavorable clinical course in smokers, though passive smokers did show a poorer course[24].

Smoking has been correlated to a lesser prevalence of inflammatory (non-stricturing, non-penetrating) behavior of the disease, thus suggesting that tobacco consumption influences progression towards fistulizing or stricturing disease[22,25-27]. Available data are contradictory when assessing the risk of perianal disease, since it was included in the definition of the fistulizing CD pattern in the initial Vienna phenotypic classification of CD[28], but not in the Montreal adaptation[29]. Of note is the fact that phenotypic characteristics may differ greatly according to the ethnical origin of the studied population. Thus, for the French Canadian population, the phenotypic pattern has been differentiated from that of other Caucasian populations, with an important trend towards aggressive fistulizing behavior[30]. In this population, the association between smoking and CD is strong enough to have possible implications. The lack of association between smoking and CD has now been established in Jewish patients in Israel. The stronger genetic tendency in CD may contribute to this discrepancy[31].

POST-SURGICAL RECURRENCE IN SMOKERS

Intestinal resection still remains a cornerstone in the management of CD despite the availability of biologic agents and the widespread use of immunomodulators. Recently, two independent studies reported a cumulative probability of undergoing abdominal surgery of about 40% and 60% within five and ten years from disease diagnosis, respectively, in an adult hospital-based and in a pediatric population-based cohort of CD patients[32,33]. However, disease recurrence is almost the rule after a "curative" resection and, for this reason, preventive algorithms have been repeatedly

proposed[34,35]. Although it is still to be established whether all patients should start immunomodulators after surgery or not, all authors agree that smoking cessation must be strongly encouraged. In fact, many studies have found that active smoking is firmly correlated with an increased risk of CD postoperative recurrence[36]. This has been recently confirmed in two large studies that found smoking to be an independent risk factor for surgical recurrence (re-operation)[37,38]. Postoperative recurrence usually occurs among patients operated on because of ileal disease, and the harmful effect of tobacco seems to be greater in patients with ileal involvement[17]. However, the results of most of these studies are handicapped by methodological aspects. Firstly, most of them are retrospective; smoking habits may vary with time and this is not always registered in medical records. Secondly, the effects of tobacco may be influenced by some potential confounding factors such as gender[39,40], daily cigarette dose[41], or even ethnicity[41,42], and these have not always been taken into account. Finally, definitions of "active smoker" or "former smoker" are heterogeneous between studies; this is especially important when evaluating smoking cessation, since the effect of giving up smoking seems to be clinically relevant from 1 year on[43].

Cottone *et al*[44] reported the results of a retrospective study which included 182 CD patients who underwent intestinal resection, 109 of whom had an endoscopic assessment for mucosal recurrence 1 year after surgery. The authors found, for the first time, that active smoking was an independent risk factor for endoscopic, clinical, and surgical recurrence. Moreover, this deleterious effect of tobacco on clinical recurrence was dose-dependent. The major drawbacks of this study were that it did not accurately assess the severity of endoscopic lesions and that preventive treatment (if any) was not taken into account. Cortés *et al*[45] recently reported the results of the first prospective study assessing factors associated with endoscopic recurrence. The study included 152 patients participating in three prospective trials that evaluated the efficacy of diagnostic procedures or different preventive strategies for postoperative recurrence and in which endoscopic and clinical monitoring was systematically performed. Smoking and thiopurine use were the only independent predictors of significant postoperative recurrence as defined by the occurrence of clinical recurrence and/or Rutgeerts grade 3 or 4 of endoscopic recurrence (Figure 1). Once again, this risk was much more marked among heavy smokers (patients who smoked > 10 cigarettes/d). It has to be noted that, despite the suggestion that the harmful effect of tobacco in CD might be neutralized by the use of immunomodulators[16], this does not seem to be the case in all clinical settings; as regards postoperative recurrence, two different studies have identified both azathioprine use and active smoking as independent factors associated with both endoscopic and surgical recurrence[38,45].

SMOKING INFLUENCING THERAPEUTIC RESPONSE

Although it has been proven that smoking increases therapeutic requirements in CD (steroids, immunomodulators, surgery), only a few studies addressing the impact of smoking on drug efficacy have been performed to date.

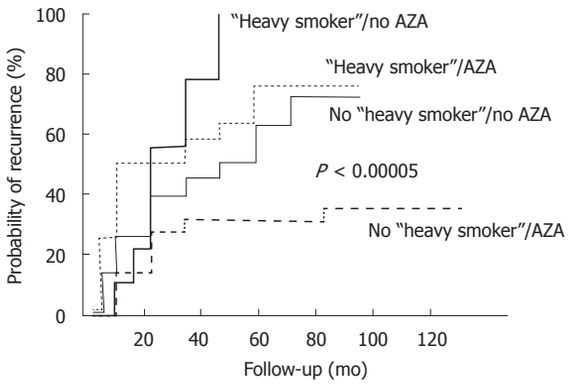

Figure 1 Cumulative probability of relevant recurrence (grade 3 or 4 of endoscopic and/or clinical recurrence) depending on preventive use of azathioprine and active smoking. Reproduced from Cortés *et al*[45] with permission. AZA: Azathioprine.

Some studies have tried to assess the influence of smoking habits on the response to infliximab. Parsi *et al*[46] aimed to identify those demographic and clinical parameters associated with the response to infliximab in 59 patients with luminal CD. Logistic regression analysis found that only smoking and the concomitant use of immunomodulators were independent predictors of response to one single infusion of the drug. Similar results were obtained in a subsequent study in 60 patients treated with a single infliximab infusion for refractory luminal CD[47]. However, a larger European study including 137 patients treated for luminal disease found that age, disease location and concomitant use of immunomodulators, but not smoking habits, were the only factors associated with clinical response in the multivariate analysis[48]. A North American study[49] performed with 122 patients who received one single infliximab infusion for refractory luminal CD did not find any predictor of response among several demographic and clinical factors, including smoking habits. Finally, an Italian multicenter study involved 382 patients who received infliximab for induction of remission in luminal CD (137 of them with a single infusion and 245 with a conventional three-infusion schedule)[50]; among several clinical parameters, only treatment with one single infusion and previous surgery were associated with a lesser probability of response in both univariate and multivariate analyses. All the above-mentioned studies also included patients with fistulizing CD, but no predictor of response (including smoking habits) was found in any of them.

In summary, despite initial data which suggested that active smoking reduced the likeliness to respond to one single infusion among patients with luminal CD, larger studies repeatedly found no association between smoking and infliximab response; however, it has to be said that most of these studies did not consider the tobacco dose.

The influence of smoking on other IBD-related drugs has been infrequently addressed. In this regard, a Spanish study evaluated for the first time the relationship between

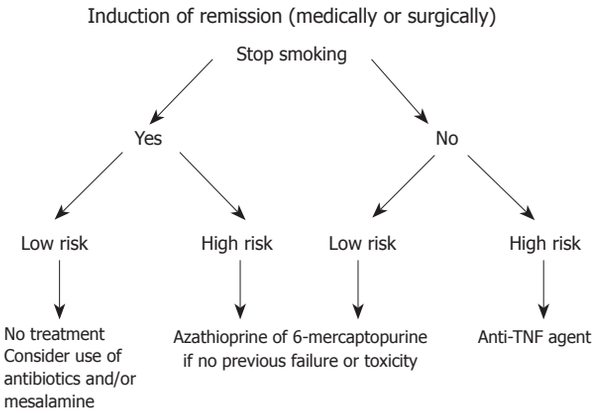

Figure 2 Algorithm for patient in medically or surgically induced remission. Low risk is defined as long-standing, short segment fibrostenotic disease without or with minimum active inflammation. TNF: Tumor necrosis factor.

smoking and the response to thiopurines[51]. The study included 163 IBD patients (103 CD and 60 UC) who started thiopurine therapy because of steroid dependency and who were followed up in two Catalonian centers. Smoking habits at the time thiopurines were started were carefully assessed. No difference in the proportion of responders was found, in CD or in UC, suggesting that, once again, tobacco does not influence the efficacy of drug therapies in IBD. Results remained the same when several exploratory sub-analyses combining gender, disease location (colonic or ileal involvement, colonic or ileal isolated disease) and smoking habits (non-smokers, smokers, heavy smokers of > 10 cigarettes/d) were performed. Of note, CD responders who continued smoking had a higher rate of relapses during follow-up, although this did not lead to higher requirements of biological agents or surgery. Surprisingly, the authors found that treatment discontinuation because of thiopurine-related side effects was independently associated with active smoking in the multivariate analysis. This led to a reduced treatment efficacy among CD patients (as compared to UC) when evaluated by intention-to-treat analysis. Similarly, the largest survey of thiopurine-related toxicity in IBD patients reported to date, which included 3900 IBD patients treated with thiopurines from the Spanish ENEIDA Register [a nationwide register of IBD patients promoted by the Spanish Working Group in IBD (GETECCU)] found that both hepatotoxicity and acute pancreatitis were significantly more frequent in CD as compared to UC, although the smoking status information when starting thiopurines was not available[52].

In summary, it seems clear that active smoking increases the risk of a more disabling course of CD, particularly in patients with ileal disease, women, and heavy smokers. This harmful effect might be genetically modulated, as seen in certain ethnic groups. Once those populations at risk are identified, interventional measures to ensure smoking cessation should be considered (Figure 2). If the patient fails in giving up smoking, more intensive CD treatment strategies such as earlier use of immunomodulators and/or biological agents should be taken into account in order to anticipate complications.

INTERVENTIONS FOR SMOKING CESSATION: ARE THEY EFFECTIVE?

The strongest evidence of the deleterious effect of tobacco consumption upon the course of CD is precisely the beneficial consequences of smoking cessation[5]. In the cohort study published by Cosnes *et al*[17], patients were followed up for 12-18 mo and a lower relapse risk was observed among those patients who stopped smoking for at least 6 mo. In ex-smokers, the clinical course was similar to that seen in patients who have never smoked.

There is no doubt regarding the beneficial effects of smoking cessation upon the clinical course of CD. Such benefit might be even greater than that afforded by the use of thiopurines as maintenance therapy. The only interventional study published to date included 474 consecutive smokers with CD who were offered a smoking cessation program[18]. Patients who stopped smoking for more than one year (quitters) were included in a prospective follow-up study comparing disease course and therapeutic requirements with two control groups - continuing smokers and non-smokers - matched for age, gender, disease location and activity. Fifty-nine patients were able to quit smoking (12%). After a median follow-up of 29 mo, the risk of flare-up in quitters did not differ from that in non-smokers, and was lower than that of smokers (Figure 3). Steroid and immunomodulator requirements were similar among quitters and non-smokers, but greater among smokers. Finally, the risk of surgery was not significantly different between the three groups. Interestingly, the authors found that the physician in charge, previous intestinal surgery, high socioeconomic status and, in women, oral contraceptive use, were predictors of quitting tobacco consumption.

Despite the negative effect of smoking on health, and specifically on the clinical course of CD, smoking cessation is not an easy matter. Management of smokers is based on two complementary interventions: behavioral intervention and drug therapy. Physicians must remember the "four As" needed to correctly address this topic: (1) asking about current smoking habit; (2) advising them to stop; (3) assisting the patient by way of the available methods; and (4) arranging follow-up. It is very important to stress the importance of smoking cessation, since patients who are aware of the strongly negative impact of the habit upon the course of the disease will find it easier to stop smoking. Unfortunately, CD patients are too often unaware of the risks that smoking poses for their disease, thus indicating the need for increased patient information with regard to the effects of smoking on CD[53,54].

Recently, an interesting study designed to increase the motivation to stop smoking involved 140 smokers without CD[55]. Individuals were informed about the characteristics of CD, and underwent a genetic study (*NOD2/CARD15* mutations) in order to classify them according to the risk of developing the disease. The results confirmed the hypothesis that increased information on the genetic burden of CD could modify the intention to quit smoking, to the extent that those individuals at higher risk showed a greater willingness to stop smoking.

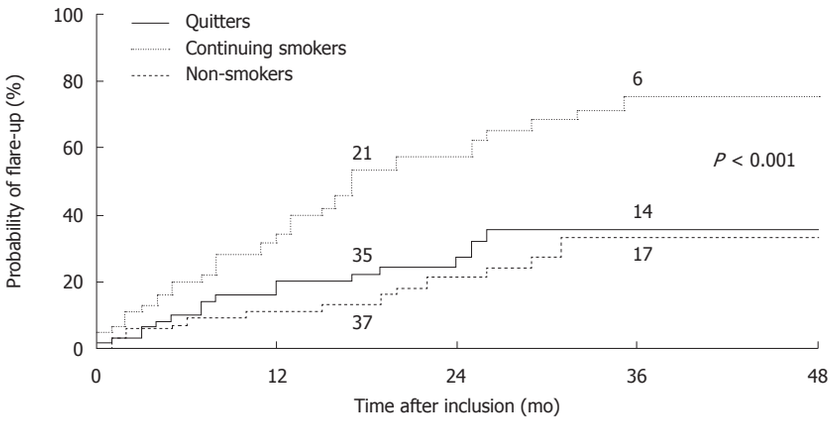

Figure 3 Relapse (flare-up) risk during follow-up of Crohn's disease in continuing smokers, ex-smokers (quitters) and patients who have never smoked. The stated *P*-value corresponds to comparison between the quitters and continuing smokers. Reproduced from Cosnes *et al*[18] with permission.

Behavioral interventions designed to stop smoking

Behavioral interventions designed to facilitate smoking cessation include specific warnings from the general practitioner, intensive advice or counseling by specialists on disease-related risks, and supportive measures in the form of written material or telephone calls[5].

Simple warning by physicians to stop smoking has shown some usefulness in the studies conducted by the Cochrane Tobacco Addiction Review Group[56], though the effect is relatively poor[57]; assuming an unassisted quitting rate of 2%-3%, a brief advice intervention can increase quitting by a further 1%-3%. Direct comparison of intensive *vs* minimal advice has shown a small advantage for intensive advice [relative risk (RR) 1.37, 95% confidence interval (95% CI): 1.20-1.56]. Additional components appear to exert only a minor effect, though a small additional benefit is derived from more intensive interventions compared to very brief interventions.

Behavioral interventions are useful, especially when combined with drug therapy, and particularly over the short term. Motivational interviewing is a directive patient-centered style of counseling designed to help people to explore and resolve ambivalence about behavior change. It was developed as a treatment for alcohol abuse, but may help smokers to make a successful attempt to quit smoking[58]. Innovative effective smoking cessation interventions are required to appeal to those who are not accessing traditional cessation services. Mobile phones are widely used and are now well integrated into daily life, particularly among young adults - as most CD patients are at disease onset. Mobile phones are a potential medium for the delivery of health programs such as those designed to facilitate smoking cessation, but current evidence shows no effect of mobile phone-based smoking cessation interventions upon long-term outcome[59]. Pooled data from the Internet and mobile phone programs show statistically significant increases in both short- and long-term self-reported quitting (RR 2.03, 95% CI: 1.40-2.94). While short-term results are positive, more rigorous studies of the long-term effects of mobile phone-based smoking cessation interventions are needed.

Many European centers have outpatient clinics specifically targeted to help smoking cessation by means of a multidisciplinary approach (with nurses, psychologists, pneumologists and general practitioners), but drug therapy is almost universally used in these clinics as a complement to behavioral interventions.

Drug therapy

Pharmacological smoking cessation aids are recommended for all smokers who are trying to quit, unless contraindicated. The available drugs include the following.

Nicotine replacement products: Such products offer a way to administer nicotine without smoking. They can be used in the form of patches, chewing gum, or nasal spray formulations. The aim of nicotine replacement therapy (NRT) is to temporarily replace much of the nicotine from cigarettes to reduce motivation to smoke and nicotine withdrawal symptoms, thereby easing the transition from cigarette smoking to complete abstinence. A recent Cochrane review[60] identified 132 trials on the use of NRT in people willing to quit smoking. Of these, 111 trials involving over 40 000 participants contributed to the primary comparison between any type of NRT and a placebo or non-NRT control group. The RR of abstinence for any form of NRT relative to control was 1.58 (95% CI: 1.50-1.66). The pooled RR for each type were 1.43 (95% CI: 1.33-1.53, 53 trials) for nicotine gum, 1.66 (95% CI: 1.53-1.81, 41 trials) for nicotine patch, 1.90 (95% CI: 1.36-2.67, 4 trials) for nicotine inhaler, 2 (95% CI: 1.63-2.45, 6 trials) for oral tablets/lozenges, and 2.02 (95% CI: 1.49-3.73, 4 trials) for nicotine nasal spray. The effects were largely independent of the duration of therapy, the intensity of the provided additional support, or the setting in which NRT was offered. The effect was similar in a small group of studies that aimed to assess the use of NRT obtained without a prescription. In highly dependent smokers there was a significant benefit of 4 mg gum compared with 2 mg gum, but weaker evidence of a benefit from higher doses in patch form. There was evidence that combining a nicotine patch with a rapid delivery form of NRT is more effective than a single type of NRT. Only one study directly compared NRT to another drug treat-

ment modality. In this study the smoking cessation rates with nicotine patch were lower than with the antidepressant bupropion.

Bupropion: While not a substitute for nicotine, this drug reduces the anxiety associated with not smoking. Bupropion is an atypical antidepressant that acts as a norepinephrine and dopamine reuptake inhibitor. Initially researched and marketed as an antidepressant, bupropion was subsequently found to be effective as a smoking cessation aid. Conversely to selective serotonin reuptake inhibitors (e.g. fluoxetine), the antidepressants bupropion and nortriptyline contribute to long-term smoking cessation. It has been suggested that the mode of action of bupropion and nortriptyline could be independent of their antidepressant effect, and that their efficacy is similar to that of nicotine replacement[61].

Varenicline: This new smoking dishabituation agent acts by blocking the nicotinic receptors and induces similar effects to that of nicotine, counteracting the craving to smoke, lessening the withdrawal syndromes, and reducing the gratifying and reinforcing effects of smoking. Varenicline was developed as a nicotine receptor partial agonist from cytisine, a widely used drug in Central and Eastern Europe for smoking cessation. Nicotine receptor partial agonists may help people to stop smoking by a combination of maintaining moderate levels of dopamine to counteract withdrawal symptoms (acting as an agonist) and reducing smoking satisfaction (acting as an antagonist). The first reports of trials with varenicline were published in 2006[62]. This drug offers higher efficacy rates (smoking cessation) than those of the existing alternatives[63]. Varenicline increased by 2- and 3-fold the chances of successful long-term smoking cessation as compared with pharmacologically unassisted quitting attempts. There is a need for independent community-based trials of varenicline to test its efficacy and safety in smokers with varying co-morbidities and risk patterns. Likewise, there is a need for further trials of the efficacy of treatment extended beyond 12 wk.

In February 2008, the United States Food and Drug Administration issued a public health advisory note, reporting a possible association between varenicline and an increased risk of behavior change, agitation, depressed mood, and suicidal ideation and behavior[64]. The possible risks of serious adverse events occurring while using varenicline or bupropion should always be weighed against the significant health benefits of quitting smoking that include not only a better prognosis for CD but also a reduction in the chance of developing lung or heart disease, and cancer.

MANAGEMENT OF SMOKING RELAPSE

Less than 10% of all patients who quit smoking without medical help are able to maintain abstinence over the long term[65]. Interventions, whether pharmacological or surgical, increase the long-term cessation rates as compared to control interventions, though there is a permanent reduction in the general success rates due to the fact that a proportion of individuals who are initially able to stop smoking subsequently relapse over time.

At present there is insufficient evidence to support the use of any specific behavioral intervention for helping smokers who have successfully quit for a short time to avoid relapse[66]. The verdict is strongest for interventions focusing on identifying and resolving tempting situations, as most studies have been concerned with these. There is little research available regarding other behavioral approaches. Extended treatment with varenicline may prevent relapse, though extended treatment with bupropion is unlikely to have a clinically important effect. Studies of extended treatment with nicotine replacement are needed.

DIFFERENT APPROACH TO CD PATIENTS WHO CONTINUE SMOKING

When compared to similar data for the general population, patients with CD are not found to be more refractory to smoking cessation[67]. CD patients must be informed of the importance of smoking cessation for the course of their disease, and individualized medical intervention should be established to reach this objective. This is particularly important in smoking women with CD. Nicotine patch replacement therapy during the first 6-12 wk of abstinence is appropriate in heavy smokers and in smokers with a high degree of tobacco dependency. If, despite reinforced medical advice, the patient is unable to quit smoking, referral to a specific smoking cessation clinic is recommendable. If this is not possible, then the physician should offer behavioral and drug treatment support.

Varenicline is likely to be clinically and cost-effective for smoking cessation, assuming that each user makes a single attempt to quit smoking. The key area of uncertainty concerns the long-term experience of subjects who have remained abstinent beyond 12 mo. Guidelines issued by the National Institute for Health and Clinical Excellence in July 2007 state that varenicline is recommended under its licensed indications as an option for smokers who have expressed their wish to quit smoking, and that varenicline should normally be prescribed only as part of a behavioral support program[68,69]. In the presence of depressive symptoms or other contraindications to the use of these drugs, NRT may be used.

Whenever a CD patient is not able to stop smoking, a close monitoring (clinical and/or even endoscopic) is recommended and early introduction of more intensive therapeutic strategies (immunomodulators and/or biological agents) should be considered, especially in women with ileal involvement.

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TOPIC HIGHLIGHT

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Management of the complications of endoscopic submucosal dissection

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Abstract

Endoscopic submucosal dissection (ESD) is currently widely accepted as a standard treatment option for early gastrointestinal neoplasms in Korea. However, ESD has technical difficulties and a longer procedure time than conventional endoscopic resection. So it may have a higher risk of complications than conventional endoscopic resection techniques. We, the ESD study group of Korean Society of Gastrointestinal Endoscopy, have experienced many complications, mostly treated by endoscopic or conservative management. Here, we introduce and share our experiences for management of post ESD complications and review published papers on the topic.

Key words: Endoscopic submucosal dissection; Complication; Management

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INTRODUCTION

Endoscopic submucosal dissection (ESD) enables complete and *en bloc* resection of large or ulcer related superficial gastric cancer. However, the complication risk can be increased due to the long procedure time of submucosal dissection[1]. The major complications include bleeding and perforation[2]. The bleeding which occurs during ESD is almost controlled. Minor bleeding does not cause any problems to patients. So it is not considered to be a complication of ESD[1]. Risk of perforation, which is another major complication, is not reduced because of extended ESD indications. However, ESD complications can be mostly treated by endoscopic or conservative management and do not affect the prognosis of patient.

COMPLICATIONS OF ENDOSCOPIC SUBMUCOSAL DISSECTION

Bleeding

The bleeding rate of endoscopic mucosal resection (EMR) has been reported as from 4% to 38%. The risk of bleeding in ESD is from 13% to 38% which is slightly higher than EMR[2]. Bleeding is categorized by acute bleeding during the procedure and delayed bleeding after procedure. Acute bleeding is defined as any bleeding which occurs during the procedure while delayed bleeding does not occur during the operation; it is defined by endoscopic evaluation at least 24 h after the operation[3].

Acute bleeding is controlled easily by endoscopic coagulation but in cases of delayed bleeding, transfusion, emergency endoscopic evaluation and even surgical procedure could be required[3]. It is manifested with hematemesis or melena. It occurs within 24 h in most cases, but it can also happen 2 wk after the procedure[1]. A considerable duration, at least 8 wk, is needed for healing of EMR related ulcers and bleeding can be detected for quite a long time[4].

Endoscopic hemostasis: It is important that accurate incision or submucosal dissection between the submucosa and muscularis propria layer is performed to prevent procedure related bleeding. There are large vessels in the middle of submucosa layer and the bleeding risk can be increased with blind dissection. Targeted dissection and precoagulation are needed to prevent bleeding due to the presence of large vessels in the dissected layer[5]. There are several kinds of endoscopic hemostasis with hemoclip, electronic coagulation and argon plasma coagulation[2]. Also it is common for proton pump inhibitors (PPI) or histamine receptor (H2) blockers to be prescribed for 4 to 8 wk after ESD to prevent bleeding and encourage healing of post ESD ulcer[6].

Hemoclips can control bleeding without any tissue damage. It is not usually recommended to use these in ESD for several reasons. It can disturb the procedure to use knives, or may obstruct the operator's field of vision and cause problems when trying to perform another endoscopic hemostasis[2]. It can be used in cases of uncontrolled massive bleeding that are not controlled with other methods. The major indication for hemoclipping is for preventing bleeding of visible vessels on post ESD ulcer (Figure 1A)[5]. It can also prevent delayed bleeding with mucosal protection from gastric acid, pepsin and mechanical damage of vessels[7].

Electrocoagulation is useful for hemostasis of visible vessels or active bleeding during or after the procedure. The electrosurgical unit can be readily used as the knife itself during the procedure. In oozing of blood, electrocoagulation controls the bleeding immediately (Figure 1B and C). However, it is not indicated for pulsating bleeding and condensed vessels. The hemostatic forcep is used for pulsatile or nonlocalized bleeding (Figure 1D and E). Repeated electrocoagulation can cause tissue damage and perforation. Pyloric obstruction can occur at the healing stage after excessive and repeated electrocoagulation[2]. Microvessels can be coagulated by applying an electric current via the knife tip itself (Figure 1F). For large vessels (larger than 1 mm), the site of bleeding is cauterized by an electric current using hemostatic forceps or hot biopsy forceps[5]. When the bleeding site is unclear, washing helps to find the bleeding focus, and then bleeding is controlled by coagulation using the lift technique of hemostatic forceps. If massive bleeding occurs or the site of bleeding focus is unclear, hot biopsy forceps are useful[7].

Oozy bleeding control with argon plasma coagulation (APC) during or after the operation is effective. Tissue damage with APC can be reduced by submucosal injection[8]. So as the APC method is a non-contact type, it is used to control bleeding with the front or side of the probe by forced APC mode, flow 1.8 L/min, 45 watt. The disadvantage of APC is it produces considerable smoke during coagulation and frequent suction of smoke is required. However it can not be used in pulsatile bleeding[4].

Perforation

Recent studies report the incidence rate about 4%, even though the rate has fallen since ESD was first used[9,10]. The rate is not decreasing any further because large and difficult lesions, which were not treated this way before, are dissected by ESD. There are no definite guidelines for treatment of perforation, even though several successful medical treatment have been reported[11]. It is important to find the perforated site and close the defect to prevent severe peritonitis with damage to other organs as soon as possible. Kaneko *et al*[12] reported several indications for a clipping method of ESD induced perforations. It suggest that if perforation size could be covered by a metal clip, the margins of the perforation site are smooth and clean of the margin, and one can get a clear endoscopic view[12]. ESD or EMR induced perforations are suitable for treatment in this way in most cases since usually these perforations are small with a linear shape and little contamination risk due to patient's fasting state[9,11,13-15].

In most cases, without profound peritoneal involvement and abnormal vital signs, localized peritonitis can be improved with conservative medical treatment. Mostly, microperforation can be improved with conservative treatment[16]. When perforation is suspected during the procedure, it should be sutured with metallic clips (Figure 1G, H and I, Figure 2). Antibiotics and fasting are also needed in these conditions[17]. Conservative medical treatment includes fasting, intravenous fluid therapy, Levine tube insertion for 12 to 24 h and antibiotics supply for 2 d[11,15,18-20]. Patients can take meals after 2 to 4 d with improvement of peritoneal irritation signs, intraperitoneal free air and white blood cell counts[15,19-21]. Surgical treatment can be considered in the cases of large perforation which cannot sutured with clips and presenting as aggravated peritonitis or unstable vital signs[14,22,23]. It

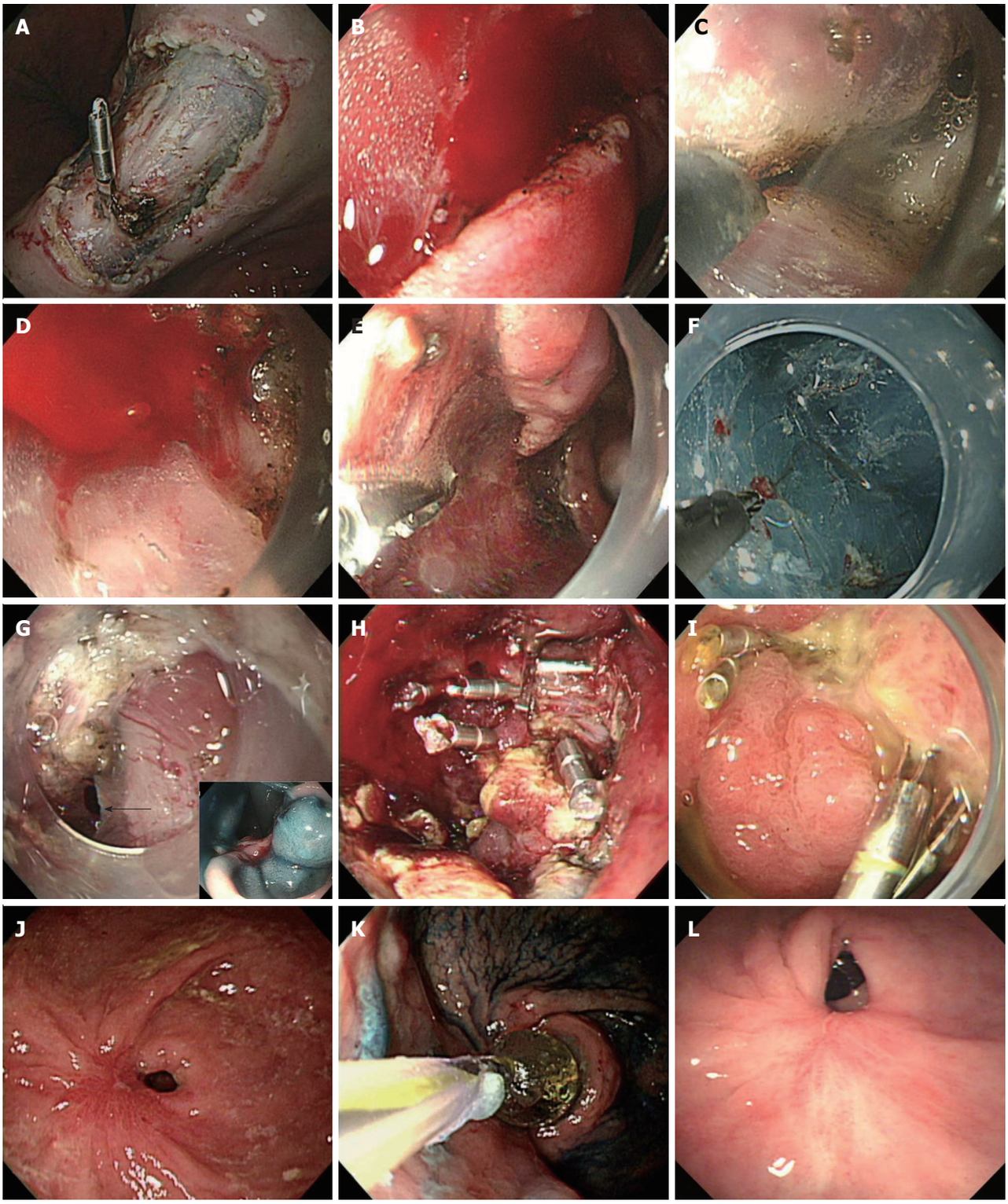

Figure 1 Endoscopic views. A: Endoscopic view of exposed vessels on post endoscopic submucosal dissection (ESD) ulcer, showing hemoclipping for prevention of delayed bleeding; B, C: Endoscopic view of oozing of blood during ESD, showing immediate electrocoagulation by IT knife itself; D, E: Endoscopic view of pulsatile bleeding during ESD (D) and showing coagulation by hemostatic forcep (E); F: Endoscopic view shows that microvessels of the submucosal layer are cauterized by flex knife; G-I: Endoscopic view of jejunal loop side of G-Jstomy showing a perforation (arrow) seen during ESD for EGC of stoma of remnant stomach (G), and the view after closure of the perforation by endoclips (H). A follow-up endoscopy showed the healed perforation 2 wk after endoscopic closure (I); J-L: Endoscopic view shows severe antral stenosis 7 wk after gastric ESD (J). Endoscopic view of balloon dilation procedure (K). A follow-up endoscopic view showed relieved stenosis without any symptoms 3 mo after balloon dilatation (L). Ulcer induced stricture was detected at 4 wk after ESD.

is an absolute indication of surgical therapy when a large amount of fluid is seen in abdominal computed tomography which suggests intraperitoneal infection by gastric contents. But surgery can be held when perforated sites

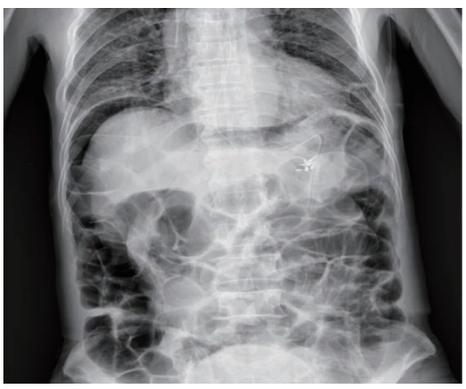

Figure 2 Abdominal radiograph showed pneumoperitoneum.

are sutured promptly and peritoneal infection does not spread extensively[24]. Delayed perforation can develop within hours or days after ESD. It is manifested with abrupt onset of abdominal pain and fever and it can progress to extensive peritonitis. Excessive and repeated electrical coagulation makes burns on the submucosa and muscularis propria, and this condition may cause delayed perforation[19,25]. It is not clear whether peritoneal seeding of cancer cells is possible through perforation, the operator should be careful to prevent dissection induced perforation[26].

Other complications

Prepyloric or pyloric stenosis: Resection of distal antral lesions can cause pyloric stenosis, especially if lesions directly invade the pylorus or cover more than two-thirds of the luminal area. Tsunada *et al*[27] reported five cases of pyloric stenosis. One case showing severe obstruction underwent surgical resection, the others were treated by balloon dilatation. Two patients improved after balloon dilatation but the other two patients underwent surgery due to perforation after dilatation. Due to the operation risk, like these cases, this study group recommended balloon dilatation within 8 wk after resection when lesions encircle more than four-fifths of the antral luminal area. We have to consider the possibility of severe stenosis after ESD of a laterally spreading tumor. A stricture after ESD in the antrum is not easily rescued by balloon dilatation (Figure 1J, K and L) and sometimes requires surgical intervention. Therefore, we must perform ESD with precise planning of the large antral lesion and patients should be followed by endoscopic observation after the procedure[25,27].

Transient bacteremia: Post ESD transient bacteremia can be improved with conservative treatment and empirical antibiotics in most cases. The American and European Society of Endoscopy recommends prophylactic antibiotics use before any endoscopic procedure. Using prophylactic antibiotics in high risk patients undergoing endoscopic variceal sclerotherapy or balloon dilatation is recommended due to the risk of endocarditis or other possible infections[28].

Aspiration pneumonia: Older patients are the main risk group for aspiration pneumonia. Onozato *et al*[29] reported this complication rate in 93 older patients, aged $\geqslant$ 75 years who underwent ESD. Aspiration pneumonia occurred in 2 patients, fever developed in 6 patients. These 6 patients did not show obvious evidence of pneumonia in radiologic findings but fever might be considered to relate with aspiration. All of them were improved with oxygen and antibiotics treatment. The risk of aspiration pneumonia is dependent on the age of patients. It can be prevented with overtube, frequent suction and regular position change[29].

CONCLUSION

The complications of ESD can be prevented and improved with endoscopic and supportive management. Several essential things are needed to prevent or cure these complications such as a skilled operator, careful consideration of the patient's underlying condition and accurate preventive management.

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TOPIC HIGHLIGHT

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Do you have what it takes for challenging endoscopic submucosal dissection cases?

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Abstract

Endoscopic submucosal dissection (ESD) is a widely accepted treatment for early gastric cancer (EGC), especially in Korea and Japan. The criteria for the therapeutic use of ESD for EGC have been expanded recently. However, attention should be drawn to the technical feasibility of the ESD treatment which depends on a lesion's location, size or fibrosis level, or operator's experience. In the case of a lesion with a high level of difficulty, a more experienced operator is required. Thus, the treatment for a lesion with a high level of difficulty should be performed according to the degree of the operator's experience. In this paper, the authors describe the ESD procedure for lesions with a high level of difficulty.

Key words: Endoscopic submucosal dissection; Anatomical location; Technical feasibility

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INTRODUCTION

Recent advances in endoscopic technology, the accumulation of vast amounts of surgical data regarding the lymph node status in early gastric cancer (EGC), and increasing operator experience in the endoscopic treatment of EGC have made endoscopic submucosal dissection (ESD) a widely acknowledged standard curative therapy for EGC[1-3]. Nonetheless, not only the histology type and lesion size but also the anatomical location must be considered in deciding whether ESD is suitable for the lesion[4,5]. Generally, ESD is difficult to perform when patient cooperation is poor (because of severe belching or vomiting); when an anatomical deformity of the stomach (due to gastroesophageal reflux disease, hiatal hernia or a previous stomach operation) makes sufficient stomach inflation difficult; when the patient is in a poor condition (old age, cardiopulmonary comorbidities, patients taking anticoagulation or antiplatelet agents); and when the lesion is located at a place where endoscopic access and submucosal dissection are difficult (cardia, high body, fundus, pylorus, and duodenum). Of these factors, an anatomically difficult location is the most common factor that makes the procedure difficult even for experienced

endoscopists. This review is thus focused on ESD for an anatomically difficult lesion. This article is part of a series of reviews on ESD.

COMMON ANATOMICALLY DIFFICULT LOCATIONS

Cardia and gastroesophageal junction

The cardia and gastroesophageal (GE) junction are susceptible to bleeding and perforation due to their abundant submucosal vasculature and thin walls. Regarding the cardia, the approaching angle is acute, and the lumen is narrow at the GE junction (Figures 1 and 2). When performing a mucosal incision at the margin, it is easier when the endoscope is retroflexed and when the incision is initially cut from the oral to the anal side of the esophagus. While dissection of the esophageal lesion is being performed, the region of dissection must be carefully watched as the esophageal wall lacks a serosa layer and has a thin muscle layer.

An insulation-tipped electrosurgical knife (IT knife) makes cutting easy and safe. When dissecting the lesion from the stomach, it is important for the flap of the lesion made by gradual dissection to be maximally turned over by gravity. Normally, the finishing is performed under retroflexion view, but when using an IT knife, it may be easier to straighten the endoscope at the GE junction and then to make the final cut.

Body

When performing ESD for a lesion located in the body of the stomach, the endoscopist has to be very cautious to prevent bleeding and perforation especially in the high body. Therefore, the endoscopist has to make sure that a sufficient visual field is secured through precoagulation. To guarantee a good visual field, the endoscopist has to bear in mind that the direction of the incision should not be disturbed by unpredictable bleeding in a blind fashion. For larger vessels, precoagulation with hemostatic forceps is needed to avoid uncontrolled bleeding during the procedure. It is also essential to understand the anatomical characteristics of this particular area for a successful and safe procedure. In the deepest muscular layer of the stomach body, especially in the anterior and posterior wall sides, lie the medial longitudinal oblique muscles, where the perforating vessels form a network. Along with this transverse vasoganglion, the attached fibrous tissue forms a so-called "myofascial layer". The fibrotic nature and abundant vasculature of this layer explain why the cutting and dissection of the high body anterior and posterior walls are difficult. Just beneath the transverse vasoganglion (i.e. just above the muscular layer), there is a layer with less vessels and fibrotic tissue. Performing the dissection in this layer is the key point to easy and safe ESD. In dissection, cutting through the above-mentioned vessel network can cause problems due to bleeding. In this situation, the simple maneuver called "coagulation mode trimming" is useful. During this maneuver, the endoscopist uses an IT or flex knife, which is moved back and forth to both coagulate the vessels and dissect through the transverse vasoganglion. Once the mucosa is cut to an adequate depth, the endoscopist should follow the general principles of the procedure. These include maintaining a good visual field of the submucosa, prophylactic coagulation of the visible vessels, and dissection with a curved movement along the direction of the gastric wall through the appropriate handling of the endoscope. In contrast to the anterior and posterior walls of the high body, the lesser curvature of the body has less perforating branches and fibrotic submucosa, which generally makes dissection easier. During the ESD procedure for a lesion in the lesser curvature of the body, precoagulation using Coagrasper hemostatic forceps is required to prevent bleeding because damage to the large blood vessels emerging from the muscle layer caused by an electrosurgical knife may induce massive bleeding.

The high body can have a normal external protrusion beside the adjacent organs, such as the liver. The muscle layer can be regionally elevated, and dissection would have to be made in a curved line to overcome the perpendicular angle between the cutting knife and the muscle layer. Without sufficient visualization of the field, the chance of perforation is high.

Fundus

The difficulty of performing ESD for a fundal lesion lies in the limited visual field because of the stomach contents, blood retention due to gravity, movement of the lesion *via* respiration or heartbeat, and the thin muscle wall in the fundus compared to other parts of the stomach. Nevertheless, when the lesion is located obliquely in between the fundus and the anterior or posterior wall of the high body, an experienced endoscopist can dissect the lesion with an IT knife (Figure 2). For lesions that can be seen only when the endoscope is turned around, and when the lesion is located at the greater curvature side between the cardia and the fundus, ESD is generally impossible to perform. When the lesion is relatively small and cutting around its edge is possible, the removal of the lesion by snare can be an alternative treatment option.

For fundal lesions, because of this highest level of difficulty, ESD is usually not recommended.

Pylorus

The incidence of ESD performance for a pyloric lesion has recently been increasing, but actually reported cases are still rare. When the distal part of the lesion is located at the pyloric channel, the incision and cutting should start from the proximal part, i.e. the antrum. As the dissection progresses and when the pyloric channel is reached, the flap can be pushed into the bulb by the tip of the endoscope, which exposes the muscle and bottom layers of the lesion. With sufficient submucosal injection, the remainder of the lesion can be cut using a needle knife if the distal margin is confined to the channel ring, and if a clear margin can be assured. In the case of the

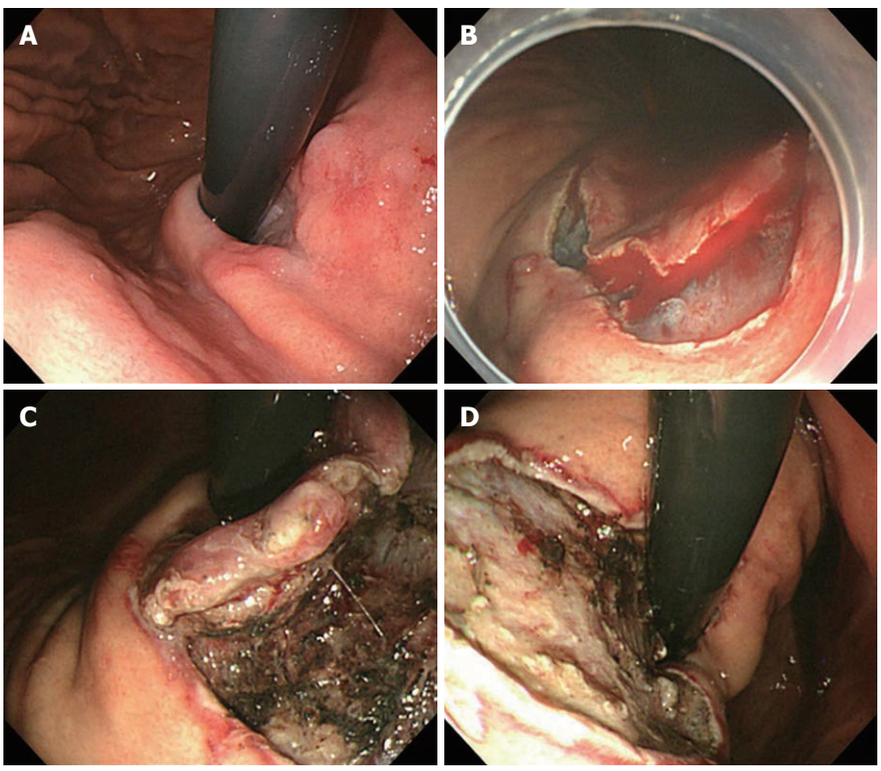

Figure 1 Lesion at the cardia, just below the gastroesophageal junction. A: Retroflexed view of the cardia; B: A circumferential incision was made from the oral to the anal side, which is then vulnerable to bleeding; C: Submucosal dissection from the anal to the oral side; D: The lesion was completely resected.

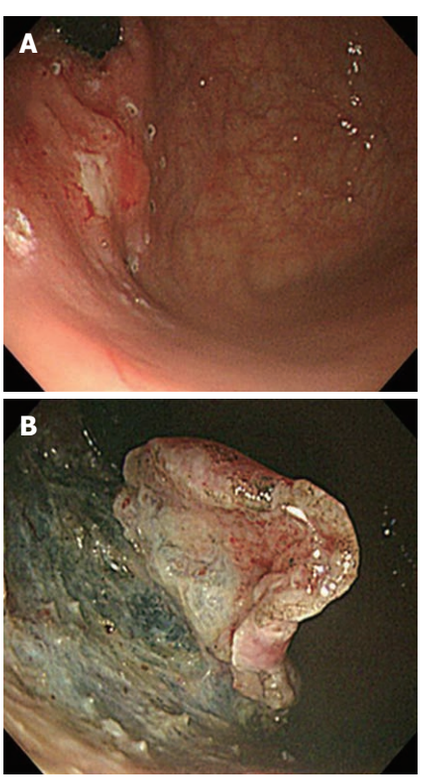

Figure 2 Lesion at the fundus. A: The lesion was located between the fundus and the anterior side of the high body; B: Submucosal dissection was performed from the cardia to the fundus.

pyloric lesion extended to the duodenal bulb, the endoscope has to be retroflexed gently at the duodenal bulb, and the dissection incision should be started from the duodenal distal end (Figure 3). Through this maneuver, a safe distal margin can be secured, but when there is an anatomical deformity of the bulb, a reverse turn of the endoscope may not always be possible, and there is a risk of perforation during the dissection of the pyloric channel. Therefore, in the event of such a case, after the distal margin in the bulb is first dissected using a retroflexed endoscope, the endoscope is straightened for the dissection of the remaining proximal margin at the antrum and for the completion of the procedure.

The duodenal wall is highly susceptible to perforation if the submucosal injection is not sufficient because mucosal and muscle layers of the duodenal wall are very thin in contrast to that of the gastric wall. Thus, great care must be taken to make an incision of the mucosal layer not too deep after sufficient submucosal injection.

Duodenum

Performing ESD for a duodenal lesion is much more challenging than performing it anywhere else because of the insufficient mucosal elevation and poor mucosal contraction. Moreover, the abundant vasculature in the submucosal layer, and the thin muscle layer, make the procedure very vulnerable to bleeding and perforation[6]. If the patient is uncooperative, it will be very hard to maintain the endoscope close to the lesion, especially at the second portion, because of the movement *via* respiration and belching. Therefore, it is very common for an endoscope inserted into the second portion to slip out to the bulb or the antral portion while being pulled with a

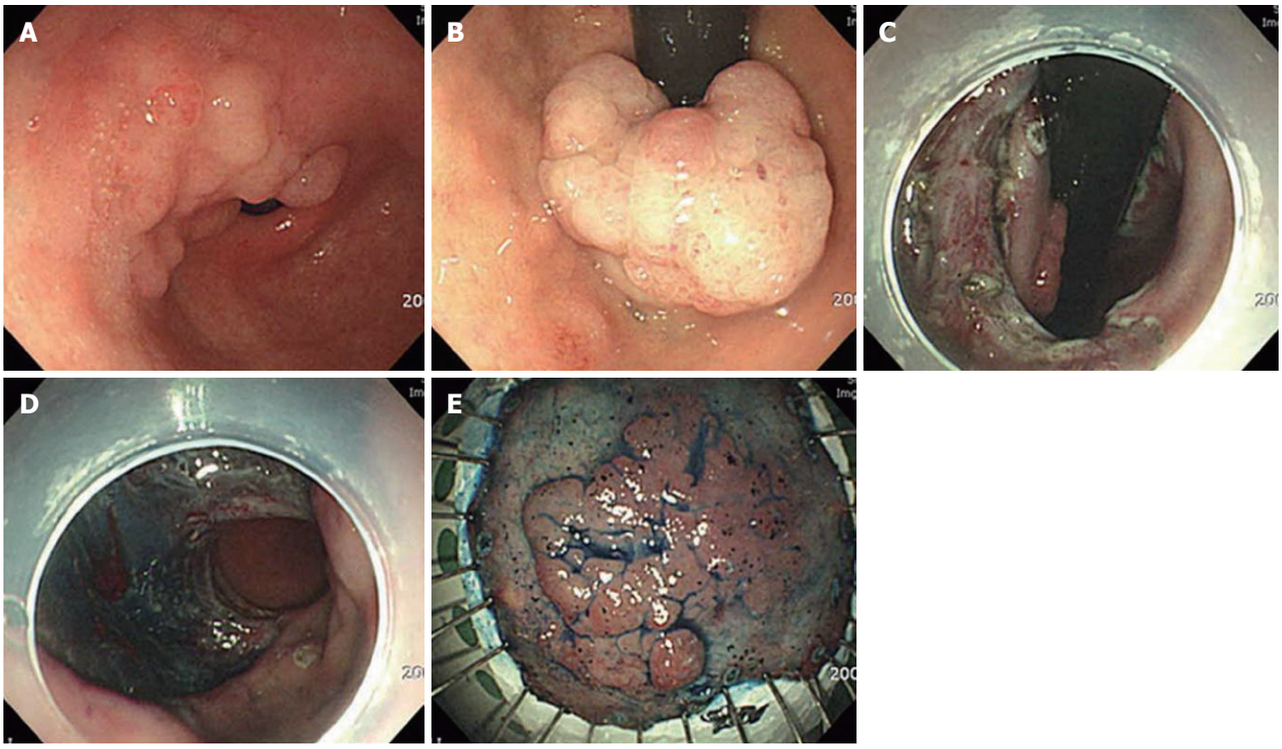

Figure 3 Lesion at the pyloric channel extending to the duodenal bulb. A: Nodular elevated lesion involving the pyloric channel; B: Polypoid mass lesion at the duodenal bulb, retroflexed view; C: Incision and submucosal dissection were performed from the duodenal bulb to the antrum; D: The 180° circumferential dissection was completed; E: The *en bloc* resection was completed.

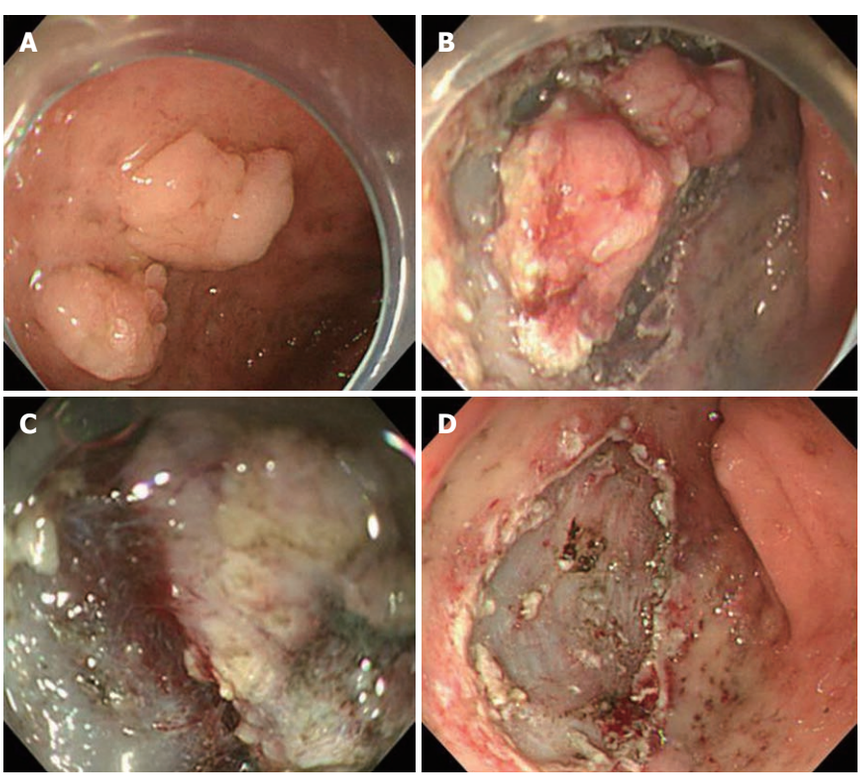

Figure 4 Lesion at the duodenum. A: Two flat elevated lesions at the duodenal bulb; B: Circumferential incision; C: Submucosal dissection (the lifting of the lesion after submucosal injection was limited); D: The *en bloc* resection was completed.

needle or IT knife during incision or dissection. Thus, in such situations, the use of a hook knife is more ideal. It is also important for the assistant to get a firm grip of the endoscope during the procedure. On account of these dif-

ficulties, the endoscopic treatment of duodenal lesions has been limited to polypectomy or mucosal resection using the endoscopic mucosal resection C or EMR sodium hyaluronate methods, and the performance of real ESD has been very rare. The thin submucosal layer, which has the unique anatomical characteristics of the duodenum, makes sufficient "cushion" formation after the submucosal injection difficult (Figure 4). Moreover, mucosal contracture after precutting does not occur as easily as in the other parts of the stomach. Therefore, submucosal dissection just above the muscle layer is an alternative solution for performing duodenal ESD.

CONCLUSION

As regards the level of difficulty of ESD procedures, the fundus is the region with the highest level of difficulty. The fundus is the most difficult area on which to perform an ESD procedure because approach is difficult, the gastric wall is thin, and it is adjacent to the diaphragm. For the duodenum, it is impossible to perform an ESD procedure except on some portions of the bulb. Moreover, endoscopists other than highly experienced experts should avoid performing ESD procedure on the bulb. The easiest area on which to perform ESD is the antrum where even novices can perform an ESD procedure.

In this article, ESD for an anatomically difficult lesion was briefly discussed. The result of the procedure varies based on the endoscopist's skill, the size of the lesion, the type of device used, and even the coagulation mode employed. This means that there is no such thing as "a magic bullet" for a difficult ESD. The recent development of the multibending and "R" scopes and of many other devices will hopefully help overcome the aforementioned difficulties, and further studies on this subject are needed. Training endoscopists to help them acquire the ability to manage an unplanned emergency situation during the procedure is also important.

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Technical issues and new devices of ESD of early gastric cancer

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Abstract

Endoscopic submucosal dissection (ESD) is a highly refined technique compared to conventional endoscopic mucosal resection. It enables complete resection of early gastric cancer (EGC) which has no possibility of lymph node metastasis. Indication for ESD of EGC generally entails early gastric cancer confined to the mucosa with well differentiated histology, though there are clinically suitable expanded criteria. As ESD requires specific skill and expertise, endoscopists need to be familiarized with basic methods and the use of special devices. The essence of the technique is to dissect the submucosal layer with direct vision and maintain the cutting plane above the underlying proper muscle layer. Although there are some differences in the detailed technical aspect, the cardinal method of ESD is now well established and standardized. Furthermore, research and development of new ESD devices that render more efficient, safe ESD are still in progress to improve the overall result of ESD on early gastric cancer.

Key words: Endoscopic submucosal dissection; Technique; Device; Early gastric cancer

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INTRODUCTION

Endoscopic submucosal dissection (ESD) is a novel endoscopic technique that enables *en bloc* resection of large superficial gastric cancer[1]. Since this technique was introduced, there has been remarkable improvement in technique and experience regarding the safety and efficiency of ESD[2-4]. Though reliable, long term results have not been obtained, there is a large body of evidence to suggest that ESD is the therapy of choice for early gastric cancer on occasions when the risk of lymph node metastasis can be excluded[5,6]. General indications for ESD for gastric cancer were proposed on the basis of Japanese studies[7,8]. The absolute indication was non-ulcerative, well-differentiated mucosal cancer less than 2 cm in diameter. Expanded criteria could encompass non-

ulcerative well-differentiated cancer over 2 cm, ulcerative well-differentiated cancer under 3 cm and non-ulcerative, well-differentiated, submucosal invading (limited to 500 μm below the lamina propria) cancer under 3 cm in diameter[9].

Individual ESD technique could vary among endoscopists, but the cardinal aspect of this revolutionary technique is quite straightforward. The following section deals with a brief contemporary summary on the subject, which includes core technical issues and new devices pertaining to ESD procedure.

THE CARDINAL TECHNIQUES OF ESD

ESD is a unique, advantageous procedure over conventional endoscopic mucosal resection in that endoscopists can determine the extent of resection through establishing a outer imaginary line around the lesion[10]. This not only helps endoscopists to control the dissection process, but also ensures complete resection of the lesion confirmed histologically after ESD. Therefore, clarifying the boundaries of the lesion by careful observation before entering the procedure is highly important. Visual enhancing methods to improve detection of the lesion, such as chromoendoscopy, narrow band imaging or magnifying endoscopy, are sometimes helpful[11-14]. Using magnified pit pattern and microvascular pattern as a reference, magnification and endoscopy with or without a narrow band imaging system can give more information about the histological differentiation, depth of invasion and clarification of the extent of the EGC[15,16]. Preoperative diagnosis by image-enhanced endoscopy may have a significant supplementary role to conventional endoscopic ultrasonography in exact localization and clarification of indication, thereby improving the overall result of the procedure (Figure1).

Marking

Marking around the lateral boundary of the target lesion is usually done by pointed devices at the coagulation setting of an electrosurgical unit. Devices such as the needle knife, flex knife, and hook knife are commonly used in careful contact with the mucosa, while coagulating force is fired only briefly to prevent deep thermal injury. Alternatively, argon plasma coagulation is conveniently used due to its non-contact thermal effect[17]. It is important to mark at least 5 mm apart from the outer circumferential margin of the lesion. After completion of marking, additional marking at the proximal or distal inner part of the lesion is usually required as a reference for determining the orientation of the resected specimen (Figure 1A).

Submucosal injection

Traditionally, hyper or isotonic saline mixed with indigocarmine and epinephrine has been used as basic injection solution. Characteristics of ideal injection materials would be to reduce submucosal blebs, have a hemostatic effect and be non-toxic to tissue. To fulfill these requirements, various injection solutions and mixtures, including hypertonic glucose, glycerol, sodium hyaluronic acid, and fibrinogen, have been tried. Some reported that high molecular hyaluronic acid solution was superior to others, albeit at high cost[18-20]. Maintaining the angle of injection needle 45 degrees tangentially for the mucosa is advised to avoid injecting into the muscle layer. It is recommended that injection starts from the anal part the lesion first and proceeds to the proximal part to avoid interruption of the visual field. For each endoscopist's preference, partial injection and simultaneous submucosal dissection, beginning from a specific area, can be done initially instead of elevating whole circumference evenly.

Mucosal incision (precut)

Mucosal incision usually involves circumferential cutting around the lesion prior to submucosal dissection. It is important to incise deep enough into the muscularis mucosal layer that it expose the underlying submucosal layer, because shallow incision leads to unexpected bleeding and make subsequent submucosal dissection difficult. On doing this, immediate trimming by coagulation force with knives on the initial incised spot is really helpful in completing incision and preventing bleeding from the incised area. Available devices are the needle knife, insulation-tipped (IT) knife, flex knife, hook knife, and triangular-tipped knife[21-23]. With a needle knife, a small incision hole is made prior to insertion of the IT knife into the submucosal layer and beginning circumferential incision[1]. On the other hand, needle knife, flex knife, hook knife and triangular knife can be used alone to complete the incision. The sequence and direction of the mucosal incision is dependent on the location of the lesion and selection of the devices (Figure 1B).

Submucosal dissection

Submucosal dissection is a continuing process related to mucosal incision (precut) and also the final stage of ESD. The entire procedure, from marking to dissection, should be carefully designed prior to ESD so that each step guarantees a smooth transition into the next. Direction of gravity, location of lesion, and presence of fibrosis and ulcer should all be taken into account thoroughly in order to apply different tactics and devices for individual lesions (Figure 1C and D).

The technique of submucosal dissection relies heavily on device selection. There are two different classes of knives currently used in clinical practice. One category is pointed tip devices such as needle, flex and hook knives, which are useful for horizontal dissection and have an easy maneuvering quality in all directions. The category contains linear blade devices such as the IT knife, which has an insulated ball tip to prevent perforation and provide fulcrum during dissection. While the IT knife has some disadvantages against fibrotic lesions and features diminished horizontal cutting ability, dissection is quicker and more efficient than other devices, especially in the stomach. Careful dissection with pointed tip knives may be the only reliable option for lesions with ulcer or dense fibrosis.

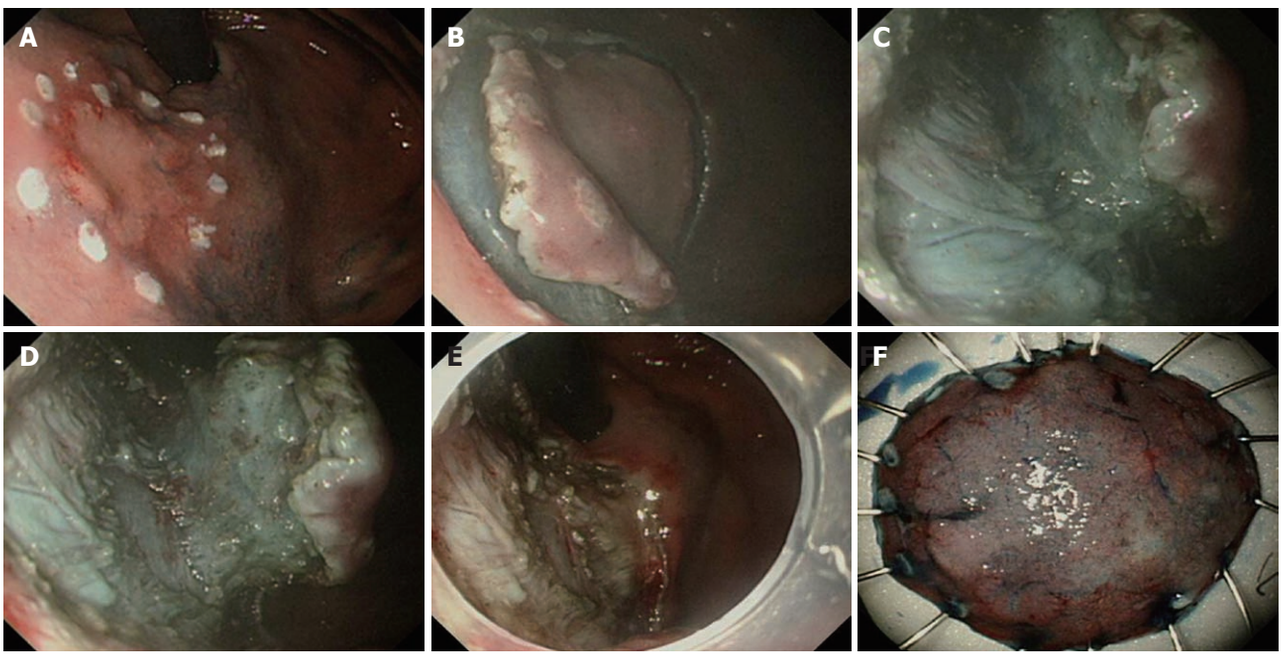

Figure 1 The cardinal steps of endoscopic submucosal dissection technique. A: Marking around early gastric cancer at fundus, marking at least 5 mm apart from the outer circumferential margin of the lesion with the argon plasma coagulation; B: Mucosal incision (precut), circumferential cutting around the lesion prior to submucosal dissection. Incision must be deep enough to expose submucosal layer fully; C, D: Submucosal dissection, early gastric cancer located in upper stomach or fundus like in this case should be dealt with great care to avoid bleeding or perforation. It is advised to always maintain a visual landmark between submucosa and underlying proper muscle layer; E: Completion of endoscopic submucosal dissection, large artificial ulcer was formed after submucosal dissection; F: Acquisition and fixation of the specimen, the specimen was fixated on the board with the pin spreading the lesion circumferentially for the preparation of the pathologic interpretation.

For successful submucosal dissection, measures to adjust to various situations, such as optimization of the operation field by repeated submucosal injection, utilization of transparent cap, adjusting air insufflations and frequent suction, are known to be essential. The cardinal aspect of dissection is maintaining an optimal dissection plane through the submucosal layer, while prudently avoiding injury to the underlying proper muscle layer. By this method, unexpected, blind dissection into the muscle layer and resultant perforation can be prevented.

Bleeding should be minimized for the clear operation field by recognition and meticulous coagulation of vessels before they are inadvertently injured by a cutting knife. Failure to do so often results in a poor field of view and a prolonged procedure time.

Acquisition and preparation of specimen (Figure 1E and F)

Dissected specimens can be dragged out of the stomach to prepare for histopathologic examination. Such specimens should be handled with care during stretching and fixation on the board. Processing the specimen should guarantee accurate analysis and correct diagnosis. Before submitting to pathology, endoscopists are obliged to determine spatial orientation and cutting direction for the preparation of pathologic specimens.

Electrosurgical unit and usage of carbon dioxide (CO_2)

For successful ESD, the understanding and proper use of electrosurgical units is essential[24]. Earlier models of electrosurgical units were composed of a simple cutting and coagulation mode with only the output being adjustable. Recent models added multiple modes that could be used on different lesion characteristics. ICC 200 and VIO300D (ERBD, Germany) are equipped with sensors that pick up the changing signals from the cutting device and tissue interaction and automatically control output and maintain quality of cutting. Cutting mode was comprised of Endocut I&Q mode, dry cut, and swift coagulation, whereas coagulation mode incorporates forced, soft and sprays coagulation.

The time taken for ESD can be longer than 1 or 2 h if the lesion is large and in a difficult location. The patient often complains of abdominal distension and an urge for belching, owing to continuous air inflation of the stomach for maintaining visual field. It appears that there was less bloating and pain after procedures using CO_2 for gut distension compared to air[25]. CO_2 was found to be superior to air insufflations during balloon enteroscopy, endoscopic retrograde cholangio-pancreatography and invasive procedure such as colonic submucosal dissection[26-28]. Usage of CO_2 clearly has a clear advantage when perforation occurs during ESD, because rapid absorption into splanchnic blood makes patients' symptoms more tolerable and helps to stabilize vital signs.

BRIEF KNACK ON ACQUIRING CLEAR FIELD OF VIEW

Unlike the esophagus or large intestine, which is a long tubular structure, the stomach is a distensible bottle-shaped organ that requires diverse approaching techniques depending on the location. For a successful pro-

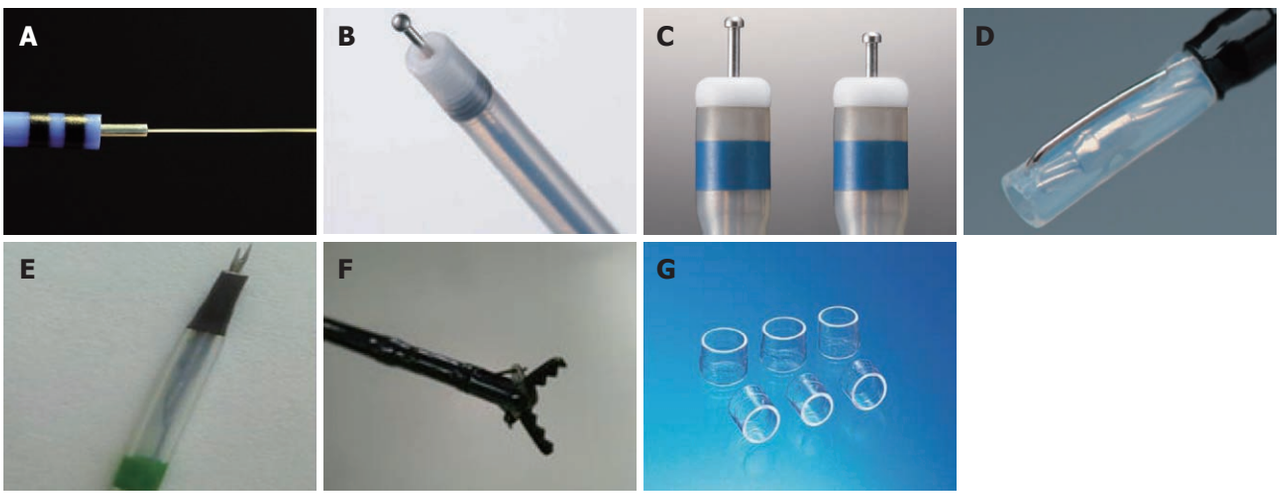

Figure 2 New devices. A: Water-jet hybrid knife allows needleless infusion and lifting of the lesion as well as cutting and coagulation at the same time without the need of changing instruments; B: Ball tipped flush knife (Flush knife-BT) features improved hemostatic efficacy and dissection speed compared with standard flush knife; C: Dual knife is a newly-devised version of pre-existing flex knife, having 0.3 mm needle tip shaped like a doorknob makes the needle less likely to slip, simplifies marking and hemostasis; D: Mucosectome is made of non-conducted tip and endo-knife which is located at the side of the non-conducted tip; E: Fork knife has two interchangeable knives, a fixed flexible snare and a forked knife, which form a single working unit, and has an inlet for material injection or saline irrigation during the procedure; F: Grasping-type scissors forceps has a 0.8-mm-wide and 6-mm-long serrated cutting edge to facilitate grasping of tissue; G: Distal attachment helps keep the field of view clear throughout the procedure and can be chosen from various sized and shaped models fitted with endoscopes.

cedure, the anatomical structures and characteristics of each region should be first acknowledged so that individualized incision and dissection techniques can be applied.

As stated earlier, it is important to maintain a constant depth of incision or dissection while securing the desired operation field during the procedure by appropriately using turns of the endoscope (J-turns and U-turns), adjustments of the left/right levers, and changes of body position. In cases where fibrosis is severe, linear incision knives (such as needle, flex or hook knives) could be more advantageous, while incidence of perforation should be minimized by moving the knives elaborately by small amounts. Certain areas of stomach, such as the cardia or angularis, could be difficult to reach with a conventional endoscope. A multi-banding endoscope (GIF-2TQ260M, Olympus, Tokyo) can sometimes provide assistance in this situation, with its additional bending section enabling easier approximation to the lesion.

New devices (Figure 2)

Knives are a basic instrument for ESD. Selection of the proper instrument influences the quality of the procedure and overall outcome. Every device has its own merits and disadvantages, with new devices usually giving specific modifications to cover up the weaknesses of earlier models.

Several knives, such as the needle, IT/IT-2 knife, hook, flex and flush knives are currently used. Constant effort has been paid to improve the dissection efficiency and safety of each knife. New devices have been devised to maximize ESD potential, while minimizing the ESD time, complication rate and patient discomfort.

The ERBE Hybrid Knife (ERBE, Tüebingen, Germany) combines an ultrafine high-pressure fluid jet with an electrocautery needle, making this device an attractive tool for performing ESD. This device allows submucosal fluid elevation with a preselected pressure and subsequent cutting or coagulation, and is used as a combination of a high-pressure water-jet and a radiofrequency surgical intervention. This allows needleless infusion and lifting of the lesion, as well as cutting and coagulation at the same time without the need to change instruments [29]. However, there is little experimental data and even less human experience with this device at the time of writing.

The ball tipped flush knife (Flush knife-BT) is the improved model of the flush knife, and was developed for the further improvement of the operability and ability of the hemostasis by the knife itself. It has a ball tip of 0.9 mm in diameter and 3 projecting parts of 1.5, 2 and 2.5 mm in length[30,31]. In one case-control study, Flush knife-BT appeared to improve hemostatic efficacy and dissection speed, compared with the standard flush knife.

The dual knife is a newly revised version of the pre-existing flex knife. Having a 0.3 mm needle tip shaped like a doorknob makes the needle less likely to slip, and simplifies marking and hemostasis. A two-step knife extrusion provides length adjustment, with no need for confirmation under endoscopic view, makes up for the weakness of the flex knife. These features enable a precise and effective cutting ability while reducing the burning effect and perforation.

Mucosectome is made of a non-conducted tip and endo-knife. Its blade is located at the side of the non-conducted tip and the tip is rotatable, so the blade can face the lumen and the non-conducted portion of the tip can face the wall of the hallow viscus[32].

The fork knife has two interchangeable knives; a fixed flexible snare and a forked knife, which forms a single working unit, and has an inlet for material injec-

tion or saline irrigation during the procedure. The knives can be changed during ESD by using two switches, the fork knob and core knob, located on the center of the instrument[33].

Grasping-type scissors forceps have a 0.8-mm-wide and 6-mm-long serrated cutting edge to facilitate grasping tissue. The outer side of the forceps is insulated so that electrosurgical current energy is concentrated at the blade to avoid burning the surrounding tissue. The forceps are also able to rotate to the desired orientation[34].

One of the most important devices in ESD is the distal attachment, which leads to safe and fast ESD. It can be fitted at the tip of the scope, making it possible to position the knives at the submucosal layer *en face*[1]. The distal attachment helps keep the field of view clear throughout the procedure and can be chosen from various sized and shaped models fitted with endoscopes. The most widely used hood is a disposable, transparent hood (D-201, Olympus, Japan). It is soft so that endoscopists can compress the submucosal layer without muscle injury and still get a good view. The small caliber tip transparent hood was reported to be useful in getting a higher complete resection rate and preventing perforation[35]. To facilitate the evacuation of blood and water that can be retained on the inner part of the hood and hinder field of the vision, hoods equipped with a irrigation port or side hole have been devised.

CONCLUSION

The technique of ESD for gastric cancer, though there can be slight differences between endoscopists, is relying on the basic concept of lifting the lesion and dissecting the submucosal layer under direct vision. Complications such as bleeding and perforation should be minimized for the invasive nature of the procedure. There are several knives and devices currently available for various purposes. However, the conclusion for which is superior is difficult to be judge because each set of devices has a unique advantage under specific circumstances. The development of new devices has focused on improving dissecting ability and shortening the procedural time while keeping safety in mind. It seems to be clear that current ESD techniques have certain limitations for the full application in all indications regarding early gastric cancer. Therefore, there should be a constant effort to refine and improve ESD devices to come up with ways to improve the ESD technique for early gastric cancer.

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TOPIC HIGHLIGHT

Hoon Jai Chun, MD, PhD, AGAF, Professor, *Series Editor*

Outcome after endoscopic submucosal dissection for early gastric cancer in Korea

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Abstract

Endoscopic treatment, such as endoscopic mucosal resection (EMR) and endoscopic submucosal dissection (ESD), has been established as one of the treatment options for selected cases with early gastric cancer (EGC). Most studies on this topic have been carried out by researchers in Japan. Recently, the experience in EMR/ESD for EGC outside Japan is increasingly reported. In Korea, gastric cancer is the most common malignant disease, and the second leading cause of cancer death. Currently, EMR for EGC is widely performed in many centers in Korea. Early results with a short-term follow-up period are very promising in Korea. The complete resection rate of EMR was 37.8%-94.3%, and that of ESD was 77.4%-93.1%. In this review, we will provide an overview of the outcomes of endoscopic treatments in Korea.

Key words: Early gastric cancer; Endoscopic mucosal resection; Endoscopic submucosal dissection; Outcome

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INTRODUCTION

Gastric cancer is the most common malignancy and the second leading cause of cancer death in Korea. The detection rate of early gastric cancer (EGC), however, has been steadily increasing in Korea. One of the main reasons for this trend is the widespread use of endoscopy as a screening tool for gastric cancer-especially in individuals without symptoms[1,2].

Endoscopic mucosal resection (EMR) and/or endoscopic submucosal dissection (ESD) is currently accepted as a standard treatment for selected cases with EGC[3,4]. Early data suggest that EMR/ESD provides a survival rate of more than 90% comparable to that of surgery if the technique is applied for the appropriate indication[5,6]. Most studies on this topic were performed in Japan, where the incidence of EGC is very high (for review, see these articles[4,7]). The philosophy and technique of endoscopy is quite different between Eastern and Western endoscopists[8]. Even among Eastern coun-

tries, the idea of the use of endoscopy for gastric cancer is quite different between Korean doctors and Japanese doctors. Therefore, extrapolation of Japanese data to other groups of patients may lead to suboptimal results.

The experience of EMR/ESD for EGC outside Japan has been increasingly reported[9-16]. The gold standard study design for evaluation of the efficacy of endoscopic treatment of EGC is a long-term, large-scale, randomized controlled trial. However, the excellent prognosis after surgical treatment of EGC, especially in cases indicated for endoscopic resection, makes randomized controlled trials unethical. Therefore, the best feasible evidence of the efficacy of EMR/ESD comes from long-term clinical follow-up data. In this review, we will provide an overview of the updated outcomes of EMR/ESD for EGC in Korea (Table 1).

Endoscopic treatment of EGC before the introduction of ESD

In Korea, EMR for EGC was first reported in 1996[9], followed by a number of clinical studies. A typical example with relatively long-term follow-up data was a study performed by Youn *et al*[17] from Yonsei University Medical Center in 2006. Between April 1996 and March 2005, 147 patients were treated by EMR. The overall rate of complete resection was 84.6% (126/149), while a complete resection rate of 93.5% was achieved in mucosal cancers (115/123). The success of complete resection was significantly affected by endoscopic gross type (depressed lesion), the degree of differentiation, and the depth of invasion, independently. There were only 5 cases (4.0%) of local recurrence during the follow-up period. There was no disease-related or treatment-related mortality.

Following endoscopic resection of EGC, the development of additional gastric cancer is a significant problem. Investigators from Yonsei University studied the factors related to the multiple synchronous and/or metachronous gastric cancers in EMR/ESD patients[18]. After endoscopic treatment of EGC(s), they followed 235 patients for 24 mo or longer. Twenty-three patients (9.8%) were found to have additional gastric cancer within 1 year. Twenty metachronous cancers (8.5%), which were defined as cancers detected after 1 year of treatment, were also found. Interestingly, initial histology of the resected specimen was related to the development of additional cancer; undifferentiated histology of the primary lesion was related to synchronous and metachronous gastric cancer ($P < 0.001$ and $P = 0.002$, respectively)[18]. This is very interesting data which should be considered in the discussion of expanding the indications of EMR/ESD.

Techniques and results of ESD for EGC

In Korea, the most commonly used endoscopic treatment modality for EGC has been changed from endoscopic mucosal resection with precutting (EMR-P) to ESD (Figure 1)[19]. The techniques of ESD used in Korea and Japan are quite similar. In brief, ESD is usually performed under conscious sedation or slightly deeper sedation using either midazolam or propofol. Cardiorespiratory function is continuously monitored during the procedure. After identifying the target lesion, marking dots were made circumferentially at about 5 mm lateral to the margin of the lesion using a needle knife or an argon plasma coagulation probe. After marking, a submucosal injection of various solutions, such as normal saline and epinephrine mixture or glycerol mixture, is performed around the lesion to make a submucosal cushion. An initial short incision of the mucosa was made with the needle knife to allow the submucosal insertion of the tip of the insulation-tipped (IT)-knife or other knives. Circumferential mucosal cutting is performed outside the marking dots to separate the lesion from the surrounding non-neoplastic mucosa. After the circumferential cutting, an additional submucosal injection is carried out. Finally, direct dissection of the submucosal layer is performed using one of the various knives. When needed, an electrocautery snare may be used at the final step. During the ESD procedure, endoscopic hemostasis is performed with a needle knife or specialized hemostatic forceps.

Jung *et al*[20] from Asan Medical Center reported early results of ESD in their institution. From 2005 to 2006, ESDs for 264 cases of EGC were performed. The median size of the tumor was 19 mm, and the median size of the resected specimen was 50 mm. The rate of complications was 14.0% (bleeding 9.8% and perforation 3.8%). The complete resection rate was 87.9% (232/264)[20]. Recently, researchers at Asan Medical Center presented their updated results of ESD for EGC as an abstract[21]. In their institution, EMR or ESD was performed on 1340 EGCs in 1187 patients from July 1994 to January 2009. The complete resection rate was 96.6% and was 86.9% in the absolute indication group and in the extended indication group ($P < 0.001$). The local recurrence rate was similar[21].

Min *et al*[22] from Samsung Medical Center reported their experience of EMR-P and ESD with short-term follow-up data. From 2003 to 2006, 346 consecutive patients with EGC were treated by either EMR-P (103 patients) or ESD (243 patients) and their clinical outcomes were compared. In the ESD group, the rate of en bloc plus R0 resection was significantly higher than the EMR-P group (88.9% *vs* 75.7%, $P = 0.002$). For small EGC (diameter < 20 mm), however, the en bloc plus R0 resection rate for EMR-P was comparable to ESD. The complication rate was slightly higher in the ESD group, but there was no statistical significance. In the case of R0 resection of intramucosal differentiated cancer, neither group showed local recurrence during the median 29 and 17 mo of follow-up[22].

Jang *et al*[23] from Dong-A Medical Center reported their follow-up data after ESD. A total of 198 patients with EGC were treated with ESD from 2004 to 2007. In EGC patients, en bloc resection was achieved in 89.7% (177/198), and the complete resection rate was 87.9% (174/198). During the median follow-up period of 30 mo, local recurrence was found in 10 patients (5.1%). Tumor size > 20 mm was significantly associated with

Table 1 Clinical outcome of endoscopic mucosal resection or endoscopic submucosal dissection for early gastric cancer in Korea (selected) (%)

Author	Year	*n*	Methods	Complete resction	Local recurrence	Bleeding	Perforation
Lee	1996	19	Strip biopsy	37.8	28.6	-	-
Cheon	2000	28	Strip biopsy	64.3	3.6	-	-
Kim	2000	20	EMR-L	85.0	5.9	0.0	0.0
Seong	2002	35	Strip biopsy	94.3	6.1	-	-
Hyun	2003	45	Strip biopsy	55.6	0.0	24.4	0.0
Kim	2005	109	Strip biopsy, EMR-C, EMR-P	67.9	1.4	8.3	2.8
Youn	2006	149	Strip biopsy, EMR-C, EMR-L, ESD	84.6	4.0	22.8	1.3
Kim	2007	514	Strip biopsy, EMR-C, EMR-L, EMR-P, ESD, polypectomy	77.6	6.0	13.8	0.6
Jung	2007	360	EMR-P	82.8	-	10.6	1.1
Jung	2007	264	ESD	87.9	-	9.8	3.8
Kang	2008	456	ESD	80.3	0.0	-	-
Park	2008	434	ESD	77.4	1.8	8.1	2.3
Min	2009	103	EMR-P	75.7[1]	0.0	3.9	1.9
Chung	2009	534	ESD	87.7[1]	-	15.6	1.2
Jang	2009	198	ESD	87.9	5.1	7.4	2.9
Lee	2010	806	ESD	93.1	0.4	4.2	3.0

[1]Complete *en bloc* resction rate; EMR-C: Endoscopic mucosal resection using a transparent cap; EMR-P: Endoscopic mucosal resection with precutting; EMR-L: Endoscopic mucosal resection with band ligation; ESD: Endoscopic submucosal dissection.

local recurrence. The 3-year cancer-free survival rate was 94.9%. Among 10 patients with local recurrence, 6 were successfully treated with a second ESD, and 4 were treated surgically after a failed attempt at ESD. Six metachronous cancers were also found, which were treated with ESD. As a whole, the 3-year cancer-free survival rate was 94.9%[23].

Kim *et al*[24] from Soonchunhyang University reported their experience using a novel device, the Fork knife (Endo FS). Although the authors did not report the long-term follow-up data after ESD, the *en-bloc* resection rate was 95.8% (254/265) using the Fork knife, and was comparable with that of 93.1% (67/72) using a more popular Flex knife. Complete ESD without tumor cell invasion of the resected margin was obtained in 81.1% (215/265). The mean procedure time was shorter in the Fork knife group compared to the Flex knife group (59.6 min *vs* 76.7 min, $P < 0.05$). The authors concluded that the Fork knife is useful for clinical practice and has the advantage of reducing the procedure time[24].

Recently, Lee *et al*[16] from Samsung Medical Center reported their updated data of ESD for EGC[16]. Before March 2009, 806 lesions of EGC in 780 patients were treated with ESD at their institution. They divided their cases into two groups: a conventional indication group ($n = 595$) and an expanded indication group ($n = 211$). The complete resection rate was 97.3% and 81.0% in the conventional indication group and in the expanded indication group, respectively. The conventional indication group and expanded indication group did not differ with regard to the rates of local recurrence (0.7% *vs* 0%), or metachronous recurrence (3.6% *vs* 3.3%). The rate of perforation was higher in the expanded indication group than in the conventional indication group (6.6% *vs* 2.4%, $P < 0.001$). When they followed 458 patients for a median of 26 mo, there were no cancer-related deaths in the two groups. Two cases (0.4%) with local recurrence and 16 cases (3.5%) with metachronous recurrence were observed. Disease-free survival rate was not different between the two groups[16].

Korean multicenter studies

Two multicenter retrospective studies on the clinical efficacy of endoscopic treatment of EGC have recently been published. The first study was published in 2007 by Kim and other members of the Korean EMR Study Group (changed to the Korean ESD Study Group in 2009)[25]. Data were collected retrospectively using the on-line database registry system. From 2000 to 2002, 514 EGCs in 506 patients were treated by various techniques in 13 institutions. EMR-P was the most commonly used technique (52.3%). ESD was used in only 6.6%. The resection was regarded as incomplete if histopathologic examination revealed a positive resection margin, submucosal invasion, positive lymphovascular invasion, or undifferentiated histologic diagnosis. The rate of complete resection was 77.6%. For completely resected mucosal cancers ($n = 399$), the median duration of follow-up was 23.5 mo (range 5-70 mo). In this group, local recurrence was detected in 24 cases (6.0%) with a median interval between EMR and recurrence of 17.9 mo (range 3.5-51.7 mo). There were 3 cases with perforation and 71 cases with bleeding. No deaths were related to recurrence of gastric cancer during the overall median follow-up period of 39 mo[25].

After the first multicenter study, ESD was widely used in different hospitals in Korea. The Korean ESD study group has carried out a second multicenter retrospective study on the safety and effectiveness of ESD[26]. From January 2006 to June 2007, 1000 EGCs in 952 patients (502 men, 450 women; mean age 62.1 years, range 43-90 years) were treated using ESD at 6 Korean ESD study group-related university hospitals in Korea. The rates of en bloc resection and complete en bloc resection were 95.3% and 87.7%, respectively. The rates of significant bleeding and perforation were 0.6% and 1.2%,

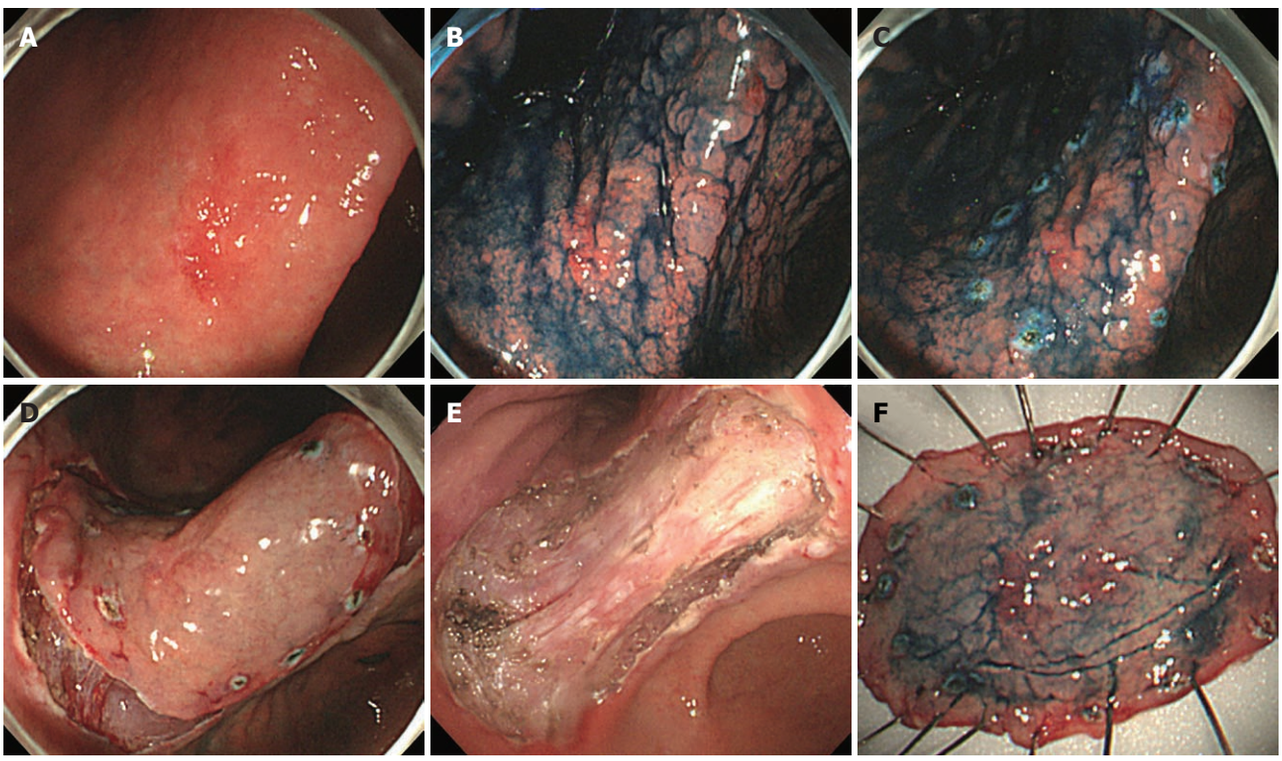

Figure 1 Endoscopic submucosal dissection procedure for early gastric cancer. A: 1.5 cm × 1.2 cm sized hyperemic slightly elevated early gastric cancer was seen at the lesser curvature side of the lower body just above the gastric angle. Previous forceps biopsy results showed moderately differentiated adenocarcinoma; B: Indigo carmine dye was sprayed onto the lesion to define the lateral margin more clearly. Gastric mucosa around the cancer lesion showed severe metaplastic change; C: Using the tip of the needle knife, marking dots were made circumferentially at about 5 mm to 10 mm lateral to the estimated margin of the lesion; D: After submucosal injection of saline mixed with epinephrine and indigo carmine, a circumferential mucosal cutting was performed outside the marking dots to separate the lesion from the surrounding non-cancerous mucosa; E: After additional submucosal injection, direct dissection of the submucosal tissue was performed using an IT-knife and endoscopic hemostasis was carried out. A large artificial ulcer was made; F: The resected specimen with a central cancerous lesion. In the pathologic examination, a 1.8 cm × 1.1 cm sized moderately differentiated tubular adenocarcinoma limited in the mucosal layer was identified. The resection margin was free of cancer, and there was no lymphovascular invasion.

respectively. The mean procedure time was 47.8 ± 38.3 min. However, multicenter long-term follow-up data after ESD have not yet been reported[26].

DISCUSSION

Endoscopic treatment of EGC was developed in Japan. However, experience in endoscopic treatment has now been reported in many other countries. As shown in this review, ESD for EGC has become quite a common procedure in Korea. In 2009, a multicenter study of ESD was reported in Taiwan[27]. In China, early experience of ESD has been reported[28]. Even in Western countries where early EGC scheduled for endoscopic treatment is uncommon, small studies evaluating the usefulness of ESD have been published[11,29,30]. Because of the advantages of ESD in terms of complete resection rate and curative resection rate, we expect that more cases of EGC will be treated by ESD not only in Korea but also in many other countries.

The complete resection rate of endoscopic treatment for EGC depends on the inclusion criteria and the definition of complete resection, so head to head comparisons are difficult. As shown in a recent meta-analysis[31], the complete resection rate of ESD is generally higher than EMR. In Korea, the complete resection rate of EMR was 37.8-94.3%, and that of ESD was 77.4-93.1% (Table 1). This is quite an important achievement, because the selection criteria for endoscopic treatment of EGC have been expanded. The size of the endoscopically treated lesions these days is larger, and technical developments have made the complete resection rate better. Although the procedure time is longer, and the complication rate is higher in ESD, most complications can be treated medically without surgery.

Early results of endoscopic treatment of EGC in Korea have been very promising. However, the duration of follow-up is rather short, which makes conclusive comments difficult. Before expanding indications for endoscopic treatment, we need to examine the reported and unreported data very carefully. In this regard, a nationwide registry of endoscopically treated EGC cases seems to be mandatory.

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ORIGINAL ARTICLE

Special diaphragm-like strictures of small bowel unrelated to non-steroidal anti-inflammatory drugs

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Abstract

AIM: To summarize clinical, endoscopic, radiologic and pathologic features of special diaphragm-like strictures found in small bowel, with no patient use of non-steroidal anti-inflammatory drugs (NSAIDs).

METHODS: From January 2000 to December 2009, 5 cases (2 men and 3 women, with a mean age of 41.6 years) were diagnosed as having diaphragm-like strictures of small bowel on imaging, operation and pathology. All the patients denied the use of NSAIDs. The clinical, endoscopic, radiologic and pathologic findings in these 5 patients were retrospectively reviewed from the hospital database. Images of capsule endoscopy (CE) and small bowel follow-through (SBFT) obtained in 3 and 3 patients, respectively, and images of double-balloon enteroscopy and computed tomography enterography (CTE) obtained in all 5 patients were available for review.

RESULTS: All patients presented with long-term (2-16 years) symptoms of gastrointestinal bleeding and varying degrees of anemia. There was only one stricture in four cases and three lesions in one case, and all the lesions were located in the middle or distal segment of ileum. Circumferential stricture was shown in the small bowel in three cases in the CE image, but the capsule was retained in the small bowel of 2 patients. Routine abdomen computed tomography scan showed no other abnormal results except gallstones in one patient. The lesions were shown as circumferential strictures accompanied by dilated small bowel loops in the small bowel on the images of CTE (in all 5 cases), SBFT (in 2 cases) and double-balloon enteroscopy (in all cases). On microscopy, a chronic inflammatory infiltrate and circumferential diaphragm were found in all lesions.

CONCLUSION: Diaphragm-like strictures of small bowel might be a special consequence of unclear damaging insults to the intestine, having similar clinical, endoscopic, radiologic and pathologic features.

Key words: Small bowel; Gastrointestinal bleeding; Diaphragm; Stricture; Endoscopy; Computed tomography; Enterography

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INTRODUCTION

Many medications, diseases and processes may cause insult to the small bowel and result in strictures of the bowel cavity, such as potassium chloride tablets, surgical anastomoses, radiation, ischemia, Crohn's disease, tuberculosis, eosinophilic enteritis, lymphoma, *etc*.

Diaphragm disease was first defined by Lang *et al*[1] in 1988, who described the pathologic findings of non-specific small-bowel disease in patients taking non-steroidal anti-inflammatory drugs (NSAIDs). The mucosal disease caused by these drugs in the small bowel is termed NSAID enteropathy. The abnormalities of NSAID enteropathy include inflammation, erosion, fibrosis, stricture, perforation, and formation of diaphragm disease. The most frequent manifestations are iron-deficient anemia, acute hemorrhage, perforation and obstruction of the small bowel. Many studies have reported cases with multiple diaphragm-like strictures in the whole gastrointestinal tract that are associated with the chronic use of NSAIDs[2-6]. In contrast, from the references we can find, only one reported case with small bowel diaphragm disease is not associated with the use of NSAIDs[7]. Multiple diaphragm-like strictures emerged in the ileum and jejunum at different times in this patient and he underwent three surgical operations.

Here, we report on a group of 5 patients who presented symptoms of gastrointestinal bleeding and characteristics of diaphragm-like strictures of small bowel that were not attributable to the utilization of NSAIDs. The purpose of this study was to summarize the clinical, radiologic, endoscopic and pathologic features of these special diaphragm-like strictures of small bowel.

MATERIALS AND METHODS

Ethics

This work has been carried out after receiving the approval from our institutional review board. All patients were not individually asked for consent to be included in this study, but each patient in the study did agree to the retrospective use of their medical records and images for research purposes during treatment at our hospital.

Patient data

From cross-referenced records in the Departments of Gastroenterology, Radiology and Pathology at Ruijin Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai, from January 2000 to December 2009, 5 patients were identified with clinically confirmed diaphragm-like strictures of small bowel. All the 5 patients denied the use or prescription of NSAIDs**,** which was confirmed by their family members and medical history records. These 5 patients included two men and three women. Clinical data from the patients including sex, age, hemoglobin level, white blood cell (WBC) count, C-reactive protein (CRP) level and major onset symptoms were obtained from the hospital database and most information is shown in Table 1. All cases underwent removal of the lesions by laparoscopically assisted enterectomy.

Endoscopic procedure

Capsule endoscopy (CE) was performed in 3 patients (cases 1, 2 and 5). The small bowel capsule, manufactured by Given Imaging (Yoqneam, Israel) measures 11 mm × 26 mm and weighs 3.7 g. The camera in the capsule moves through the gastrointestinal (GI) tract *via* peristalsis and transmits 2 images per second to a data recorder located on the waist of the patient.

All 5 patients underwent double-balloon endoscopy (Fujinon, En-450P5/20, Fujinon Inc, Saitama, Japan). When the location of the lesion could be predicted in advance by the color of the feces or other examination findings, an insertion approach close to the lesion was selected, either oral or anal. When the location could not be predicted, the anal approach was selected first, in principle. However, whether the responsible lesion was specified or not by the first insertion approach, insertion with the other approach was performed later to observe the entire small bowel.

All the images were reviewed by two gastroenterologists who had no knowledge of the final radiologic, endoscopic, or pathologic findings. The endoscopic evaluation included number, location, size, shape, color and texture.

Radiologic examination

Routine abdominal computed tomography (CT) scan was performed in one patient. CT enterography (CTE) was performed in all five patients. For CTE examination, all patients underwent an intestinal preparation according to the following plan: the day before a light diet free of fruit and vegetables; 500 mL of a mixture of Sennosides and tea at 5:00 pm, another 500 mL followed at 6:00 pm. All patients accepted oral administration of 2.5% mannitol solution (1500-2000 mL) over 30-45 min. Thirty minutes after oral administration, CT scans were performed on a multi-slice multidetector computed tomography (MDCT) scanner [two patients had scans performed on a 16-slice MDCT scanner (LightSpeed-16, GE Medical Systems, Milwaukee, WI) and the other three patients were on a 64-slice MDCT scanner (LightSpeed Volume CT, GE Medical Systems, Milwaukee, WI)]. Section thickness ranged between 5 mm and 7.5 mm. Intramuscular injection of 20 mg anisodamine was administered 10 min before CT scan. After unenhanced CT scan, all patients received an intravenous injection of 1.5 mL/kg of iohexol (Omnipaque300; Amersham, Shanghai, China) at a rate

Table 1 Clinical data of study patients

Case	Sex	Age at onset (yr)	Age at diagnosis (yr)	Past history	Main symptoms		Physical examination	HGB (g/dL)	WBC count ($\times 10^9$/L)
					GI bleeding	Abdominal pain			
1	F	30	33	Gallbladder stone	P	P	Normal	9.0	3.2
2	F	48	64	No	P	P	Normal	5.8-9.6	4.1
3	M	24	26	Appendectomy	P	N	Normal	10.9	5.3
4	F	40	44	Hysteromyoma	P	P	Normal	9.2	5.7
5	M	32	41	No	P	N	Normal	9.7	5.0

F: Female; M: Male; P: Positive; N: Negative; GI: Gastrointestinal; HGB: Hemoglobin; WBC: White blood cell.

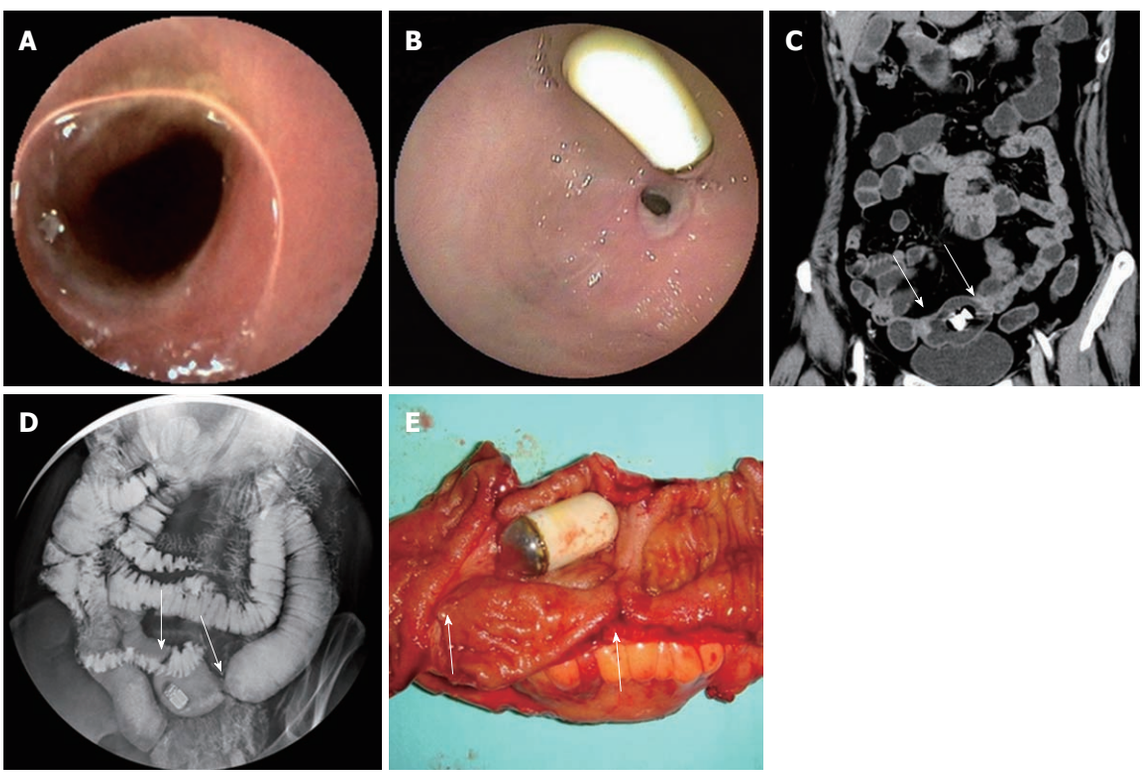

Figure 1 Sixty-four-year-old woman presenting with 16 years of intermittent black stools. A: Circumferential stricture seen on capsule endoscopy image; B: Double-balloon enteroscopy image shows an unusual diaphragm-like stricture in the ileum and the retained capsule; C: Computed tomography enterography image (oblique MPR) showing two diaphragm-like strictures and capsule retention in a dilated small bowel loop in the ileum (arrows); D: Diaphragm-like strictures and capsule retention in a dilated small bowel loop shown by small bowel follow-through (arrows); E: Longitudinal section of specimen containing laterigrade diaphragms and a retained capsule endoscope in the middle, and a laterigrade ulcer can be seen in one of the diaphragms (arrows).

of 3 mL/s. Contrast-enhanced CT images were acquired in arterial phase (25-30 s) and venous phase (60-65 s). Multiplanar reconstructions and maximum intensity projection were performed at the workstation (ADW4.2 and ADW4.4).

In addition, small-bowel follow-through (SBFT) was performed in 3 patients (cases 2, 4 and 5). Selective mesenteric angiography examination and bowel isotope scans using ^{99m}Tc-labeled red blood cells (^{99m}Tc-RBC) were also performed for case 2.

The radiologic images were reviewed in consensus by two radiologists who had no knowledge of the final endoscopic, radiologic, or pathologic results. At the workstation, the CT images from each patient were reviewed to analyze the following criteria: (1) location of lesion; (2) number of lesions; (3) thickness of bowel wall; (4) lumen cavity; (5) mesenteric vessels; and (6) lymph nodes. At non-enhanced CT, attenuation in the lesions was classified as hypodense, isodense, or hyperdense compared with the normal bowel wall. After contrast enhancement, the degree of enhancement was classified into no enhancement, mild (10-20 Hu), moderate (20-50 Hu), or marked (> 50 Hu) enhancement. These findings were used to characterize the imaging and the gross pathological features of the lesions.

Pathological technique

All the specimens were fixed in 10% neutral-buffered formaldehyde solution and were embedded in paraffin wax. Hematoxylin and eosin staining was performed in all the pathologic specimens. All the pathologic specimens were reviewed retrospectively by two pathologists. The macroscopic appearances of each resected segment were analyzed with photomacrographs; the analysis in-

Table 2 Data of enteroscopic findings

Case	*n*	Location (distance to ileocecal valve) (cm)	Type of lesion	Stricture		Edema in mucosa	Pass-through of scope
				Mild to moderate	Severe		
1	1	80	Ulcer	1	0	P	P
2	3	100-115	Ulcer and erosion	1	2	P	P
3	1	100	Ulcer	1	0	P	P
4	1	80	Ulcer	1	0	P	P
5	1	150	Erosion	0	1	P	P

P: Positive; N: Negative.

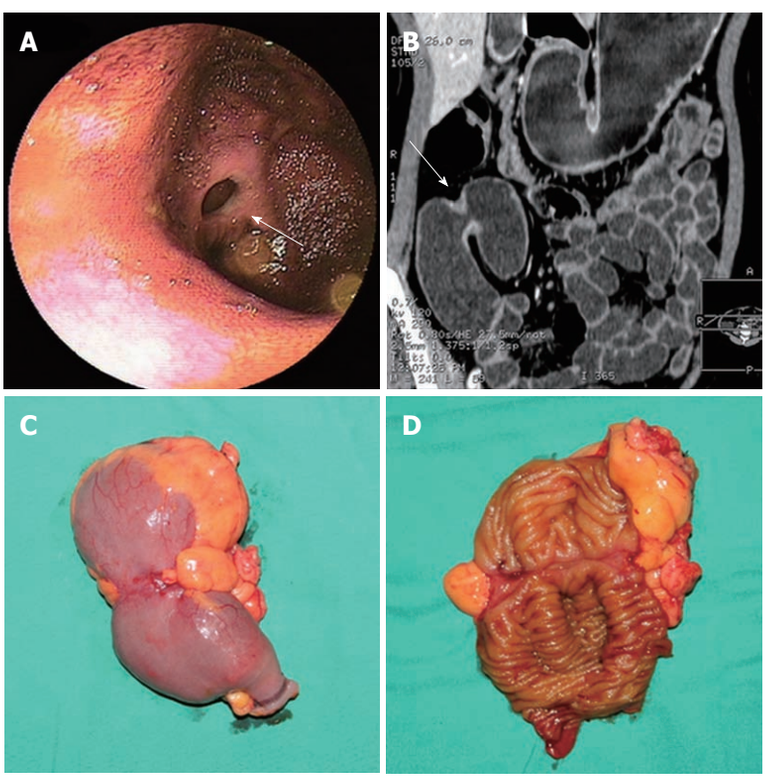

Figure 2 Thirty-one-year-old woman presenting with a 3-year history of recurrent episodes of abdominal pain, incomplete intestinal obstruction and intermittent black stools. A: Double-balloon enteroscopy image showing circumferential diaphragm-like stricture in the ileum (arrow); B: Computed tomography enterography image (oblique multiplanar reconstruction) showing mild bowel expansion in the ileum with a diaphragm-like stricture in the middle (arrow); C: A diaphragm-like stricture in the middle can be seen in the iliac specimen; D: Longitudinal section of specimen containing a laterigrade ulcer.

cluded number, location, size, shape depth and edge.

RESULTS

Clinical information

All the 5 patients presented long-term (2-16 years) symptoms of gastrointestinal bleeding (hematochezia appeared in case 4 and intermittent black stools occurred in the other four patients) and varying degrees of anemia, accompanied by obscure abdominal pain in three patients (cases 1, 2 and 4) (Table 1) and incomplete intestinal obstruction in one case. No significant changes were detected on physical examination, gastroscopy and colonoscopy. Three patients (cases 1, 2 and 3) were suspected of having small bowel Crohn's disease and received mesalazine medication. However, this medication was ineffective in these cases. In addition, administration of hemostyptic agents and iron supplementation eliminated bleeding and improved the condition of all patients. Three lesions were detected in case 2 and the remaining 4 cases had one lesion in the ileum. WBC count was not elevated and the proportion of eosinophils did not increase in all cases. One patient had a CRP level test with normal result. All cases had removal of the lesions by laparoscopically assisted enterectomy and remained well at 1 to 3.5 years follow-up with no signs of gastrointestinal bleeding.

Endoscopic findings

On the images of capsule endoscopy, a mild circumferential stricture in the ileum was shown in two lesions which the capsule could pass through (Figure 1A). In another two cases, the capsule was stopped by markedly circumferential stricture of the lumen.

All the 7 strictures in five patients were found in the double-balloon enteroscopy (DBE) examination undergone by all five patients. All lesions appeared in the middle or distal segment of ileum which was about 80 cm to 150 cm away from the ileocecal valve and the scope could pass through. Circumferential ulcers or erosions with clear margin on the surface of stenoses were found in all lesions. All ulcers were covered by faint white mucous exu-

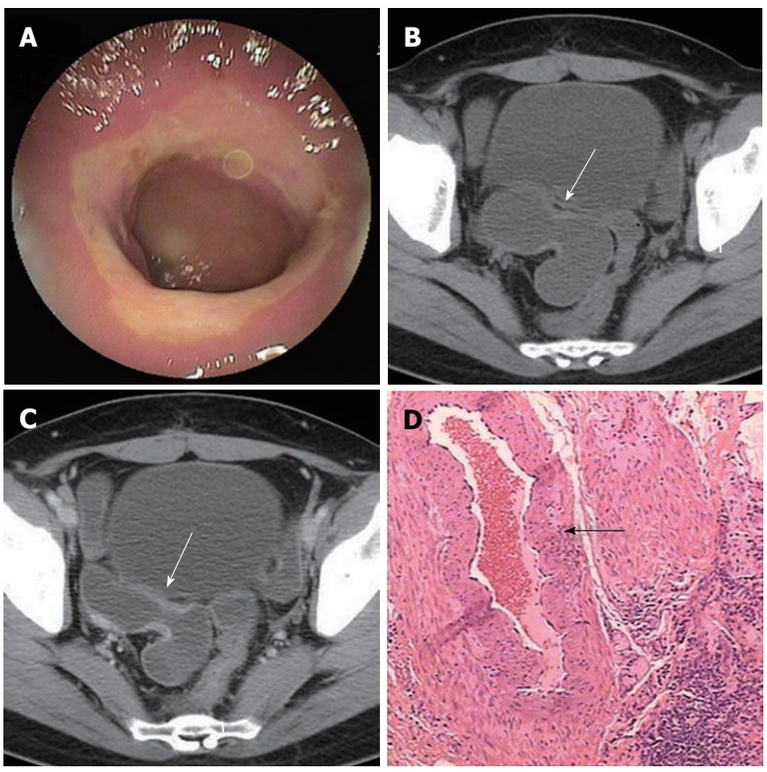

Figure 3 Forty-four-year-old woman presenting with a 4-year history of recurrent episodes of hematochezia and abdominal pain. A: Double-balloon enteroscopy image showing circumferential stricture in the ileum; B: Computed tomography (CT) plain scan image showing mild bowel wall thickening and circumferential stricture of the lumen in the ileum (arrow); C: Contrast-enhanced CT scan image showing that contrast enhancement of thickened bowel wall is homogenous and moderate (arrow); D: Blood vessel with thickened wall and expanded lumen shown in the submucosa (hematoxylin and eosin, orig. mag × 40).

dates. Congestion and edema in the neighboring mucosa and dilated small bowel loop adjacent to the stricture were also observed in all lesions (Table 2, Figure 1B, 2A and 3A).

Radiologic findings

Routine abdominal CT scan was performed in only one patient and showed no other abnormal results except gallstones. The main features of diaphragm-like strictures of small bowel seen at CTE include thickening of bowel wall, circular stricture in the middle or distal segment of the ileum and dilated small bowel loop adjacent to the stricture. These features were observed in three patients. In case 2, only two out of three lesions were detected by CTE. A minor stricture could not be shown. In case 3, CTE could only show the thickened bowel wall, the significant dilated small bowel loop and several enlarged lymph nodes. However, the stricture was not shown in CT images. Bowel wall thickening appeared as mild, symmetrical isodensity with respect to the normal bowel wall with homogenous and moderate contrast enhancement. The thickened wall ranged from 2.5 mm to 5.0 mm in thickness and reached 7.0 mm in one lesion (case 2) which turned the lumen into a tight stricture and caused retention of the capsule. The diameter of the lumen in the dilated small bowel loop ranged from 2.7 cm to 5.0 cm in 4 patients and reached 7.0 cm in one case (Table 3, Figure 1C, 2B, 3B and C). There was no abnormality in the mesenteric vessels.

SBFT was performed in 3 patients (cases 2, 4 and 5). Two strictures in case 2 and 1 stricture in case 5 with dilated bowel loop were observed (Figure 1D). The pass-through of barium was blocked and stenoses of the lumen were still at the fixed location at a later time point observation after compression. Capsules floated in the dilated small bowel loops and could not move to the bottom with the downward movement of barium. Selective mesenteric angiography was performed in case 2 and showed no positive findings. One bowel isotope scan using ^{99m}Tc-RBC revealed intestinal bleeding in the distal ileum.

Pathologic findings

On gross examination, circumferential strictures were found in all the lesions of the resected small intestine in all five patients. These strictures were perpendicular to the long axis of the intestine. The lesions appeared as laterigrade diaphragms with laterigrade pittings in the mucosa, 0.2 cm to 0.5 cm in width, with edema in the neighboring mucosa in the longitudinal section of the specimens in four lesions from four patients (cases 1, 2, 3 and 4) (Figure 1E, 2C and D). They were approximately 0.5 cm in width in case 5 and in two lesions of case 2. Edema in the neighboring mesentery and enlargement of several lymph nodes were found in case 3. No abnormality was shown in the adjacent mesentery in the other cases. On microscopy, a chronic inflammatory infiltrate was found in all five subjects. Depth of ulcer reached to the muscularis propria in local areas in cases 1 and 2. The ulcer was limited to the submucosa in cases 3 and 4. Villous adenomatous hyperplasia in the mucosal layer was found in case 5 and in two lesions of case 2. Moderate local inflammatory cell infiltration was found in the submucosal layer of cases 1, 5 and two lesions of

Table 3 Data of computed tomography findings

Case	Location	*n*	Stenosis	Lumen expansion	Bowel wall			Mesenteric vessels	Lymph node enlargement
					Thickness (mm)	Attenuation	Enhancement		
1	Ileum	1	P	P	2.5	Isodense	Moderate	Normal	N
2	Ileum	2	P	P	7	Isodense	Moderate	Normal	N
3	Ileum	1	N	P	3	Isodense	Moderate	Normal	P
4	Ileum	1	P	P	2.5	Isodense	Moderate	Normal	N
5	Ileum	1	P	P	5	Isodense	Moderate	Normal	N

P: Positive; N: Negative.

Table 4 Data of histologic findings

Case	Size[1] (cm)	Type	Depth[1]	Inflammatory infiltrate	Fibrosis	Mucosal atrophy	Thickening of muscularis mucosa	Edema in submucosa
1	2.0 × 0.5	Ulcer	Muscular layer	Moderate	Mild	N	Mild	N
2[2]	2.0 × 0.2	Ulcer	Muscular layer	Severe	Mild	N	Moderate	N
3	3.0 × 0.2	Ulcer	Submucosa	Severe	Moderate	N	N	P
4	3.0 × 0.5	Ulcer	Submucosa	Severe	Moderate	N	Moderate	P
5	2.0 × 0.5	Erosion	Mucosa	Moderate	None	N	N	N

[1]Means the size and depth of ulcer or erosion of specimen; [2]Information of the most narrow lesion. P: Positive; N: Negative.

case 2. Inflammatory cell infiltration was obvious and reached to the serosa in cases 3, 4 and one lesion of case 2. Fibrosis in the submucosal layer was mild in cases 1 and 2 and moderate in cases 3 and 4 (Table 4). The rupture of the muscularis mucosa under the ulcer was also found in case 3. Specifically, proliferation of blood vessel with thick wall and expanded lumen appeared in the submucosa and distorted muscularis propria in two cases (cases 3 and 4) (Figure 3D). Granulomatous lesion was not found in any patient. No cytomegalic inclusion associated with cytomegalovirus (CMV) infection was found in any lesion.

DISCUSSION

This retrospective study showed the clinical, endoscopic, radiologic and pathologic features of distinctive small bowel diaphragm-like strictures. All patients presented with long-term (2-16 years) symptoms of gastrointestinal bleeding and varying degrees of anemia. All cases were not associated with the use of NSAIDs and had similar clinical, endoscopic, radiologic and pathologic features.

The cause of these special diaphragm-like strictures remains uncertain. Diaphragm disease induced by NSAIDs characterized by inflammatory strictures of the small intestine has previously been recognized as an uncommon complication of NSAID enteropathy[1]. The abnormalities of NSAID enteropathy include inflammation, erosion, fibrosis, stricture, perforation, and formation of diaphragm disease. Diaphragm disease most frequently affects the ileum. It can also affect the jejunum and colon, as well as stomach and duodenum. There are usually multiple diaphragms. The depth of ulcer is restricted to the submucosal layer and it never extends to the proper muscular layer[1-6]. Improvement in clinical findings (signs and symptoms) and/or endoscopic findings appears on cessation of NSAID utilization, except for diaphragm disease. Diaphragm disease coupled with the use of NSAIDs is a pathognomic feature of NSAID enteropathy because of its non-specific histological findings[1-6]. Regarding clinical manifestations, the imaging findings in this study group share some common features with those observed in NSAID enteropathy, namely, concentric stenosis and circular ulcers. Diaphragm-like strictures in our study group are, however, different to those induced by NSAIDs with regard to many aspects such as the location, number, fibrosis and the disease process (Table 5).

Santolaria *et al*[7] reported a male patient with diaphragm disease who had a 25-year history of relapsing abdominal pain and edema and who did not have long-term use of NSAIDs. However, the symptoms and history of this case were different to those in our group. Shimizu *et al*[8] reported a case with diaphragm-like stricture of the small intestine related to CMV infection. Multiple erosions and small ulcers in the ileum and a circumferential diaphragm-like stricture were seen in this patient, with increased C-reactive protein level, and a cytomegalic inclusion was found in the strictured lesion on biopsy. CMV infection usually occurs in immunosuppressed patients. In our group, immune response of all patients was normal and no cytomegalic inclusion was found in pathology findings. Pasha *et al*[9] reported a case with eosinophilic gastroenteritis (EGE) mimicking diaphragm disease of the small bowel. Multiple ulcerated stenoses were present and capsule retention occurred in this case. Mucosal eosinophilia (> 20/HPF) can be found in EGE cases, usually accompanied by increased level of peripheral blood eosinophil count, signs which were not found in our group. Specifically, dilated blood

Table 5 Differences between diaphragm disease of non-steroidal anti-inflammatory drug-enteropathy and diaphragm-like strictures in study group

	History	Location	*n*	Fibrosis	Lesions in other area of bowel	Disease process
Diaphragm disease	Long term NSAID use	Whole GI tract, most frequently in ileum	Multiple	Obvious	Exist, can be inflammation, erosion, fibrosis, stricture and perforation	Improvement in clinical findings by cessation of NSAID utilization
Diaphragm-like strictures	No NSAID use	Middle or distal segment of ileum	Usually single, no more than three	Mild or moderate	No	Non-self-limiting

NSAID: Non-steroidal anti-inflammatory drug; GI: Gastrointestinal.

vessel with thickened wall appeared in the submucosa in two cases in our group. This was different to angiodysplasia or hemangioma, in which the dilated blood vessel usually has a thin wall. Moreover, the manifestations of CT angiography in arterial phase and DBE also excluded the existence of vascular malformation. The history and pathologic examination of the lesions also excluded other potential causes of intestinal strictures, including use of potassium chloride tablets, surgical anastomoses, radiation, ischemia, Crohn's disease, tuberculosis, and lymphoma.

Diaphragm-like strictures can also be found in the small bowel in congenital cases in adults, though this is rare. Congenital atresias, diaphragm-like strictures or stenoses are well documented occurrences in the stomach, duodenum and small bowel. Small intestinal atresia/stenosis most frequently affects the duodenum, followed by the jejunum, and least often the ileum, and can affect multiple sites of the intestine[10-16]. Intestinal obstruction is described as the main symptom. Cases that occur in the ileum and lead to bleeding of the small bowel have not been found in the literatures. All seven diaphragm-like strictures of the five cases were found in the middle or distal segment of the ileum in our group. It may be possible that congenital diaphragm-like stenoses may firstly have existed in the ileum, inflammation and ulcers then occurring after a long-term limitation of intestinal motility in the stenoses and friction between food and stenoses. This could explain why there was only one lesion in most cases, the stricture was obvious even if there was no marked fibrosis in the lesion, and the symptoms disappeared after operation. However, more evidence is needed to test this hypothesis.

Diagnosis of special diaphragm-like strictures of small bowel in non-NSAID patients may be difficult. No significant findings were detected on physical examination. Routine abdominal CT scan in general could not show any abnormal results. CE, DBE, CTE and SBFT may be helpful in making a diagnosis and may facilitate preoperative evaluation of the lesions. CE has been mainly used to evaluate patients with obscure GI bleeding[17]. There have also been many reports demonstrating diaphragm-like strictures in CE examinations[18-20]. Sometimes, diaphragms may be misinterpreted as exaggerated plicae circulares. CE carries a risk of obstruction in patients with tight stenoses. Therefore, it should not be used if a stricture is present. DBE may show circumferential diaphragm-like strictures with ulcers or erosions. DBE has been used to successfully diagnose diaphragm disease in patients with GI bleeding and ileus in many literature reports[21-23]. DBE can also be used for treatment of the diaphragm disease[22,23]. As there are no specific pathological changes, endoscopic biopsy could not help much in the diagnosis. Three patients in our study group were initially suspected of having small bowel Crohn's disease. CTE can show thickening of bowel wall, dilated small intestinal loops and circular or diaphragm-like strictures. CTE can also show the adjacent mesentery, mesenteric vessels and lymph nodes, which could help to exclude other potential causes of intestinal strictures[24-27]. Adequate distension of the entire small intestine is crucial to display the lesions. It must be mentioned here that it is necessary to combine CTE with other inspections such as SBFT or DBE to make a correct diagnosis. This is due to the fact that the images of CT are static and may misinterpret the lesion as plicae circulares especially when the bowel lumen is not distended completely. SBFT can show diaphragm-like strictures with dilated small bowel loop in the adjacent segment. The diaphragm lesions are thin and do not distort the bowel wall[28]. The greatest advantage of SBFT is its dynamic view of the small intestine motility. The diaphragms which are not shown distinctly might be misinterpreted as exaggerated plicae circulares[3]. Mesenteric angiography may have no positive findings, which implies that the symptom of recurrent gastrointestinal bleeding is not caused by mucosal vascular abnormalities or vasculitis, *etc*. Bowel isotope scan may be not informative and may only reveal the intestinal bleeding. Though there are many imaging examinations to help make a diagnosis, in some cases surgical intervention might be necessary to make the definitive diagnosis.

Our study had several limitations. For example, the sample size is small due to the rarity of the disease. It is meaningless to conduct statistical analysis for this small number of cases. In addition, the cases were a select surgical series which had radiological and/or endoscopic presurgical work-up. Obviously, non-surgical cases and those patients who did not have imaging or endoscopic work-up would have been excluded.

Although the number of subjects was small, our results indicate that special diaphragm-like strictures

characterized by ulcers and circular stenosis can also occur in the small intestine in patients without the use of NSAIDs and might be a special consequence of unclear damaging insults to the intestine. They have similar clinical, endoscopic, radiologic and pathologic features. The diaphragm-like strictures tend to be intractable because this is a non-self-limiting condition with little tendency toward mucosal healing. Currently, enterectomy of the diseased bowel segment is the only useful therapy. Clinicians should be aware that small-bowel diaphragm-like strictures might be a cause of chronic small bowel bleeding in patients receiving no NSAID therapy.

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COMMENTS

Background

Diaphragm disease induced by non-steroidal anti-inflammatory drugs (NSAIDs), as inflammatory strictures of the small intestine, has previously been recognized as an uncommon complication in patients taking NSAIDs. The most frequent manifestations are iron-deficiency anemia, acute small-bowel hemorrhage and perforation, and obstruction of the small bowel. However, diaphragm-like strictures in the small bowel can also not be associated with the use of NSAIDs. Here, the authors illustrate a group of five patients presenting with bleeding of small bowel and in whom diaphragm-like strictures in small bowel were present that were not attributed to NSAID use.

Research frontiers

Bleeding within the small bowel is often difficult to diagnose. Currently, the first diagnostic procedure in patients with small bowel hemorrhage is capsule endoscopy (CE) or double-balloon enteroscopy (DBE). CE has been mainly used to evaluate patients with obscure gastrointestinal (GI) bleeding, but carries a risk of obstruction in patients with tight stenoses such as diaphragm-like strictures. DBE can show circumferential diaphragm-like strictures with ulcers or erosions and can also be used for treatment of the diaphragm disease. Contrast-enhanced computed tomography (CT) scanning is establishing itself as a rapid, noninvasive, and accurate diagnostic method in gastrointestinal bleeding. Arterial phase CT scanning is an excellent diagnostic tool for fast and accurate detection and localization of GI hemorrhage. The combination of a variety of inspection methods can help to confirm the diagnosis.

Innovations and breakthroughs

The unique aspect of this study is that it is the largest series of intestinal diaphragm-like strictures which are not associated with NSAID use. To the best of our knowledge, there are still no descriptions about the imaging features of this disease in the English literature. The results indicate that special diaphragm-like strictures characterized by ulcers and circular stenosis can also occur in the small intestine in patients without the use of NSAIDs and might be a special consequence of unclear damaging insults to the intestine. They have similar clinical, endoscopic, radiologic and pathologic features and tend to be intractable for this is a non-self-limiting condition with little tendency toward mucosal healing. Findings at endoscopic and radiologic imaging of this disease may help to make a diagnosis and facilitate preoperative evaluation of the lesions.

Applications

Small-bowel diaphragm-like strictures characterized by ulcers and circular stenosis might be a cause of chronic small bowel bleeding in patients without NSAID therapy and might be a special consequence of unclear damaging insults to the intestine. They have similar clinical, endoscopic, radiologic and pathologic features. The diaphragm-like strictures tend to be intractable because it is a non-self-limiting condition with little tendency toward mucosal healing. Currently, enterectomy of the diseased bowel segment is the only useful therapy.

Terminology

Diaphragm disease is inflammatory strictures of the small intestine and is an uncommon complication of non-specific small-bowel disease, caused by mucosal and submucosal fibrosis and thickening in patients taking NSAIDs. Computed tomography enteroclysis (CTE) is to perform contrast-enhanced CT scanning and image post-processing after small intestine distension by administering a high volume of contrast medium into the small intestine orally or *via* a nasojejunal catheter. CTE can display the cavity and wall of small intestine, parenteral lymph nodes, mesentery, mesenteric vessels and the adjacent structures, etc.

Peer review

In this study, the authors summarized the characteristics of diaphragm-like strictures of the small bowel without use of NSAIDs. Their report contained 5 cases and described the clinical, endoscopic, radiographic and pathologic features. Although there are many papers that have described diaphragm disease of the small bowel associated with NSAIDs, diaphragm disease unrelated to NSAIDs rarely exists in clinical settings. It is necessary to accumulate many more clinical cases to reveal the clinical significance of this disease phenotype. Although this report is preliminary as it stands, it might see the light in this field.

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ORIGINAL ARTICLE

Ginsenoside Rg3 inhibit hepatocellular carcinoma growth *via* intrinsic apoptotic pathway

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Abstract

AIM: To investigate the anti-tumor function of ginsenoside Rg3 on hepatocellular carcinoma (HCC) *in vitro* and *in vivo*, and its mechanism.

METHODS: Hep1-6 and HepG2 cells were treated by Rg3 in different concentrations (0, 50, 100 and 200 μg/mL) *in vitro*. After incubation for 0, 6, 12, 24 and 48 h, cell viability was measured by 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide assay. Apoptosis was identified by terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling. Caspase-3 activity was measured by chromophore p-nitroanilide and flow cytometry. Bcl-2 family proteins were ascertained by Western-blotting. Mitochondria membrane potential was detected by 5, 5′, 6′ 6′ - tetrachloro-1, 1′, 3, 3′ - tetraethylbenzimidazolylcarbocyanine iodide. Forty liver tumor-bearing C57Bl6 mice were divided randomly into 4 groups for intra-tumor injection of saline, ginsenoside Rg3, cyclophosphamide (CTX) and ginsenoside Rg3 + CTX combination.

RESULTS: The survival time was followed up to 102 d. The mice in the Rg3 + CTX group showed significant increased survival time compared with those in the control group ($P < 0.05$). Rg3 could inhibit HCC cell proliferation and induce cell apoptosis *in vitro* in the concentration and time dependent manner. It also induced mitochondria membrane potential to decrease. Caspase-3 activation can be blocked by the inhibitor z-DEVD-FMK. Bax was up-regulated while Bcl-2 and Bcl-XL were down-regulated after Rg3 treatment.

CONCLUSION: Our data suggested that Rg3 alone or combined with CTX inhibited tumor growth *in vivo* and prolonged mouse survival time by inducing HCC cell apoptosis *via* intrinsic pathway by expression alterations of Bcl-2 family proteins.

Key words: Ginsenoside Rg3; Apoptosis; Hepatocellular Carcinoma; Bcl-2 family proteins; Cyclophosphamide

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INTRODUCTION

Hepatocellular carcinoma (HCC) is the fifth most common fatal human malignancy worldwide[1]. HCC is highly resistant to chemotherapeutic drugs and there is no single effective chemical against it. Two or three agents are often combined to enhance the efficacy of chemical agents. Chemotherapy causes serious toxic effects[2]. Thus, there is an urgent need to develop novel treatment modalities.

Ginseng is a traditional herbal medicine well known for its wide spectrum of pharmacological effects[3]. Recently researchers have found ginsenoside Rg3 can inhibit growth of several cancer cell lines[4-9]; however, the mechanism is not fully understood so far. In this study, two liver cancer cell lines, Hep1-6 and HepG2 cells were treated with ginsenoside Rg3 *in vitro* to explore the possible molecular mechanism. Ginsenoside Rg3 was also injected into tumor-bearing mice to investigate the anti-tumor effect in a long-term way.

MATERIALS AND METHODS

Ginsenoside Rg3

Ginsenoside Rg3 was purchased from Fusheng Pharmaceutical Ltd. Rg3 was dissolved in dimethyl sulfoxide (DMSO) and filtered by 0.2 μm membrane. It was diluted by cell culture media to various final concentrations (0, 50, 100, 200 μg/mL).

Cell lines and cell culture

Hep1-6 and HepG2 cells were purchased from the Institute of Biochemistry and Cell Biology, Academy of Science (Shanghai, China) and cultured in Dulbecco's Modified Eagle's Medium and Eagle's Minimum Essential Medium (ATCC, Manassas, VA, United States) supplemented with 10% fetal bovine serum (FBS) (Atlanta Biologicals), 4 mmol/L 1-Glutamine (Cellgro) and 2% penicillin-streptomycin solution (Cellgro). The cells were incubated at 37 ℃ in a mixture of 5% CO_2 and 95% air.

HCC animal model

Forty female C57BL/6 mice (4 wk, 16g ± 3 g, purchased from Shanghai Experimental Animal Center of the Chinese Academy, Shanghai, China) were divided randomly into 4 groups of 10 mice in each group: control (saline), ginsenoside Rg3, cyclophosphamide (CTX) and Rg3 + CTX combination. After being transplanted with 1×10^6 Hep1-6 cells in 50 μL PBS on the flank, the mice were given an intra-tumor injection of ginsenoside Rg3 (3.0 mg/kg) and CTX (20.0 mg/kg) or Rg3 + CTX for 10 d following inoculation of Hep1-6 cells. The negative control was saline injection (1.5 mg/kg). Mice were euthanized according to IACUC proposals when the tumor was larger than 20 mm in diameter. The survival days were recorded. Mouse weight and tumor weight were measured.

After treatment, the survival study began. The animal technician, who was blind to the study, monitored the mouse weight and tumor size every day. When the diameter of tumor was larger than 2 cm on the tumor-bearing mouse, or the mouse weight loss was more than 20% on the tumor free mouse, the mouse was euthanized by cervical dislocation according to the animal experiment protocol and the date was determined as endpoint of survival dates.

Cell viability analysis

The viability of Hep1-6 and HepG2 cells treated with and without Rg3 was determined by the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay. Briefly, cells in logarithmic growth phase were seeded in 96-well plates. Rg3 was added to the medium to different final concentrations: 0, 50, 100 and 200 μg/mL. After 0, 6, 12, 24 and 48 h incubation, 20 μL medium containing 5 mg/mL MTT was added to each well. After another 3 h incubation, DMSO (100 μL) was added to dissolve the formazan crystals. Light absorbance at 540 nm was measured. To determine the percentage of surviving cells, absorbance values of indicated concentrations were normalized to the values obtained from the cells without Rg3 treatment. Each assay was performed in 3 replicates.

Apoptosis detection

The HCC cells were incubated on the 8-well chamber slides (Nalge Nunc Corp, IL, United States) in medium with 0, 50, 100, 200 μg/mL Rg3. After 0, 6, 12, 24 and 48 h cell chambers were removed and the slides were fixed for hematoxylin and eosin (HE) stain and terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) fluorescent detection kit (Chemicon, United States). All the nuclei were stained blue by 2-(4-Amidinophenyl)-6-indolecarbamidine dihydrochloride (DAPI) while the apoptotic cells were stained as red fluorescent by apoptotic probe. The apoptotic cells were counted and statistically analyzed by the software Image J.

Caspase 3 activity assay

Caspase 3 activity was tested by colorimetric assay kit (Genscript, NJ, United States, Cat. No. L00289). The HCC cells were treated by Rg3 in different concentrations (0, 50, 100, 200 μg/mL) for 24 h. Then the cells were lysed for detection of the chromophore p- nitroanilide (pNA) after cleavage from the labeled substrate DEVD-pNA. The result was quantified as the *A* value at 405 nm. The relative increase of caspase-3 activity was determined by comparing the absorbance of pNA from Rg3 treated HCC cells to non-treated control.

Z-DEVD-FMK inhibitory assay

Cells were pretreated for 1 h with 20 mmol/L z-DEVD-FMK (R&D, Catalog Number: FMK004) prior to Rg3 treatment. The cells were then treated with Rg3 in different concentrations (0, 50, 100, 200 μg/mL) for 24 h. The cells were lysed for caspase activity measurement. Then z-DEVD-FMK was added to measure the caspase activity. The inhibitory rate was calculated by comparing caspase activity with/without z-DEVD-FMK.

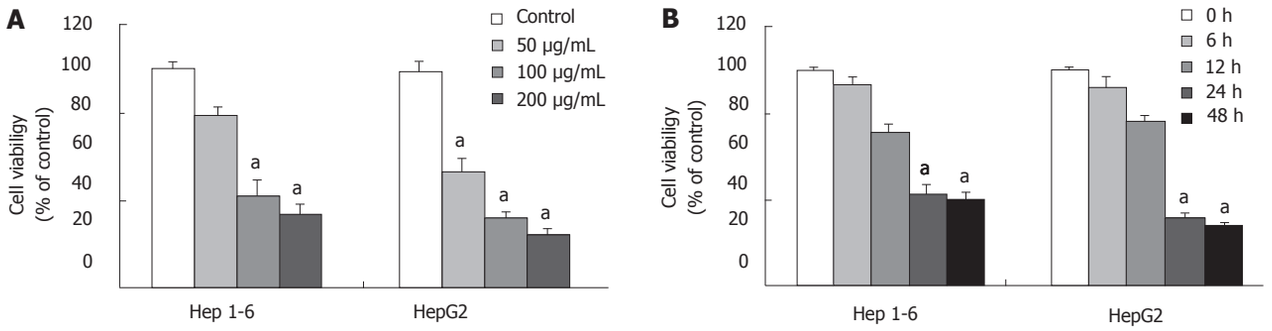

Figure 1 Ginsenoside Rg3 inhibits cell viability of human and murine liver cancer cells. A: Concentration-dependent inhibitory effects of Ginsenoside Rg3 on cell viability in Hep1-6 and HepG2 cell lines. Cells were treated with Rg3 at 0, 50, 100, 200 μg/mL in 10% fetal bovine serum-supplemented medium for 24 h; cell viability was determined by 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide assay (n = 6 for Hep1-6, n = 6 for HepG2). B: Time-dependent inhibitory effects of Rg3 on cell viability in Hep1-6 and HepG2 cell lines. Cells were treated with Rg3 100 μg/mL for 0, 6, 12, 24, 48 h. One-way ANOVA was performed to test the concentration and time-dependent effects. $^aP < 0.05$ *vs* untreated controls.

Western blot analysis

After being treated with 0, 50, 100, 200 μg/mL Rg3 for 24 h, Hep1-6 and HepG2 cells were washed with ice-cold PBS twice and lysed on ice. Mitochondrial fraction and cytosolic fraction were extracted by Cytosol/Mitochondria Fractionation Kit (Calbiochem, United States). Extracted proteins were separated by 12% SDS-PAGE and transferred onto PVDF membrane. The membrane was incubated with primary antibodies: procaspase 8, cytochrome c, Bcl-2, Bax, Bad, Bcl-XL (Santa Cruz Biotechnology Inc. dilution: 1:200) and beta-actin (Cell Signaling Technology, dilution: 1:500) in blocking buffer for 1 h at room temperature followed by incubation with secondary antibodies conjugated with horseradish peroxidase (Santa Cruz Biotechnology Inc. dilution: 1:500). The protein expression was detected by X-ray film.

Measurement of transitions in mitochondrial transmembrane potential

Hep1-6 and Hep G2 cells were grown in 4-well cover glass chambers (Nalge Nunc) and treated with Rg3 100 μg/mL containing DMEM supplemented with 5% FBS. After incubation for 24 h, cells were stained with 5 μg/mL of 5, 5', 6, 6' - tetrachloro-1, 1', 3, 3' - tetraethylbenzimidazolylcarbocyanine iodide (JC-1), a widely used dye for measuring membrane potential of mitochondria. Cells were irradiated at an excitation wavelength of 488 nm, and the irradiated field was photographed using a confocal microscope equipped with an emission filter of 533 nm (100 magnification, Leica). Depolarized mitochondrial membranes were detected by the presence of a diffuse green fluorescence in cells.

Flow cytometry

After treatment with Rg3 100 μg/mL or saline for 24 h, 105 Hep1-6 and Hep G2 cells were suspended in 50 μL HBSS containing propidium iodide (PI) and fluorescein isothiocyanate (FITC) caspase-3 (Bioss LTD, Beijing, China) to identify apoptosis and necrosis, respectively. Fluorescent dyes were diluted to 1 μg/mL in HBSS containing 1% FBS. Incubations were carried out for 30 min on ice. After staining, the cells were washed twice in HBSS/1% FBS and then analyzed by a flow cytometry (LSR Ⅱ, BD).

Tumor histopathology

When the tumor was as large as 20 mm in diameter, the animal was euthanized and the tumor was dissected and fixed in 40 g/L neutral formaldehyde. After 24 h it was embedded in paraffin, cut into 3 μm sections, stained with HE, and examined under light microscopy.

Statistical analysis

The present data are expressed as mean ± SD. For statistical comparison of values, a Student's t test was used. P values less than 0.05 were deemed to indicate statistical significance. The Kaplan-Meier method is used to analyze the cumulative survival and draw the survival curve by software SPSS 11.0. Log rank statistic and significance were also presented by SPSS.

RESULTS

Rg3 represses liver cancer cell viability in a dose- and time-dependent way

MTT assay was done to examine if Rg3 could affect liver cancer cell growth in culture. Hep1-6 and HepG2 cells were treated with increasing concentrations of Rg3 (0, 50, 100, 200 μg/mL) for 24 h. The viable Hep1-6 and HepG2 cells consistently decreased with the higher concentrations of Rg3 as shown in Figure 1A. When Hep1-6 cells were treated by 100 and 200 μg/mL Rg3, the cell viability was significantly decreased ($P < 0.005$ *vs* control). HepG2 cells had significantly decreased viability when they were treated by 50, 100 and 200 μg/mL Rg3. When both cell lines were treated by 100 μg/mL Rg3 for 0, 6, 12, 24, 48 h, the cell viability declined significantly over 24 h ($P < 0.005$ *vs* control, Figure 1B).

Rg3 induced liver cancer cells apoptosis in vitro

To determine if Rg3 causes apoptosis in Hep1-6 and HepG2 cells, DNA degradation and cleavage were detected in Rg3-treated liver cancer cells. When the HCC cells

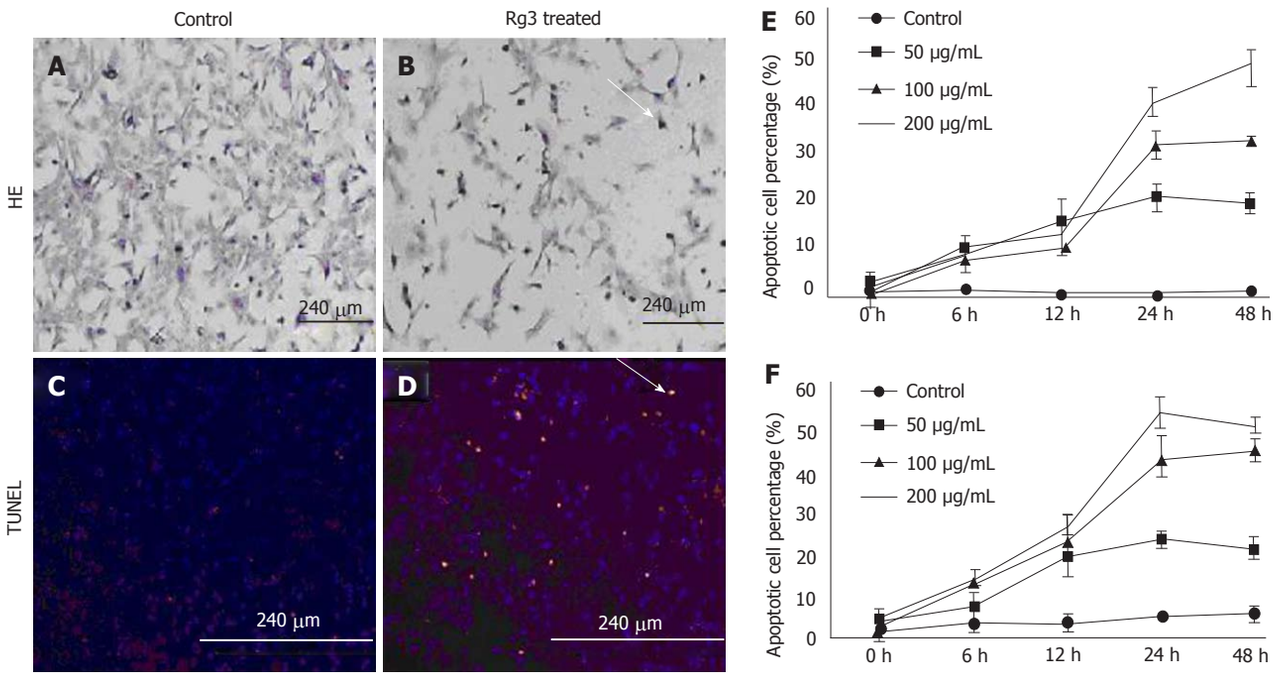

Figure 2 Ginsenoside Rg3 caused hepatocellular carcinoma morphological changes of apoptotic cells. A, B: Cell apoptosis morphology was observed by hematoxylin and eosin (HE) stain. After 50 μg/mL Ginsenoside Rg3 (Rg3) incubation for 12 h, the Hep1-6 cells (B) indicate less survival cells compared to control group (A); C, D: DNA fragmentation *in situ* was detected by transferase-mediated dUTP-biotin nick end labeling. The control cells was stained blue (C) and the Rg3 treated group present apoptotic cells stained red (D); E, F: The apoptotic cells showed reduced volume and condensed chromatin. In both Hep1-6 and HepG2, the apoptotic induction effect is dose and time-dependent. Rg3: Ginsenoside Rg3; HE: Hematoxylin and eosin; TUNEL: Transferase-mediated dUTP-biotin nick end labeling.

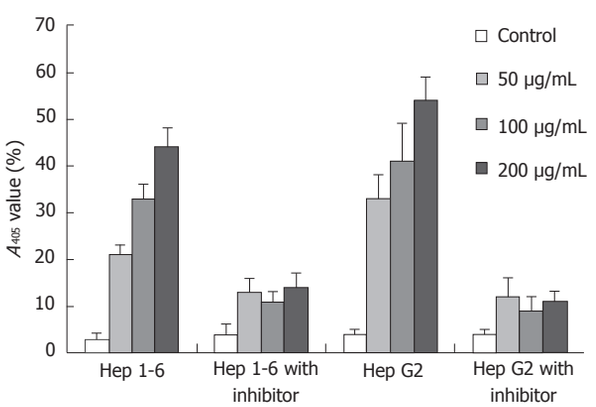

Figure 3 The caspase activity was measured by the chromophore p-nitroanilide. Hep1-6 and HepG2 cells were pretreated with or without 20 mm z-DEVD-FMK for 1 h, and then cultured with 0, 50, 100, 200 μg/mL ginsenoside Rg3 for 24 h. The caspase activity was measured by the chromophore p-nitroanilide (pNA) after cleavage from the labeled substrate DEVD-pNA by a plate reader A_{405} nm.

were treated with 100 μg/mL Rg3 for 24 h, both Hep1-6 and HepG2 cells displayed typical apoptotic morphology, including reduced volume and condensed chromatin (Figure 2A) compared to the control cells without Rg3 treatment (Figure 2B). To further specify apoptotic cell death, we stained nuclei with DAPI, a DNA-specific fluorescent dye. We also detected *in situ* DNA fragmentation in Hep1-6 and HepG2 cells by using TUNEL method. All the Hep1-6 cell nuclei were stained blue by DAPI while the apoptotic cells were stained red by apoptotic probe (Figure 2D). Hep1-6 cells treated by Rg3 showed a higher percentage of apoptotic cells (Figure 2D) compared to the control group (Figure 2C). The apoptotic Hep1-6 and HepG2 cells were counted and statistically analyzed by the software Image J. Rg3-induced apoptosis occurred in Hep1-6 and HepG2 cells when treated by 50-200 μg/mL Rg3 for 12-24 h (Figures 2E and F).

Induction of apoptosis by Rg3 depends on mitochondria-medicated caspase cascade

To determine whether Rg3-induced apoptosis in Hep1-6 and HepG2 cells was mediated *via* caspase cascade, caspase-3 activity was measured by pNA. The relative increase of caspase-3 activity was determined by comparing the absorbance of pNA from Rg3 treated HCC cells to the non-treated control. In both Hep1-6 and HepG2, caspase-3 was activated by Rg3 treatment in a dose dependent manner (the means and SDs in Hep1-6 cells were 5% ± 1%, 22% ± 4%, 32% ± 3%, 43% ± 5% when Rg3 concentration were 0, 50, 100, 200 μg/mL, respectively; the HepG2 cells were 4% ± 0.5%, 32% ± 6%, 41% ± 7%, 54% ± 4%) (Figure 3). However, when both cell lines were pretreated with 20 mmol/L z-DEVD-FMK for 1 h, the caspase-3 activity was decreased significantly (4% ± 1%, 13% ± 3%, 11% ± 1%, 14% ± 4% when Rg3 concentration were 0, 50, 100, 200 μg/mL, respectively; the HepG2 cells were 4% ± 0.5%, 11% ± 1%, 11% ± 3%,10.5% ± 1%), suggesting Rg3 induce apoptosis *via* caspase-3 dependent apoptotic pathway in Hep1-6 and HepG2 cells. The caspase-3 activity is still elevated even in the presence of z-DEVD-FMK (Figure 3).

Because the activation of caspase-3 could be preceded

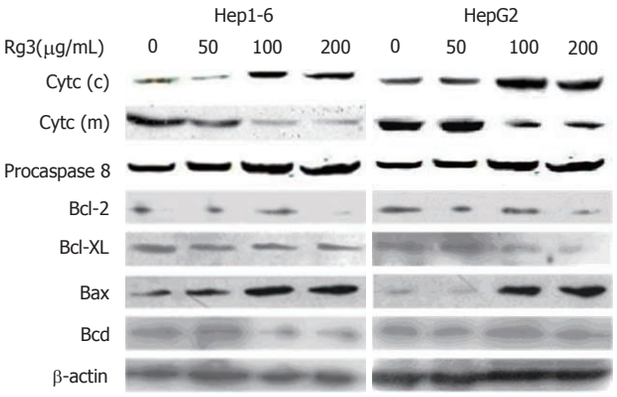

Figure 4 Effects of ginsenoside Rg3 on the cytochrome c release, caspase 8 cleavage and Bcl2-family. Hep1-6 and HegG2 cells were treated with 0, 50, 100, 200 μg/mL ginsenoside Rg3 (Rg3) for 24 h, and western-blot was used to detect the pro-casepase-8, cytochrome c in cytosolic fractions (c) and mitochondria fractions (m), Bcl-2, Bcl-XL, Bax, and Bad. β-actin is the protein loading control. Pro-caspase-8 remains static with or without Rg3 treatment. Cytochrome c decreased in the mitochondrial fraction and increased in the cytosolic fraction. Bcl-2 and Bcl-XL were down-regulated while Bax was up-regulated. Bad remains unchanged in the cells with and without Rg3 treatment. Rg3: Ginsenoside Rg3.

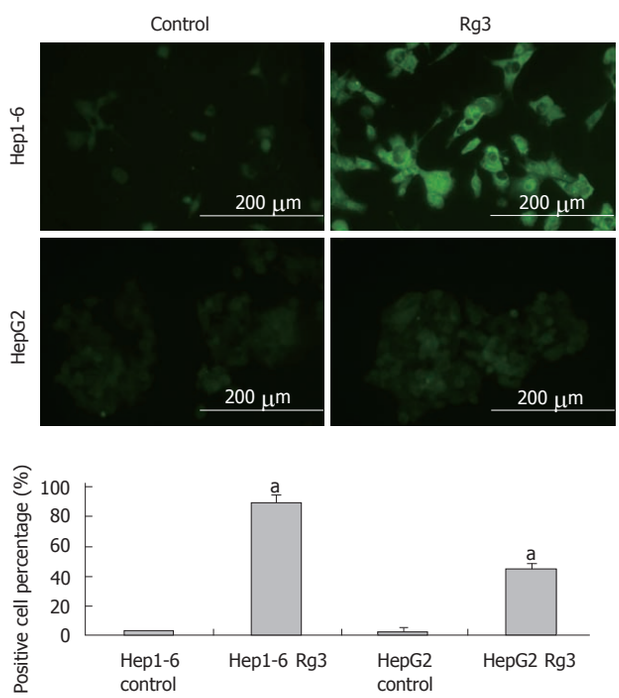

Figure 5 Hep1-6 and HepG2 cells were treated with Rg3 100 μg/mL or saline for 24 h then cells were stained with 5, 5', 6, 6' - tetrachloro-1, 1', 3, 3' - tetraethylbenzimidazolylcarbocyanine iodide dye. Depolarized mitochondrial membranes were detected by the presence of a diffuse green fluorescence. Ginsenoside Rg3 treated groups had a significantly higher percentage of green fluorescent cells: Hep1-6 (87% ± 6% *vs* control 2% ± 1%) and HepG2 (46% ± 4% *vs* control 3% ± 2%). Rg3: Ginsenoside Rg3. $^{a}P < 0.05$ *vs* control group.

by either caspase-8 *via* the death receptor pathway or caspase-9 *via* the mitochondria pathway, we tested pro-caspase-8 and cytochrome c to determine which pathway is dominant in Rg3-induced apoptosis. As illustrated in Figure 4, cleavage of caspase-8 was not evident, but cytochrome c decreased in the mitochondrial fraction and increased in the cytosolic fraction, which suggested that Rg3 mainly induced cytochrome c release.

Rg3 altered apoptotic related gene expression

To further investigate the molecular mechanism of mitochondria pathway activation, Bcl-2 family protein expression in Hep1-6 and HegG2 cells was detected by western-blot after they were treated by Rg3 in different concentration (0, 50, 100, 200 μg/mL) for 24 h. As shown in Figure 4, Bcl-2 and Bcl-XL, the anti-apoptotic members of Bcl-2 family, were reduced by Rg3 treatment. Bax, the pro-apoptotic member, was increased. Bad was unchanged in the cells with and without Rg3 treatment.

In order to determine whether the increase in mitochondrial Bax was associated with altered mitochondrial transmembrane potential, we measured changes in JC-1. JC-1 is a lipophilic cationic dye that can selectively enter into mitochondria and reversibly change color as illustrated in Figure 5; depolarized mitochondrial membranes were detected by the presence of a diffuse green fluorescence in cells. The green fluorescence shift indicates loss of mitochondrial function, which suggested that Rg3 activated the mitochondrial pathway by decreasing mitochondrial trans-membrane potential.

To further characterize the observed apoptotic phenotype, we carried out double staining of caspase-3-FITC and PI in two cell lines treated with saline and Rg3. Caspase-3-FITC can be detected in apoptosis. PI enters the cell in late apoptosis or necrosis. Viable cells were negative for both caspase-3-FITC and PI; early apoptotic cells were positive for caspase-3-FITC and negative for PI; late apoptotic or necrotic cells displayed both positive caspase-3-FITC and PI; non-viable cells which underwent necrosis were positive for PI and negative for caspase-3-FITC (Figure 6). After Rg3 treatment for 24 h, the percentage of early apoptotic cells induced by Rg3 in Hep1-6 and HepG2 were 85% ± 9%, 71% ± 8%, respectively. Their controls were 9% ± 3%, 11% ± 2%, respectively (Figure 6).

Rg3 improved HCC tumor bearing animals' survival time

The survival study was carried out up to one hundred and two days after the last intra-tumor injection. The number of living mice in the ginsenoside Rg3 group, CTX group, combined treatment and saline control group are summarized in Figure 7. Mice in the control group were euthanized within 20 d when tumors were larger than 20 mm in diameter. The tumor of the mice in the Rg3 treated group reached 20 mm in diameter within 102 d. There were no significant abnormalities in mental state, activities, or response to stimulus. The survival time of mice in the ginsenoside Rg3 group, CTX group and combined treatment group was significantly longer than that in the control group ($P < 0.001$), which demonstrated that ginsenoside Rg3 inhibited the tumor growth and prolonged survival time of tumor-bearing mice.

Tumor growth and pathology

Tumors reached 20 mm in diameter on day 14 (± 6.3), day 87 (± 9), day 93 (± 11) and day 95 (± 7) in the control

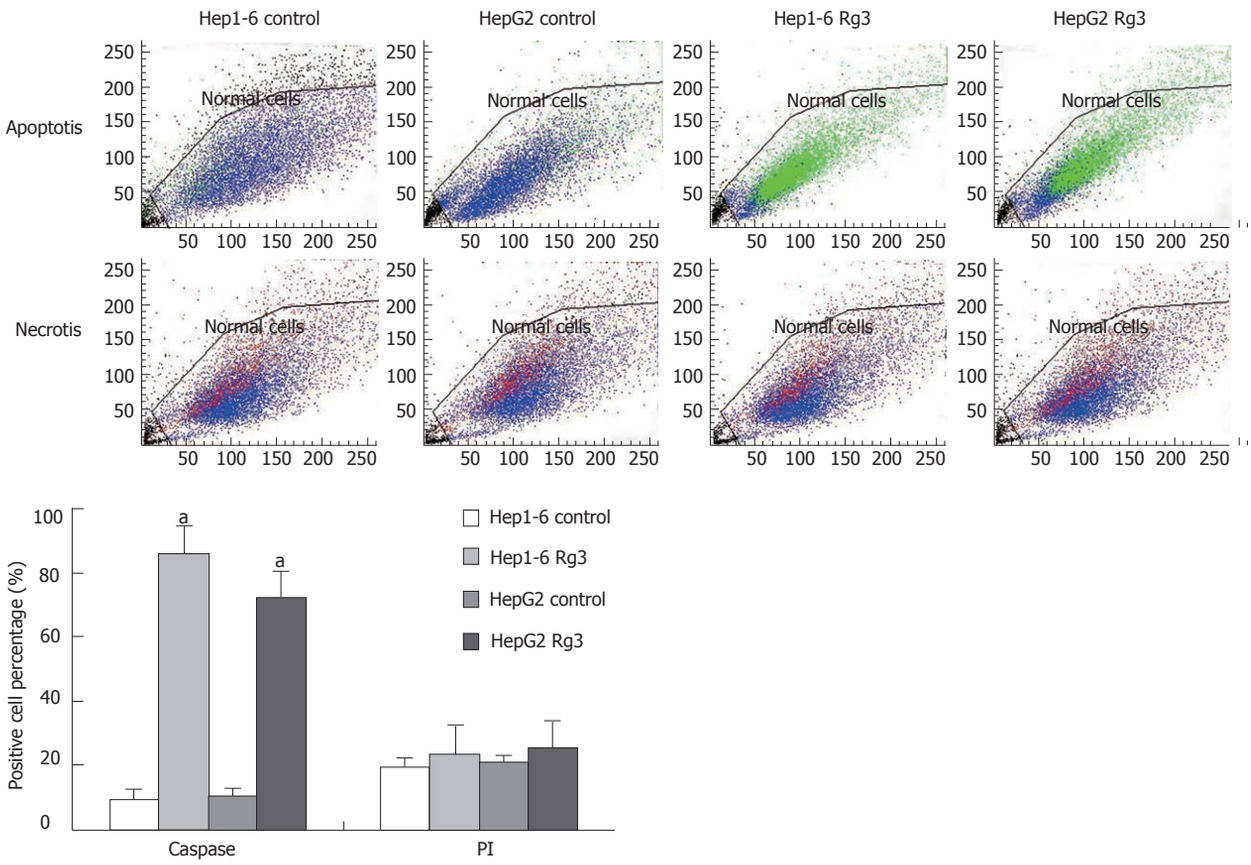

Figure 6 Flow cytometry after caspase-3-fluorescein isothiocyanate/propidium iodide staining. After being treated by ginsenoside Rg3 (Rg3) 100 μg/mL or saline for 24 h, Hep1-6 and HepG2 cells were stained by caspase-3-fluorescein isothiocyanate (FITC) and propidium iodide (PI). Viable cells are shown as blue and early apoptotic cells are green (caspase-3-FITC). The non-viable necrotic cells are red (PI). Rg3 treated groups had a significantly higher percentage of caspase-3 positive cells: Hep1-6 (85% ± 9% *vs* 9% ± 3%) and HepG2 (71% ± 8% *vs* 11% ± 2%). There are no statistical difference in PI staining between Rg3 treated group and control group: Hep1-6 (23% ± 3% *vs* 19% ± 2%) and HepG2 (25% ± 4% *vs* 21% ± 3%). Rg3: Ginsenoside Rg3. $^{a}P < 0.05$ *vs* control group.

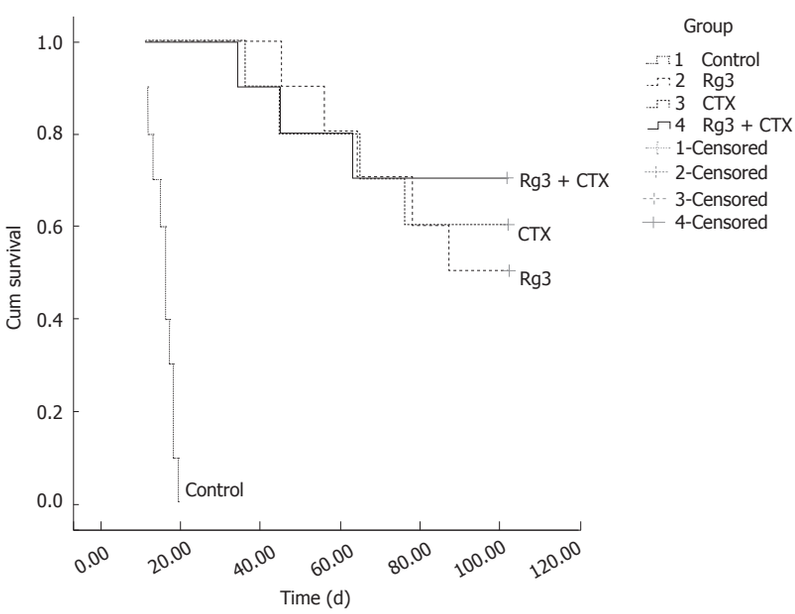

Figure 7 Survival time and survival rate of mice bearing hep1-6 tumor. The forty mice were divided into 4 groups with ten in each group, and inoculated with 1 × 106 Hep1-6 cells in each mouse. The mice were given an intratumoral injection of ginsenoside Rg3 (Rg3) (3.0 mg/kg) and cyclophosphamide (CTX) (20.0 mg/kg) or saline (1.5 mg/kg) for 10 d following inoculation of Hep1-6 cells. The animal survival study was followed up to 102 d, to observe and compare their survival time and survival rate. The Rg3 and CTX combination group, Rg3 group, and CTX group were statistically significant compared with the saline injected control group. Rg3: Ginsenoside Rg3; CTX: cyclophosphamide.

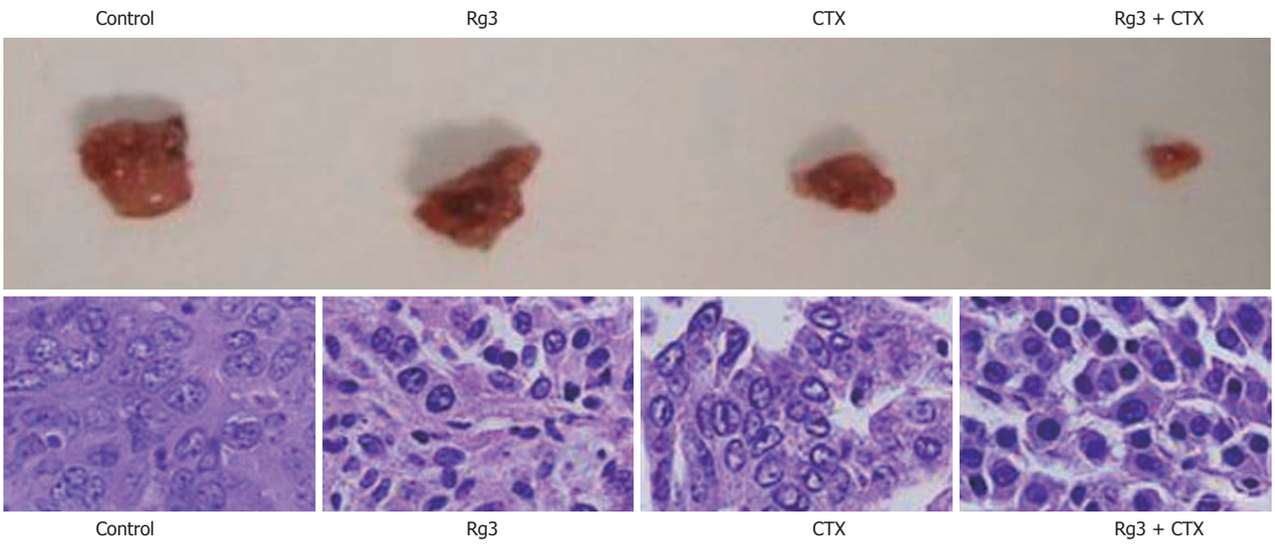

Figure 8 Tumor grosses dissection and histology. Tumors were dissected immediately after the mice were euthanized. Tumor blood vessels and their wall were abundant in the control group. The Ginsenoside Rg3 (Rg3) and cyclophosphamide (CTX) group showed smaller tumor size. The CTX group had the necrosis in the center. Rg3 + CTX had the smallest volume without necrosis. Samples were stained with hematoxylin and eosin in succession. In the control group, moderately differentiated hepatocellular carcinoma cells were surrounded by thick fibrous capsule. The nuclei were large. In the Rg3 treated group, tumor cells were irregular and condensed. In the CTX treated group, the tumor cells lost connective tissue or blood supply. In Rg3 + CTX group, there was obvious chromatin condensation in the hepatocyte. Rg3: Ginsenoside Rg3; CTX: Cyclophosphamide.

Table 1 Comparison of body weight, tumor weight, and the ratio of tumor weight/body weight in the different groups

Groups	Control	Rg3 alone	CTX	Rg3 + CTX
Body weight (g)	18.3 ± 1.8	19.1 ± 2.3	18.1 ± 1.6	21.9 ± 1.4
Tumor weight (g)	1.7 ± 0.5	0.9 ± 0.2[a]	0.5 ± 0.3[a]	0.3 ± 0.1[a]
Tumor weight/ Body weight (%)	0.09 ± 0.004	0.05 ± 0.003	0.03 ± 0.004	0.01 ± 0.002[a]

Values were pressed by Mean ± SD, [a]$P < 0.05$ in Student's *t* test *vs* the control group. Rg3: Ginsenoside Rg3; CTX: Cyclophosphamide.

group, Rg3 group, CTX group and Rg3 + CTX group, respectively. The Rg3, CTX and Rg3 + CTX treatment resulted in a delayed tumor growth compared with the control group ($P < 0.01$). Tumors observed in the control group, Rg3, CTX and Rg3 + CTX treated groups were dissected and sent for HE stain. Dissected tumors are shown in Figure 8. Tumor weights at the time of sacrifice are present in Table 1. The inhibitory effects of Rg3 + CTX on tumor growth were comparable and significant *vs* control ($P < 0.05$).

Ultra structure and nuclear change were revealed by HE. The tumors in the mice of the control group showed aggressive growth and a regular nest shape with a rich blood supply. Tumor cells featured clear and regular nuclei with prominent nucleoli. The cytoplasm was characteristically pink and clear. In Rg 3 alone treated tumors, the nuclei dramatically shrink. CTX treated tumors lost the cord-like supporting structure on which tumor cells extend. In Rg3 + CTX treated tumors, individual cells elongated and condensed, nuclear to plasma ratio decreased with obvious chromatin condensation in the hepatocytes.

DISCUSSION

Ginseng, the root of panax *ginseng*, has been widely used in Asian medicine for more than 2000 years. *Ginseng* contains many active components such as ginsenosides, polysaccharides, peptides, fatty acids and mineral oils[2]. Among these components, ginsenosides were found most responsible for the pharmacological and immunological activities such as tonic, immunomodulatory, anti-mutagenic, adaptogenic, anti-aging activities, function and immune improvement[3]. Recently Rg3 has been suggested to inhibit cancer cell growth, invasion and metastasis, e.g. lung carcinoma[4], prostate cancer[5], colorectal cancer[6], ovarian cancer[7,8] and breast cancer[9]. Our present study in liver cancer cell lines demonstrated that ginsenoside Rg3 can also inhibit Hep1-6 and HepG2 growth. TUNEL and HE stain suggest Rg3 can induce apoptosis in a concentration and time dependent manner.

Understanding of the mechanism of Rg3-induced apoptosis will shed some light on the intracellular function of Rg3 in HCC cells. Caspases are a family of proteases regulating apoptosis[10,11] which includes upstream initiator caspases, such as caspase-8 and 10, and downstream executor caspases, such as caspase-3[12,13]. In our study, we examined the involvement of caspase-3 and found that Rg3 could activate caspase-3 in a concentration dependent way. Confirming caspase-3 is essential for Rg3-induced HCC cell apoptosis, HCC cells were pretreated by an irreversible pan-caspase inhibitor, z-DEVD-FMK, and then caspase-3 activation was blocked, suggesting Rg3-induced apoptosis is caspase-3 dependent.

There are two possible pathways that can lead to caspase-3 activation[14], either through caspase-8 *via* the death receptor pathway or caspase-9 *via* the mitochondria path-

way[15,16]. Thus we tested pro-caspase-8 and cytochrome c to determine which pathway is dominant in Rg3-induced apoptosis. As illustrated in Figure 4, cleavage of caspase-8 was not evident, but cytochrome c decreased in the mitochondrial fraction and increased in the cytosolic fraction, which suggested that Rg3 induced cytochrome c release from the intermembrane space of mitochondria. Our results suggested that mitochondria probably acted as the main switch of Rg3-induced apoptosis in Hep1-6 and HepG2 cells.

The BCL-2 family regulates the apoptotic mitochondrial pathway[17,18] and can be divided into two types: anti-apoptotic proteins and pro-apoptotic proteins[19]. Many agents for cancer chemotherapy target the balance of pro- and anti-apoptotic proteins[20,21]. Bcl-2 and Bcl-XL are pro-survival proteins of the BCL-2 family, and BAX is an apoptotic protein. Our results showed that Rg3 down-regulated Bcl-2 and Bcl-XL, but up- regulated BAX. As an overall result, Rg3 altered the Bcl-2 family protein expression by shifting the balance towards cell death.

There are primarily two major events involved in apoptosis *via* the mitochondrial pathway. The first event is a change in mitochondrial membrane permeability, which leads to decreased mitochondrial membrane potential. Our data demonstrated Rg3 reduced mitochondrial membrane potential as indicated by JC-1 staining. The second event in the mitochondria-induced apoptotic pathway is the release of cytochrome c from the intermembrane space of the mitochondria into the cytosol[16]. As shown by western blot, Rg3 increased the release of cytochrome c in the cytosol.

In summary, we demonstrate that Rg3 induces HCC cell apoptosis *via* the mitochondrial pathway: (1) Rg3 induces HCC cell apoptosis by triggering Bax translocation to the mitochondria; (2) Rg3-treated HCC cells causes the release of cytochrome c into the cytosol from the mitochondria; and (3) over expression of Bcl-2 attenuated Rg3-induced apoptosis, while down-regulating Bcl-2 expression also enhances cell apoptosis.

Results on cell lines often represent a distorted and incomplete picture of the in situ physiopathology of cancer where the tumor microenvironment and neovascularization play a critical role in tumor growth and progression, thus we expanded our study to matched primary tumors using xenograft models. Hep1-6 cells were transplanted into mice. Animal survival time was prolonged by Rg3, CTX and Rg3 + CTX treatment. Tumor formation was delayed and its growth was significantly slowed down by Rg3, CTX and Rg3 + CTX treatment. Furthermore, Rg3 + CTX resulted in a significantly smaller ratio of tumor weight/ body weight. The combination of low-dose CTX and Rg3 suppresses growth of experimental tumors more effectively than CTX therapy or Rg3 alone. The possible reason for this is that the occurrence of side effects was also considerably lower. Therefore, the combination of ginsenoside Rg3 and CTX has a better effect on antitumor than ginsenoside Rg3 or CTX alone.

In conclusion, in this study, Rg3 treatment inhibited Hep1-6 and HepG2 growth by inducing apoptosis *via* the intrinsic apoptotic pathway. Ginsenoside Rg3 alone suppressed the growth of Hep1-6 tumor and combination with CTX was more effective than conventional CTX alone. Therefore, ginsenoside Rg3 is able to block the caspase-dependent signaling cascade and is valuable for developing new pharmaceutical means that will decrease the side effect of chemotherapy and increase the survival rate.

COMMENTS

Background

Liver tumor is the fifth most fatal human malignancy worldwide. It is highly resistant to chemotherapeutic drugs. Two or three agents are often combined to enhance the efficacy of chemical therapy. Chemotherapy causes serious toxic effects. Thus, there is an urgent need to develop novel treatment modalities. *Ginseng* is a traditional herbal medicine and its anti-tumor effect was recently discovered. This study investigates the anti-tumor effect of ginsenoside Rg3 on liver tumors and also explores its molecular mechanism.

Research frontiers

Ginseng is a popular herbal medicine in China and Korea. Thousands of years of clinical practice have proven its wide spectrum of pharmacological effects, but the herb extract was not quantitative and was hard to repeat. The mechanism was also unclear. This study selected *Ginseng* Rg3, a standard quantitative chemical by which the experiment could be repeated. This study focuses on apoptosis- the focus of tumor therapy, indicating the molecular mechanism of how Rg3 triggers the tumor cells to clear themselves from the normal cells.

Innovations and breakthroughs

This study found the antitumor effect of *Ginseng* Rg3 alone is not as effective as the combination of Rg3 and cyclophosphamide (CTX). Together, they could inhibit liver tumor cell proliferation, induce cell apoptosis and prolong mouse survival time. Its molecular mechanism is by inducing hepatocellular carcinoma cell apoptosis *via* the intrinsic pathway by alternating Bcl-2 family proteins and activating Caspase-3.

Applications

This study provides the experimental data for clinical application of Rg3 combined with CTX to treat liver tumor. The study screened the proper drug dosage and optimal functional time.

Terminology

Rg3 is a chemical compound isolated from the traditional Chinese herb *ginseng*. Apoptosis is also called programmed cell death or cell suicide. It is different from another form of cell death called necrosis, in which uncontrolled cell death leads to lysis of cells. Apoptosis is a process in which cells play an active role in their own deaths.

Peer review

The current paper falls within the scope of the journal, its research objectives are clearly stated, study design and methology are clearly described and the conclusions are based on the results.

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ORIGINAL ARTICLE

Bimodal visualization of colorectal uptake of nanoparticles in dimethylhydrazine-treated mice

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Abstract

AIM: To investigate colorectal uptake of solid lipid nanoparticles (SLNs) in mice receiving different doses of 1,2-dimethylhydrazine (DMH) using magnetic resonance (MR) and laser-scanning confocal fluorescence microscope (LSCFM) imaging.

METHODS: Eight mice were sacrificed in a pilot study to establish the experimental protocol and to visualize colorectal uptake of SLNs in normal mice. Gadopentetate dimeglumine and fluorescein isothiocyanate (FITC)-loaded SLN (Gd-FITC-SLN) enemas were performed on mice receiving DMH for 10 wk (group 1, n = 9) or 16 wk (group 2, n = 7) and FITC-SLN enema was performed on 4 DMH-treated mice (group 3). Pre- and post-enema MR examinations were made to visualize the air-inflated distal colorectum. Histological and LSCFM examinations were performed to verify colorectal malignancy and to track the distribution of SLNs.

RESULTS: Homogeneous enhancement and dense fluorescence (FITC) deposition in colorectal wall were observed in normal mice and 1 DMH-treated mouse (group 1) on fluid attenuated inversion recovery (FLAIR) and LSCFM images, respectively. Heterogeneous mural enhancement was found in 6 mice (4 in group 1; 2 in group 2). No visible mural enhancement was observed in the other mice. LSCFM imaging revealed linear fluorescence deposition along the colorectal mucosa in all groups. Nine intraluminal masses and one prolapsed mass were detected by MR imaging with different enhancement modes and pathologies. Interstitial FITC deposition was identified where obvious enhancement was observed in FLAIR images. Bladder imaging agent accumulations were observed in 11 of 16 DMH-treated mice of groups 1 and 2.

CONCLUSION: There are significant differences in colorectal uptake and distribution of SLNs between normal and DMH-treated mice, which may provide a new mechanism of contrast for MR colonography.

Key words: Solid lipid nanoparticles; Colorectal cancer; Magnetic resonance colonography

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INTRODUCTION

Colorectal imaging examinations consist of double-contrast barium enema (DCBE), colonoscopy, computed tomography (CT) colonography and magnetic resonance (MR) colonography. The four methods have their advantages and disadvantages. Colonoscopy, however, is the dominant technique for colorectal examination due to its high diagnostic sensitivity and capability for immediate intervention and histological evaluation. Despite the merits and continuous technical innovation, colonoscopy, as an invasive test, is not well accepted in a screening setting and cannot be performed on certain conditions such as inflammatory or malignant bowel stenosis. MR colonography, introduced in the last decade, has demonstrated encouraging initial results in the detection of polyps greater than 1 cm in diameter. Unlike CT colonography, MR colonography is a "pure noninvasive" test because it is ion-free[1-3].

High contrast between the bowel wall and lumen, implemented by either "dark-lumen" or "bright-lumen" technique, is essential for successful MR colonography. The contrast mechanisms depend on the combination of ultrafast MR sequences and an appropriate rectal enema recipe[3-7]. In most cases, a low uptake of bowel contrast agents means low toxicity. To the best of our knowledge, no radiological research aiming at visualizing the uptake of bowel contrast agents has been described.

Recently, however, intestinal uptake of particulate matter in the micro- and nanometer range has been a hot topic in pharmacological research. Studies on oral delivery of insulin, vaccine and a set of hydrophobic drugs using various nano-vehicles are under way[8-13]. Solid lipid nanoparticles (SLN) are colloidal carriers for controlled drug delivery introduced after the development of emulsions, liposomes, polymer-based microparticles and nanoparticles. SLN combines the advantages of polymeric nanoparticles and oil/water fat emulsions for drug delivery, such as good tolerability, high oral bioavailability and feasibility, for large-scale production[14] .

The aim of this study is to exemplify the feasibility of using colorectal uptake of SLNs as an extra source of contrast in colonography. To model non-familial colorectal carcinoma (CRC) in rodents, 1,2-dimethylhydrazine (DMH), a specific colon carcinogen, was administered to the mice to produce CRC and impair the bowel wall. Colorectal uptake of SLNs in mice receiving different doses of DMH was investigated using MR and laser-scanning confocal fluorescence microscope (LSCFM) imaging.

MATERIALS AND METHODS

Synthesis of SLNs

Fluorescein isothiocyanate-labeled octadecylamine (ODA-FITC) and gadopentetate dimeglumine (Gd-DTPA) loaded SLNs (Gd-FITC-SLN) were synthesized by "solvent diffusion method in a nano-reactor system," as described previously [15]. Briefly, Gd-DTPA (25 mg) and Tween 80 (18 mg) were dissolved in water (1 mL) to prepare the "aqueous phase". The water in an oil mini-emulsion was obtained by mixing, stirring and ultrasonic treatment of the "aqueous phase" and the "oil phase," which consisted of Span 80 (200 mg) and n-Hexane (10 mL). A mixture of 45 mg monostearin and 5 mg ODA-FITC, dissolved in 1 mL ethanol in a 60 ℃ water bath, was quickly dispersed into the mini-emulsion under mechanical agitating at 400 for 5 min. The dispersion was centrifuged for 15 min at 20 000 r/min to precipitate SLNs, which were subsequently washed twice with n-hexane and re-dispersed in Poloxamer 188. The resultant SLNs, dispersed to equal milligrams of manicol, were freeze-dried and kept away from light at 4 ℃. Both Gd-DTPA and ODA-FITC were omitted to produce blank SLN; only one of the imaging agents, Gd-DTPA or ODA-FITC, was added to synthesize Gd-SLN or FITC-SLN. The physicochemical properties of the SLNs were characterized as documented previously [11,15].

Pilot study

All animal experiments were approved by the institutional animal care and use committee and performed in accordance with the committee's regulations. The mice were deprived of food and allowed to drink 5% glucose saline 24 h before the examination to clean the gastrointestinal tract. An intra-peritoneal injection of pentobarbital (50 mg/kg body weight) was performed before any surgical manipulation. Eight male Kunming mice (22-25 g) were sacrificed in the pilot study. An operative procedure was established to limit enema within the distal colorectum.

MR pulse sequence (SE T2WI and FLAIR) and microscopic fluorescence imaging techniques were evaluated. The concentration of enema agents, including Gd-DTPA solution, Gd-SLN, FITC-SLN and Gd-FITC-SLN suspensions, were adjusted according to MR and fluorescence image findings. Qualified data from the pilot study were included into the study results.

Animal model and groups

Subcutaneous injection of DMH (20 mg/kg body weight) was performed wkly on 5-wk-old Kunming mice for 10 (n = 15) and 16 wk (n = 15) to induce colorectal tumors. Ten mice were excluded from the study due to DMH- and anesthesia-related mortality (n = 7) and operation failures (n = 3).

Gd-FITC-SLN (40 mg/mL) enema was performed on 9 mice receiving DMH for 10 wk (group 1) and 7 mice receiving DMH for 16 wk (group 2). FITC-SLN (40 mg/mL) enemas were performed on 4 mice (group 3) receiving DMH for 10 (n = 2) and 16 wk (n = 2).

Operative procedure

After anesthesia, an abdominal incision was made into the peritoneal cavity, and the sigmoid colon was ligated. The peritoneal cavity was then closed by two layers of continuous sutures. Subsequently, the distal colorectum was slightly inflated by infusing about 0.3 mL room air *via* the anal orifice and gently ligating tissues around to prevent air leakage. Thus, the mouse was ready for the

pre-enema MR examination. After the pre-enema MR test, an SLN enema was performed for 20 min by infusing 0.3-0.4 mL of the dispersion into the rectal lumen and ligating tissues around the anus. In-enema MR imaging was performed during the enema process. After the enema was performed and the anal ligate was removed, the enema agents were cleared by warm saline coloclysis. The distal colorectal lumen was then inflated by air again for the post-enema MR examinations, performed 25 and 60 min after the SLN enema was started. The mice were warmed by placing a hot water bag aside during the experiment.

MR imaging and analysis

Image acquisition was performed with a 1.5 T clinical MR device (Signa 1.5 T; GE Medical Systems, Milwaukee, Wis). A 5-cm custom-built coil was used for signal emission and reception. Animals were examined in the supine position. Transverse FLAIR MR images from sigmoid colon to the anus were acquired using the following parameters: repetition time, 2000 ms; echo time, 11.1 ms; inversion time, 750 ms; section thickness, 2 mm; intersection gap, 0 mm; field of view, 6-8 cm; matrix, 320 × 192; number of signals acquired, one. Transverse T2WI imaging (repetition time, 3860 ms; echo time, 106.0 ms) with the same section thickness and image size was also performed. Multi-planar FLAIR and T2WI imaging were performed continuously if colorectal masses had been detected in initial imaging.

The MR images of the colorectal wall and masses were at first interpreted in consensus by two radiologists with 20 and 10 years of experience, respectively. Colorectal masses were located by measuring the mass to anus distance. Then, quantitative analysis was performed based on the recommended procedure[16]. First, identical axial FLAIR slices before and after enema were selected for region of interest (ROI) definition. Second, a curved ROI encompassing the colorectal wall or an irregular ROI encompassing the intraluminal mass, a round ROI on the back or pelvic muscle and an oval ROI along the phase encoding direction encircling air were defined; the signal intensity (SI) values were recorded (Image J, version 1.38; National Institutes of Health, Bethesda, MD). Third, Third, the the SI difference–to-noise ratios (SDNRs) for the colorectal wall or tumors were calculated using the following formula: SDNR = (SIt-SIm)/SDN, where SIt is the mean SI value of target (the colorectal wall or intraluminal mass); SIm, the mean SI of the muscle; and SDN, the standard deviation of the background noise (air).

Histopathologic and fluorescent evaluation

Animals were euthanized by an overdose of pentobarbital immediately after MR examination. The colorectum was harvested. The macroscopic morphology of the bowel as well as the location and size of the masses within the ligated distal colorectum were recorded. The colorectal wall and masses were then sampled, frozen with liquid nitrogen, and cut into 5-7 μm slices with a microtome for LSCFM (Leica TCS-SP5, Wetzlar, Germany) evaluation and HE slice preparation. Diamidinophenyl-indole (DAPI 1:15 000 dilution, Sigma, St. Louis, MO, United States) staining was performed on slices of one normal mouse to visualize the nuclei of intestinal cells. FITC carried by SLNs was excited at 488 nm and detected at 500-535 nm wavelengths. DAPI was excited at 405 nm and detected at 430-550 nm. The remaining tissues were sampled and immerged in 10% buffered formalin to prepare the standard hematoxylin and eosin (HE)-stained slices.

Statistical analysis

SDNR data of colorectal wall, intraluminal mass size and other observations were expressed as means ± standard deviations. Statistical analysis was performed with software (SPSS for Windows, release 16.0; SPSS, Chicago). One-way analysis of variance with least significant difference tests was applied for multiple comparisons of pre- and post-enema SDNRs of the colorectal wall (groups 1-3) and SDNRs of intraluminal masses (groups 2 and 3). P value < 0.01 was considered a significant difference.

RESULTS

Characterization of SLNs

SLNs exhibited bimodal particle sizes ranging from 50 to 300 nm and zeta potentials ranging from - 29.3 ± 3.4 to - 39.1 ± 2.0 mV. The particle size increased slightly as ODA-FITC was loaded. Entrapment efficiency for Gd-DTPA in Gd-SLNs or in Gd-FITC-SLNs was 55.8% or 55.0%, respectively. Loading capacity of Gd-DTPA in Gd-SLNs or Gd-FITC-SLNs was about 50%. Hence, 40 mg Gd-FITC-SLN or Gd-SLN freeze-dried powder dispersed in 1 mL water, as used in the current study, contains about 10 mg Gd-DTPA and 20 mg Mannitol. MR images of SLN dispersions and pure water are shown in Figure 1.

Pilot study findings

Four mice were sacrificed to establish the experimental protocol. The colorectal wall was detectable on FLAIR (low-SI) and T2WI (iso- to high-SI) images. However, bowel layers could not be differentiated. While LSCFM (Leica TCS-SP5, Germany) provided fine fluorescent images, the fluorescence microscope (Zeiss Axioskop 2, Carl Zeiss, Marburg, Germany) seemed applicable in tracking the distribution of FITC-loaded SLNs.

Homogeneous mural enhancement on post-enema FLAIR images was observed after Gd-SLN (n = 1) and Gd-FITC-SLN (n = 2, 20/22 slices) retention enema. Dense FITC deposition was observed in fluorescence imaging after FITC-SLN (n = 1) and Gd-FITC-SLN enema (Figure 2). No positive MR or fluorescence image finding was observed after Gd-DTPA solution (n = 1) enema.

MR and LSCFM image features of colorectal wall and pathologic correlation

MR data of only 20 of the 30 DMH-treated mice were

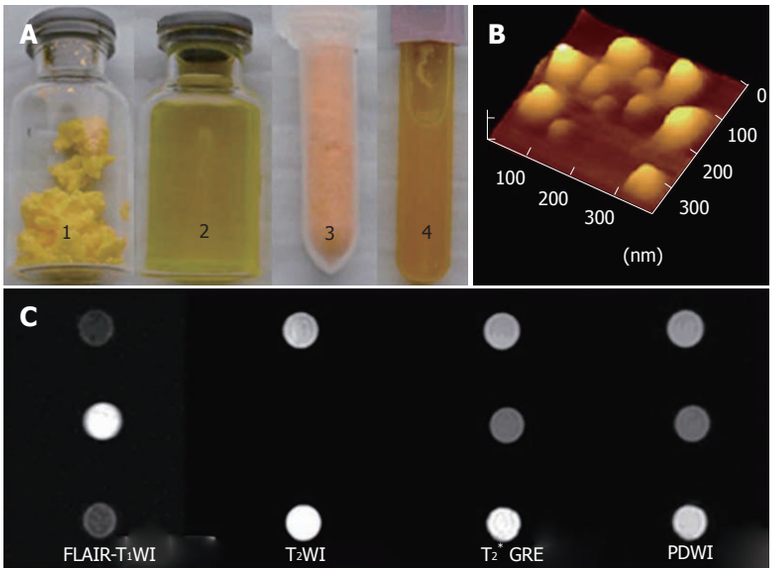

Figure 1 Characterization of solid lipid nanoparticles. A: Gadopentetate dimeglumine and fluorescein isothiocyanate-loaded solid lipid nanoparticles (Gd-FITC-SLNs) freeze-dried powder (1) and dispersion (2); Fluorescein isothiocyanate solid lipid nanoparticles (FITC-SLNs) freeze-dried powder (3) and dispersion (4); B: Atomic force microscopy images of blank solid lipid nanoparticles; C: Magnetic resonance (MR) images of FITC-SLNs dispersions (top). Gd-FITC-SLN suspension (middle). Water (bottom) obtained with fluid attenuated inversion recovery (FLAIR) (left), T_2WI (middle left), T_2* GRE (middle right) and PDWI (right). FLAIR was obtained with the following parameters: 2000/11.1/750/2, TR/TE/TI/NEX; T2WI: 3860/106/2 (TR/TE/NEX); T_2* GRE: 550/14/2/200 (TR/TE/NEX/Flip); PDWI: 3220/12/1 (TR/TE/NEX). Both sequences used a 256 × 160 matrix, a 140 mm FOV, and 4-mm-thick sections.

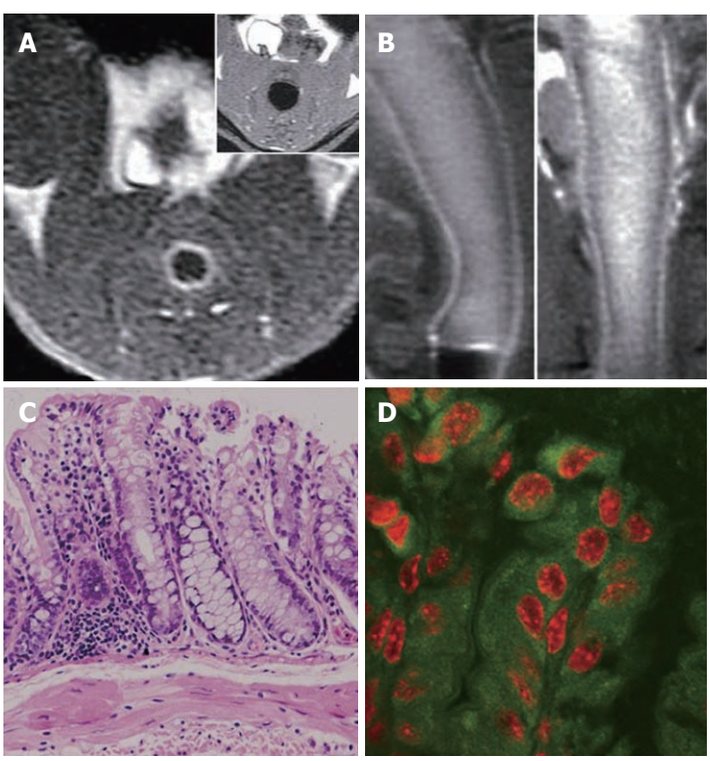

Figure 2 Magnetic resonance and fluorescent images of colorectal wall in normal mouse and bright rim sign in 1,2-dimethylhydrazine treated mouse. A: Axial fluid attenuated inversion recovery (FLAIR) image 5 min after gadopentetate dimeglumine and fluorescein isothiocyanate (FITC)-loaded solid lipid nanoparticle enema, showing the homogeneous mural enhancement; B, C: No abnormity was found in hematoxylin and eosin slices of the mouse; D: Laser-scanning confocal fluorescence microscope image of the post-enema colorectal wall of normal mouse with diamidino-phenyl-indole (DAPI) stain, showing the dense cytoplastic FITC deposition and red DAPI-stained nucleus.

available due to DMH- and anesthesia-related mortality (n = 7) and operation failures (n = 3). In group 1, homogeneous enhancement and dense FITC deposition were observed in 1 mouse (8/10 slices) with no histological abnormity; the enhanced colorectal wall manifested as a bright rim sign on in-enema FLAIR images (Figure 2B). Heterogeneous enhancement was observed in 4 of the other 8 mice (26/48 slices). No visible mural enhancement was identified in the other 4 mice; mild dysplasia was identified in HE slices of the 8 mice with linear FITC deposition observed along the intestinal lumen in LSCFM images (Figure 3). In group 2, heterogeneous enhancement was shown in 2 of the 7 mice (6/16 slices); no other mural enhancement was observed. In group 3, no enhancement was found on FLAIR images after the FITC-SLN enema. Obvious dysplasia and intraepithelial neoplasia (low to high grade) were found in HE slices of mice receiving DMH for 16 wk (groups 2 and 3) with linear FITC deposition along the intestinal lumen observed in LSCFM images. Non-enhanced colorectal walls manifested as low signal rings around the "bright lumen" in in-enema FLAIR images, which was observed in 4 of the 7 mice in group 2 (Figure 4B).

MR and LSCFM findings of colorectal mass in DMH groups and pathologic correlation

MR detected 9 intraluminal masses (short axis 2.06 ± 0.98 mm) and 1 prolapsed mass (well-differentiated squamous

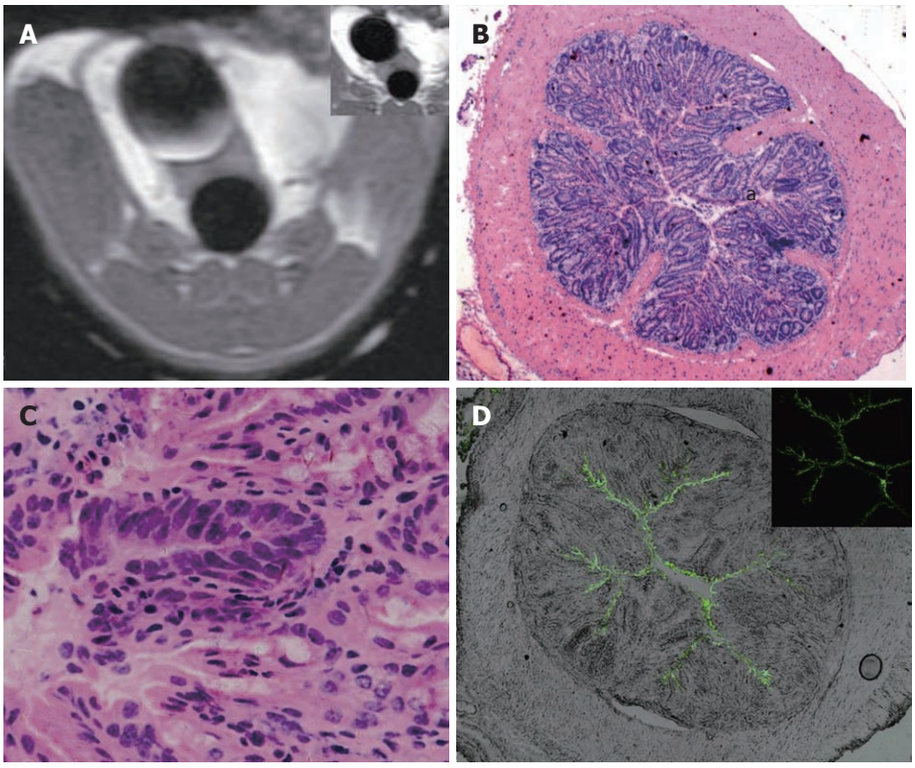

Figure 3 Magnetic resonance and fluorescent images of 1,2-dimethylhydrazine impaired colorectal wall. A: Axial fluid attenuated inversion recovery (FLAIR) image 5 min after gadopentetate dimeglumine (Gd-DTPA) and fluorescein isothiocyanate (FITC)-loaded solid lipid nanoparticle enema. No mural enhancement was identified. Note the bladder Gd-DTPA deposition (top right: pre-enema FLAIR image); B, C: Hematoxylin and eosin image of the same slice; nuclear atypia identified; B: × 40; C: × 400. D: Laser-scanning confocal fluorescence microscope image of the post-enema colorectal wall, showing the linear extracellular FITC deposition along the mucosa.

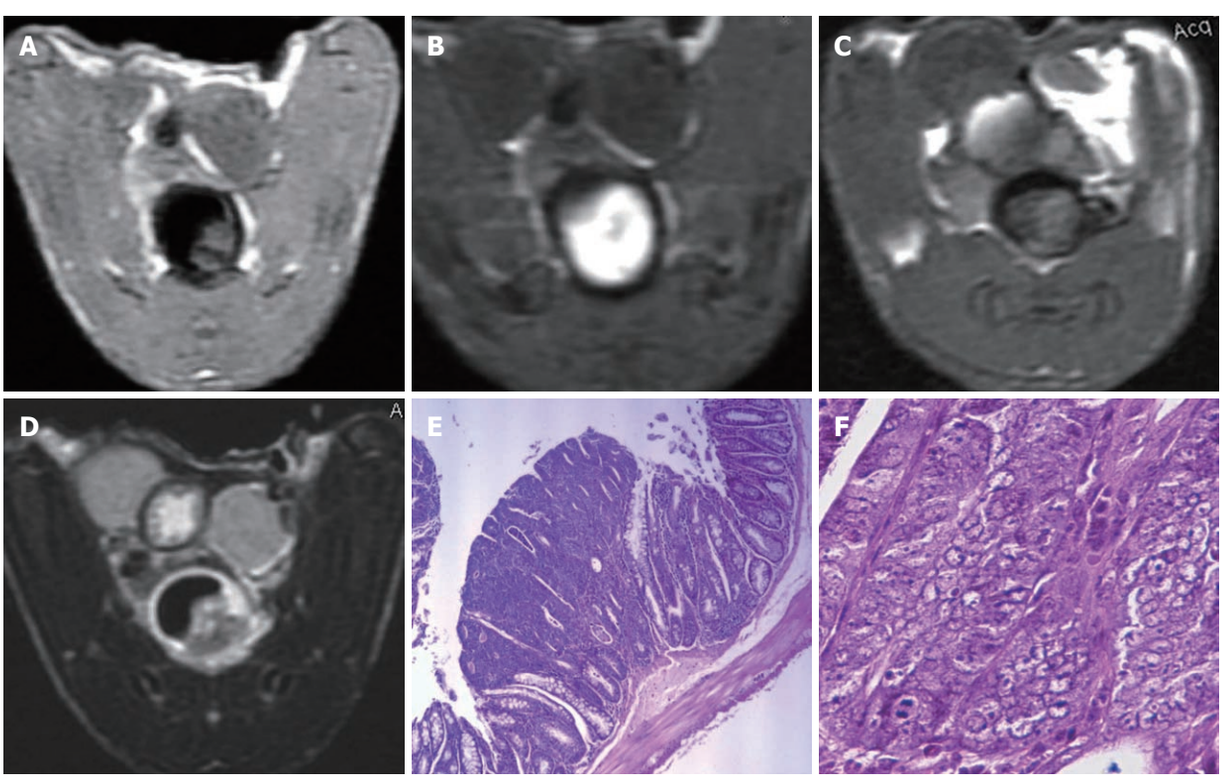

Figure 4 Magnetic resonance and histological images of adenocarcinoma. A: An irregular-shaped mass in the pre-enema fluid attenuated inversion recovery (FLAIR) image; B: Low signal ring around the bright lumen (halo sing) in the in-enema FLAIR image; C: Tumor enhancement and bladder imaging agents accumulation both occurred in the post-enema FLAIR image; D: T2 weighted post-enema image, heterogeneous signal intensity within the tumor; E, F: Hematoxylin and eosin images of the tumor, adenocarcinoma cells identified.

carcinoma) in groups 2 and 3. Eight of the 9 intraluminal masses were adenomas with different levels of malignancy; one of them was histologically proven as adenocarcinoma. The intraluminal masses manifested as filling deficits on Gd-FITC-SLN inflated bright-lumen FLAIR images. No visible enhancement was found in post-enema

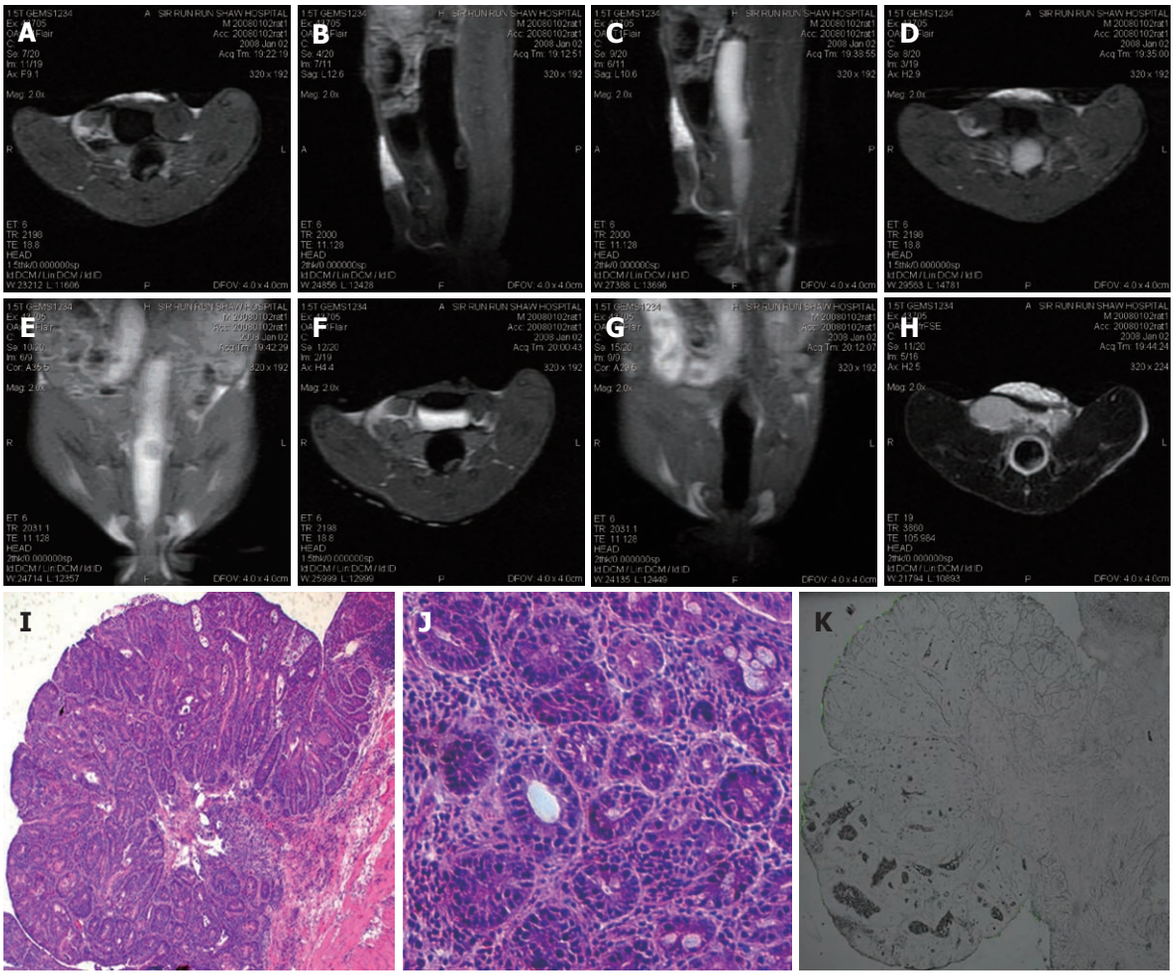

Figure 5 Magnetic resonance and fluorescent images of a non-enhanced adenoma. A, B: Axial and sagittal pre-enema fluid attenuated inversion recovery (FLAIR) images, note the inner low signal of the mass; C-E: The mass manifests as a filling deficit in the gadopentetate dimeglumine (Gd-DTPA) and fluorescein isothiocyanate-loaded solid lipid nanoparticle (Gd-FITC-SLN) enema inflated FLAIR images; F, G: No enhancement identified after the enema. Note the bladder Gd-DTPA deposition; H: Axial T2 weighted image after the Gd-FITC-SLN enema. Iso-signal observed in the adenoma; high signal observed for the bowel wall; I-K: Hematoxylin and eosin and laser-scanning confocal fluorescence microscope images of the adenoma.

FLAIR images in one narrow-based adenoma with minimum FITC deposition along the edge and within the mass (Figure 5). Various degrees of enhancement were found in the other masses with interstitial FITC depositions in LSCFM images (Figure 6).

Other MR and pathohistologic findings

Colorectum stiffness was observed in 3 of 9 mice (groups 2 and 3) receiving DMH for 16 wk. Macroscopic evaluation of the animal's distal colorectum revealed an extra adenoma measuring 2 × 3 mm in size in the sigmoid colon, which was missed in MR examination. No false-positive MR findings were observed by postmortem evaluation. Bladder imaging agent accumulation was accidently found in 11 of the 16 DMH-treated mice (groups 1 and 2) 3 - 28 (11.2 ± 9.6) min after the Gd-FITC-SLN enema was started (Figures 3-6).

Statistical results

Pre- and post-enema SDNR values of colorectal wall in each group and intraluminal masses in DMH-treated mice were plotted against time (Figure 7) with brief annotation.

DISCUSSION

We used MR and LSCFM to study the colorectal uptake of SLNs. The bimodal imaging used elsewhere for tumor angiogenesis and lymphatic vessel imaging[17,18] can mutually confirm the information both macroscopically and microscopically. We used FLAIR for colorectal imaging. An early study reported that FLAIR sequences are more sensitive to low gadolinium concentrations than T1-weighted sequences[19]. Another study showed that post-contrast FLAIR imaging may improve the lesion depiction when a higher lesion SI exists on the T2-weighted images[20]. A set of methods for quantitative MR imaging analysis were evaluated, and a standardized method was proposed[16]. We followed the suggested procedure in terms of ROI definition and SDNR calculation.

We reviewed the studies on intestinal particulate substance uptake, colon-specific drug delivery and DMH-induced intestinal and renal impairment in order to explain

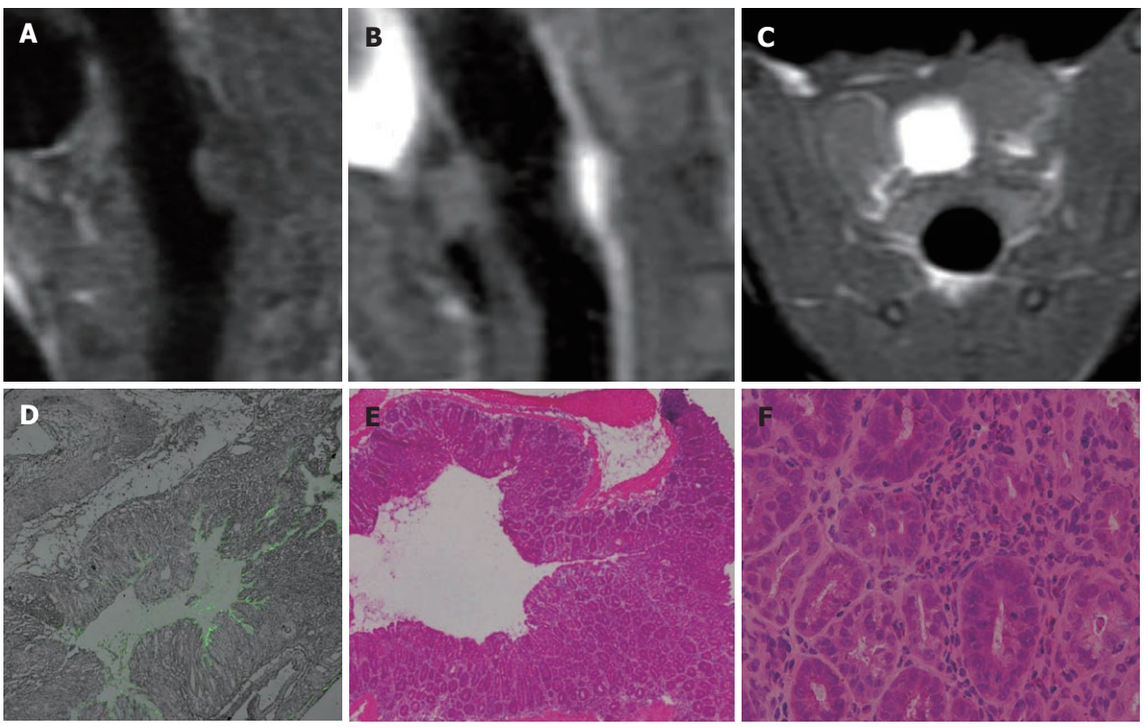

Figure 6 Peri-tumor interstitial fluorescein isothiocyanate deposition. A: A broad-based mass (high-level adenoma) in the pre-enema fluid attenuated inversion recovery (FLAIR) image; B, C: Tumor and peri-tumor mural enhancement, together with bladder imaging agent accumulation, in the post-enema FLAIR image; D: Interstitial linear fluorescein isothiocyanate deposition in the peri-tumor colorectal wall; E, F: Hematoxylin and eosin images at the same slice, obvious nuclear atypia identified.

the current results. It was proven, by lymph and plasma analysis, that more than 70% of the absorbed SLN was transported into systematic circulation *via* lymph, which is a major SLN transport pathway in the gastrointestinal tract[11]. Recent studies have further verified that the oral bioavailability of poorly water soluble contents (insulin, nitrendipine, tobramycin) increased significantly when encapsulated in the inner lipid matrix of SLNs[21,22].

The intestinal uptake of inert particles of the micrometer and nanometer range has been intensively studied in pharmaceutical research. The particles, usually used as oral drug delivery systems, include SLN (50-1000 nm), chitosan microspheres (2.1-12.5 μm), latex (2 μm), dendrimer and polymers, etc. The major conclusions of the studies[10,23-27] are summarized as follows. First, inert particulate uptake takes place along the entire length of the small and large intestines. Second, the process occurs not only *via* the M cells in the Peyer's patches and the isolated follicles of the gut-associated lymphoid tissue, but also *via* the normal intestinal enterocytes. Third, factors affecting the uptake include particle size, surface charge and surface modification. Larger (micrometer range) and surface modified particles may be retained for longer periods in the Peyer's patches, while smaller particles are transported to the thoracic duct. Fourth, an *in vitro* study using a Caco-2 cell model showed that 2 Gy X-irradiation increased particle (2 μm latex particles) uptake and translocation through the epithelium.

We hypothesize, based on the current results and earlier studies, that two different routes exist for the rectally administered SLN particles to enter the systematic circulation. In normal mice, the SLNs are mainly taken up by enterocytes and transferred to the lymphatic vessels and finally transported to the systematic circulation from the thoracic duct. In DMH-treated mice, however, the dominant route is from the carcinogen impaired colorectal mucosa, *via* the submucosal capillary network, to the mesenteric vein and then to the liver. As observed in this study, colorectal uptake and drainage of SLNs are an intracellular process; the transportation of SLNs in DMH-treated mice is a pathological process that occurs through an extracellular or interstitial route.

DMH and its metabolite azoxymethane (AOM) are specific colon carcinogens to model non-familial CRC in rodents[28]. Previous studies documented that DMH is also a renal carcinogen in mice[29-31]. The bladder gadolinium accumulation observed in this study may result from renal and intestinal epithelial impairment caused by DMH and its metabolites and the diuresis effect of manicol contained in SLN freeze-dried powder (20 mg /mL).

We believe that the bright rim sign in post-enema FLAIR images and corresponding cytoplastic FITC deposition in normal and group 1 mice is a manifestation of normal intestinal uptake function. Likewise, the halo sign observed in group 2 mice and linear extracellular FITC deposition is attributed to the impairment of intestinal epithelial barrier function. As functional changes always precede morphological lesions, further experiments are necessary to exemplify the early diagnostic potential by clarifying the mechanism at both cellular and

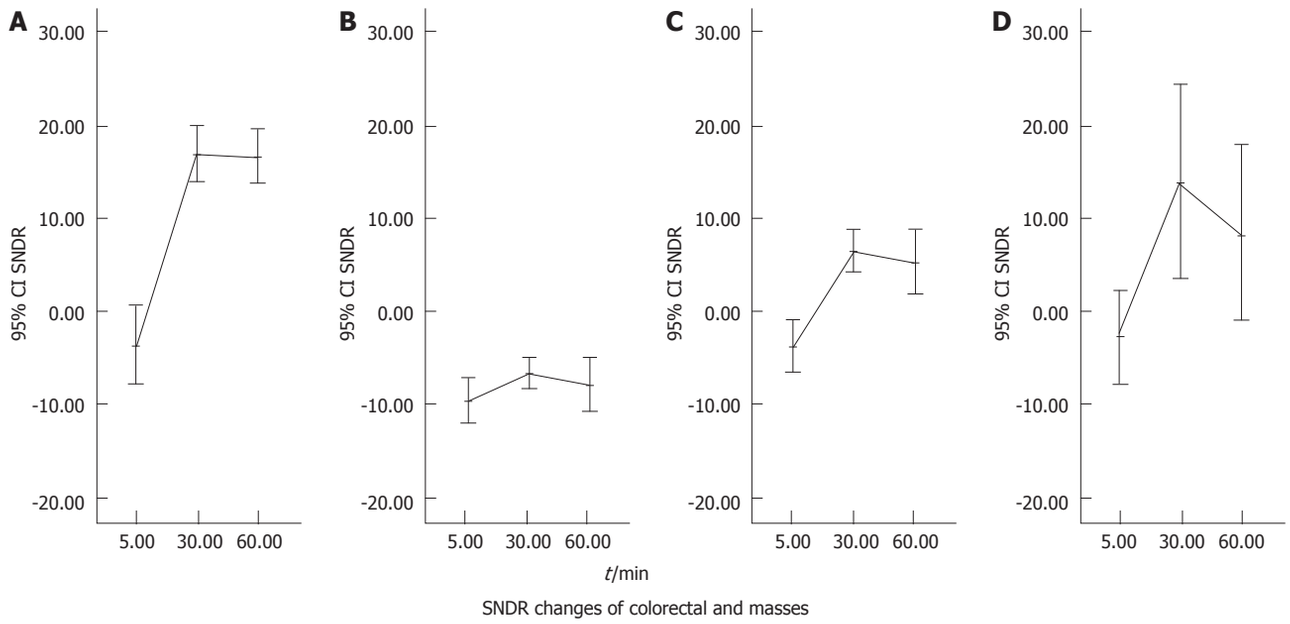

Figure 7 The signal intensity difference-to-noise ratios changes of colorectal wall and masses before and after the gadopentetate dimeglumine and fluorescein isothiocyanate-loaded solid lipid nanoparticle enema. A: The signal intensity difference-to-noise ratios (SDNRs) of colorectal wall in normal mice increased sharply after 20 min of the gadopentetate dimeglumine and fluorescein isothiocyanate-loaded solid lipid nanoparticle (Gd-FITC-SLN) enema ($P < 0.01$) and remain at a high level in the following 30 min with a minimum decrease ($P > 0.05$); B: The SDNRs of colorectal wall in group 1 [1,2-dimethylhydrazine (DMH) treated for 10 wk] increased significantly, with about one half of the amplitude compared with that of the normal mice, after the Gd-FITC-SLN enema; a visible decrease with no statistical significance ($P > 0.05$) of SDNRs occurred in the following 30 min; C: The SDNRs of colorectal wall in group 2 (DMH treated for 16 wk) increased and decreased non-significantly in post-enema images ($P > 0.01$); D: The SDNRs of intraluminal masses in DMH treated mice increased significantly ($P < 0.01$) and decreased non-significantly in the post-enema images ($P > 0.01$). SDNR: The Signal Intensity Difference–to-noise ratios.

molecular levels.

There were limitations in our study. First, layers of murine colorectal wall could not be differentiated on MR images due to the small animal size. We noticed that layers of the bowel and tumor invasion in the excised human colon cancer specimen were clearly depicted in one study[32]. Optimal delineation of layers of colorectal wall may likely be achieved if a porcine model was adopted. Second, sharp contrast in post-enema MR images (partial enhancement) existed but was rare, which may be explained by the diffuse impairment caused by DMH and its metabolite azoxymethane (AOM), delivered to the distal colorectum *via* the biliary system. Adoption of another colorectal tumor model, focally administered AOM, may improve the post-enema contrast between normal and cancerous tissues. Third, the long acquisition times of FLAIR pulse sequence may not be suitable for clinical MR colonography. More researches are, therefore, needed to optimize the technique in a clinical context.

COMMENTS

Background

High contrast between the bowel wall and lumen is essential for successful magnetic resonance (MR) colonography, which has been intensively studied in recent years. However, there has been no research aiming at MR visualizing the colorectal uptake of contrast medium.

Research frontiers

In this study, colorectal uptake of solid lipid nanoparticles (SLNs) in normal and dimethylhydrazine (DMH)-treated mice was visualized by MR and laser-scanning confocal fluorescence microscopic imaging.

Innovations and breakthroughs

Significant differences in colorectal uptake and distribution of SLNs were revealed in normal and DMH-treated mice, which may provide new mechanisms of contrast for MR colonography.

Applications

Direct and *in vivo* imaging of colorectal uptake of nanoparticles could be translated into radiological and pharmaceutical applications. Further work is needed to explore the potential value of current findings for personalized therapy and radiographic follow-up.

Terminology

SLNs are colloidal drug delivery systems with mean particle diameters ranging from 50 up to 1000 nm. SLNs combine the advantages of polymeric nanoparticles and fat emulsions for drug delivery administration, such as good tolerability, high oral bioavailability and large-scale production by high pressure homogenization. Magnetic resonance imaging is a cross-sectional imaging technique that does not utilize radiation and provides excellent tissue differentiation. MR colonography, based on the use of ultrafast MR sequences and relevant bowel contrast agents, is a less invasive colon imaging tool compared with optic colonoscopy.

Peer review

The authors concluded that the uptake of SLNs into the colon wall was significant difference between normal and 1, 2-DMH, specific colon carcinogens, treated mice. This paper has very interesting results, but the objective is not clear.

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BRIEF ARTICLE

Combined inhibitors of angiogenesis and histone deacetylase: Efficacy in rat hepatoma

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Abstract

AIM: To evaluate the antitumoral effect of combined inhibitors of angiogenesis and histone deacetylases in an experimental rat hepatoma model.

METHODS: MH7777A hepatoma cells were injected into the liver of male Buffalo rats. After 7 d treatment with the vascular endothelial growth factor receptor antagonist PTK787/ZK222584 (PTK/ZK), the histone deacetylase inhibitor MS-275, tamoxifen (TAM) and/or retinoic acid was initiated ($n \geqslant 8$ animals/group). Natural tumor development was shown in untreated control groups (control 1 with $n = 12$, control 2 with $n = 8$). The control groups were initiated at different time points to demonstrate the stability of the hepatoma model. For documentation of possible side effects, we documented any change in body weight, loss of fur and diarrhea. After 21 d treatment, the rats were euthanized. Main target parameters were tumor size and metastasis rate. Additionally, immunohistochemistry for the proliferating cell nuclear antigen (PCNA) and TdT-mediated dUTP-biotin nick end labeling (TUNEL) assay were performed.

RESULTS: The control groups developed large tumor nodules with extrahepatic tumor burden in the lung and abdominal organs (control 1: 6.18 cm^3 ± 4.14 cm^3 and control 2: 8.0 cm^3 ± 4.44 cm^3 28 d after tumor cell injection). The tumor volume did not differ significantly in the control groups ($P = 0.13$). As single agents MS-275 and PTK/ZK reduced tumor volume by 58.6% ± 2.6% and 48.7% ± 3.2% *vs* control group 1, which was significant only for MS-275 ($P = 0.025$). The combination of MS-275 and PTK/ZK induced a nearly complete and highly significant tumor shrinkage by 90.3% ± 1% ($P = 0.005$). Addition of TAM showed no further efficacy, while quadruple therapy with retinoic acid increased antitumoral efficacy (tumor reduction by 93 ± 1%) and side effects. PCNA positive cells were not significantly reduced by the single agents, while dual therapy (MS-275 and PTK/ZK) and quadruple therapy reduced the PCNA-positive cell fraction significantly by 9.1 and 20.6% *vs* control 1 ($P < 0.05$). The number of TUNEL-positive cells, markers for ongoing apoptosis, was increased significantly by the single agents (control 1: 6.9%, PTK/ZK: 11.4%, MS-275: 12.2% with $P < 0.05$ *vs* control 1). The fraction of TUNEL-positive cells was upregulated highly significantly by dual therapy (18.4%) and quadruple therapy (24.8%, $P < 0.01$ *vs* control 1). For the proliferating (PCNA positive) and apoptotic cell fraction, quadruple therapy was significantly superior to dual therapy ($P = 0.01$).

CONCLUSION: Combined PTK/ZK and MS-275 were highly effective in this hepatoma model. Quadruple therapy enhanced the effects microscopically, but not macroscopically. These results should be investigated further.

Key words: PTK787; ZK222584; MS-275; Hepatocellu-

lar carcinoma; Histone deacetylase inhibitor

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INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common tumor entities in Asia and Africa, but not in western countries[1,2]. However, the increasing rate of hepatitis C virus infection and alcohol abuse has led to a rising incidence of HCC in industrial countries[1,2].

Life-prolonging HCC therapy is based on surgical tumor elimination (liver transplant, resection) or interventional local ablation[3,4]. For large tumor volume or metastasis, therapeutic options are limited, because HCC is resistant to systemic chemotherapy[3,4]. A wide range of experimental compounds, such as vitamin D or tamoxifen (TAM) were not effective in placebo-controlled trials[1,3,4]. Therefore, not single, but combination treatment should be evaluated. We have shown that combined application of TAM and cis-retinoic acid (CRA) induces moderate antitumoral effects in a rat model, while single agents are ineffective[5,6]. Current investigations have put combination molecular targeted therapy into focus[7-9]. Sorafenib is a molecular targeted agent with antiproliferative as well as antiangiogenic activity. It is the first effective compound in HCC patients, which proves the concept of combination treatment. However, the antitumoral and life-prolonging effects of sorafenib are very limited[3,7-9]. Nevertheless, the strategy of combined molecular targeted agents has to be further investigated.

In most HCCs, a high grade of vascularity has been demonstrated. Folkman *et al*[10-12] have demonstrated that any tumor larger than a few millimeters in diameter induces angiogenesis for self-supplementation. Furthermore, connection with the host's vessel system and degradation of the extracellular matrix leads to metastasis[11]. Therefore, inhibitors of angiogenesis have been shown to reduce hepatoma volume *in vivo* and *in vitro*[9-12]. In recent years, highly effective synthetic angiogenesis inhibitors, such as PTK787/ZK222584 (PTK/ZK), have been developed for cancer therapy. These are currently being evaluated in clinical trials or approved for therapy of advanced colorectal cancer[12,13].

Histone deacetylase (HDAC) inhibitors suppress the post-translational deacetylation of histone proteins. In consequence, these histone proteins are hyperacetylated and the DNA structure is loosened, which mediates enhanced binding of transcription factors to certain gene loci and higher gene expression[14]. HDAC inhibitors have been shown to induce growth inhibitory genes and pro-apoptotic factors *in vivo* and *in vitro*[7,14,15]. MS-275 is a benzamide with activity against HDAC class 1 and 2. Ongoing phase Ⅰ-Ⅲ trials have displayed no adverse effects, therefore, further clinical development is ongoing[16,17].

In this experimental setting, we evaluated a combination of the angiogenesis inhibitor PTK/ZK and the HDAC inhibitor MS-275[12,16] in a syngeneic rat model of hepatoma. PTK/ZK is an aminophthalocine and a potent inhibitor of vascular endothelial growth factor (VEGF) receptor tyrosine kinases, which impairs VEGF-induced responses and tumor growth after oral administration (Vatalanib®)[12,16,18]. VEGF receptors mediate an increase in vascular permeability, angiogenesis and lymphogenesis. Mainly, the expression of VEGF receptor 1 is enhanced in tumor tissue[16,18]. PTK/ZK inhibits VEGF receptors 1-4 with highest potency towards receptors 1 and 2. Additionally, we added TAM and CRA, which have been shown to act *via* intracellular receptors and to be effective combination partners in this HCC model[5].

MATERIALS AND METHODS

Reagents and cell culture

For *in vivo* experiments MS-275 was suspended in methanol, while 9-cis retinoic acid (cRA), TAM and PTK787/ZK222584 (Vatalanib®, PTK/ZK) were dissolved in DMSO. Any of these stock solutions were diluted at least 20-fold with Aqua injectabile (Baxter, Deerfield, IL, United States) to a final maximum concentration of 5% DMSO or 0.5% methanol before injection. MS-275 and PTK/ZK were kindly donated by Schering AG (Berlin, Germany).

Morris hepatoma of the rat

Male Buffalo rats (200-350 g) from Charles River Laboratories (Schweinfurt, Germany) were kept as pairs in polycarbonate cages (Eurostandard Ⅲ H; Techniplast, Berlin, Germany). Room temperature was kept at 27 ℃ and room humidity maintained at 30%. The rats were fed a standardized gluten-poor diet (Altromin, Frankfurt, Germany) and water. Animal maintenance and experimental procedures were approved by the government of middle Franconia and carried out according to "The 1996 Guide for the Care and Use of Laboratory Animals" as published in ILAR[19].

For tumor induction, MH7777A cells (DSMZ, Braunschweig, Germany) were grown in Dulbecco's Modified Eagle's Medium (Biochrom, Berlin, Germany) that contained 10% fetal calf serum (Gibco BRL, Karlsruhe, Germany), penicillin (100 U/L), streptomycin (10 mg/L), insulin and dexamethasone at 37 ℃ under 5% CO_2 (5-10 passages). The cells were trypsinized, suspended in PBS (Biochrom) at a concentration of 10^6 cells/100 μL. Buffalo rats were anesthetized using ethyl ether. After a median laparotomy (2 cm), the liver was embedded into wet sterile compresses. One hundred microliters of the cell suspension was injected into the subcapsular space

of the left liver lobe and leakage of tumor cells was prevented by compression and a hemostatic (Tabotamp; Ethicon, Johnson and Johnson, Norderstedt, Germany). The animals received metamizol for analgesia (7 d). They were controlled for diarrhea, loss of hair, food intake and unusual behavior daily, and body weight was measured weekly. On postoperative day 7 (tumor size 5-7 mm diameter), treatment with single or combined drugs was started. The drugs were administered at the recommended dose of 50 mg/kg per day i.p. for PTK/ZK, 3 mg/kg per day i.p. for MS-275, 10 mg/kg per day i.p. for TAM and 6 mg/kg per day i.p. for CRA. After 21 d treatment (day 28 after tumor implantation), the rats were euthanized with ether anesthesia. At least eight animals were evaluated per group.

Macroscopic evaluation

The liver was removed and the tumor volume calculated using the formula (largest diameter a × smallest diameter b^2)/2 as recommended in the literature[6]. The following organs were inspected for tumor nodules: lungs, spleen, kidneys, peritoneum and diaphragm. The primary tumor and both lungs were fixed in 5% buffered formalin.

Microscopic analysis

TdT-mediated dUTP-biotin nick end labeling (TUNEL)-positive cells were analyzed using the *in situ* Cell Death Detection Kit (Roche, Mannheim, Germany) according to the manufacturer's instructions. Briefly, formalin-fixed tissues were permeabilized with proteinase K (30 min, 37 ℃) and peroxidase blocked in methanol containing 0.3% H_2O_2. Fluorescent nucleotides mixed with terminal deoxynucleotide transferase were added for 60 min at 37 ℃, followed by incubation with converter-peroxidase (POD) conjugated anti-fluorescein antibody (provided in the kit) for 30 min at 37 ℃. Slides were developed using diaminobenzidine (DAB) substrate for 10 min and counterstained using methylene green (7.5%, 7 min at room temperature).

Proliferating cell nuclear antigen (PCNA)-positive cells in formalin-fixed tissue were detected after blocking of endogenous biotin with chicken egg and 1.5% fat milk for 15 min at room temperature. Mouse PCNA antibody (Novo Laboratories, Newcastle, United Kingdom) was diluted 1:50 in Tris buffer and added for 2 h, followed by 30 min incubation with the biotinylated second antibody. Color was developed with streptavidin-alkaline phosphatase complex (DAKO, Mannheim, Germany) and FAST Red (Sigma, Frankfurt, Germany).

The stained sections were examined using a light microscope (Axiophot, Nikon coolpix 99; Zeiss, Jena, Germany) and the CellExplorer 2001 software (BioSciTec, Frankfurt/Main, Germany). For quantification of TUNEL- and PCNA-positive cells, 10 high power fields per slide were investigated at 400 × magnification. Four of eight animals were analyzed per experimental group. All cell nuclei were related to the specifically stained cells to obtain the percentage of positive cells per slide.

For qualitative validation of the anti-angiogenic activity cryofixed sections (6 μm, lysine-coated slides) were blocked (buffer containing 2% BSA, 0.2% low fat milk, 2% mouse serum and PBS) and incubated with a rabbit anti-von Willebrand factor (vWF) antibody (Santa Cruz Biotechnology, Santa Cruz, CA, United States; 1:200 dilution) for 1 h at 37 ℃. After several washing steps and addition of a biotinylated second antibody (30 min, room temperature), color was developed with streptavidin–peroxidase complex (DAKO) and DAB. Counterstaining was done using standard haemalaun.

Statistical analysis

Statistical analysis was performed using SPSS for Windows version 16.0. Significance was calculated using the *t* test or Wilcoxon test for paired samples (if not otherwise stated *vs* control 1). $P < 0.05$ was regarded as significant, and $P < 0.01$ as highly significant.

RESULTS

In vivo studies

In untreated animals (control 1, $n = 12$) the tumor volume was 6.18 cm^3 ± 4.14 cm^3 after 28 d. In a second untreated control group (control 2, $n = 8$), which was started at an independent time point, tumor volume was 8.0 cm^3 ± 4.44 cm^3. This was not significantly different to control 1 ($P = 0.13$) and may confirm the reproducibility of the animal model.

Twenty-eight days after tumor implantation 80%-90% of the animals suffered from diffuse lung metastases and tumor spread to kidneys, spleen and peritoneum. Eighty percent of the animals developed ascites. Bleeding from the eyes and the nose showed impaired coagulation. The body weight was increased by 17% ± 15% (due to ascites and tumor burden).

As stated before, treatment with TAM or CRA as single agents showed no significant antitumoral efficacy, while a combination of both showed moderate tumor reduction (14%, 8.5% and 60% for TAM, CRA and TAM/CRA)[7].

Even the single agents PTK/ZK and MS-275 induced some antitumoral effects and reduced tumor volume by 50 and 60% (Figure 1). However, PTK/ZK failed to show significant antitumoral efficacy ($P = 0.212$), while MS-275 showed significant tumor growth reduction even as a single agent ($P = 0.025$). Dual therapy with PTK/ZK and MS-275 reduced tumor growth by > 90% in a highly significant manner ($P = 0.005$, Figures 1 and 2). PTK/ZK + MS-275 + TAM showed no additional effect on tumor volume, while the quadruple therapy enhanced the efficacy slightly, but not significantly ($P = 0.007$ and 0.002 for triple and quadruple therapy *vs* control 1; $P = 0.49$ and $P = 0.039$ *vs* dual therapy, Figure 1).

Monotherapy did not change the extent of extrahepatic tumor burden, while combination therapy reduced rate of metastases significantly (Table 1). Again, quadruple therapy failed to enhance the effects of combined

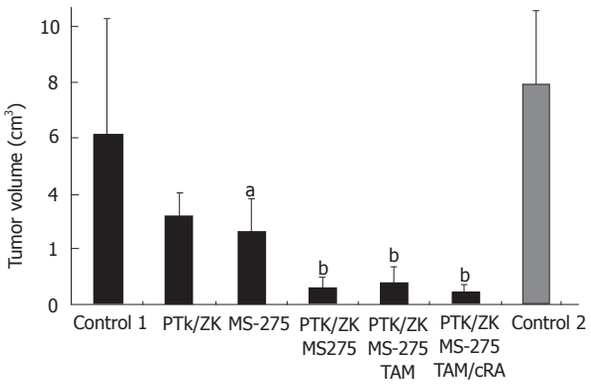

Figure 1 Macroscopic tumor growth. The results are given as absolute values. [a]*P* = 0.025, MS-275 *vs* control 1; [b]*P* = 0.005, ZK/PTK/MS *vs* control 1. PTK/ZK: PTK787/ZK222584 (Vatalanib®); TAM: Tamoxifen; cRA: 9-cis-retinoic acid.

PTK/ZK + MS-275 (data not shown).

Compared to placebo treatment, monotherapy increased the rate of diarrhea and loss of fur. Combination therapy intensified the number of these side effects significantly (Table 1). Subgroup analysis showed fewer side effects for dual therapy compared to triple and quadruple therapy. However, this particular result was not significant due to the low number of animals/group (data not shown).

Any effective treatment (monotherapy, dual therapy and quadruple therapy) induced loss of weight, which can be explained by the increased rate of diarrhea and the reduced amount of ascites in these treatment groups (Table 1). No animal had to be euthanized due to the number or severity of side effects.

Microscopic results

After treatment with PTK/ZK alone and in combination, vessel density decreased qualitatively, which was exemplified by staining with vWF antibody in a subgroup of animals (data not shown). Quantification of microvessel density was not performed, because the antiangiogenic efficacy of PTK/ZK has been well described[12,19].

In hematoxylin and eosin (HE) and immunohistochemically stained tissue, any effective treatment went along with an increase in areas of necrosis (Figure 3). Depending on the extent of the necrotic areas, we detected 200-500 cells/field. The number of TUNEL-positive cells as a marker for ongoing apoptosis increased significantly after monotherapy (P = 0.04 and 0.02 for PTK/ZK and MS-275 *vs* control 1). As expected, the number of apoptotic cells increased even more markedly after dual therapy (highly significant *vs* control 1 with P = 0.002). The results of quadruple therapy were significantly higher even if compared to dual therapy (P = 0.01 for quadruple therapy *vs* dual therapy; Table 2). The signal for proliferating cells (PCNA positive) remained stable for monotherapy, but decreased significantly for dual therapy (P = 0.04 *vs* controls). Again, quadruple therapy showed a significant difference compared to the combination of PTK/ZK + MS-275 (P = 0.01 for quadruple *vs* dual therapy) (Table 2).

Table 1 Extrahepatic tumor burden and side effects in untreated and treated tumor-bearing rats (%)

	Control group (n = 12)	Monotherapy (n = 17)	Combination therapy (n = 24)	P value[2]
Tumor burden - pulmonary	83.3	82.4	70.8	0.586
Tumor burden - abdominal	91.7	100.0	66.7	0.013
Ascites	83.3	29.4	0.0	< 0.001
Behavior[1]	41.7	0.0	25.0	0.019
Loss of fur	16.7	41.2	66.7	0.015
Loss of weight (> 10% body weight)	0.0	47.1	79.2	< 0.001
Diarrhea	16.7	29.4	62.5	0.015

[1]Reduced food consumption, isolation, stereotypic movements, apathia; [2]*P* values for combined *vs* single therapy.

Table 2 Histological analysis of the tissue

	Control 1	PTK/ZK	MS-275	PTK/ZK MS-275	PTK/ZK MS-275 TAM cRA
PCNA pos. cells	44.2 ± 12.4	42.7 ± 9.4	43.7± 5.5	35.1 ± 3.6[a]	23.6 ± 3.8[a,c]
TUNEL pos. cells	6.9 ± 4.4	11.8 ± 6.6[a]	12.2 ± 7.7[a]	18.4 ±5.5[c]	24.8 ± 10[b,c]

[a]P < 0.05 *vs* control 1; [b]P < 0.01 *vs* control 1; [c]P = 0.01 dual therapy *vs* quadruple therapy for TdT-mediated dUTP-biotin nick end labeling and proliferating cell nuclear antigen staining. PTK/ZK: PTK787/ZK222584; TAM: Tamoxifen; cRA: 9-cis-retinoic acid; PCNA: Proliferating cell nuclear antigen; TUNEL: TdT-mediated dUTP-biotin nick end labeling.

DISCUSSION

Extensive screening procedures and better imaging techniques have increased the mean survival time of HCC patients[2-4]. The curative or life-prolonging therapeutic options are based on tumor ablation *via* liver transplantation, resection or local instillation of heat or ethanol[3,4]. To date, sorafenib is the only systemic treatment option. Sorafenib acts *via* antiproliferative and antiangiogenic effects and is the proof of concept for systemic treatment in HCC. However, the achieved life-prolonging effect of a few months is a very limited benefit, and a wide range of side effects is induced[7-9]. The majority of patients have advanced-stage tumors and are beyond effective therapy when diagnosed[3,4].

Basic science studies have shown that HCC develops distinct molecular changes and patterns. It has been hypothesized that a couple of mutations leads to a change from cirrhotic nodules to invasive carcinoma. Mutations are observed for the tumor suppressor gene p53 or for members of signal transduction pathways such as insulin receptor substrate-1 or β-catenin[2,20-22]. Different subtypes of HCC have been classified. Each subtype displays distinguishable genetic alterations and characteristics in clinical behavior[20-22].

This may explain why systemic treatment with a single agent fails to be effective for HCC, and why some compounds display their antitumoral efficacy only in specific subgroups of HCCs. Therefore, combination therapy

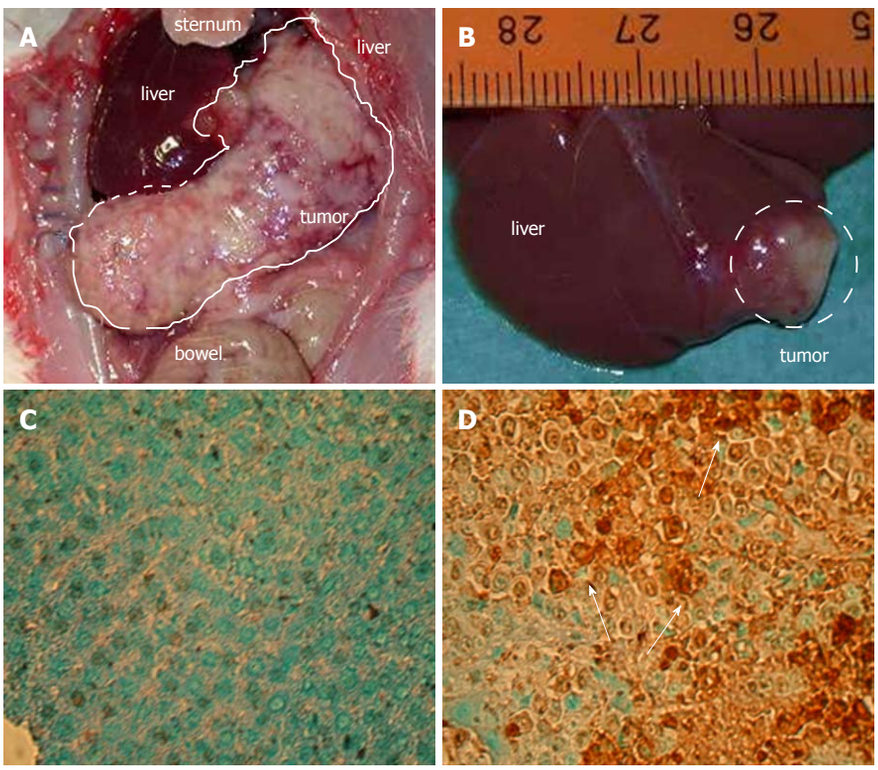

Figure 2 Macroscopic tumor growth and TdT-mediated dUTP-biotin nick end labeling assay. A: Control 1, large tumor volume. B: PTK787/ZK222584 **(PTK/ZK) + MS-275,** small tumor volume. C: Control 1, (mediated dUTP-biotin nick end labeling) TUNEL assay for apoptotic cells (dark brown). D: PTK/ZK + MS-275: TUNEL assay for apoptotic cells (dark brown).

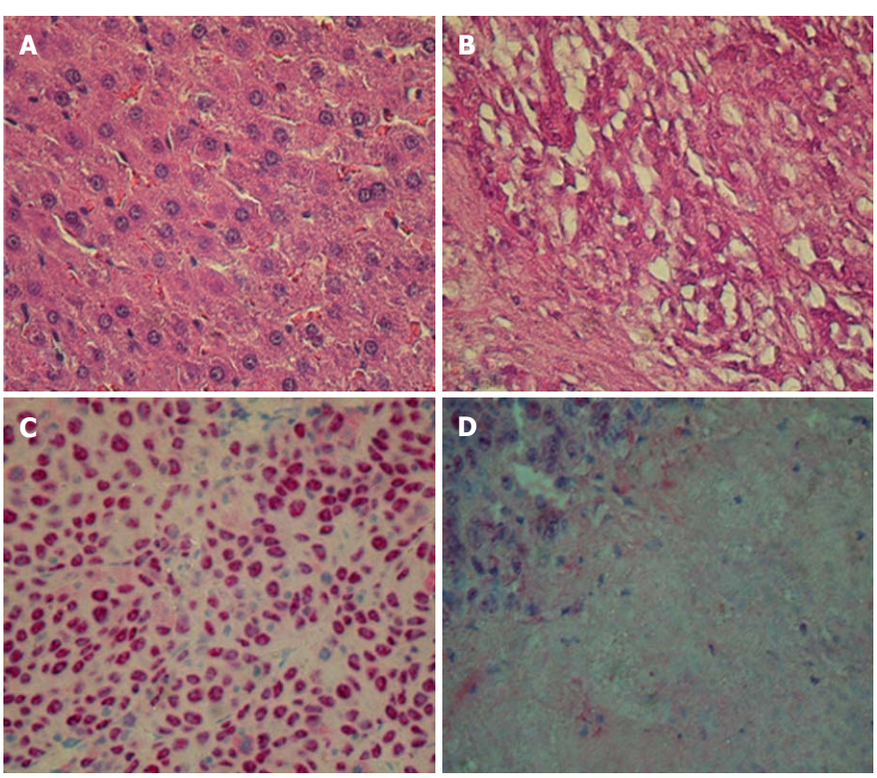

Figure 3 Hematoxylin-eosin and proliferating cell nuclear antigen staining. A: Control 1, hematoxylin-eosin staining. B: Quadruple therapy, disintegrating cells, necrosis. C: Control 1, proliferating cell nuclear antigen (PCNA) staining for proliferating cells. D: Quadruple therapy, PCNA staining, reduced number of cells, and large necrotic area.

should represent a possible treatment option as shown in other malignancies, such as colorectal cancer. We have shown that HDAC inhibitors combined with retinoids or conventional chemotherapeutic agents induce apoptosis and decrease growth of hepatoma cells in an additive manner[5,23]. Furthermore, we have confirmed that retinoids or TAM as monotherapy have no effect *in vivo*, while the combined agents are moderately effective[7].

In the current setting, we evaluated combination therapy in a syngeneic rat hepatoma model. HCC is known to be highly vascularized and to produce a wide range of proangiogenic factors[4,10]. In an experiment by Yao *et al*[24], 70% of resected HCC nodules showed increased expression of VEGF, which correlates with metastasis rate and poor prognosis. Therefore, we chose PTK/ZK, which selectively inhibits the tyrosine kinase domains of VEGF

receptors, platelet-derived growth factor receptors and c-KIT[12,18,24]. PTK/ZK is an accepted antiangiogenic partner in the treatment of colorectal cancer. In the preceding clinical evaluation only minor adverse effects occurred, such as headache, vertigo and arterial hypertension[12].

Here, we observed a remarkable but nonsignificant effect with reduction of tumor burden. As expected, no animal was cured by monotherapy with PTK/ZK. Inhibition of angiogenesis does not reduce the tumor mass completely. Small aggregations of malignant cells can exist without a vessel system[11], therefore, inhibition of angiogenesis can never represent a monotherapeutic option.

As a combination partner we chose MS-275, an HDAC inhibitor. HDAC inhibitors are known to change gene expression *via* hyperacetylation of histones, which are transcription-regulatory intranuclear proteins. Subsequently, upregulation of genes induces growth arrest and cell differentiation and maturation (e.g. p21, transforming growth factor β and gelsolin)[5,14-17]. Therefore, HDAC inhibitors may be of value in antitumoral therapy, as shown *in vitro* and *in vivo*[14]. MS-275 has shown acceptable results in phase I trials and has proceeded to phase II evaluation[16,25].

In the current experimental setting, the single agent MS-275 showed significant antitumoral effects. Combination with PTK/ZK induced an excellent reduction of tumor volume in this aggressive tumor model, which was even highly significant when compared to the effects of the single agents. Histological evaluation showed necrotic areas as a sign of tumor destruction. An unproved explanation could be an increase of toxic radicals and reduced oxygen supplementation. PCNA staining and TUNEL assay confirmed the superiority of dual therapy. This supports the hypothesis that combination therapy exceeds the efficacy of monotherapy significantly and should be further evaluated.

Since HDAC inhibitors are known to interfere with intracellular retinoid and estrogen receptors and to enhance their antiproliferative effects at least *in vitro*[4,5,21], we decided to evaluate triple and quadruple therapy. The combination of PTK/ZK + MS-275 + TAM did not increase the macroscopic antitumoral effect compared to dual therapy.

Quadruple therapy (PTK/ZK + MS-275 + TAM + CRA) induced a slight, but nonsignificant benefit in tumor volume, while the results for PCNA staining and TUNEL assay were enhanced significantly. Additionally, HE staining revealed large necrotic areas in these tumor samples (after quadruple therapy). Similar results have been reported for VEGF and epidermal growth factor inhibition in other tumor entities (e.g. colorectal cancer), which did not reduce the absolute tumor volume, but increased the areas of necrosis within the tumor[11,12]. Unfortunately a 3D analysis of the necrotic tumor regions in untreated controls *vs* animals with single or combined treatment was not done in this study. We can only postulate a similar mechanism and recommend dynamic imaging (magnetic resonance imaging or computed tomography) for the estimation of necrotic *vs* vital tumor regions in future studies. The same goes for the effects on angiogenesis: due to the proven antiangiogenic effect of PTK/ZK, we did not quantify the microvessel density. However, effects of certain histone deacetylases on the extracellular matrix have recently been shown[26]. Therefore, the changes in microvessel density after combination therapy compared to those with PTK/ZK monotherapy would be particularly interesting, and could explain the enhanced effects of combination therapy.

Analysis of the side effects showed diarrhea and loss of fur in animals treated with single agents, which was intensified by combined therapy. Subgroup analysis did not reach significance, but showed fewer side effects for dual therapy compared to triple and quadruple therapy. The observed loss of weight may be explained by diarrhea and the reduced amount of ascites after tumor treatment. Altogether, no single or combined treatment induced unacceptable side effects.

The relatively small number of animals in this study did not allow evaluation of the increased side effect profile *vs* the additional benefit of triple and quadruple therapy. Investigations with a higher number of animals and a longer treatment period are necessary to assess the benefit of this quadruple therapy *vs* dual therapy.

In summary, we showed that combination therapy is superior to monotherapy. At least in this rat model for HCC, PTK/ZK and MS-275 were highly effective, which justifies further investigation. The antitumoral effects were seen by macroscopic evaluation of tumor volume and evaluation of proliferation and apoptotic cells, which was especially marked in relation to decreasing tumor mass. The effects of triple and quadruple therapy need to be analyzed in further experiments. In the next step, the efficacy of dual therapy should be evaluated in different genetic, well-defined hepatoma models, which could possibly provide insight into the triggered pathways. If dual therapy (PTK/ZK and MS-275) is successful in this additional experimental setting, clinical development seems feasible.

COMMENTS

Background

The incidence of hepatocellular carcinoma (HCC) is increasing. Curative and life-prolonging therapeutic options for early tumor stages are resection, transplantation and interventional treatment. Sorafenib is the first systemic treatment option. However, the life-prolonging effect of sorafenib is limited to a few months. Effective systemic treatment for far-advanced hepatoma is still lacking.

Research frontiers

Tumor cells show signs of dedifferentiation, reduced apoptosis, and an increase in proliferation rate. They induce angiogenesis and changes in the extracellular fibers. Biomodulators are directed against these tumor-cell-specific patterns. Histone deacetylase (HDAC) inhibitors change the expression of proliferation and apoptosis-inducing factors, and lead to normalization of protein expression in tumor cells. Vascular endothelial growth factor (VEGF) receptor antagonists reduce tumor-cell-induced neoangiogenesis and destruction of the extracellular matrix. The antitumoral efficacy of these biomodulators, such as HDAC inhibitors and VEGF receptor blockers, can be evaluated *in vitro* and *in vivo*. The authors showed that combination therapy was far superior to monotherapy *in vitro* (pro-

liferation rate, induction of apoptosis). However, to date, there are not sufficient data to prove this principle *in vivo*. We used a syngeneic rat model. Morris hepatoma cells were implanted into the liver. The endpoint was macroscopic tumor growth and microscopic changes in proliferation, apoptosis and chemotaxis.

Innovations and breakthroughs

The authors evaluated monotherapy and combination therapy with four different agents: the HDAC inhibitor MS-275, the VEGF receptor blocker PTK787/ZK222584 (PTK/ZK), tamoxifen and retinoic acid. We reported significant antitumoral efficacy. Combined treatment was superior to the single agents. The side effect profile was acceptable even after combination therapy.

Applications

Combination therapy should be compared to the gold standard sorafenib in an *in vivo* model. The agents have been well described, and the next step could be a phase I trial.

Peer review

In this study, the authors examined the effects of combination therapy using PTK/ZK and MS-275 in a rat HCC model, and showed that combined therapy was highly effective. This study is significant because development of systemic chemotherapy for advanced HCC is an important subject.

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BRIEF ARTICLE

Ketamine and midazolam sedation for pediatric gastrointestinal endoscopy in the Arab world

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Abstract

AIM: To evaluate the safety and effectiveness of intravenous ketamine-midazolam sedation during pediatric endoscopy in the Arab world.

METHODS: A retrospective cohort study of all pediatric endoscopic procedures performed between 2002-2008 at the shared endoscopy suite of King Abdullah University Hospital, Jordan University of Science & Technology, Jordan was conducted. All children were > 1 year old and weighed > 10 kg with American Society of Anesthesiologists class 1 or 2. Analysis was performed in terms of sedation-related complications (desaturation, respiratory distress, apnea, bradycardia, cardiac arrest, emergence reactions), adequacy of sedation, need for sedation reversal, or failure to complete the procedure.

RESULTS: A total of 301 patients (including 160 males) with a mean age of 9.26 years (range, 1-18 years) were included. All were premedicated with atropine; and 79.4% (239/301) had effective and uneventful sedation. And 248 (82.4%) of the 301 patients received a mean dose of 0.16 mg/kg (range, 0.07-0.39) midazolam and 1.06 mg/kg (range, 0.31-2.67) ketamine, respectively within the recommended dosage guidelines. Recommended maximum midazolam dose was exceeded in 17.6% patients [34 female (F):19 male (M), $P = 0.003$] and ketamine in 2.7% (3 M:5 F). Maximum midazolam dose was more likely to be exceeded than ketamine ($P < 0.001$). Desaturation occurred in 37 (12.3%) patients, and was reversible by supplemental oxygen in all except 4 who continue to have desaturation despite supplemental oxygen. Four (1.3%) patients had respiratory distress and 6 (2%) were difficult to sedate and required a 3rd sedative; 12 (4%) required reversal and 7 (2.3%) failed to complete the procedure. None developed apnea, bradycardia, arrest, or emergence reactions.

CONCLUSION: Ketamine-midazolam sedation appears safe and effective for diagnostic pediatric gastrointestinal endoscopy in the Arab world for children aged > 1 year and weighing > 10 kg without co-morbidities.

Key words: Pediatric endoscopy; Sedation; Ketamine; Arab

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INTRODUCTION

Although progress is being made in improving medical services in modern third world countries, limited financial resources are a critical concern. This environment necessitates effective but cost conscious approaches to medical care. Sedation for medical procedures is a potential area for intervention. For example, ketamine-based intravenous sedation has proven effective in suboptimal circumstances, such as the wartime battlefield, avoiding the need for general anesthesia[1-2].

Endoscopic procedures are frequently required for the diagnosis and treatment of gastrointestinal diseases in children. Since such procedures can cause considerable anxiety and distress, many children find the procedures worse than disease itself.

The goal of sedation is to provide a patient who is only lightly sedated, cooperative on demand, free from anxiety and amnesic after the procedure[3]. It must have a rapid onset, short duration of action, and should be safely administered by a non-anesthesiologist without significantly increased risk of potential complications[4]. Unfortunately, there is no ideal sedation protocol for gastrointestinal (GI) endoscopy that is agreed upon by pediatric gastroenterologists as confirmed by a recent survey by the North American Society for Pediatric Gastroenterology, Hepatology and Nutrition (NASPGHAN)[5] and by another survey by the Francophone Pediatric Hepatology, Gastroenterology and Nutrition Group[6]. It appears that there is a wide variety of sedation techniques used by practicing pediatric gastroenterologists.

Although ketamine-based sedation provides many of the desired effects of an ideal sedative, it is not widely used in pediatric gastrointestinal endoscopy. There is limited published research regarding its efficacy and safety, particularly in developing countries. The aim of this study is to assess the safety and efficacy of ketamine + midazolam sedation for GI endoscopy in pediatric patients in Jordan.

MATERIALS AND METHODS

A retrospective cohort study of all pediatric endoscopic procedures done under intravenous sedation by a combination of ketamine and midazolam over a period of six years (August 1st, 2002 - July 31st, 2008) done at the endoscopy suite of King Abdullah University Hospital (affiliated with Jordan University of Science and Technology) in Jordan was conducted.

All procedures were performed in the endoscopy suite which is shared with adult gastroenterologists and pulmonologists and a part of a general university hospital. All procedures were performed by a single pediatric gastroenterologist. A registered nurse provided constant patient monitoring (before, during and after the administration of sedatives) including continuous cardiac monitoring, respiratory rate, pulse oximetry and blood pressure monitoring. During the study period, the same gastroenterologist performed all the procedures, calculated and administered the sedative medications. The operating physician and nursing staff assessed the adequacy of sedation and documented all the above. The operating physician is Pediatric Advance Life Support certified.

Pre-sedation risk assessment included a detailed history and complete physical examination, review of current medications and drug allergies as well as an assessment of the cardiopulmonary status. All patients in the study were at American Society of Anesthesiologists (ASA) class 1 or 2. Procedures in patients with a higher ASA class were excluded from the study and performed under general anesthesia[7,8]. General anesthesia was used in the following groups of patients: (1) children below the age of one year or weighing less than 10 kg; (2) patients underwent the therapeutic endoscopy (e.g. esophageal dilation, variceal treatment and polypectomy) under general anesthesia for their safety, the need to be motionless during the procedure as these procedures are typically associated with more pain and discomfort; (3) patients with known neurologic disorders (seizures), developmental delay (cerebral palsy), and psychiatric disorders (phobia); (4) increased intracranial pressure (hydrocephalus); and (5) patients known to have abnormal anatomy of the upper airways (Pierre Robin sequence).

All patients were premedicated with atropine at a dose of 0.01 mg/kg - 0.02 mg/kg (a minimum dose of 0.1 mg to avoid paradoxical bradycardia, and a maximum dose of 0.4 mg). Standard dosage guidelines for sedatives are: midazolam 0.05 mg/kg - 0.20 mg/kg and ketamine 0.5 mg/kg - 2.0 mg/kg used at each dose[4,8]. Midazolam was always administered first.

In our study, three types of endoscopic procedures were used: upper (U), lower (L), and combined upper and lower (U&L). If an upper and lower endoscopy was performed in the same sedation session, they were counted as a single sedation session. If the same procedure was repeated at a later time, it was counted as a separate sedation session.

Analysis of the data included demographic details (age, gender) weight, procedure (s) performed, doses of each medication/kg body weight, effectiveness of sedation, need for other sedatives, side effects and complications.

Sedation-related complications were defined as a drop in oxygen saturation to equal or less than 94%, respiratory distress (stridor or wheezes), apnea, bradycardia, cardiac arrest and emergence reactions. The operating gastroenterologist and nursing staff assessed the adequacy of sedation; this was defined as lack of agitation, ability to complete the procedure comfortably, and no need to add other sedatives. The need of an antidote for reversal medications was also included.

Table 1 Types of endoscopic procedures

	n (%)	Males	Females	Age range (yr)	Mean age (yr)
Upper (U)	218 (72.4)	106	112	1-18	8.86
Lower (L)	16 (5.3)	14	2	1-16	9.09
U & L	67 (22.3)	40	27	2-18	10.89
Total	301	160	141		

Table 2 Midazolam and ketamine doses

	Midazolam doses			Ketamine doses		
	All patients	Males	Females	All patients	Males	Females
Min dose (mg/kg)	0.07	0.07	0.07	0.31	0.31	0.46
Max dose (mg/kg)	0.39	0.32	0.39	2.67	2.00	2.67
Mean dose (mg/kg)	0.16	0.15	0.16	1.06	1.03	1.08

Table 3 Sedative doses

	Midazolam doses (mg/kg)			Ketamine doses (mg/kg)		
	Min	Max	Average	Min	Max	Average
Upper (U)	0.07	0.39	0.15	0.33	2.67	1.02
Lower (L)	0.07	0.32	0.17	0.60	1.84	1.12
U & L	0.08	0.27	0.18	0.31	2.11	1.17

Table 4 Distribution of patients receiving doses exceeding recommended max dose

Procedure	Midazolam		Ketamine	
	n (%)	% per specific procedure	*n* (%)	% per specific procedure
Upper (U)	30 (57)	14	4 (50)	1.8
Lower (L)	5 (9)	31	0 (0)	0
U & L	18 (34)	27	4 (50)	6
Total	53 (17.6)		8 (2.7)	

Table 5 Sedation failure

Sedative-related complications	*n* (%)
Desaturation < 94% in RA	37 (12.3)
Desaturation < 94% on supplemental O_2	4 (1.3)
Respiratory distress	4 (1.3)
Apnea	0 (0.0)
Bradycardia	0 (0.0)
Cardiac arrest	0 (0.0)
Emergence reaction	0 (0.0)
Difficult to sedate/third medication	6 (2.0)
Need for reversal medications	12 (4.0)
Need for overnight stay	1 (0.3)
Failure to complete the procedure	7 (2.3)

RA: Ruba abdelhadi.

Failure of sedation was defined as: (1) The occurrence of sedative-related complications: (a) Oxygen desaturation < 94%; (b) Respiratory distress wheezes or stridor; (c) Apnea; (d) Bradycardia; (e) Cardiac arrest; and (f) Emergence reactions; (2) Difficult to sedate, as judged by the physician or nursing staff, requiring a third sedative medication; (3) The need for reversal medications; (4) Need for overnight stay because of sedation-related issues; and (5) Failure to complete the procedure.

RESULTS

A total of 560 procedures were performed over the study period (August 1st, 2002 - July 31st, 2008), 12 patients were excluded because of incomplete medical records. Of the 548, 247 were performed under general anesthesia, and 301 were done utilizing ketamine + midazolam. All 301 patients included in the study who had conscious sedation received combined midazolam and ketamine in addition to atropine. The sedatives were given in small boluses and titrated to achieve the desired effect. Not infrequently, patients required extra doses during the procedure; this was especially noted in longer procedures. For dosage calculations, we used the cumulative dose.

There were 160 males and 141 females (1.13:1), age ranged from 1 to 18 years, with a mean age of 9.26 years in males and 10 years in females. Among the three types of endoscopic procedures, upper endoscopy was the most frequently performed procedure (218 patients or 72.4%) followed by combined upper and lower (67 patients or 22.3%). Details are shown in Table 1.

The average dose of midazolam used in all procedures was 0.16 mg/kg, (range, 0.07 mg/kg - 0.39 mg/kg), while the average dose of ketamine was 1.06 mg/kg (range, 0.31 mg/kg - 2.67 mg/kg) (Table 2). There was no statistically significant difference in the average dose used between males and females for either of the two medications.

Analysis of sedative dosage according to the type of procedure is shown in Table 3. In general, patients require similar doses of sedatives regardless of the type of procedure.

Most patients received a dose within the recommended dosage guidelines of both medications (248 patients or 82.4%). The maximum dose for either medication was exceeded in 53 patients (17.6%) (Table 4).

The recommended maximum dose of midazolam was exceeded in 53 (17.6%) patients (19 M:34 F), which was more likely to be exceeded in females ($P = 0.003$). The recommended maximum dose of ketamine was exceeded in only eight patients (2.6% of all patients) (3 M:5 F). The dose of midazolam was more likely to be exceeded than ketamine ($P < 0.001$).

Maximum dose of midazolam was significantly exceeded in combined upper and lower endoscopic procedures when compared to upper endoscopies (27% *vs* 14%; $P = 0.02$). Maximum midazolam dose was also exceeded more in lower endoscopic procedures when compared to upper endoscopies (31% *vs* 14%), but the limited number of lower endoscopic procedures precluded sta-

Table 6 Effects of midazolam and ketamine dosing on development of desaturations

Procedure	Midazolam average dose (mg/kg)			Ketamine average dose (mg/kg)		
	Patients with desaturation	Patients without desaturation	*P* value	Patients with desaturation	Patients without desaturation	*P* value
Upper (U)	0.26	0.14	< 0.001	2.2	0.86	< 0.001
Lower (L)	NA	0.17	NA	NA	1.12	NA
U & L	0.24	0.168	< 0.001	2	1.01	< 0.001
n	37	264	NA	37	264	NA

NA: Not available.

tistical significance ($P = 0.126$). The maximum ketamine dose was also exceeded more in combined upper and lower endoscopies (6% *vs* 1.8%), but was not statistically significant ($P = 0.170$)

Sedation failure

Two hundred and thirty-nine patients (79.4%) had effective and uneventful sedation. Sedation failure is summarized in Table 5.

Sedative-related complications: (1) Desaturation < 94% in room air occurred in 37 (12.3%) patients, (26 U, 11 U&L, none of L). The average doses of both medications were higher than the maximum dose. The patients received a higher dose of both medications in comparison with the patients who did not develop desaturation; the difference was statistically significant (Table 6). Oxygen by nasal cannula was administered to these patients, and normal saturation was achieved in 33/37 (89%). In four patients, the oxygen saturation did not improve and the procedure was terminated and rescheduled under general anesthesia later; and (2) respiratory distress (stridor or wheezes) developed in four patients (1.3%) after termination of procedure. All four were U, one recovered spontaneously while the other three required Albuterol (Ventolin) nebulizer treatment. None of the patients who developed respiratory distress exceeded the recommended dose of either medication, and none of them developed desaturation. No apnea, bradycardia, cardia arrest or emergence reactions occurred in any patient.

Difficult to sedate: As judged by the physician or nursing staff, six patients (2%) (3 M, 3 F) required a third sedative medication and meperidine was given. Five were U&L, and one was L. The maximum dose of midazolam was exceeded in four but none of them exceeded the maximum dose of ketamine.

Need for reversal medications: During recovery, 12 (4.0%) patients were judged to be excessively sedated and required reversal of benzodiazepines using flumazenil (3 U, 8 U&L, 1 L). None of them had desaturation or respiratory distress. The average dose of midazolam in those patients was 0.23 mg/kg (range, 0.18 mg/kg - 0.27 mg/kg). The average dose of ketamine in those patients was 1.4 mg/kg (range, 0.80 mg/kg - 2.11 mg/kg).

Need for overnight stay: One.

Failure to complete the procedure: Seven (2.3%) patients failed to complete the procedure. Four patients had desaturation despite oxygen supplementation and all were U. The other three did not complete the procedure because of lack of cooperation of the patient.

DISCUSSION

Intravenous ketamine + midazolam sedation for gastrointestinal endoscopy is safe and effective in most patients. Routine use of general anesthesia for endoscopic procedures is not necessary, which increases cost and is often not readily available in some developing countries. According to a recent NASPGHAN survey, 23% of the respondents described the difficulties and inconvenience in the process of scheduling a procedure in the operating room[5]. Only half (55%) reported their endoscopy suites with general anesthesia equipment. In developing countries, cost is a detrimental factor. To the best of our knowledge, this is the first study that documents the safety and effectiveness of ketamine + midazolam sedation for pediatric gastrointestinal endoscopy in an Arab country.

Ketamine is a non-barbiturate dissociative agent with a rapid onset of action (peak intravenous concentrations occur within one minute) that induces profound sedation, analgesia and amnesia, with a short duration of action (15-30 min) which is adequate for routine diagnostic endoscopy, allowing fast recovery[9,10]. It induces functional dissociation between the limbic and the cortical systems. This cataleptic state impairs sensory recognition of painful stimuli and memory inducing a state referred to as "dissociative anesthesia"[9]. Protective airway reflexes are maintained during sedation with ketamine, with minimal cardiovascular and respiratory side effects. This paramount advantage over other categories of sedatives (narcotics) lies in maintaining airway reflexes with minimal cardiovascular and respiratory side effects[11].

The high therapeutic index of ketamine makes it useful in children with less predictable response to sedatives[12]. This might explain the low incidence of sedative-related complications in our study.

While midazolam has been used in procedural sedation in children extensively, ketamine has not. Mid-

azolam provides sedation and amnesia but it lacks any analgesic effect. The analgesic properties of small-dose ketamine have been rediscovered. Available data strongly suggest that the preemptive administration of ketamine can have profound effects on postoperative analgesic requirements with minimal risk and side effects[13,14]. The use of ketamine for procedural (endoscopic and other procedures) sedation is increasing in the developed countries[15]. There are only a handful of studies that looked at ketamine's value in the endoscopic sedation in children[13,16] but none of them in developing countries.

A prospective randomized study by Varadarajulu *et al*[17] evaluated the use of ketamine for endoscopic procedures and concluded that ketamine is a useful adjunct to conscious sedation in patients who are difficult to sedate. Its use results in better quality and depth of sedation with shorter recovery than in patients sedated using benzodiazepines and meperidine alone. This was confirmed in our study as patients who did not respond to midazolam + ketamine did not benefit from adding meperidine.

Endoscopy was completed in 97.7% (294/301) of our study patients, confirming that midazolam-ketamine is an effective sedation for such procedure. A third medication was mainly needed in the combined procedures (U&L); the ketamine dose could be increased rather than adding an additional sedative. It may be more prudent to maximize ketamine dose before adding a third medication. For example, five of the six patients who were difficult to sedate by the endoscopist and nursing staff, remained inadequately sedated even after adding a third sedative.

Respiratory distress (stridor or wheezes) was rare (1.3%). This low rate of respiratory distress could be explained by the fact that both ketamine and atropine have a bronchodilator effect which adds another advantage in the pediatric age group where reactive airway disease is common.

Like any other sedatives, the sedative response to ketamine is not uniform and may be unpredictable; hence it is prudent to increase a small dose slowly, tittering to the desired effect (typically horizontal nystagmus). Dosing of ketamine has a wide safety margin. Reported unintentional administration of overdoses (up to ten times that of recommended dosage) has been followed by prolonged but complete recovery[1,12,18,19].

Bradycardia is a known side effect of sedatives and may be further augmented by vagal stimulation during upper endoscopy. The lack of bradycardia in our patients could be related to the fact that all patients received atropine prior to the procedure[20].

One of the major drawbacks of ketamine is the occurrence of emergence reactions (psychological manifestations vary in severity between pleasant dream-like state, vivid imaginary, hallucinations and emergent delirium). None of our patients developed any of these reactions. The explanation for that is probably multifactorial. First, emergence reactions are more common in adults than in children. Second, this phenomenon is more common in patients known to have psychiatric or neurological disorders. In our study, those were excluded and their endoscopies were done under general anesthesia. Third, these reactions are usually more pronounced if ketamine is used alone, in large doses and if rapidly administered[21]. All our patients received a combination of midazolam and ketamine; both medications were given slowly and in small boluses. They were observed for the development of emergence reactions for at least 2 h after the procedure. Our findings are supported by a recent study by Gilger *et al*[9]. In that study, the authors concluded that emergence phenomena were more common in those not receiving ketamine, and suggested that true ketamine-associated emergence phenomena are either rare or that midazolam does reduce the frequency of emergence reactions[9].

The biggest worry of the endoscopist administering ketamine is the lack of an antidote[12]. One reassuring fact about ketamine is its short duration of action of 15-30 min[1]. If a patient develops an unexpected adverse reaction, he can be managed by supportive care until the drug effect wears off. This was supported by our findings among the 301 patients; only one child required an overnight stay for observation due to over-sedation. In this particular patient, the recommended doses of midazolam and ketamine were exceeded and required meperidine.

Midazolam + ketamine is not routinely used in children at KAUH outside the operating room. This "procedural sedation phobia" by non-anesthesiologists is noted among other medical institutions in other parts of the world as there is still significant resistance to pediatric sedation techniques used outside the operating room by non-anesthesiologists , as reported in a recent study by Krauss *et al*[15]. The lack of familiarity with ketamine significantly affects the comfort level of the physicians and nursing staff and brings more hesitation to use it outside the operating room by non-anesthesiologists.

In conclusion, ketamine + midazolam is a safe and effective sedative regimen for diagnostic pediatric GI endoscopy in the Arab world for children over the age of one year and weighing more than 10 kg without comorbidities. Side effects of hypoxia and respiratory distress are uncommon. None of our patients developed a serious complication (apnea, bradycardia, cardiac arrest, or emergence psychosis). Pre-sedation risk assessment and proper patient evaluation and selection are of paramount importance and cannot be over-emphasized to minimize potential complications.

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COMMENTS

Background

Endoscopic procedures are frequently required for the diagnosis and treatment

of gastrointestinal diseases in children. Such procedures can cause considerable anxiety and distress, and many children find the procedures worse than disease itself. Ketamine-based sedation is not widely used in pediatric gastrointestinal endoscopy. There is limited published research regarding its efficacy and safety, particularly in developing countries.

Research frontiers

Ketamine-based intravenous sedation has been studied and proven to be effective when used in the emergency room for children requiring painful interventions.

Innovations and breakthroughs

This research focuses on the endoscopic sedation in a pediatric population using ketamine-based sedation avoiding the need for general anesthesia.

Applications

About 80% of children had effective and uneventful sedation using intravenous midazolam and ketamine. Side effects were uncommon and reversible, and there was no mortality associated with this type of sedation. This should encourage more clinicians to use this type of sedation, thus avoiding general anesthesia for pediatric endoscopic procedures.

Peer review

This research confirms the safety and efficacy of ketamine-based sedation for endoscopic procedures in a third world country.

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BRIEF ARTICLE

IL28B polymorphisms associated with therapy response in Chilean chronic hepatitis C patients

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Abstract

AIM: To analyze the association of three IL28B single nucleotide polymorphisms with response to therapy in Chilean patients infected with hepatitis C virus (HCV).

METHODS: We studied two groups of patients with chronic HCV infection (genotype 1), under standard combined treatment with pegylated interferon plus ribavirin. One group consisted of 50 patients with sustained virological response, whereas the second group consisted of 49 null responders. In order to analyze the IL28B single nucleotide polymorphisms rs12979860, rs12980275 and rs8099917, samples were used for polymerase chain reaction amplification, and the genotyping was performed by restriction fragment length polymorphism.

RESULTS: The IL28B rs12979860 CC, rs12980275 AA and rs8099917 TT genotypes were much more frequently found in patients with sustained virological response compared to null responders (38%, 44% and 50% *vs* 2%, 8.2% and 8.2%, respectively). These differences were highly significant in all three cases ($P < 0.0001$).

CONCLUSION: The three IL28B polymorphisms studied are strongly associated with sustained virological response to therapy in Chilean patients with chronic HCV (genotype 1).

Key words: IL28B; Hepatitis C virus; Chile; Pegylated interferon; Ribavirin

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INTRODUCTION

Chronic infection with hepatitis C virus (HCV) is a global health problem that affects more than 170 million people worldwide, with 3-4 million new cases each year[1]. Most (70%-80%) HCV infections persist, and about 30% of individuals with a persistent infection develop chronic liver diseases, including cirrhosis, and hepatocellular carcinoma[2].

The most effective current standard therapy for chronic HCV infection consists of subcutaneous injections of long-acting pegylated interferon-α (PEG-IFN) plus oral treatment with ribavirin (RIB). This therapy, however, yields a sustained virological response (SVR) in only 40%-50% of patients who are infected with HCV genotype 1, the most common viral genotype[3]. In Chile, HCV genotype 1 is also the most prevalent[4]. Thus, since a significant number of patients will fail to respond, or will experience significant side-effects, the identification of host and viral determinants predicting virologic response is of major interest.

Recently, three independent research groups have reported the results of separate genome-wide association studies (GWAS), supporting the association of SVR in HCV genotype 1 with single nucleotide polymorphisms (SNPs) near the gene region IL28B encoding interferon lambda 3. In the first study, performed with European-American, African-American, and Hispanic individuals, the rs12979860 SNP was most strongly associated with SVR, which is located 3 kilobases upstream of the *IL28B* gene. The minor allele (T) was associated with a lower rate of SVR (26% in those with genotype TT and 79% in those with genotype CC)[5]. In the second study, carried out with 293 Australian patients, a significant association between the SNP rs8099917 and SVR was found. This was further validated by an independent cohort of 555 European individuals. From 392 patients who achieved SVR, 247 (63%) were homozygotes for the allele T, which was significantly higher than genotype GG (SVR of 3.8%)[6]. Similar findings were also reported in a Japanese study. Results of a GWAS showed a significant association between treatment response with two SNPs (rs12980275 and rs8099917), both located in the IL28B gene region, with the latter being the same SNP found by Australian researchers. In this case, for the SNP rs8099917, the G allele was associated with a significantly lower SVR (0% for genotype GG and 78% for genotype TT). For the SNP rs12980275, homozygotes for the allele A had a SVR rate of 85%, which was significantly higher than genotype GG[7].

The aim of this study was to investigate the association between these three IL28B polymorphisms and the virological response in treatment-naïve Chilean patients infected with HCV genotype 1, which is the most prevalent viral isolate within Latin-American populations.

MATERIALS AND METHODS

Patient samples

The present study involved serum samples collected (January 2002-July 2010) at the Clinical Hospital University of Chile (Santiago, Chile) from 99 Chilean patients with chronic HCV infection. Patients who received at least 80% of the recommended dose of PEG-IFNα2a and RIB were considered assessable for response to treatment. We included 50 patients that achieved SVR (defined as an undetectable HCV RNA in serum more than 24 wk after treatment termination), and 49 null responder patients (NR) (defined as those who did not achieve an early virological response, < 2 $\log_{10}$ decrease in viral load at week 12 of treatment). Among these patients, no co-infections with human immunodeficiency virus or hepatitis B virus were included. Both groups of patients were similar in terms of basal viral load, age, gender, and clinical degree of the liver disease. This study was approved by the ethics committee of the Clinical Hospital University of Chile (protocol number 394/10).

Genotyping of SNPs rs12979860, rs12980275, rs8099917, and RFLP analyses

Genomic DNA was prepared from peripheral blood lymphocytes. The rs12979860, rs12980275, and rs8099917 SNPs genotyping was carried out by polymerase chain reaction (PCR), and restriction fragment length polymorphism (RFLP). For rs12979860, oligonucleotide primers were: 5'- AGG GCC CCT AAC CTC TGC ACA GTC T -3' (sense), and 5'- GCT GAG GGA CCG CTA CGT AAG TCA CC -3' (antisense). For rs12980275, primer sequences were: 5'- GAG AGC AAG AGG AGG GAA GGA A -3' (sense), and 5'- GTG TGC CAT TAG CCA GTC AGA T -3' (antisense). For rs8099917, oligonucleotide primers were: 5'- TTC ACC ATC CTC CTC TCA TCC CTC AT -3' (sense) and 5'- TCC TAA ATT GAC GGG CCA TCT GTT TC -3' (antisense). PCR reaction conditions (30 μL) were: initial denaturation at 94 ℃ for 10 min, followed by 40 cycles of: denaturation at 94 ℃ for 1 min, annealing at 58 ℃ for 40 s, and extension at 72 ℃ for 1 min. The PCR product for rs12979860, rs12980275 and rs8099917 was of 403, 441 and 401 base pairs, respectively.

In order to perform RFLP assay for the rs12979860 genotype, 20 μL of amplicons were digested with 5U of *Bst*U I restriction endonuclease (New England Biolabs, MA, United States) at 60 ℃ for 2 h. *Bst*U I digestion of allele CC yields fragments of 184, 105, 89 and 25 base pairs, whereas DNA containing the allele TT polymorphism yields fragments of 184, 130 and 89 base pairs. For the RFLP assay for the rs12980275 genotype, 20 μL of amplicons were digested with 5U of *Bsl* I restriction endonuclease (New England Biolabs, MA, United States) at 55 ℃ for 2 h. *Bsl* I digestion of allele AA yields fragments of 121 and 320 base pairs, whereas DNA containing the allele GG polymorphism yields fragments of 121, 30 and 290 base pairs. For the RFLP assay for the rs8099917 genotype, 20 μL of amplicons were digested with 1U of *Mae* Ⅲ restriction endonuclease (Roche Molecular Systems, Branchburg, NJ, United States) at 55 ℃ for 2 h. *Mae* Ⅲ digestion of allele TT yields fragments of 105, 110 and 186 base pairs, whereas DNA containing the allele GG polymorphism yields fragments of 105, 110, 39 and 147 base pairs. Restriction digestion products for each were separated on agarose gels stained with ethidium bromide for visualization on a UV transilluminator.

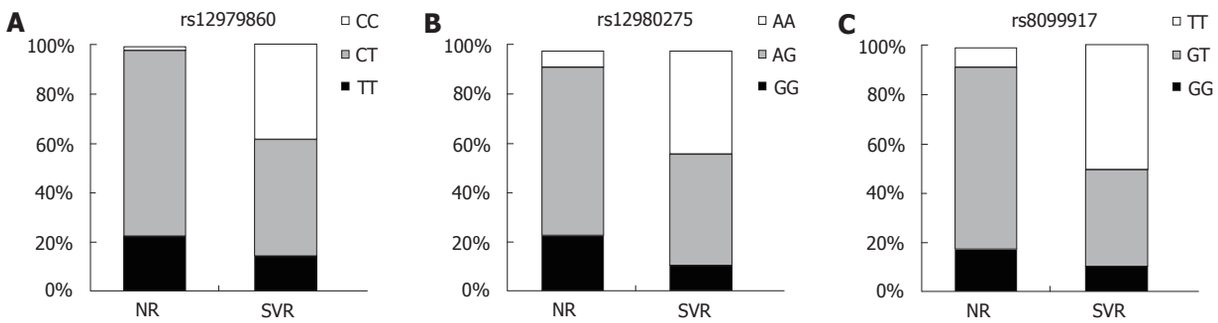

Figure 1 Distribution of *IL28B* single nucleotide polymorphisms by response to combined therapy with pegylated interferon-α plus oral treatment with ribavirin, in Chilean patients with chronic hepatitis C (genotype 1). A: rs12979860 genotype; B: rs12980275 genotype; C: rs8099917 genotype. SVR: Sustained virological response (n = 50); NR: Null responders (n = 49).

Statistical analysis

Genotypic frequencies were obtained by direct counting, and statistical analysis was performed by the χ^2 test [calculated on 2 × 2 contingency tables, assuming a recessive model (CC *vs* CT + TT for rs12979860; AA *vs* AG + GG for rs12980275; TT *vs* GT + GG for rs8099917)]. P values less than 0.05 were considered statistically significant.

RESULTS

In the current study, results from all three recently known *IL28B* polymorphisms influencing the therapy response against HCV, rs12979860, rs12980275 and rs8099917, were available for all Chilean patients with SVR and NR, as shown in Figure 1. For the rs12979860 genotype (Figure 1A), the homozygous CC was found in 19 of 50 patients with SVR, *vs* 1 of 49 in NR patients ($P < 0.0001$). The proportion of patients with the rs12979860 CC, CT and TT genotypes was 38%, 48% and 14%, respectively, in those with SVR. In NR patients, this proportion was 2%, 76% and 22%, respectively. For the rs12980275 genotype, the homozygous AA was found in 22 of 50 cases with SVR, *vs* 4 of 49 in patients NR ($P < 0.0001$). The proportion of patients with the rs12980275 AA, AG and GG genotypes was 44%, 46% and 10% in those with SVR. In NR patients, this proportion was 8.2%, 69.4% and 22.4%, respectively, as indicated in Figure 1B. For the rs8099917 genotype, as shown in Figure 1C, the homozygous TT was found in 25 of 50 patients with SVR, *vs* 4 of 49 in patients NR ($P < 0.0001$). The proportion of patients with rs8099917 TT, GT and GG genotypes was 50%, 40% and 10% in those with SVR. In patients NR, this proportion was 8.2%, 75.5% and 16.3%, respectively.

DISCUSSION

Throughout the results shown herein, we have confirmed that the three recently identified genetic polymorphisms in the interferon λ3 gene region are strongly associated with the response to treatment with PEG-IFN/RIB in Chilean patients infected with HCV genotype 1. Moreover, our current study also represents the first analysis of these SNPs from Latin-American regions, where the genotype 1 of HCV is the most prevalent.

The significant genetic results on common IL28B polymorphisms with respect to treatment response in individuals with chronic hepatitis C infection may open the possibility of a personalized medicine for the treatment of this progressive disease. Further studies are now required to determine whether patients infected with genotype 1 of HCV, and bearing a favorable SNP, will benefit or not from a shorter treatment duration with the current therapy scheme. This might reduce the cost and side effects associated with longer term treatment[8]. The way in which SNP responder genotypes influence the outcomes of anti-viral strategies including those based upon protease and polymerase inhibition, requires immediate investigation. Understanding the clinical implications of these findings will be a major research goal for the immediate future.

COMMENTS

Background

The current standard therapy for chronic **hepatitis C virus (HCV) infection** genotype 1 consists of pegylated interferon alfa plus ribavirin for a period of 48 wk. This regimen, however, yields a sustained virological response in only 40%-50% of patients. **Because a significant number of patients will fail to respond or will** have significant side effects, it is of major interest for both patient care and economic approach to predict non response.

Research frontiers

Recently, several independent research groups have reported results of genome-wide association studies, supporting the association of sustained virological response in HCV genotype 1 with single nucleotide polymorphisms (rs12979860, rs12980275 and rs8099917) near the gene region IL28B, encoding interferon lambda 3.

Innovations and breakthroughs

This study represents the first analysis of these well-know IL28B polymorphisms in patients with chronic hepatitis C infection from Latin-American regions, where genotype 1 is the most commonly found. The report shows that the three IL28B polymorphisms are associated with the sustained virological response in Chilean patients treated with standard therapy.

Applications

This study may contribute to better treatment strategies of hepatitis C. Genotyping of these IL28B polymorphisms will aid clinical decisions, improve current standard of care, and potentially lead to the integration of other agents in the future, providing an opportunity for clinicians to individualize treatment regimens

for hepatitis C patients.

Peer review

The authors investigated the association between genetic IL28B polymorphisms, which encode interferon lambda 3, with the response to the treatment against hepatitis C with standard combined therapy: pegylated interferon-α plus ribavirin. They described that the rs12979860 CC, rs12980275 AA and rs8099917 TT genotypes were much more frequently found in patients with sustained virological response compared to null responder patients. This study may contribute to better treatment strategies for hepatitis C.

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BRIEF ARTICLE

A nested case-control study of maternal-neonatal transmission of hepatitis B virus in a Chinese population

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Abstract

AIM: To examine the determinants of maternal-neonatal transmission of hepatitis B virus (HBV).

METHODS: A nested case-control study was conducted in Changsha, Hunan, People's Republic of China from January 1, 2005 to September 31, 2006. To avoid potential maternal blood contamination, we collected vein blood of newborns immediately after birth and before initial hepatitis B vaccination to determine the HBV infection status of the newborn. For each HBsAg-positive infant, one HBsAg-negative infant born to an HBsAg-positive mother was matched by hospital at birth (same), gender (same), and date of birth (within 1 mo). A face-to-face interview was conducted to collect clinical and epidemiological data. Conditional logistic regression analysis was used to estimate the independent effects of various determinants on maternal-neonatal transmission of HBV.

RESULTS: A total of 141 HBsAg-positive infants and 141 individually matched HBsAg-negative infants were included in the final analysis. Maternal first-degree family history of HBV infection, intrahepatic cholestasis, and premature rupture of membranes were risk factors for perinatal transmission of HBV, whereas systematic treatment and HBV immunoglobulin injections for mothers with HBV infection were protective factors for maternal-neonatal transmission of HBV, after adjustment for potential confounding factors.

CONCLUSION: For HBsAg-positive mothers, systematic treatment, HBV immunoglobulin administration, and controlling intrahepatic cholestasis and pregnancy complications may reduce the incidence of perinatal transmission of HBV.

Key words: HBsAg-positive; Hepatitis B virus; Perinatal transmission: Nested case-control study

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INTRODUCTION

China has a high incidence of hepatitis B infection. The positive rate of serum hepatitis B surface antigen (HBsAg) is about 10%-15%, and accumulated hepatitis B virus (HBV) infection rate is 60%-70% in China[1-3]. The reported positive rate of serum HBsAg in Chinese pregnant women varies from 5.9% to 21.3%[4,5]. Perinatal transmission is the most important vertical transmission route of chronic infection by HBV. About one third of HBV infections are through perinatal transmission, and mostly occur in asymptomatic carriers[6]. There are three ways to realize perinatal transmission of HBV: (1) intrauterine transmission; (2) labor transmission; and (3) postnatal transmission. Intrauterine transmission, including infections that are blood-borne and cell, spread mainly *via* the placenta[7]. This may be because of uplink vaginal infections and other infections. Wang proposed that HBV can be integrated into placental tissue leading to the infection[8]. The mechanisms of intrauterine transmission of HBV are not fully understood. Current theories include infection through the placenta, placental leakage, peripheral blood mononuclear cells, and paternal transmission. In general, pregnant women who are HBV-DNA positive are at increased risk of perinatal transmission of HBV[9-11]. Among HBsAg-positive pregnant women, newborn infection rate in the United States is lower than 15%, while it is higher than 40% in China and Japan[12,13]. If there is TORCH infection, this may result in placental cracks, or placental barrier damage, and therefore the risk of neonatal HBV infection is increased. HIV infection will also increase the risk of HBV infection[14-16]. There is no effective prevention of intrauterine transmission. It remains controversial whether injection of three to four doses of hepatitis B immune globulin (HBIG) can prevent vertical transmission[17,18]. Labor transmission occurs mainly through the HBV contaminated maternal blood, amniotic fluid, and vaginal secretions, which are either swallowed by the fetus or get into the fetal blood circulation by placental rupture[19]. As little as 10^{-8} HBV per mL of contaminated maternal blood entering a fetal body can result in fetal infection[20,21]. A small proportion of perinatal transmission is attributable to postpartum transmission, through HBV contaminated maternal material such as breast milk and saliva. If mothers are positive for HBsAg, HBeAg, and anti-HBc, HBV-DNA can be detected from almost all mothers' breast milk, but if only HBsAg is positive, HBV-DNA can be detected in only 46% of the subjects[22,23].

Since 1992, HBV vaccination for newborn infants has been implemented in China. The vaccination rate in urban areas has reached 90%, and the HBsAg-positive rate in these areas has been reduced to below 1%[24]. However, joint neonatal HBV vaccine and HBIG still have an immunization failure rate of 20%-30% in infants born to HBsAg-positive mothers[4,25]. Wu *et al* found that neonatal T cell function has not yet been fully developed, and newborns have immune tolerance to HBsAg. It is easier for them to become chronic carriers, and the younger the age infected, the higher probability of becoming chronic carriers[26]. It is important to identify the determinants of perinatal transmission of HBV in this era of immunization. Moreover, previous studies in this field have largely relied on cord blood samples to determine HBV infection status of the newborn. False positives may have occurred in the diagnosis of neonatal HBV infection in these studies where cord blood sample was used because contamination from maternal blood cannot be avoided; therefore, the validity of the study findings is compromised. The objective of this study was to assess the determinants of perinatal HBV transmission in a group of Chinese pregnant women with HBV infection, using vein blood of newborns immediately after birth and before initial hepatitis B vaccination to determine the HBV infection status of the newborn.

MATERIALS AND METHODS

This study was conducted in Xiangya Hospital and Xiangya Second Hospital of the Central South University, Yiyang Municipal Hospital, and Yiyang Maternal and Infant Hospital in Hunan, China. This study has been approved by REB of the Central South University.

All consenting HBsAg-positive pregnant women in the participating hospitals with a singleton live-born infant during the period of January 1, 2005 to September 31, 2006 were recruited into the study. Mothers with serious mental illness were excluded.

All HBsAg-positive newborns were selected as cases of the study. For each HBsAg-positive newborn, an HBsAg-negative newborn matched for hospital at birth (same) and gender (same) and date of birth (within 1 mo) was selected as the control. A questionnaire designed specifically for this study was used to collect clinical and epidemiological data, using face-to-face interview with the mother during postpartum hospital stay after childbirth.

Elbow blood of pregnant women prior to delivery and vein blood of newborns immediately after birth and before initial hepatitis B vaccination was taken for laboratory investigations. ELISA was used to detect HBsAg; Test Kits were purchased from the Shanghai Kehua Bioengineering Technology Company, Limited. All laboratory processes were strictly followed according to the instructions provided by the company. A HITACHI 7600-automatic biochemical analyzer was used to test liver functions for HBsAg-positive pregnant women.

We first compared the baseline maternal and infant characteristics between cases and controls. Then we estimated the odds ratios (ORs) and 95% confidence intervals (CIs) of maternal-neonatal transmission of HBV. Conditional logistic regression analysis was used to estimate the independent effects of various determinants on maternal-neonatal transmission of HBV, adjusting simultaneously for several potential confounding factors. Independent variables included in the logistic regression model were maternal education, family income, maternal first-degree family history of HBV infection, liver function, systematic treatment of patients with liver function abnormality, hypertension in pregnancy, intrahepatic cholestasis, premature

Table 1 Comparison of baseline characteristics between cases and controls (Hunan, China, 2005-2006)

Research factor	Cases number (%)	Controls number (%)
Education of mother		
< College	81 (57.45)	57 (40.43)
> College	60 (42.55)	84 (59.57)
Income (yuan/mo)		
< 1500	90 (63.83)	92 (65.25)
> 1500	51 (36.17)	49 (34.75)
First-degree family history		
No	69 (48.94)	107 (75.89)
Yes	72 (51.06)	34 (24.11)
Liver function		
Normal	99 (70.21)	117 (82.98)
Abnormal	42 (29.79)	24 (17.02)
Systematic treatment		
No	119 (84.40)	94 (66.67)
Yes	22 (15.60)	47 (33.33)
EHP		
No	127 (90.07)	133 (94.33)
Yes	14 (9.93)	8 (5.67)
Intrahepatic cholestasis		
No	101 (71.63)	121 (85.82)
Yes	40 (28.37)	20 (14.18)
Premature rupture of membranes		
No	99 (70.21)	119 (84.40)
Yes	42 (29.79)	22 (15.60)
Anti-hepatitis B immunoglobulin injection		
No	100 (70.92)	68 (48.23)
Yes	41 (29.08)	73 (51.77)
Fetal distress		
No	68 (48.23)	91 (64.54)
Yes	73 (51.77)	50 (35.46)

EHP: Edema hypertension proteinuria syndrome.

Table 2 Determinants of perinatal transmission of hepatitis B virus (Hunan, China, 2005-2006)

Research factor	OR (95% CI)	
	Single factors analysis	Adjust
Education of mother		
< College	Reference	Reference
> College	0.50 (0.31-0.81)	1.17 (0.56-2.45)
Income (yuan/mo)		
< 1500	Reference	Reference
> 1500	1.06 (0.65-1.73)	1.16 (0.72-1.87)
First-degree family history		
No	Reference	Reference
Yes	3.28 (1.98-5.46)	2.84 (1.47-5.48)
Liver function		
Normal	Reference	Reference
Abnormal	2.07 (1.17-3.65)	1.11 (0.48-2.55)
Systematic treatment		
No	Reference	Reference
Yes	0.36 (0.21-0.66)	0.36 (0.17-0.76)
EHP		
No	Reference	Reference
Yes	1.83 (0.74-4.52)	0.88 (0.28-2.75)
Intrahepatic cholestasis		
No	Reference	Reference
Yes	2.40 (1.32-4.36)	2.71 (1.01-7.27)
Premature rupture of membranes		
No	Reference	Reference
Yes	2.29 (1.28-4.10)	2.25 (1.08-4.68)
Anti-hepatitis B immunoglobulin		
No	Reference	Reference
Yes	0.38 (0.23-0.62)	0.27 (0.12-0.59)
Fetal distress		
No	Reference	Reference
Yes	1.95 (1.21-3.15)	1.70 (0.93-3.10)

OR: Odds ratios; CI: Confidence intervals; EHP: Edema hypertension proteinuria syndrome.

rupture of membranes, maternal administration of HBIG, and fetal distress. Definition of systematic treatment in this study followed the Chinese national guideline for chronic hepatitis B prevention and treatment, which included using drugs to reduce enzyme levels, to protect the liver, and to enhance immune function in mothers with HBV infection and liver function abnormality[27]. All analyses were performed using Statistical Analysis System, Version 9.1 (SAS Institute Inc., Cary, North Carolina, United States).

RESULTS

A total of 590 HBsAg-positive mothers were recruited into the study, of which 151 HBsAg-positive newborns were defined as cases. Ten cases were excluded because no suitable controls could be identified. A total of 141 HBsAg-positive newborns and 141 individually matched HBsAg-negative newborns were included in the final analysis.

Compared with HBsAg-negative newborns, HBsAg-positive newborns tended to be born to mothers with lower education level, or with abnormal liver function, or with intrahepatic cholestasis, or with premature rupture of membranes, or who less frequently received systematic treatment for abnormalities of liver function, or who were less likely to receive HBIG (Table 1). HBsAg-positive newborns were also more likely to develop fetal distress or to be born from mothers with first-degree family history of HBV (Table 1).

The result of the conditional logistic regression analysis showed that maternal first-degree family history of HBV, intrahepatic cholestasis, and premature rupture of membranes were risk factors for perinatal transmission of HBV, whereas antiviral treatment for maternal HBV and maternal administration of HBIG were protective factors for maternal-neonatal transmission of HBV, after adjustment for potential confounding factors (Table 2).

DISCUSSION

Our nested case-control study, based on 141 pairs of HBsAg-positive and HBsAg-negative infants born to mothers with HBV infection in China, found that maternal first-degree family history of HBV, intrahepatic cholestasis, and premature rupture of membranes were associated with an increased risk of maternal-neonatal

transmission of HBV, whereas systematic treatment for mothers with HBV and maternal HBIG injection at late gestation were associated with decreased risk, after simultaneous adjustment for several potential confounding factors. The main strength of our study is that we used vein blood obtained from the newborns for laboratory tests of markers of HBV infection. Previous studies have largely relied on cord blood samples to determine HBV infection in the newborn. Because contamination from maternal blood cannot be avoided when cord blood samples are used, false positives can occur in the diagnosis of neonatal HBV infection, which therefore will compromise the validity of the study findings.

Our study showed that maternal first-degree family history of HBV was an independent risk factor of perinatal HBV transmission[28]. This may be caused by gene polymorphisms which result in familial aggregation of HBsAg carriers. In order to reduce perinatal HBV transmission, enhanced surveillance and additional interventions may be needed for newborns born to mothers with a first-degree family history of HBV. Intrahepatic cholestasis was an independent risk factor of perinatal transmission of HBV. This finding makes biological sense. When there is an intrahepatic cholestasis in pregnancy, bile salt deposition can cause pathological changes in placental villi, weakening the protective effect of the immune system or causing abnormal immune response[29], which may lead to increased risk of perinatal transmission of HBV. Premature rupture of membranes was associated with increased risk of perinatal transmission of HBV, which was similar to the findings of the study by Yue *et al*[7]. HBV infection of the fetus may happen through HBV contaminated vaginal secretions by premature rupture of membranes. Our results show that systematic treatment of HBsAg-positive mothers whose liver function was abnormal protected their offspring from HBV infection (OR = 0.36), suggesting that active and systematic treatment can improve and stabilize liver function, leading to reduction in perinatal transmission of HBV. Firstly, the risk of perinatal HBV transmission increases as the mother's viral load increases[30]; treatments such as lamivudine can reduce HBV load and thus transmission from mothers to their infants[16,31]. Secondly, improving and stabilizing maternal liver function can also reduce the risk of perinatal HBV transmission[32].

Previous studies have found that HBIG can combine with HBsAg, forming antigen-antibody complexes, and promptly mobilizing the immune system to remove HBV[33]. Our study showed that prenatal injection of HBIG had a strong protective effect on perinatal transmission of HBV (OR = 0.38). The Chinese chronic hepatitis B prevention guidelines published in 2005 do not advocate the use of HBIG for pregnant women in advanced stages of pregnancy to prevent mother-to-infant transmission of HBV[27]. This is contrary to what happens in France, where after 6 mo of pregnancy every pregnant woman must be tested for HBsAg, and HBIG injection is mandated for all HBsAg-positive pregnant women[34].

In summary, our study found that maternal first-degree family history of HBV, intrahepatic cholestasis, and premature rupture of membranes were risk factors for perinatal transmission of HBV, whereas systematic treatment for pregnant women with HBV infection and maternal HBIG administration were protective factors. Except for maternal first-degree family history of HBV, other factors are modifiable, suggesting that there are large areas for improvement in terms of reducing maternal-neonatal transmission of HBV.

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COMMENTS

Background

Hepatitis B is endemic in China and other parts of Asia. Most people in the region become infected with HBV during childhood and perinatal transmission is the most common route of HBV transmission.

Research frontiers

Maternal screening programs and universal vaccination in infants with active and passive immunoprophylaxis have reduced perinatal HBV transmission rates dramatically. However, perinatal transmission may still be occurring despite the use of effective active and passive immunoprophylaxis. More studies are needed to assess the potential risk reduction associated with treatment of high maternal-neonatal transmission during pregnancy.

Innovations and breakthroughs

Previous studies have largely relied on cord blood samples to determine HBV infection in the newborn in which contamination from maternal blood cannot be avoided and false positives can occur. The authors' study used vein blood obtained from the newborns for laboratory tests of markers of HBV infection, and found maternal first-degree family history of HBV infection, intrahepatic cholestasis, and premature rupture of membranes were risk factors for perinatal transmission of HBV, whereas systematic treatment and HBV immunoglobulin injection for mothers with HBV infection were protective factors for maternal-neonatal transmission of HBV.

Applications

According to the findings, the authors suggest that clinicians consider risk factors and protective factors when a pregnant woman's HBsAg test is positive in order to prevent maternal-neonatal transmission of hepatitis B virus.

Terminology

The nested case-control study design is used here. In the nested case-control study, cases of a disease that occur in a defined cohort are identified and, for each, a specified number of matched controls is selected from among those in the cohort who have not developed the disease by the time of disease occurrence in the case.

Peer review

The authors' investigation was to identify the determinants of perinatal transmission of HBV in the era of immunization using venous blood of newborns immediately after birth and before initial hepatitis B vaccination to determine HBV infection status. The study design and methods seem appropriate.

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BRIEF ARTICLE

Potentially predictive microRNAs of gastric cancer with metastasis to lymph node

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Abstract

AIM: To detect the expression of 60 microRNAs (miRNAs) in gastric cancer tissues and find new predictive biomarkers of gastric cancer with metastasis.

METHODS: The expressions of 60 candidate miRNAs in 30 gastric cancer tissues and paired normal tissues were detected by stem-loop real-time reverse transcription-polymerase chain reaction. After primary screening of miRNAs expression, 5 selected miRNAs were further testified in another 22 paired gastric tissues. Based on the expression level of miRNAs and the status of metastasis to lymph node (LN), receiver-operating-characteristic (ROC) curve were used to evaluate their ability in predicting the status of metastasis to LN.

RESULTS: Thirty-eight miRNAs expressions in gastric cancer tissues were significantly different from those in paired normal tissues ($P < 0.01$). Among them, 31 miRNAs were found to be up-expressed in cancer tissues and 1 miRNAs were down-expressed $\geqslant$ 1.5 fold *vs* paired normal gastric tissue. Five microRNAs (miR-125a-3p, miR-133b, miR-143, miR-195 and miR-212) were differently expressed between different metastatic groups in 30 gastric cancer biopsies ($P < 0.05$). Partial correlation analysis showed that hsa-mir-212 and hsa-mir-195 were correlated with the status of metastasis to LN in spite of age, gender, tumor location, tumor size, depth of invasion and cell differentiation. ROC analysis indicated that miR-212 and miR-195 have better sensitivities (84.6% and 69.2%, respectively) and specificities (both 100%) in distinguishing biopsies with metastasis to LN from biopsies without metastasis to LN.

CONCLUSION: miR-212 and miR-195 could be independent biomarkers in predicting the gastric cancer with metastasis to LN.

Key words: MicroRNA; miR-212; MiR-195; Gastric cancer; Metastasis to lymph node

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INTRODUCTION

Gastric cancer is the forth common malignancy and the second cause of death world widely[1]. Although radical gastrectomy with systemic lymph node (LN) dissection has saved many gastric cancer patients, the 5-year survivals are still far satisfactory[2]. Many evidences from large sample studies have shown that LN metastasis, the same to depth of invasion, histological differentiation, distant metastasis and tumor node metastasis stage, is one of the prognostic factors[3-6]. Currently we could get a little information of the status of LN metastasis before the operation while get most information from the histopathological diagnosis after the operation. Whether we could find a new way to predict LN metastasis became a new problem that faced us.

Accumulating evidences demonstrates that gastric cancer is a multigene-related disease with abnormal multistep developing progress of associated oncogenes and tumor suppressor, including various genetic and epigenetic alterations of oncogenes, tumor suppressor genes, DNA repair genes, cell cycle regulators and cell adhesion molecules take part in LN metastasis[7]. Thousands of genes have been reported involved in the process of gastric cancer, such as p53, muc1, cea, E-cadherin, p16 and CD44[8-11]. Large-scale molecular techniques as DNA microarrays have been widely utilized in investigation of the molecular complexity of gastric cancer and prognostic classification based on gene expression profile[12,13]. Recently, development of MicroRNAs (miRNAs) technique may complement and enhance our current understanding of the development and progression of gastric cancer and may evolute our way to predict the status of LN metastasis before the surgical operation.

miRNAs are an abundant class of endogenous noncoding RNAs (about 18~24 nt) that regulate the stability and expression efficiency of target mRNAs at the posttranscription level. By entirely or partially base-pairing to the 3'-untranslated region of target mRNAs, the miRNAs induce translation repression or degradation of the mRNAs[14], which plays a crucial role during various biological progresses, such as cell development, proliferation, differentiation and apoptosis[15]. Hypothetically, miRNAs are contributors to oncogenesis, functioning as tumor suppressors or oncogenes[16]. miRNAs can influence cancer development in many ways, such as the regulation of cell proliferation, cell transformation, cell death, and so forth. Recently studies have shown that some miRNAs were aberrantly expressed in many cancer tissues compared with paired normal tissues, such as the significant down-regulation of mir-143 and mir-145 in colon cancer[17] and up-regulation of mir-21 in breast cancer[18], thus underscoring the tremendous diagnostic and therapeutic potential of miRNAs in cancer. Furthermore, some miRNAs were closely associated with the prognosis of some specific cancers, for example, up-regulated mir-155 is linked to poor survival in pancreatic cancer[19]. A large-scale study of miRNAs in gastric cancer showed that two miRNAs (mir-143 and mir-145) were down-regulated[17,20] and one miRNA mir-27a correlated with LN metastasis[21], which implied that miRNAs were crucial markers for diagnosis and prognosis of cancer[22]. However, whether miRNA could predict the status of gastric cancer with metastasis to LN has not been identified.

In the present study, we first selected 60 miRNAs, which had been reported to participate in the regulation of cell growth, cell proliferation, cell differentiation, cell apoptosis, tumor cell invasion, migration in other cancers (The function of candidate miRNAs is shown in the appendix), as candidate, then, detected the expressions of 60 miRNAs in 30 paired gastric cancer tissues to select target miRNAs which were differently expressed in different status of metastasis to LN. The candidate miRNAs which expressions correlated with metastasis to LN were further testified in another 22 paired gastric tissues, and the correlationships between miRNAs expression and metastasis to LN were analyzed.

MATERIALS AND METHODS

Patients and biopsies

Paired specimens of gastric cancer tissues and corresponding normal gastric tissue (5 cm from cancer lesion without pathologically proven tumor cells), were obtained from patients with gastric cancer who underwent surgical resection at the First Affiliated Hospital of Wenzhou Medical College (Zhejiang Province, China) from December 2008 to April 2009. All the tissues were snap-frozen and stored in liquid nitrogen until total RNA was extracted. The histopathological diagnosis of gastric cancer was made by Department of Pathology, the First Affiliated Hospital of Wenzhou Medical College according to the criteria of the World Health Organization. All the patients did not received radiation therapy or chemotherapy before the surgical operation. Patients' characteristics of clinical-pathologic features were listed in Table 1.

Informed written consent was obtained from each patient and the study was approved by the Human Research Ethics Committee from the First Affiliated Hospital of Wenzhou Medical College.

RNA isolation

Total RNA was extracted from gastric cancer tissues and corresponding normal gastric tissues using Trizol Reagent (Invitrogen Life Technologies, United States) according to the manufacturer's instructions with some modifications. Briefly, the extracted RNAs re-suspended in isopropanol were incubated at -20 °C for at least 2 h (instead of 5 min at room temperature) to enhance precipitation efficiency of low-molecular-weight RNAs. Following a wash with 80% ethanol, RNA was re-suspended in diethylpyrocarbonate (DEPC)-treated water and stored at -80 °C. The concentration and purity of total RNA were qualified by the ultraviolet spectrophotometer at 260 nm and 280 nm. Only the RNA samples with ratio of $A_{260}/A_{280} > 1.8$ were used for the experiment.

Table 1 Clinical-pathological features of 30 biopsies

Clinicopathological variables	*n*
Gender	
Male	16
Female	14
Age	
< 60 yr	14
⩾ 60 yr	16
Tumor location	
Upper third	7
Middle third	16
Lower third	7
Tumor size	
< 5 cm	12
⩾ 5 cm	18
Histological type	
Well and moderately differentiated	11
Poorly differentiated	19
[1]Depth of invasion	
T1	4
T2	8
T3	18
Lymph node involvement	
N0	15
N1	8
N2	7
[1]Tumor node metastasis stage	
Stage Ⅰ	7
Stage Ⅱ	8
Stage Ⅲ	11
Stage Ⅳ	4

[1]According to the tumor node metastasis staging of Union for International Cancer Control.

Quantification of gastric specimen's miRNAs expression

Stem-loop real-time reverse transcriptase polymerase chain reaction (RT-PCR) was used according to Chen *et al*[23], Tang *et al*[24] and Xue *et al*[25], and miRNA hsa-mir-let7a as internal control in this study[26]. Briefly, 4 μg total RNA was reverse transcribed to synthesize cDNA. The 20 μL reverse transcription reaction system includes 4 μL RT Buffer (Toyobo), 0.5 μL RT ACE (Toyobo), 0.5 μL RNase inhibitor (Toyobo), 1 μL dNTP, 4 μg total RNA, 2 μL 1 μmol/μL stem-loop RT specific primer and RNase-free ddH_2O. Four internal controls including U6, let7a, hsa-mir-191 and hsa-mir-103 were reverse transcribed in parallel. The reaction condition was as follows: incubated at 16 ℃ for 30 min, 42 ℃ for 30 min, and 70 ℃ for 15 min finally. The synthesized cDNA was diluted up to 40 μL and preserved at -20 ℃ until use. The qRT-PCR reaction was performed on Applied Biosystems 7500 detection system by a 20 μL reaction system including 10 μL SYBR green real-time PCR Master Mix-plus (Toyobo, Japan), 2 μL Plus solution (Toyobo, Japan), each 2 μL specific Forward Primer and Reverse Primer, 1 μL RT product of total RNA and 3 μL DEPC water. All reactions were triplicate. The reaction was performed at 95 ℃ for 2 min, then followed by 40 amplification cycles of 95 ℃ for 15 s and 60 ℃ for 1 min. Melting curves were generated for each real-time RT-PCR to verify the specificity of each PCR reaction.

Data analysis

The Ct value (threshold cycle) is defined as the fractional cycle number at which the fluorescence passed the fixed threshold. Delta Ct (ΔCt) represent the expression difference between the target miRNA and the normalizer: $\Delta Ct = Ct_{mir} - Ct_{normalizer}$. Then delta delta Ct (ΔΔCt) was calculated using the equation: $\Delta\Delta Ct = \Delta Ct_{cancer\ tissue} - \Delta Ct_{normal\ tissue}$. The normalized miRNA in a sample is $2^{-\Delta\Delta Ct}$. For the matched normal tissue control sample ΔΔCt equal to zero and $2^{-\Delta\Delta Ct}$ equals to one. The expression levels of normalized miRNAs were characterized by their median and range (25th-75th percentile) because they did not fit the Gaussian distribution. Paired sample *t* test was used to evaluate the difference of miRNA expression between GC tissue and paired normal tissue and $P < 0.05$ was considered to have significant difference. Non-parameter tests were used to evaluate the differences of the miRNA expression between different groups: the Wilcoxon test for 2 paired groups (the tumor group and paired normal group) and the Mann-Whitney *U* test for the 2 independent groups. The partial correlation analysis was used to evaluate the relationship between some miRNA expression and the status of LN metastasis eliminating age, gender, tumor location, tumor size, invasion depth and cell differentiation. $P < 0.05$ was considered to be statistically significant. Receiver-operating-characteristic (ROC) curves was used to evaluate the sensitivity and specificity in predicting the LN metastasis based on the miRNA expression. The area under the ROC curve (AUC) and 95 percent confidence intervals were calculated. An AUC with a confidence interval that did not include the 0.5 value was considered that the miRNA had some ability to distinguish between the two groups. All calculations were performed with the software SPSS16.0.

RESULTS

Expression of 60 candidate miRNAs in gastric cancer specimens

The expressions of 60 candidate miRNAs were detected in 30 gastric cancer specimens by SYBR-green-based stem-loop real-time RT-PCR. As shown in Table 2, the expressions of the miRNAs in cancer tissues were very different from those of corresponding normal tissues. The relative expression of 38 miRNAs expressions ($2^{-\Delta\Delta Ct}$) in gastric cancer tissues were significantly different from those in paired normal tissues which set at 1.000 ($P < 0.01$), suggesting that those 38 miRNAs might be involved in the process of gastric tumorigenesis. Among them, 31 miRNAs were found to be up-expressed in cancer tissues and 1 miRNAs were down-expressed ⩾ 1.5 fold *vs* paired normal gastric tissue. Also, 5 miRNAs (hsa-mir-221, hsa-mir-15b, hsa-mir-181b, hsa-mir-199a-3p and hsa-mir-155) were in the highest expression levels, whereas hsa-mir-30b expression was the lowest.

Among the 30 candidate gastric cancer cases, 15 cases

Table 2 The relative expression of 60 candidate microRNAs ($2^{-\Delta\Delta Ct}$) in 30 gastric cancer specimens

MiRNA	Median	Percentile		[1]*P* value
		25%	75%	
Hsa-mir-106b	1.129	0.783	1.982	0.889
Hsa-mir-143	1.231	1.095	1.378	0.779
Hsa-mir-125a-5p	1.255	0.963	1.392	0.889
Hsa-mir-145	1.084	0.931	1.280	1.000
Hsa-mir-25	1.727	1.152	2.103	0.208
Hsa-mir-133b	1.127	0.938	1.314	0.779
Hsa-mir-195	0.989	0.754	1.534	0.889
Hsa-mir-374	1.005	0.800	1.599	0.779
Hsa-mir-451	0.594	0.442	1.636	1.000
Hsa-mir-1	1.228	0.950	2.135	0.327
Hsa-mir-141	0.973	0.663	1.125	0.208
Hsa-mir-200a	0.954	0.757	1.038	0.123
Hsa-mir-29c	0.867	0.664	1.042	0.123
Hsa-mir-29b	1.217	0.964	1.500	0.674
Hsa-mir-30b	0.600	0.542	0.782	0.017
Hsa-mir-26a	1.062	0.787	1.211	0.779
Hsa-mir-26b	1.257	0.838	1.369	0.779
Hsa-mir-144	1.548	1.251	2.118	0.123
Hsa-mir-103	2.103	1.259	2.339	0.050
Hsa-mir-h450b	1.500	1.061	2.601	0.208
Hsa-mir-191	1.770	1.007	2.216	0.161
Hsa-mir-200b	1.314	0.951	1.556	0.401
Hsa-mir-200c	1.151	1.101	1.545	0.161
Hsa-mir-203	1.790	1.464	2.498	0.069
Hsa-mir-429	1.944	1.250	2.028	0.050
Hsa-mir106a	1.279	1.139	1.917	0.069
Hsa-mir-15a	1.782	1.209	1.961	0.093
Hsa-mir-16a	1.145	1.112	1.338	0.017
Hsa-mir-17	1.603	1.396	1.921	0.017
Hsa-mir-155	2.663	1.873	3.557	0.012
Hsa-mir-18a	2.149	2.040	4.214	0.012
Hsa-mir-181b	2.535	2.195	2.635	0.012
Hsa-mir-421	2.141	1.518	2.498	0.012
Hsa-mir-92a	1.848	1.524	2.092	0.012
Hsa-mir-20a	1.596	1.037	2.071	0.036
Hsa-mir-125a-3p	1.729	1.191	2.997	0.036
Hsa-mir-199a-5p	1.645	1.157	2.624	0.069
Hsa-mir-93	2.583	1.250	3.139	0.025
Hsa-mir-222	1.373	1.309	2.488	0.025
Hsa-mir-15b	3.162	1.655	3.821	0.017
Hsa-mir-199a-3p	2.346	1.872	2.779	0.012
Hsa-mir-212	1.543	1.230	2.702	0.050
Hsa-mir-221	2.961	2.440	3.609	0.025
Hsa-mir-147	2.162	1.928	6.019	0.012
Hsa-mir-205	2.008	1.727	15.946	0.017
Hsa-mir-30d	1.614	1.346	2.17	0.017
Hsa-mir-363	1.732	1.612	1.928	0.012
Hsa-mir-23b	1.744	1.339	2.116	0.017
Hsa-mir-214	1.768	1.69	1.902	0.012
Hsa-mir-497	1.520	1.204	2.328	0.017
Hsa-mir-let7c	1.759	1.276	2.300	0.012
Hsa-mir-99a	1.286	1.220	2.734	0.012
Hsa-mir-193b	1.616	1.387	2.554	0.012
Hsa-mir-31	1.749	1.355	3.234	0.012
Hsa-mir-let7b	1.287	1.155	2.071	0.017
Hsa-mir-487b	1.598	1.424	2.855	0.017
Hsa-mir-h450a	1.435	1.116	1.609	0.123
Hsa-mir-18b	1.691	1.413	3.164	0.069
Hsa-mir-19b	1.411	1.248	1.726	0.093
Hsa-mir-19a	1.687	1.209	1.823	0.093
Hsa-mir-let7e	1.195	1.061	1.459	0.123

The relative expression of miRNA ($2^{-\Delta\Delta Ct}$) in tumor samples, with that in nontumor control samples set at 1.000. [1]*P*: The Mann-Whitney *U* test for the different expression of microRNA in different group.

Table 3 Five miRNAs expression in different lymph node metastasis groups

MiRNA	LN Negative (n = 15)	LN Positve (n = 15)	[1]*P* value
miR-125a-3p	1.87 (1.27, 2.32)	0.81 (0.49, 1.15)	0.02
miR-133b	1.31 (1.08, 1.59)	0.74 (0.41, 0.97)	0.04
miR-143	1.38 (1.23, 1.57)	0.57 (0.16, 1.03)	0.04
miR-195	1.53 (1.18, 2.52)	0.46 (0.31, 0.64)	0.02
miR-212	2.70 (2.14, 3.16)	1.06 (0.93, 1.18)	0.02

[1]*P*: The Mann-Whitney *U* test for the different expression of microRNA in different groups. LN: Lymph node.

Table 4 Partial correlation analysis of 5 miRNAs expressions and lymph node metastasis

MicroRNA	Correlation coefficient	*P* value
miR-125a-3p	-0.451	0.091
miR-133b	-0.014	0.961
miR-143	-0.25	0.369
miR-195	-0.57	0.026
miR-212	-0.616	0.014

associated with LN metastasis, whereas 15 did not. Using the Mann-Whitney U test, we found that 5 miRNAs (hsa-mir-125a-3p, hsa-mir-133b, hsa-mir-143, hsa-mir-195 and hsa-mir-212) in gastric cancer patients with LN metastasis were quite different from those in patients without LN metastasis (Table 3). hsa-mir-125a-3p and hsa-mir-212 in patients with LN metastasis were lower than those in patients without LN metastasis while hsa-mir-143 and hsa-mir-195 were higher than those in patients without LN metastasis. But the expressions of the miRNAs did not correlate with age, gender, tumor location, tumor size and depth of invasion (data not shown).

Correlation between the miRNAs expressions and lymph node metastasis of gastric cancer

As the expressions of hsa-mir-125a-3p and hsa-mir-212 in patients with LN metastasis were lower than those in patients without LN metastasis while hsa-mir-143 and hsa-mir-195 were higher than those in patients without LN metastasis, partial correlation analysis for eliminating age, gender, tumor location, tumor size, invasion depth and cell differentiation showed that hsa-mir-212 and hsa-mir-195 were correlated with LN metastasis in spite of the status of cell differentiation (Table 4).

Predicting value based on miRNAs expression in lymph node metastasis

Five candidate miRNAs which correlated with metastasis to LN were selected and re-examined in another 22 gastric cancer biopsies to evaluate the predicting value in metastasis to LN (Figure 1). ROC analysis indicated hsa-mir-212 yielded an AUC of 0.915 (95% CI: 0.790-1.039). At the cutoff value of 1.439, hsa-mir-212 had 84.6% sensitivity and 100% specificity in discriminating gastric cancer biopsies with metastasis to LN. Whereas, hsa-mir-195 yielded AUC of 0.821 (95% CI: 0.634-1.007) with 69.2%

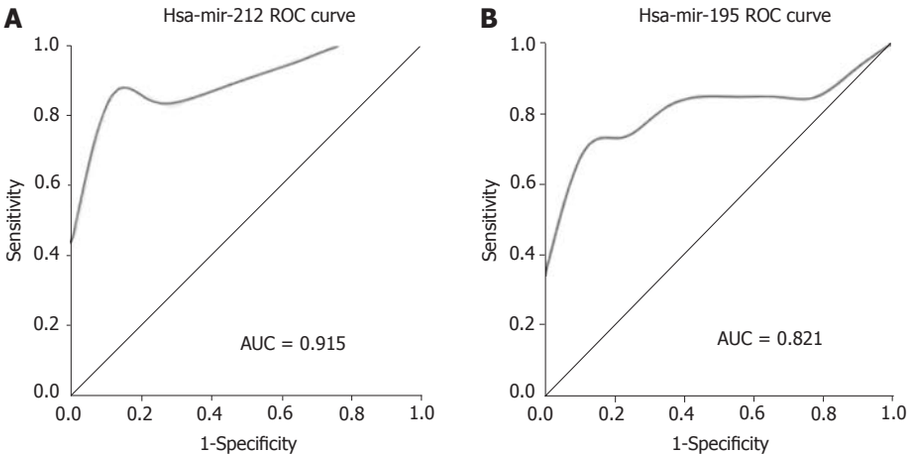

Figure 1 Receiver operating characteristics curve analysis using hsa-mir-212 and hsa-mir-195 for discriminating gastric cancer biopsies with or without metastasis to lymph node. A: Hsa-mir-212 yielded an AUC (the areas under the receiver operating characteristics curve) of 0.915 (95% CI: 0.790-1.039) with 84.6% sensitivity and 100% specificity in discriminating gastric cancer biopsies with metastasis to lymph node (LN); B: Hsa-mir-195 yielded AUC of 0.821 (95% CI: 0.634-1.007) with 69.2% sensitivity and 100% specificity of in discriminating gastric cancer biopsies with metastasis to LN.

sensitivity and 100% specificity of in discriminating gastric cancer biopsies with metastasis to LN.

DISCUSSION

Recently, many researches have demonstrated that miRNAs played an important role as either an oncogene or tumor suppressor gene in the initiation and progression. Though a few studies on miRNAs expression of gastric cancer have been carried out, and aberrant expressions in gastric cancer have been identified. However, few reports had screened the miRNAs expression specifically associated with LN metastasis, which was a prognostic factors to gastric cancer patients. In this study, firstly the expressions of 60 candidate miRNAs were detected in 30 gastric cancer specimens by SYBR-green-based stem-loop real-time RT-PCR, and 38 of 60 miRNAs expressions in gastric cancer tissues were found to be significantly different from those in paired normal tissues ($P < 0.01$). Among them, 31 miRNAs were found to be up-expressed in cancer tissues and 1 miRNA were down-expressed $\geqslant$ 1.5 folds *vs* paired normal gastric tissue, suggesting that abnormal expression of those miRNAs may play a role in the development of gastric tumorigenesis.

The abnormal expressions of some miRNAs had been reported in several cancers, for example, hsa-mir-155 was over-expressed in pancreatic tumor[27], thyroid tumor[28], cervical cancer[29] and the up-regulated expression of hsa-mir-155 was related to a poor prognosis of patients with pancreatic cancer[19]. hsa-mir-15b was up-regulated in pancreatic cancer[30], colorectal cancer[31] and cervical cancer[29]. The highly expressed hsa-mir-221, one member of mir-221/222 cluster, was up-regulated in glioblastoma[32], bladder cancer[33]. Three reports demonstrated that mir-199a was down-regulated in bladder tumor[34], ovarian cancer[35] while up-regulated in hepatoblastoma[36]. hsa-miR-143 and hsa-miR-145 were down-regulated in colon cancer[37] and nasopharyngeal cancer[38] and gastric cancer[17]. hsa-mir-143 and hsa-mir-145 have been identified to have a suppressive effect on cell growth and the reduction in the level of mir-143 and mir-145 positively contributed to the proliferation in gastric cancer cell[17]. hsa-mir-212 was downregulated and repressed growth by targeting methyl-CpG-binding protein MeCP2 in gastric cancer cell line[39]. Therefore, the abnormal expression of these miRNAs may be correlated to the development cancers.

Recently, miR-373 and miR-520c have been reported to be as metastasis-promoting miRNAs that promote tumor invasion and metastasis, whereas miR-335, miR-206, and miR-126 have been as suppressors of breast cancer metastasis[40,41]. The down-regulation of has-mir-195 has been observed in primary peritoneal carcinoma[42] and bladder tumor[34]. Introduction of miR-195 dramatically suppressed the ability of hepatocellular carcinoma and colorectal carcinoma cells to form colonies *in vitro* and to develop tumors in nude mice[43]. However, our data showed that hsa-mir-195 and hsa-mir-212 were down-regulated in gastric cancer with LN metastasis in spite of the status of tumor cell differentiation.

Li *et al*[44] recently reported that seven microRNAs (miR-10b, miR-21, miR-223, miR-338, let-7a, miR-30a-5p, miR-126) as a risk signature was an independent predictor of overall survival and relapse-free survival. Here we reported that the down-regulation of hsa-mir-212 and hsa-mir-195 were not only associated with lymph metastasis for the first time but also had better predicting value in LN metastasis.

Taken together, abnormal expression of miRNAs may be correlated with the development and progression of gastric cancer, the down-regulation of hsa-mir-212 and hsa-mir-195 were correlated with LN metastasis and couuld distinguish patients with LN metastasis from patients without LN metastasis. Further works and large samples of gastric cancer are needed to validate the diagnostic criteria of miRNA for gastric cancer with LN metastasis and identify target mRNAs from candidate.

COMMENTS

Background

Gastric cancer is one common solid tumor world widely. Almost one million people die from it every year. Although patients received radical gastrectomy with systemic lymph node (LN) dissection the 5-year survival is still far satisfactory. Gastric cancer with metastasis to LN is one important prognostic factor. As surgeons could get little information of metastasis to LN before the operation some patients who should receive radical gastrectomy received operation style of endiscopic mucosal reseceion or laparoscopy-assisted gastrectomy. So patients

could benefit from the evaluation of metastasis to LN before surgical operation.

Research frontiers

Accumulating evidence indicate that gastric cancer metastasized to LN is the results of various genetic and epigenetic alternations of oncogens, tumor supressor genes, DNA repair genes, cell cycle regulators and cell adhension molecules. Hundreds of genes have been reported involved in the process of metastasis and this made the study of metastasis a more complex problem. Recently researches have focused their study on the aberrantly expressed microRNA in gastric caner. However, there haven't been a report about the preditive value in evaluating the status of gastric cancer metastasized to LN basing on the expression level of microRNA.

Innovations and breakthroughs

Recent reports have demonstrated many microRNAs were upregulated or downregulated in gastric cancer. This is the first study to report that hsa-mir-212 and hsa-mir-195 had better sensitivity and specificity in distinguishing gastric cancer biopsies with metastasis to LN from gastric cancer biopsies without metastasis to LN.

Applications

By understanding the predictive value of microRNA in evaluating the status of gastric cancer metastasized to LN, this study approached the problem to identify a diagnostic criteria for gastric cancer biopsies with metastasis to LN employing miRNA as biomarkers and help surgeons to evaluate the status of metastasis to LN before surgical operation and choose reasonable operation style for patients.

Terminology

microRNAs are an abundant class of endogenous non-coding RNAs that regulate the stability and expression efficiency of target mRNAs at the post-transcription level. Almost 50% microRNAs located at or near to the fragile site of tumor-associated genes. MicroRNAs which were near to the gene promoting metastasis of gastric cancer should be aberrantly expressed. This signature make it feasible that microRNA could distinguish gastric cancer biopsies with metastasis to LN from gastric cancer biopsies without metastasis to LN.

Peer review

This is a very interesting study in which the investigators approached the problem to identify a diagnostic criteria for gastric cancer biopsies with LN metastasis employing miRNA as biomarkers.

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BRIEF ARTICLE

Two clinically relevant pressures of carbon dioxide pneumoperitoneum cause hepatic injury in a rabbit model

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Abstract

AIM: To observe the hepatic injury induced by carbon dioxide pneumoperitoneum (CDP) in rabbits, compare the effects of low- and high-pressure pneumoperitoneum, and to determine the degree of hepatic injury induced by these two clinically relevant CDP pressures.

METHODS: Thirty healthy male New Zealand rabbits weighing 3.0 to 3.5 kg were randomly divided into three groups (n = 10 for each group) and subjected to the following to CDP pressures: no gas control, 10 mmHg, or 15 mmHg. Histological changes in liver tissues were observed with hematoxylin and eosin staining and transmission electron microscopy. Liver function was evaluated using an automatic biochemical analyzer. Adenine nucleotide translocator (ANT) activity in liver tissue was detected with the atractyloside-inhibitor stop technique. Bax and Bcl-2 expression levels were detected by western blotting.

RESULTS: Liver functions in the 10 mmHg and 15 mmHg experimental groups were significantly disturbed compared with the control group. After CDP, the levels of alanine transaminase and aspartate transaminase were 77.3 ± 14.5 IU/L and 60.1 ± 11.4 IU/L, respectively, in the 10 mmHg experimental group and 165.1 ± 19.4 IU/L and 103.8 ± 12.3 IU/L, respectively, in the 15 mmHg experimental group, which were all higher than those of the control group (P < 0.05). There was no difference in pre-albumin concentration between the 10 mmHg experimental group and the control group, but the pre-albumin level of the 15 mmHg experimental group was significantly lower than that of the control group (P < 0.05). No significant differences were observed in the levels of total bilirubin or albumin among the three groups. After 30 and 60 min of CDP, pH was reduced (P < 0.05) and $PaCO_2$ was elevated (P < 0.05) in the 10 mmHg group compared with controls, and these changes were more pronounced in the 15 mmHg group. Hematoxylin and eosin staining showed no significant change in liver morphology, except for mild hyperemia in the two experimental groups. Transmission electron microscopy showed mild mitochondrial swelling in hepatocytes of the 10 mmHg group, and this was more pronounced in the 15 mmHg group. No significant difference in ANT levels was found between the control and 10 mmHg groups. However, ANT concentration was significantly lower in the 15 mmHg group compared with the control group. The expression of hepatic Bax was significantly increased in the two experimental groups compared with the controls, but there were no differences in Bcl-2 levels among the three groups. Twelve hours after CDP induction, the expression of hepatic Bax was more significant in the 15 mmHg group than in the 10 mmHg group.

CONCLUSION: A CDP pressure of 15 mmHg caused more substantial hepatic injury, such as increased levels of acidosis, mitochondrial damage, and apoptosis;

therefore, 10 mmHg CDP is preferable for laparoscopic operations.

Key words: Carbon dioxide pneumoperitoneum; Hepatic injury; Rabbit; Mitochondria; Apoptosis

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INTRODUCTION

Developments in technology and medicine and improvements in anesthetic and surgical techniques have led to the extensive use of laparoscopic procedures for different patient groups, including high-risk patients[1-4]. The advantages of laparoscopic surgery compared with open surgery include a shorter hospital stay, early return to work, and decreased cost. Other advantages of laparoscopic surgery include less perioperative blood loss, a reduction in, or absence of, postoperative pain, and better cosmetic healing[5].

The creation and maintenance of a pneumoperitoneum to create space for dissection is one of the basic requirements for laparoscopic procedures, and carbon dioxide is the most commonly used gas for inducing a pneumoperitoneum. However, insufflation of carbon dioxide into the abdominal cavity for a short time may significantly and adversely impact respiration, circulation, and the acid-base balance in patients because of carbon dioxide absorption and persistently high intra-abdominal pressure[6-7]. Increased intra-abdominal pressure has some potential side effects, such as impairments of liver, kidney, and heart functions. The severity of such impairments is directly related to the degree of intra-abdominal pressure. Uncomplicated laparoscopic cholecystectomy can be performed reasonably safely with a low-pressure pneumoperitoneum. However, if available space is needed for extended resections or complicated reconstructive operations, such as laparoscopic colorectal surgery, a high-pressure pneumoperitoneum is induced. To maintain sufficient intra-abdominal space for surgical procedures, 15 mmHg carbon dioxide pneumoperitoneum (CDP) is routinely used instead of 10 mmHg to 12 mmHg. An increasing number of cases presenting hepatic injury after laparoscopic surgery have been reported, but the number of studies assessing this complication under clinical levels of intra-abdominal pressure is very limited[8-14]. Therefore, in this study we investigated hepatic injury in response to these two clinically relevant levels of intra-abdominal pressure to investigate the safety of CDP.

MATERIALS AND METHODS

Animals

Thirty healthy male New Zealand rabbits weighing 3.0 to 3.5 kg were randomly divided into three groups (n = 10 in each group) and submitted to different CDP pressures: a control group (no gas), 10 mmHg group (carbon dioxide pressure was 10 mmHg), and 15 mmHg group (carbon dioxide pressure was 15 mmHg). Rabbits were given no water or food for 8 h prior to the experiments. They received 1 mg/kg midazolam and 20 mg/kg ketamine before surgery. A tracheal incision was made, and a 4.5 F canal was inserted for mechanical ventilation. The tidal volume was maintained at 10 mL/kg at a frequency of 30 times per minute. Anesthesia was sustained with injections of ketamine (5 μg/kg per minute) and vecuronium bromide (0.1 mg/kg per minute). All chemical treatments were halted 10 min before CDP induction. The rabbits in the experimental groups underwent CDP for 1 h. Mechanical ventilation was stopped once the rabbits recovered from CDP. All operations were approved by the animal care guidelines of the General Hospital of Chengdu Military Command.

Reagents

^{3}H-ADP and atractyloside were obtained from Sigma Corporation (United States). Rabbit anti-Bax polyclonal IgG and anti-Bcl-2 polyclonal IgG were purchased from Santa Cruz Biotechnology (United States). The goat anti-rabbit horseradish peroxidase-conjugated antibody was purchased from Zhongshan Golden Bridge Biotechnology Co. (China).

Liver function assay

Blood samples were collected from ear-edge veins 12 h after the commencement of CDP and allowed to clot, and sera were isolated by centrifugation at 1000 r/min for 10 min and stored at -20℃ until the assay. Serum levels of alanine transaminase (ALT), aspartate transaminase (AST), and albumin were determined by routine laboratory methods using a Hitachi Automatic Analyzer (Hitachi, Inc., Japan).

Blood gas analysis

Femoral artery blood samples were collected at 0 min, 30 min, 60 min, and 12 h after the CDP operation and analyzed using a blood gas analyzer (AVL 995). When the samples were collected at 0 min, 30 min, and 60 min, the rabbits underwent mechanical ventilation.

Histological examinations of liver tissue

Liver biopsies were collected 12 h after the beginning of CDP, and the specimens were fixed in 10% formalde-

Table 1 Changes in liver function (mean ± SD)

Group	TB	ALT	AST	A	Pre-A
Control	1.5 ± 0.3	52.4 ± 9.6	41.0 ± 9.1	32.6 ± 2.1	154.5 ± 17.7
10 mmHg	1.6 ± 0.3	77.3 ± 14.5[a]	60.1 ± 11.4[a]	31.7 ± 2.0	146.9 ± 15.7
15 mmHg	1.7 ± 0.5	165.1 ± 19.4[a]	103.8 ± 12.3[a]	30.5 ± 1.5	118.0 ± 14.9[a]

[a]$P < 0.05$ *vs* control group. TB: Total bilirubin; ALT: Alanine transaminase; AST: Aspartate transaminase; A: Albumin; Pre-A: Pre-albumin.

hyde for 12 h to 24 h, embedded in paraffin, sliced into 5-μm-thick sections, and stained with hematoxylin and eosin. Histological changes in the liver tissues were observed using a micrographic system (Olympus).

Transmission electron microscopy

Liver tissues were fixed using 3% glutaraldehyde, post-fixed in 1% osmium tetroxide in 0.1 mol/L cacodylate buffer, dehydrated with acetone, and embedded in EPON 812. After location by semi-thin sectioning, the samples were sectioned to a thickness of 50-80 nm and poststained with 2% aqueous uranyl acetate. All samples were examined and photographed by transmission electron microscopy (PHILIPS TECNAI 10, Netherlands) at an accelerating voltage of 100 kV.

Atractyloside-inhibitor block technique

Mitochondria in the liver tissues were isolated by centrifugation. The activity of adenine nucleotide translocator (ANT) in the liver tissue was detected using the atractyloside-inhibitor stop technique. Mitochondrial function was initiated by adding ^{3}H-ADP and terminated after 12 s by adding adriamycin. The radioactivity in each group was measured, and ANT activity was expressed as 10^{-9} mol/min per g protein.

Western blotting assay

Liver tissue samples (100 mg) were homogenized in a liquid nitrogen-cooled grinding bowl and lysed in cold RIPA buffer (25 mmol/L Tris-HCl pH 7.6, 150 mmol/L NaCl, 1% NP-40, 1% sodium deoxycholate, and 0.1% SDS) supplemented with Halt™ Protease Inhibitor Cocktail. Whole cell lysates were obtained by subsequent centrifugation at 15 000 *g* for 10 min at 4℃. Protein concentrations were determined using a Bradford Protein Assay Kit with bovine serum albumin as a standard. Protein extracts (40 μg) were subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to a Protran nitrocellulose membrane. This membrane was incubated with rabbit anti-Bax polyclonal antibody or rabbit anti-Bcl-2 polyclonal antibody at 4℃ overnight after being blocked with a 10% bovine serum albumin solution. The membrane was washed with TBST buffer (20 mmol/L Tris-HCl pH 7.4, 150 mmol/L NaCl, and 0.1% Tween-20), incubated with a secondary goat anti-rabbit horseradish peroxidase-conjugated antibody for 2 h at room temperature, and finally detected with a DAB Kit. Beta-actin was used as an internal control for data analysis.

Statistical analysis

All experimental data are expressed as the means ± SD and were analyzed by a *t*-test using SPSS 10.0 statistical software. Probability values of < 0.05 were considered to be statistically significant.

RESULTS

CDP operation disturbs liver function

Liver function in both CDP groups was disturbed compared with the control group (Table 1). After the CDP operation, the ALT and AST levels were 77.3 ± 14.5 and 60.1 ± 11.4 IU/L, respectively, in the 10 mmHg group and 165.1 ± 19.4 and 103.8 ± 12.3 IU/L, respectively, in the 15 mmHg group; each of these were higher than the control group ($P < 0.05$). Compared with the control group, there was no significant difference in the serum concentration of pre-albumin in the 10 mmHg group; however, it was significantly lower in the 15 mmHg group compared to the control group ($P < 0.05$). No significant difference was observed in the levels of total bilirubin or albumin among the three groups.

Blood gas analysis

Blood pH in the 10 mmHg group was significantly decreased compared with the control group at 30 min and 60 min after CDP induction (Figure 1A), and $PaCO_2$ was significantly increased (Figure 1B). Blood pH and $PaCO_2$ levels were much higher in the 15 mmHg group than in the controls at these time points (Figure 1A and B). However, there were no significant differences in PaO_2, SpO_2, or pH levels for the experimental groups at 12 h post-CDP induction compared with the control group (Figures 1C and D).

Histological changes in liver tissue

Hematoxylin and eosin staining and transmission electron microscopic images were analyzed for each group. The morphological changes of liver tissues in the 10 mmHg and 15 mmHg groups were similar and included mild hyperemia and mitochondrial swelling (Figures 2B, C, E and F). The hyperemia was more severe in the 15 mmHg group than in the 10 mmHg group, and the mitochondrial swelling was more apparent in the 15 mmHg group. In addition, the rough endoplasmic reticulum was slightly expanded in cells of the 15 mmHg group (Figure 2F).

ANT Activity in liver mitochondria

In the control group, ANT activity was 10.83 ± 1.11 (10^{-9} mol ADP/min per g protein), while the activity of ANT was 9.03 ± 0.89 in the 10 mmHg group; there was no significant difference between these two groups. In the 15 mmHg group, ANT activity was only 6.64 ± 0.77, which was significantly lower than the control group ($P < 0.05$), indicating that the energy metabolism of liver mitochondria was damaged by CDP (Table 2).

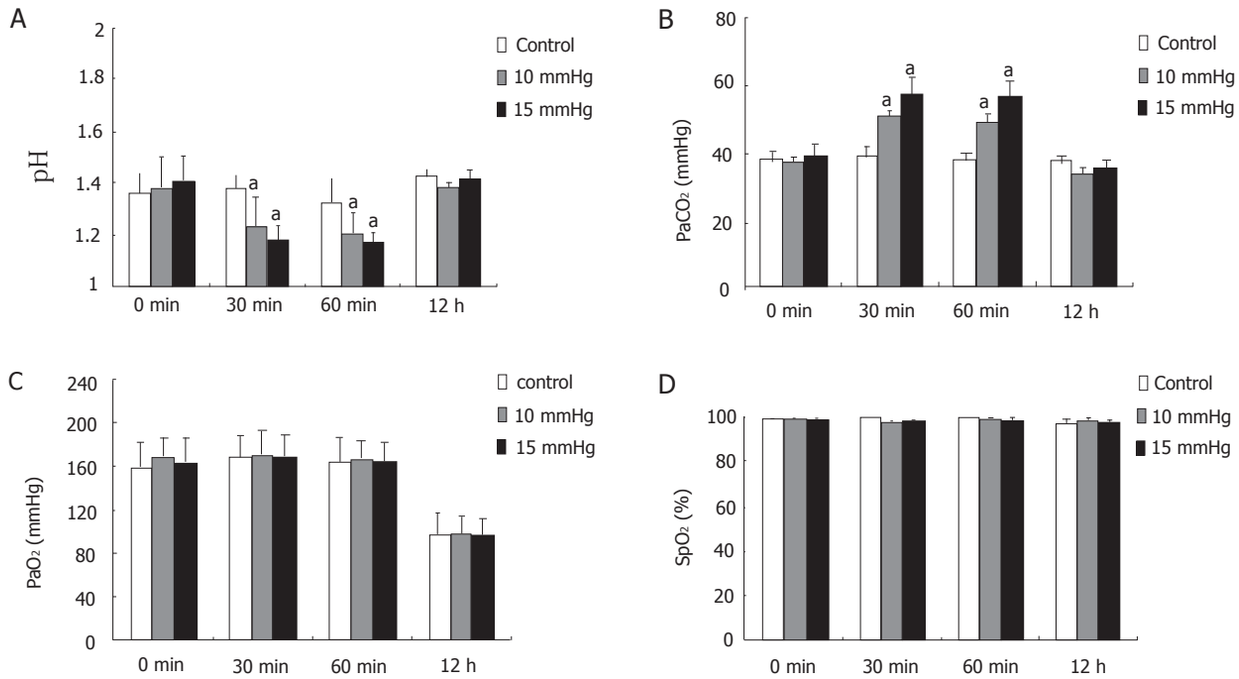

Figure 1 Arterial blood gas analysis. Data are presented as the mean ± SE (n = 10). $^{a}P < 0.05$ *vs* control.

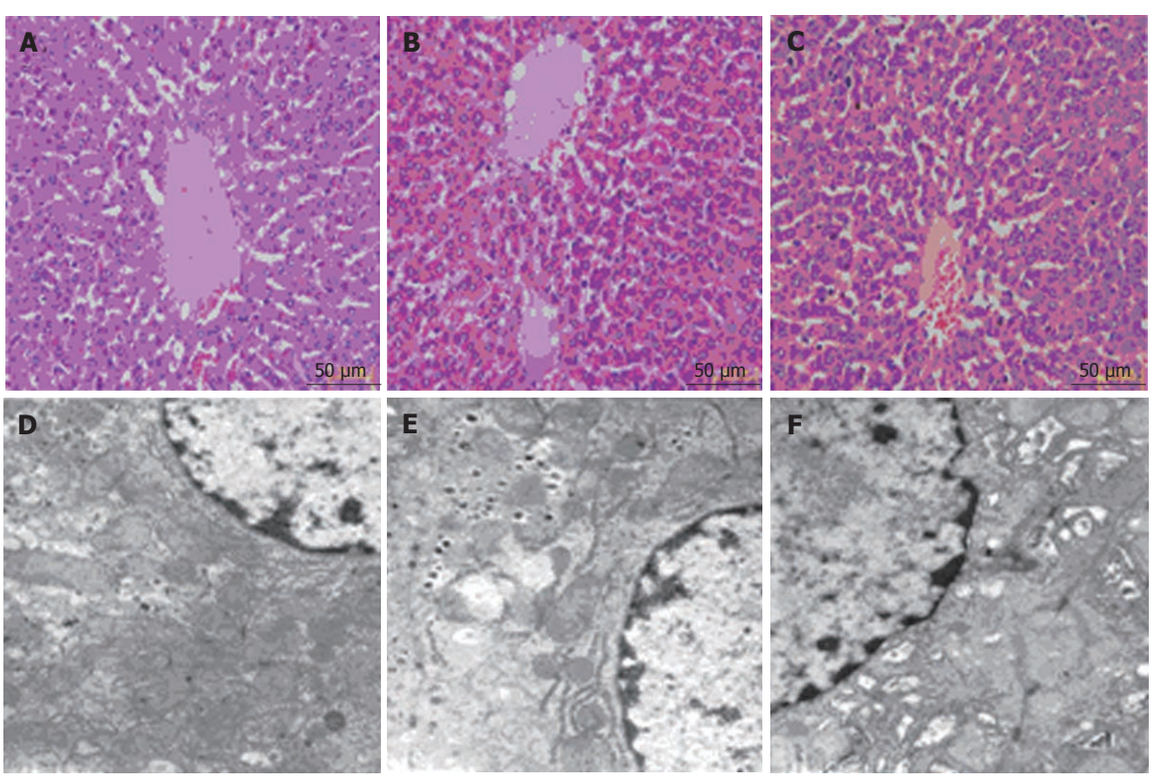

Figure 2 Histological changes in liver tissue. A-C: Hematoxylin eosin observation;D-F: Transmission electron microscopic observation. A, D: The normal structure of hepatocytes and mitochondria in the control group; B, E: Mild hyperemia in liver tissue and mitochondrial swelling were observed in the 10 mmHg group. C, F: The hyperemia was more serious: Mitochondrial swelling and expanded rough endoplasmic reticulum were observed in the 15 mmHg group.

Expression of Bax and Bcl-2 in liver tissue

Bax and Bcl-2 protein levels were analyzed by western blot assay. The expression of Bax was significantly elevated in the 10 mmHg and 15 mmHg groups, but there was no significant change in Bcl-2 expression among the three groups. Compared with the 10 mmHg group, hepatic Bax expression in the 15 mmHg group was more significantly increased 12 h after the initiation of CDP (Figure 3).

Table 2 Activity of adenine nucleotide translocator in liver mitochondria (mean ± SD)

Group	ANT (9-10 mol/min per g protein)
Control	10.83 ± 1.11
10 mmHg	9.03 ± 0.89
15 mmHg	6.64 ± 0.77[a]

[a]$P < 0.05$ *vs* control group; ANT: Adenine nucleotide translocator.

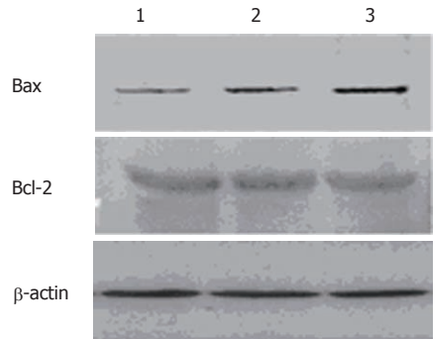

Figure 3 Expression of Bax and Bcl-2 in rabbit liver tissues. 1: Control group; 2: 10 mmHg group; 3: 15 mmHg group.

DISCUSSION

Laparoscopic procedures are favored by both surgeons and patients because of convenience, the minor degree of trauma, rapid healing, and a good cosmetic prognosis[5]. A pneumoperitoneum is a necessary requirement during laparoscopic operations, and carbon dioxide is the most frequently used gas to create a pneumoperitoneum. Previous studies have noted that the increased abdominal pressure caused by CDP in a short time span can impact the hemodynamics, hemoperfusion, and function of critical abdominal organs, such as the liver[15-17]. Technical advancements have led to laparoscopic surgery becoming more complicated, and a lower-pressure CDP (e.g., 10-12 mmHg) is no longer sufficient. To create a sufficient operation space and decrease complications during laparoscopic procedures, many surgeons have increased the CDP abdominal pressure to 15 mmHg. However, the safety and effects of this pressure on abdominal organs have not been fully elucidated. The liver is one of the most important abdominal organs, and it is quite sensitive to harm. Therefore, in this study we investigated the effects of two clinically relevant CDP pressures on liver function, hepatocyte morphology, and protein expression.

Serum ALT and AST are two most commonly used markers of hepatocyte damage. In our study, a 1-h CDP operation resulted in hepatocyte injury at both 10 mmHg and 15 mmHg CDP. Increased ALT and AST levels were observed, suggesting that hepatocytes were damaged by both carbon dioxide pressures. More pronounced changes were detected in the 15 mmHg CDP group, indicating a more severe level of hepatocyte injury. Albumin and pre-albumin levels are markers of hepatocyte protein synthesis. Our data show that 10 mmHg CDP did not impact serum albumin levels, suggesting that 10 mmHg does not affect the rate of hepatocyte protein synthesis, despite its influence on ALT and AST levels. However, 15 mmHg CDP resulted in a decrease in pre-albumin, indicating that this CDP pressure could disturb hepatocyte activity as well as liver function. No significant changes in albumin levels were observed in the two CDP groups compared with the control group. The relatively long half-life of albumin might have been responsible for this phenomenon. In this experiment, we only measured albumin levels up to 12 h after the operation, which is quite short given the 14-d half-life of albumin.

The mechanisms underlying the influence of CDP on liver function might be related to hemodynamic changes and imbalanced acid-base levels. Many studies have shown diminished portal venous flow during increased intra-abdominal pressures, which possibly leads to decreased liver blood supply and impaired hepatic function[9,15,18-21]. Hepatic perfusion is characterized by a unique autoregulatory mechanism known as the hepatic arterial buffer response. Under physiological and pathological conditions, alterations in portal venous flow are counteracted by changes in hepatic arterial flow, thereby maintaining total hepatic blood flow and preserving a sufficient supply of oxygen to the liver[22,25,26]. However, several studies have demonstrated that during CDP, hepatic arterial blood flow does not compensate for the reduction in portal venous inflow[9]. Furthermore, there is a linear relationship between intra-abdominal pressure and portal venous pressure as well as a reciprocal correlation between increased intra-abdominal pressure and decreased portal venous flow[9,20,21,27]. In this study, CDP resulted in increased $PaCO_2$ levels and decreased blood pH, and this effect was more pronounced at 15 mmHg than at 10 mmHg. These results indicate that increased abdominal pressure leads to more severe acidosis. These changes are thought to result from the absorption of insufflated carbon dioxide or ventilation-perfusion mismatching during the procedure[28,29]. Absorption of carbon dioxide through the peritoneum may result in an accumulation of carbon dioxide and subsequent acidosis. However, increased abdominal pressure during CDP could reduce abdominal blood flow and result in local hypoxia, which is another cause of acidosis[21,30]. Some researchers have adopted this view, which has been further discussed in studies demonstrating splanchnic hypoperfusion, regardless of whether intra-abdominal pressure was increased without the use of gas[20,21] or insufflation of CO_2, N_2O, helium, or argon was used to induce pneumoperitoneum[17,19,31-33]. Neither PaO_2 nor SpO_2 was affected by the two CDP pressures, possibly because of the use of intermittent positive-pressure mechanical ventilation.

In addition to influencing hemodynamics and the acid-base balance, CDP operations resulted in changes in hepatocyte morphology. Neither pressure led to ap-

parent tissue damage (based on hematoxylin and eosin staining), but both pressures resulted in mild liver hyperemia, the severity of which was related to CDP pressure. CDP also impacted hepatocyte ultrastructure, including mitochondrial swelling and expanded rough endoplasmic reticulum. The activity of ANT, a marker of mitochondrial energy metabolism[34], was reduced after 15 mmHg CDP, but not by 10 mmHg CDP. This suggests that a relatively lower pressure might not impact hepatocyte energy metabolism, despite mitochondrial swelling.

Bcl-2 and Bax are 2 important apoptotic regulatory genes. They are widely distributed in tissues and cells, and they coordinate with each other to regulate apoptosis. When Bax expression is upregulated, Bax/Bax homodimers are formed to induce apoptosis. However, increased Bcl-2 expression results in isodimers of Bcl-2 and Bax that inhibit apoptosis[35]. In this study, neither 10 mmHg nor 15 mmHg CDP resulted in increased Bcl-2 expression. However, both pressures led to elevated Bax expression, especially in the 15 mmHg group, suggesting that CDP procedures promote hepatocyte apoptosis in a pressure-dependent manner. Elevated levels of hepatocyte apoptosis might be responsible for the disturbed liver function caused by CDP.

To summarize, we investigated the presence and mechanisms underlying liver damage caused by two clinically relevant CDP pressures. Liver injury has been shown to be pressure-dependent[11]. Although a relatively high CDP pressure is required for laparoscopic procedures, such as the 15 mmHg pressure used in this study, we must bear in mind that this pressure can cause serious damage to liver function. The liver damage resulting from CDP may not cause severe complications, but the potential for such damage in patients with liver diseases is of particular importance. Some reports have shown that a shorter duration of carbon dioxide pressure during pneumoperitoneum might help to alleviate liver injury[8]. Stepwise increases in carbon dioxide insufflation might also be an ischemic preconditioning method to reduce liver injury[36]. Future studies are needed to elucidate the mechanisms underlying CDP-induced liver function damage, and the safety of CDP under different surgical conditions should be carefully evaluated[1-4,37].

COMMENTS

Background

Laparoscopic surgery is widely used, and the traditional low carbon dioxide pneumoperitoneum (CDP) pressure no longer meets the requirements of complicated operations. The safety of high CDP pressure in clinical practice is the subject of much attention.

Research frontiers

Some studies have shown that liver injury is pressure-dependant, and hepatocyte apoptosis was observed in the present study.

Innovations and breakthroughs

The liver is a critical organ that is sensitive to many harmful factors. Liver changes during CDP operation were evaluated in this study by assaying several different markers. The data suggest that the different pressures cause hepatic injury.

Applications

As indicated by the experimental data, although higher pressure provides more space for CDP operation, the resulting hepatic injury must be considered. Therefore, the appropriate CDP pressure should be carefully chosen for laparoscopy.

Terminology

CDP is the abdominal space created by insufflating carbon dioxide to provide operation space for laparoscopy.

Peer review

This is an interesting study even if on a well studied subject.

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CASE REPORT

A giant gas-filled abdominal mass in an elderly female: A case report

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Abstract

We report an extremely rare case of gas-filled abdominal mass caused by an ovarian teratoma fistulating to the sigmoid colon. The patient was an 85-year-old female, who presented with severe abdominal distension. Urgent computed tomography scan showed a huge abdominal mass with air fluid level and fecal matter inside. Communication between the mass and the sigmoid colon was suspected. She underwent emergency laparotomy. The mass was resected with the involved segment of colon. Pathology confirmed squamous cell carcinoma arising from mature cystic teratoma of the ovary.

Key words: Mature cystic teratoma; Fistula; Squamous cell carcinoma

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INTRODUCTION

Abdominal distension is one of the most common presenting symptoms encountered in general surgery. Although there are numerous differential diagnoses, most of the diagnoses would become apparent after radiological workup. We report here a very interesting and extremely rare cause of abdominal distension, which posed a great diagnostic challenge.

CASE REPORT

An 85-year-old lady was known to have had a sizable cystic lesion of undetermined nature for more than 40 years, causing abdominal distension. Though surgical excision was once offered, she refused intervention and defaulted clinic follow-up.

On this occasion, 7 years after default, she presented to the Accident and Emergency Department for severe abdominal pain and fever. She had experienced progressive constipation and weight loss for 6 mo prior to the presentation and had become homebound. Physical examination revealed a frail, malnourished lady with a very tense and tender abdomen. Abdominal X-ray (Figure 1) showed a well demarcated gas-filled spherical shadow. Contrast-enhanced computed tomography (CT) scan showed a huge gas-filled abdominal mass occupying the whole abdomen and pelvis, with fecal matter inside (Figure 2A). Direct communication between the mass

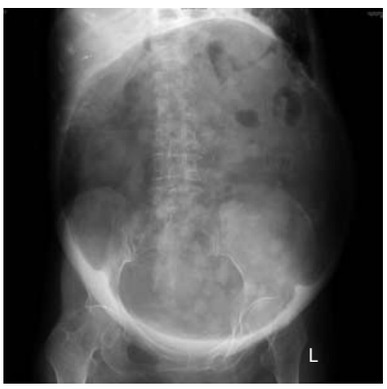

Figure 1 Abdominal X-ray.

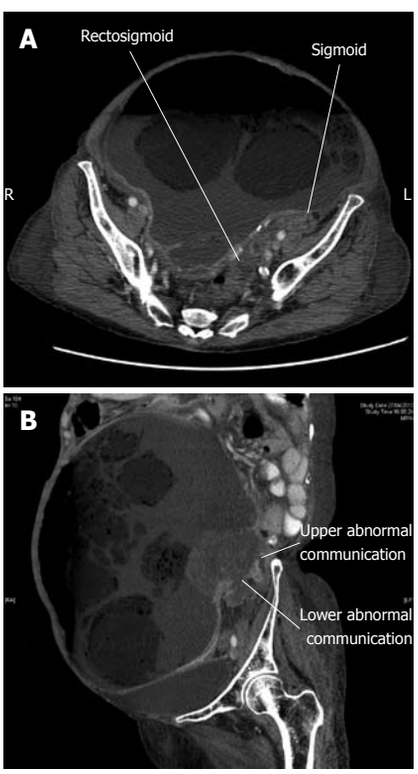

Figure 2 Computed tomography scan. A: Cross-sectional view; B: Sagittal view.

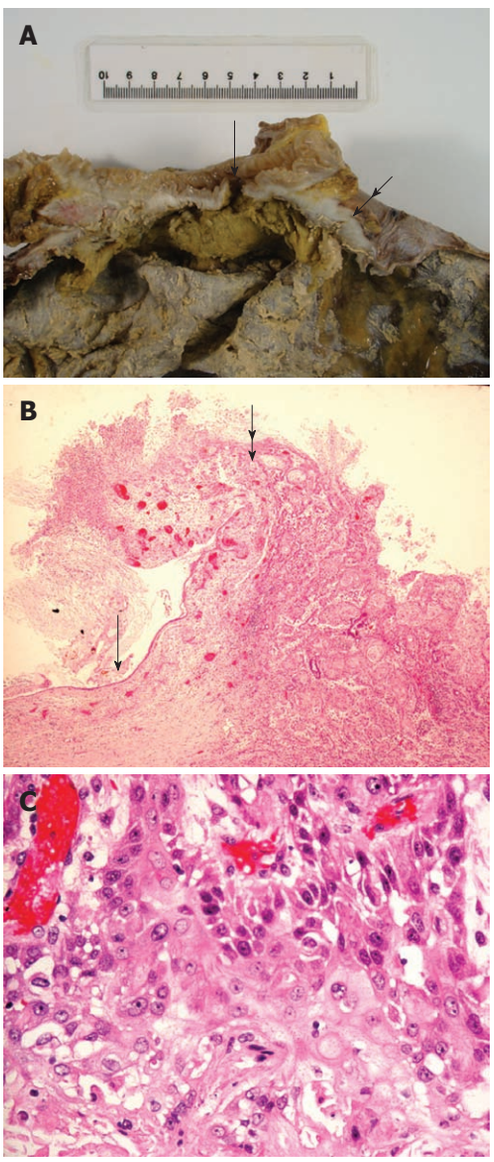

Figure 3 Pathology of the fistula and cyst wall. A: The fistula (arrow) led directly from the colon (upper part of the specimen) into the cyst wall (lower part). The tissue at the interface between the cyst wall and the colon was fleshy (double arrow); B: The benign, attenuated, ulcerative squamous cell lining of the original mature cystic teratoma (arrow) abruptly transformed to well to moderately differentiated squamous cell carcinoma (double arrow) (magnification: × 100); C: Higher magnification of the malignant component of the cyst wall showing typical squamous cell carcinoma (× 400).

and the left-sided colon was demonstrable on sagittal-reformatted image (Figure 2B). Based on the radiological features, giant colonic diverticulum and duplication cyst of colon were suspected.

Urgent laparotomy was arranged. Intra-operatively, there was a huge cystic lesion occupying the abdomen and pelvis; the lesion was densely adhered to the anterior abdominal wall and the pelvic organs. The right ovary could not be visualized but the left ovary was grossly normal. The cyst was inseparable from the colon at the sigmoid-descending junction, where solid tumor mass was found over the cyst wall. The cyst wall was completely intact at the time of laparotomy. In order to facilitate dissection, gaseous content of the cyst was decompressed by needle aspiration. The whole lesion was completely excised *en bloc* with the involved colonic segment. End colostomy was fashioned over the left upper quadrant.

Macroscopic examination of the specimen revealed a huge complex cystic lesion containing feces, gas and necrotic material inside. It had a smooth serosal surface with focal gangrenous change. The size of the colonic fistula was 5 mm in diameter. Microscopic examination confirmed poorly differentiated squamous cell carcinoma, arising from mature cystic teratoma (MCT) of the ovary (Figure 3). Direct tumor invasion into the submucosa of the colon was evident. Most of the cyst wall was eroded and replaced by granulation tissue and fibrous tissue with

focal calcification, indicating previous episodes of cyst wall rupture, inflammation and organization.

The patient's condition gradually improved after the laparotomy. However, she was noticed to have left lower limb swelling on D5. Above knee deep vein thrombosis was confirmed by Doppler ultrasound examination and anti-coagulation therapy was initiated. Taking into consideration the advanced stage of the tumor and the poor pre-morbid state of the lady, completion hysterectomy, salpingo-oophorectomy and lymphadenectomy were not offered. She was discharged home after a 3-wk period of mobilization exercise.

Four months later, she presented to our unit again with abdominal pain. On admission, her lower abdomen was diffusely tender and distended but abdominal X-ray did not reveal any dilated bowel loops. Urgent CT scan showed features suggestive of peritoneal carcinomatosis with a 5-cm heterogenous tumor in the pelvis. She was confirmed to have urinary tract infection and treated with antibiotics according to the culture result. Despite medical treatment, her condition continued to deteriorate and she finally succumbed 1 wk after admission. Though no macroscopic peritoneal nodules were noticed during the initial operation, disease dissemination likely had occurred at the time of fistula formation, which would account for her subsequent rapid deterioration.

DISCUSSION

The differential diagnosis of a gas-filled abdominal mass containing fecal matter includes giant colonic diverticulum[1-3], sigmoid or cecal volvulus, and duplication of colon[4]. MCT with fistula formation to colon is an extremely remote cause. This diagnosis requires a very high index of suspicion. In hindsight, this should have been considered in our patient who had a long history of cystic lesion.

MCT is the most common type of ovarian germ cell tumor, accounting for about 10% of all ovarian neoplasms[5]. However, malignant transformation is a rare event in MCT, with an incidence of less than 1%-2%[6,7]. Any of the tissues derived from the three embryonic germ layers have the potential to undergo malignant transformation. The vast majority (> 80%) are squamous cell carcinomas[8]. In our hospital, there were 7 patients with 8 episodes of malignant transformation out of 563 patients with ovarian teratoma in the years from 1995 to 2010 (1.24%). Six of these incidents were squamous cell carcinoma, one being papillary carcinoma of thyroid tissue and one being neuroendocrine carcinoma. Preoperative diagnosis of malignant transformation is challenging. Reported risk factors include patient's age older than 45 years, tumor size more than 10 cm and elevated tumor markers, especially the serum squamous cell carcinoma antigen[9-11].

Fistulation is a rare complication of MCT and urinary bladder is the most commonly affected organ[12,13]. Malignant transformation is not a pre-requisite for fistulation. Inflammation related to previous subclinical leakage of cyst content is believed to be the etiology of fistulation in some of the previous reports[12,14]. In our patient, both inflammation and direct infiltration by malignant cells might have contributed to the formation of fistulation. The presence of significant tumor bulk around the area of fistulation suggested tumor invasion being the predominant factor.

Because of its rarity, management of squamous cell carcinoma arising from MCT is not well defined. In a recent review[15], hysterectomy, bilateral salpingo-oopherectomy and lymphadenectomy were associated with better outcome; whereas omentectomy did not improve survival. Striking differences in survival existed between stage 1 disease and all other tumor stages. The 5-year survival rates reported by Chen *et al*[16] were 75.7%, 33.8%, 20.6% and 0% for stage I -IV disease, respectively.

In conclusion, squamous cell carcinoma arising from ovarian teratoma can rarely present as a surgical emergency with gross abdominal distension resembling bowel pathology such as giant colonic diverticulum and duplication cyst. Surgical resection remains the mainstay of treatment while adjuvant chemotherapy may improve survival in early stage diseases. Unfortunately, the overall prognosis is grave except for stage I disease.

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Gastrointestinal Cancers Symposium 2011, San Francisco, CA 94143, United States

January 27-28, 2011
Falk Workshop, Liver and Immunology, Medical University, Franz-Josef-Strauss-Allee 11, 93053 Regensburg, Germany

January 28-29, 2011
9. Gastro Forum München, Munich, Germany

February 4-5, 2011
13th Duesseldorf International Endoscopy Symposium, Duesseldorf, Germany

February 13-27, 2011
Gastroenterology: New Zealand CME Cruise Conference, Sydney, NSW, Australia

February 17-20, 2011
APASL 2011-The 21st Conference of the Asian Pacific Association for the Study of the Liver
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February 22, 2011-March 04, 2011
Canadian Digestive Diseases Week 2011, Vancouver, BC, Canada

February 24-26, 2011
Inflammatory Bowel Diseases 2011-6th Congress of the European Crohn's and Colitis Organisation, Dublin, Ireland

February 24-26, 2011
2nd International Congress on Abdominal Obesity, Buenos Aires, Brazil

February 24-26, 2011
International Colorectal Disease Symposium 2011, Hong Kong, China

February 26-March 1, 2011
Canadian Digestive Diseases Week, Westin Bayshore, Vancouver, British Columbia, Canada

February 28-March 1, 2011
Childhood & Adolescent Obesity: A whole-system strategic approach, Abu Dhabi, United Arab Emirates

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42nd Annual Topics in Internal Medicine, Gainesville, FL 32614, United States

March 7-11, 2011
Infectious Diseases: Adult Issues in the Outpatient and Inpatient Settings, Sarasota, FL 34234, United States

March 14-17, 2011
British Society of Gastroenterology Annual Meeting 2011, Birmingham, England, United Kingdom

March 17-19, 2011
41. Kongress der Deutschen Gesellschaft für Endoskopie und Bildgebende Verfahren e.V., Munich, Germany

March 17-20, 2011
Mayo Clinic Gastroenterology & Hepatology 2011, Jacksonville, FL 34234, United States

March 18, 2011
UC Davis Health Informatics: Change Management and Health Informatics, The Keys to Health Reform, Sacramento, CA 94143, United States

March 25-27, 2011
MedicReS IC 2011 Good Medical Research, Istanbul, Turkey

March 26-27, 2011
26th Annual New Treatments in Chronic Liver Disease, San Diego, CA 94143, United States

April 6-7, 2011
IBS-A Global Perspective, Pfister Hotel, 424 East Wisconsin Avenue, Milwaukee, WI 53202, United States

April 7-9, 2011
International and Interdisciplinary Conference Excellence in Female Surgery, Florence, Italy

April 15-16, 2011
Falk Symposium 177, Endoscopy Live Berlin 2011 Intestinal Disease Meeting, Stauffenbergstr. 26, 10785 Berlin, Germany

April 18-22, 2011
Pediatric Emergency Medicine: Detection, Diagnosis and Developing Treatment Plans, Sarasota, FL 34234, United States

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9th International Gastric Cancer Congress, COEX, World Trade Center, Samseong-dong, Gangnam-gu, Seoul 135-731, South Korea

April 25-27, 2011
The Second International Conference of the Saudi Society of Pediatric Gastroenterology, Hepatology & Nutrition, Riyadh, Saudi Arabia

April 25-29, 2011
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April 28-30, 2011
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May 7-10, 2011
Digestive Disease Week, Chicago, IL 60446, United States

May 12-13, 2011
2nd National Conference Clinical Advances in Cystic Fibrosis, London, England, United Kingdom

May 19-22, 2011
1st World Congress on Controversies in the Management of Viral Hepatitis (C-Hep), Palau de Congressos de Catalunya, Av. Diagonal, 661-671 Barcelona 08028, Spain

May 21-24, 2011
22nd European Society of Gastrointestinal and Abdominal Radiology Annual Meeting and Postgraduate Course, Venise, Italy

May 25-28, 2011
4th Congress of the Gastroenterology Association of Bosnia and Herzegovina with international participation, Hotel Holiday Inn, Sarajevo, Bosnia and Herzegovina

June 11-12, 2011
The International Digestive Disease Forum 2011, Hong Kong, China

June 13-16, 2011
Surgery and Disillusion XXIV SPIGC, II ESYS, Napoli, Italy

June 14-16, 2011
International Scientific Conference on Probiotics and Prebiotics-IPC2011, Kosice, Slovakia

June 22-25, 2011
ESMO Conference: 13th World Congress on Gastrointestinal Cancer, Barcelona, Spain

June 29-2, 2011
XI Congreso Interamericano de Pediatria "Monterrey 2011", Monterrey, Mexico

September 2-3, 2011 Falk Symposium 178, Diverticular Disease, A Fresh Approach to a Neglected Disease, Gürzenich Cologne, Martinstr. 29-37, 50667 Cologne, Germany

September 10-11, 2011
New Advances in Inflammatory Bowel Disease, La Jolla, CA 92093, United States

September 10-14, 2011
ICE 2011-International Congress of Endoscopy, Los Angeles Convention Center, 1201 South Figueroa Street Los Angeles, CA 90015, United States

September 30-October 1, 2011
Falk Symposium 179, Revisiting IBD Management: Dogmas to be Challenged, Sheraton Brussels Hotel, Place Rogier 3, 1210 Brussels, Belgium

October 19-29, 2011
Cardiology & Gastroenterology | Tahiti 10 night CME Cruise, Papeete, French Polynesia

October 22-26, 2011
19th United European Gastroenterology Week, Stockholm, Sweden

October 28-November 2, 2011
ACG Annual Scientific Meeting & Postgraduate Course, Washington, DC 20001, United States

November 11-12, 2011
Falk Symposium 180, IBD 2011: Progress and Future for Lifelong Management, ANA Interconti Hotel, 1-12-33 Akasaka, Minato-ku, Tokyo 107-0052, Japan

December 1-4, 2011
2011 Advances in Inflammatory Bowel Diseases/Crohn's & Colitis Foundation's Clinical & Research Conference, Hollywood, FL 34234, United States

World Journal of Gastroenterology

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Acknowledgments

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Chinese journal article (list all authors and include the PMID where applicable)

2 **Lin GZ**, Wang XZ, Wang P, Lin J, Yang FD. Immunologic effect of Jianpi Yishen decoction in treatment of Pixu-diarrhoea. *Shijie Huaren Xiaohua Zazhi* 1999; **7**: 285-287

In press

3 **Tian D**, Araki H, Stahl E, Bergelson J, Kreitman M. Signature of balancing selection in Arabidopsis. *Proc Natl Acad Sci USA* 2006; In press

Organization as author

4 **Diabetes Prevention Program Research Group**. Hypertension, insulin, and proinsulin in participants with impaired glucose tolerance. *Hypertension* 2002; **40**: 679-686 [PMID: 12411462 PMCID:2516377 DOI:10.1161/01.HYP.0000035706.28494.09]

Both personal authors and an organization as author

5 **Vallancien G**, Emberton M, Harving N, van Moorselaar RJ; Alf-One Study Group. Sexual dysfunction in 1, 274 European men suffering from lower urinary tract symptoms. *J Urol* 2003; **169**: 2257-2261 [PMID: 12771764 DOI:10.1097/01.ju.0000067940.76090.73]

No author given

6 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303 DOI:10.1136/bmj.325.7357.184]

Volume with supplement

7 **Geraud G**, Spierings EL, Keywood C. Tolerability and safety of frovatriptan with short- and long-term use for treatment of migraine and in comparison with sumatriptan. *Headache* 2002; **42** Suppl 2: S93-99 [PMID: 12028325 DOI:10.1046/j.1526-4610.42.s2.7.x]

Issue with no volume

8 **Banit DM**, Kaufer H, Hartford JM. Intraoperative frozen section analysis in revision total joint arthroplasty. *Clin Orthop Relat Res* 2002; (**401**): 230-238 [PMID: 12151900 DOI:10.1097/00003086-200208000-00026]

No volume or issue

9 Outreach: Bringing HIV-positive individuals into care. *HRSA Careaction* 2002; 1-6 [PMID: 12154804]

Books

Personal author(s)

10 **Sherlock S**, Dooley J. Diseases of the liver and billiary system. 9th ed. Oxford: Blackwell Sci Pub, 1993: 258-296

Chapter in a book (list all authors)

11 **Lam SK**. Academic investigator's perspectives of medical treatment for peptic ulcer. In: Swabb EA, Azabo S. Ulcer disease: investigation and basis for therapy. New York: Marcel Dekker, 1991: 431-450

Author(s) and editor(s)

12 **Breedlove GK**, Schorfheide AM. Adolescent pregnancy. 2nd ed. Wieczorek RR, editor. White Plains (NY): March of Dimes Education Services, 2001: 20-34

Conference proceedings

13 **Harnden P**, Joffe JK, Jones WG, editors. Germ cell tumours V. Proceedings of the 5th Germ cell tumours Conference; 2001 Sep 13-15; Leeds, UK. New York: Springer, 2002: 30-56

Conference paper

14 **Christensen S**, Oppacher F. An analysis of Koza's computational effort statistic for genetic programming. In: Foster JA, Lutton E, Miller J, Ryan C, Tettamanzi AG, editors. Genetic programming. EuroGP 2002: Proceedings of the 5th European Conference on Genetic Programming; 2002 Apr 3-5; Kinsdale, Ireland. Berlin: Springer, 2002: 182-191

Electronic journal (list all authors)

15 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/index.htm

Patent (list all authors)

16 **Pagedas AC**, inventor; Ancel Surgical R&D Inc., assignee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 20020103498. 2002 Aug 1

Statistical data

Write as mean ± SD or mean ± SE.

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Express *t* test as *t* (in italics), *F* test as *F* (in italics), chi square test as χ^2 (in Greek), related coefficient as *r* (in italics), degree of freedom as υ (in Greek), sample number as *n* (in italics), and probability as *P* (in italics).

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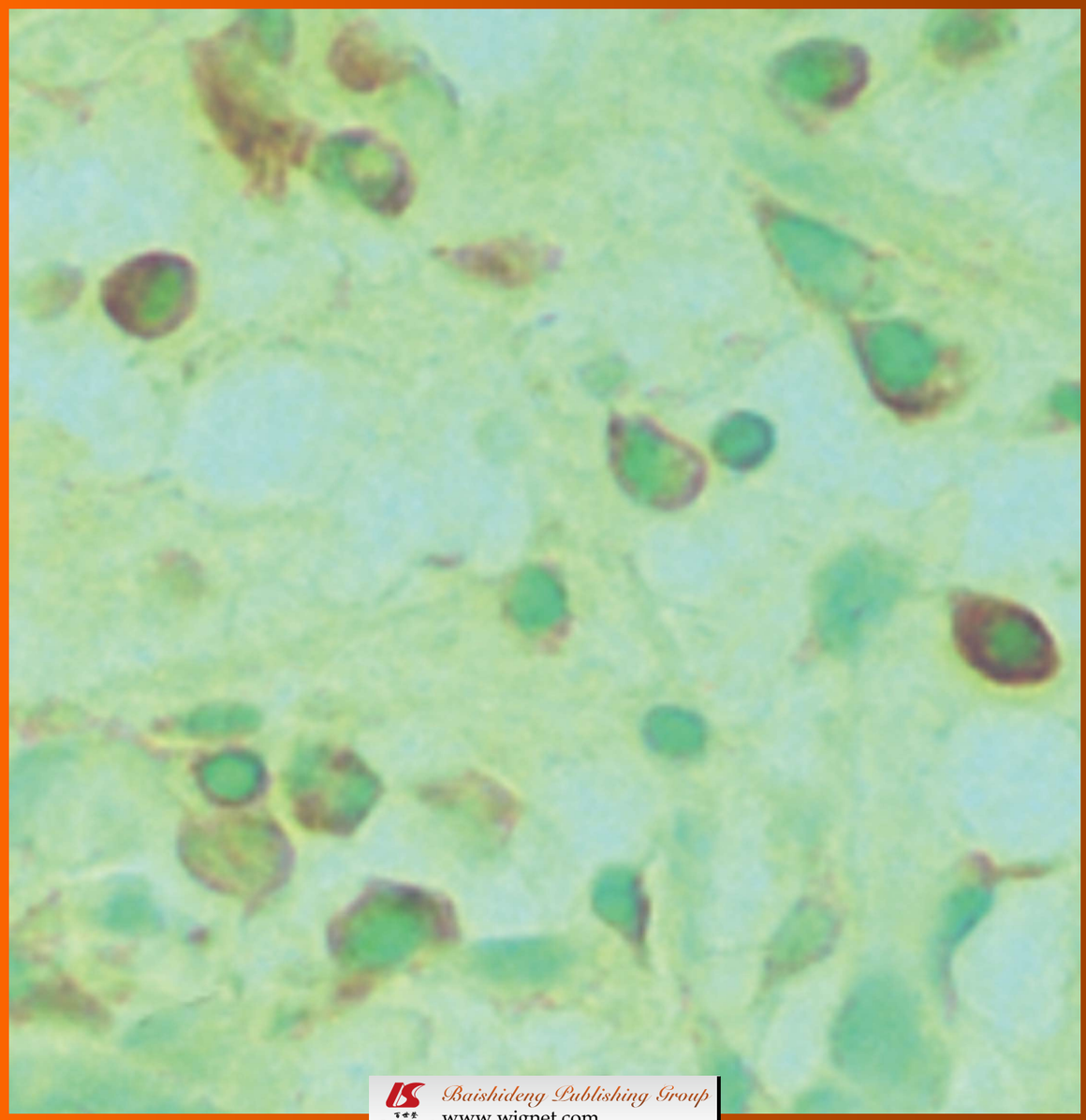

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EDITORIAL

Betaine and nonalcoholic steatohepatitis: Back to the future?

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Abstract

Nonalcoholic steatohepatitis (NASH) is an important indication for liver transplantation in many Western countries. Obesity and insulin resistance are the two most common risk factors for NASH, which can lead to recurrent NASH after liver transplantation. There is currently no approved therapy for NASH, and treatment is directed at risk factor modification and lifestyle changes. Betaine has been used for NASH, with mixed results, and may show promise in conjunction with other agents in clinical trials.

Key words: Betaine; Nonalcoholic steatohepatitis; Cirrhosis; Obesity; Insulin resistance

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INTRODUCTION

Nonalcoholic steatohepatitis (NASH), a subtype of nonalcoholic fatty liver disease, can lead to cirrhosis and is an increasingly important cause of liver transplantation in the United States[1]. It is thus subject to intense translational research. Despite a number of clinical trials on the treatment of NASH, there is still no approved therapy, and management is often directed at aggressive reduction of the two most common risk factors: obesity and insulin resistance. These limitations in management have led to renewed interest in the pathophysiology of this epidemic, as a prerequisite to embarking upon further clinical trials. However, most data in this area has been derived from animal models.

Betaine, a naturally occurring dietary compound, originally discovered in sugar beet juice, would appear to be an ideal agent for treating NASH. It is synthesized *in vivo* from the oxidation of choline, and has several effects that may impact the natural history of NASH. These include: (1) its role as a methyl donor for the conversion of homocysteine to methionine; (2) direct substitution for S-adenosylmethionine (SAM) for the direct methylation of phosphatidylethanolamine to phosphatidylcholine; (3) its downstream effects on oxidative stress and transsulfuration reactions; (4) activation of AMP-activated protein kinase; and (5) its properties as a lipotrope and osmolyte[2]. As a naturally acting agent, side effects with betaine would be expected to be minimal; however, in reality, this depends on whether it is administered as betaine anhydrous oral solution or as capsules.

Four clinical trials of betaine for the treatment of NASH have been reported. The first study by Miglio *et al*[3] was of limited value because histopathology was not used to diagnose NASH. Abdelmalek *et al*[4] first reported their experience with betaine in a pilot study of 10 patients treated for one year. Biochemical and histological improvement were noted, although three patients did not complete the study. Mukherjee *et al*[5] reported statistically significant improvement in liver function tests and histopathological

scores in their series of 35 patients treated with betaine for one year; however, this study was limited by the absence of a control arm. Abdelmalek *et al*[6] subsequently reported the results of their second study, which remains the largest and most robustly designed trial evaluating betaine for NASH. The primary aim of this study was to determine if 20 grams per day of anhydrous betaine improved liver function tests after one year of therapy. The secondary aim was to assess its impact on histology. Thirty-five patients completed this randomized placebo-controlled trial (17 betaine *vs* 18 placebo), which included pre- and post-measurement of serum anti-oxidant activity, adipokines, cytokines, homocysteine, S-adenosylhomocysteine (SAH), methionine, and liver biopsies scored according to the Brunt criteria[7]. These variables were analyzed according to the paired *t* test. At the conclusion of the study, betaine had no effect on aminotransferases, and of those patients who did show normalized aminotransferases, the proportion was similar with the placebo group. Betaine also had no effect on adiponectin, cytokine, and SAH levels. The impact of betaine on histology was also disappointing, with no change in fibrosis observed during the study. In addition, fewer patients treated with betaine versus placebo (29% *vs* 61%, $P < 0.01$) improved the steatosis grade by > 1 point. However, more betaine-treated patients compared to placebo (71% *vs* 22%, $P < 0.005$) had no change in steatosis over the study duration.

Such negative findings would appear to shut the door on betaine's therapeutic potential for NASH; however, several limitations in the study, rightfully acknowledged by the investigators, merit review. Probably the most important was the high number of patients who dropped out, which simply meant this randomized controlled study lacked power to detect a difference between the two groups. A large number of patients also had advanced fibrosis (stage 3-4), although descriptive statistics are lacking. As NASH is a chronic condition that normally takes several years to progress into cirrhosis, it is not surprising that no effect was noted after only 1 year of treatment[8]. Furthermore, it remains unclear what optimum dose and preparation of betaine are required for NASH, as the investigators extrapolated data used for homocystinuria. For example, study patients had a significantly higher incidence of gastrointestinal side effects (33% *vs* 9%, $P < 0.05$), which contributed to study withdrawal. It is plausible that a lower dose of anhydrous betaine or betaine capsules, which do not require addition with a solution before administration, might have led to improved compliance and fewer side effects. Serum betaine levels were also not measured and, although the ideal range remains to be determined, documentation would have confirmed compliance rather than accepting a subject's qualitative response in a possible attempt to appease an investigator[9].

A study by Kathirvel *et al*[10] aimed at understanding how betaine reverses hepatic insulin resistance in an animal model of nonalcoholic fatty liver disease may also provide support for re-considering betaine in future trials of NASH. It is more than likely that future trials of NASH will need to be of longer duration to fully assess the impact of treatment, and multiple medications may be required, given the multifactorial processes involved in its pathogenesis. However, betaine, by virtue of its multiple effects and low cost, strongly needs to be reconsidered in larger, prospective studies for NASH as monotherapy will enhance compliance during treatment which is likely to be prolonged in the majority of patients. Risk factor medication remains the mainstay of NASH management, but it is being increasingly recognized that NASH may develop in their absence, re-emphasizing the necessity of well- funded trials of appropriate duration (including cost-effectiveness analyses) for this silent epidemic[11].

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EDITORIAL

Ages of celiac disease: From changing environment to improved diagnostics

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Abstract

From the time of Gee's landmark writings, the recent history of celiac disease (CD) can be divided into many ages, each driven by a diagnostic advance and a deeper knowledge of disease pathogenesis. At the same time, these advances were paralleled by the identification of new clinical patterns associated with CD and by a continuous redefinition of the prevalence of the disease in population. In the beginning, CD was considered a chronic indigestion, even if the causative food was not known; later, the disease was proven to depend on an intolerance to wheat gliadin, leading to typical mucosal changes in the gut and to a malabsorption syndrome. This knowledge led to curing the disease with a gluten-free diet. After the identification of antibodies to gluten (AGA) in the serum of patients and the identification of gluten-specific lymphocytes in the mucosa, CD was described as an immune disorder, resembling a chronic "gluten infection". The use of serological testing for AGA allowed identification of the higher prevalence of this disorder, revealing atypical patterns of presentation. More recently, the characterization of autoantibodies to endomysium and to transglutaminase shifted the attention to a complex autoimmune pathogenesis and to the increased risk of developing autoimmune disorders in untreated CD. New diagnostic assays, based on molecular technologies, will introduce new changes, with the promise of better defining the spectrum of gluten reactivity and the real burden of gluten related-disorders in the population. Herein, we describe the different periods of CD experience, and further developments for the next celiac age will be proposed.

Key words: Antibodies; Autoimmunity; Celiac disease; Diagnostics; History; Intestinal mucosa

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INTRODUCTION

The first descriptions of celiac disease (CD) refer to a disorder of the gut (*koiliakos* in Greek), mainly characterized by fatty stools. While diarrhea was a symptom common to a number of diseases, fatty stools or steatorrhea was an uncommon symptom, characteristic of only a few dis-

eases, such as cystic fibrosis. The finding of steatorrhea in weaned children and in adults without cystic fibrosis was described as a single nosological entity by Samuel Gee, in a rapidly developing England at the end of the 19th century[1]. A similar disease was actually described by Aretaeus of Cappadocia, a physician active in Anatolia almost 2000 years earlier, during another period of rapid development, when agriculture had spread to the so-called region of the Fertile Crescent in the Middle-East.

We can refer to the Age from the first description by Aretaeus of Cappadocia to that of his English colleague 2000 years later as the "The Origins of the Celiac Age" (Figure 1 and Table 1). The cause of the disease was unknown, and the role of foods was conjecture. Gee described CD as a "chronic indigestion which is met with in persons of all ages, yet is especially apt to affect children between one and five years old. Signs of the disease are yielded by the feces; being loose, not formed, but not watery; more bulky than the food taken would seem to account for; pale in colour, as if devoid of bile; yeasty, frothy, an appearance probably due to fermentation; stinking, stench often very great, the food having undergone putrefaction rather than concoction". Gee described, for the first time, that the only cure for the disease would be dietary, even if he failed to identify the foods causing the disorder. With his description, we can start the second age of CD: a disease of the gut, diagnosed on the basis of clinical features and curable with diet.

THE AGE OF STEATORRHEA: FAT IN STOOLS AND DIETARY ADJUSTMENT AS A TREATMENT

The first decades after the initial description by Samuel Gee were characterized by a clear medical description of the gastroenterological symptoms and signs of CD, and by an increasing recognition and identification of new cases, both in children and in adults. Fatty stools, together with abdominal bloating and failure to gain weight were the leading symptoms of the disease, which suggested a malabsorption of food nutrients. Since that time, different attempts were made to cure CD by employing different types of diets. Although the frequent onset of the disorder occurring in babies immediately after weaning suggested a role for cereals as the offending food, the first hypothesis focused on amides, and not on the protein content of flours. In fact, in 1921 the disease was still considered an intolerance to carbohydrates. In 1949, the success of a banana-based diet eliminated carbohydrates as the cause (Sydney Haas). However, scientific methods to identify the specific offending food were applied only around the mid 20th century, thanks to advances in chemical sciences.

THE AGE OF GLUTEN: STILL A VERY RARE DISORDER

In 1950, Dicke observed for the first time, that many children with celiac syndrome might be successfully treated with a diet free from wheat and rye flours. Two years later Anderson demonstrated that the gluten in wheat and rye was the harmful factor[2]. The ability to measure the fatty acids in stools permitted the systematic evaluation of the efficacy of different diets, and eventually confirmed that wheat, barley and rye were harmful to those with CD[3].

This is not the end of this period. In fact, in the following years, the picture of CD continued to change every time improvements were made in diagnostics, which revealed new aspects of the disease. It will soon become evident that it is not just our knowledge changing over time, but CD itself, which may be due to the coincidence of several factors, including availability of large amounts of wheat with a high content of gluten, and changes in the epidemiology of gastro-enteric infections. In this Age, CD is still considered a rare disorder, affecting the gut directly as a consequence of a chronic indigestion of gluten. Late diagnosis, coincidence of malnutrition, and/or infections may account for the more severe form of the disease, the "celiac crisis", which can lead to shock and death. Currently, this form is extremely rare, while other types of presentation are increasingly described in the literature. This age can be also remembered as the age of the Crosby-Kugler capsule[4]. This instrument assisted in the diagnosis of CD allowing a mini-invasive withdrawal of fragments of the small bowel mucosa for histological analysis. The description of the typical picture of flat mucosa and *criptae* hypertrophy constituted at the same time a confirmation of diagnosis, and a tool for investigating the pathogenesis of the intestinal damage in CD. Repeat biopsies could confirm the healing of mucosa after a period of being on a gluten-free diet and the relapse after a new challenge with wheat, suggesting that sensitivity to gluten is a permanent condition in CD.

On the evolutionary scenario, the observation that mucosal damage greatly diminished the available surface for nutrient absorption could suggest a different expression of the disease depending on the available food supply. Indeed, some changes in the clinical expression of CD in different countries or in different periods may be related to the amount of food available, as well as to different epidemiology of infectious diseases, which can synergize with gluten to induce gut damage.

THE AGE OF GLIADIN ANTIBODIES: CELIAC DISEASE IS AN IMMUNE DISORDER RECALLING THE IDEA OF A "CHRONIC INFECTION BY GLUTEN"

The identification of gluten antibodies (AGA) in those affected by CD revolutionized the view of the disease in 1964[5]. Similar to what was found in the first years of the 20th century by von Pirquet in allergic diseases, CD appeared to be due to the immune response to gluten rather than to a direct action of the protein. However, it

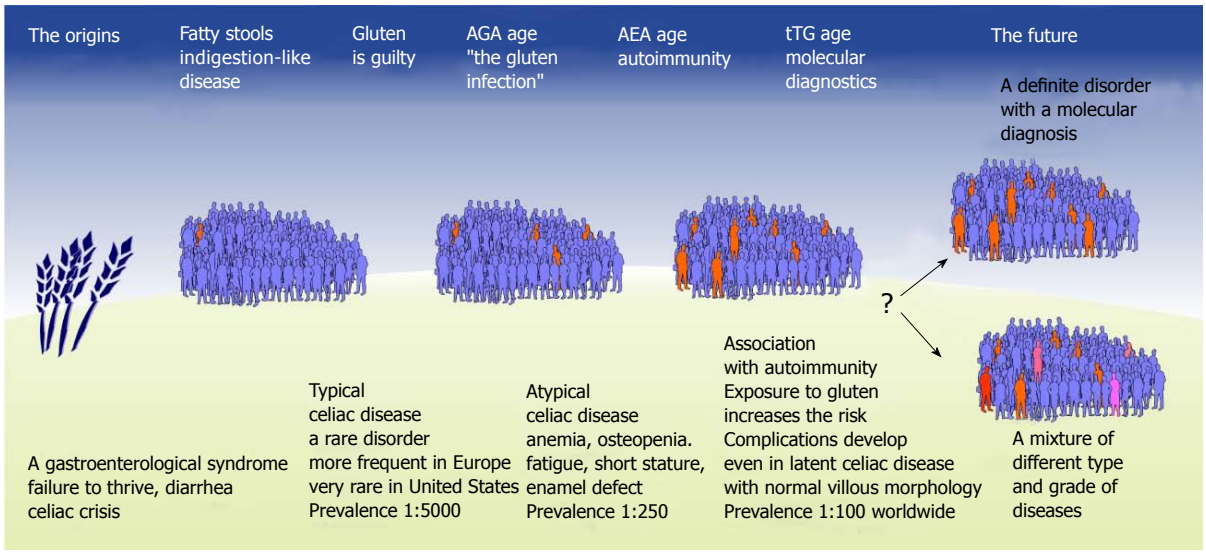

Figure 1 Changing epidemiology and clinics of celiac disease though the different ages.

Table 1 The ages of celiac disease

Age	Date	Clinics	Diagnosis	Pathogenesis
The origins	-1888	At the borders of the fertile crescent		
The age of steatorrhea Fat in stools and diet as a treatment	1888-1952	Intestinal syndrome	Fatty stools	Chronic indigestion Diet attempts
The age of gluten. An intolerance to a protein	1952-1965	Gut disease Celiac crisis	Crosby-kugler capsule assisted diagnosis Acid fat measure in feces allows diet follow-up A very rare disease	Wheat, barley and rye gluten is guilty Flat mucosa is responsible for a malabsorption syndrome The "three biopsies" approach suggested that gluten susceptibility in celiacs is a permanent condition
The age of AGA. CD as an immune disorder	1965-	AGA assay allowed characterization of the atypical form or even the diagnosis of asymptomatic subjects founded the celiac society	AGA Three biopsies	CD is an immune disorder, like a chronic infection by gluten Association to specific HLA variants
The Age of AEA. CD is associated with autoimmunity.	1973-	Definition of silent and latent CD The risk of autoimmunity in CD is, at least in part, related to the duration of exposure to gluten	AEA Three biopsies CD screening by means of AGA and AEA testing A disease more frequent than expected	Gluten is just the trigger, endomysium the target AEA and other autoantibodies in CD are gluten-dependen HLA DQ2 restricted anti-gluten T cells in biopsies The "celiac iceberg" model
The Age of transglutaminase: from target to diagnostic tool.	1997-	Widening spectrum of CD associated disorders	tTG antibodies One biopsy Screening on a few blood drops Anti-deaminated gluten peptides (DGP) antibodies ESPGHAN guidelines for diagnosis without biopsy	Tissue transglutaminase (tTG) is the autoantigen in endomysium tTG increase the affinity of gluten peptides for HLA DQ2 Interaction between tTG and gluten peptides could be responsible for autoimmune reactions and "antigen spreading"
The future. Will new tools identify new diseases?	2011-	A new definition for gluten intolerance with normal serum tTG antibodies	Mucosal tTG in potential CD	Mucosal assay for local tTG antibodies Phage display libraries to unravel CD pathology

CD: Celiac disease; AGA: Age of gliadin antibodies; AEA: Age of anti-endomysium anti-bodies; ESPGHAN: European Society for Paediatric Gastroenterology, Hepatology and Nutrition; HLA: Human leukocyte antigen.

was not an allergy, as it involved different mechanisms, which resemble more strictly the response to gut infections. Thus, the analogy of a chronic "gluten infection" substituted the definition of gluten "indigestion" previously used. The finding of gluten antibodies in CD was even more revolutionary, as it became evident that the measurement of these antibodies could allow an easier diagnosis of the disease, and a convenient follow-up by dietary change.

As a further confirmation of the role of the immune

system in the pathogenesis of CD, a close association between particular human leukocyte antigen (HLA) variants and the disease was observed. More importantly, the measurement of AGA, being a relatively non-invasive and low cost assay, allowed researchers to widen the search for CD in subjects with different clinical complaints, and to find that the disease could be associated with atypical, non-gastroenterological symptoms, such as anemia, short stature, or dermatitis herpetiformis[6-8]. Intolerance to gluten was in fact more frequent than previously expected, and could even be diagnosed in people without any evident symptoms (silent CD), but presenting the typical jejunal lesion of the disease.

THE AGE OF ANTI-ENDOMYSIUM ANTIBODIES: CELIAC DISEASE IS CONNECTED TO AUTOIMMUNITY

As a result of the AGA assay, CD was found to be more common in individuals with type 1 diabetes, and other autoimmune disorders, than in the general population. It was thus not surprising to find that serum from a person diagnosed with CD could contain autoantibodies. Anti-reticulin antibodies were identified in CD in 1971[9]. The finding that these antibodies behaved in a gluten-dependent manner, similar to AGA, was of particular interest as it represented an autoimmune reaction induced by foods[10]. Years before the relationship between gluten and reticulin was clarified, different assays for identifying CD-related autoantibodies entered clinical practice including reticulin antibodies in rat kidney; endomysium antibodies in monkey esophagus; and, later human umbilical cord sections (AEA)[11]. AEA were soon considered a specific sign of CD permitting the definition of a new kind of gluten intolerance, in the absence of overt mucosal lesion ("latent celiac disease")[12]. In these patients, the mucosal inflammation induced by gluten was only revealed by an infiltration of CD3-positive lymphocytes with an increase in the TCR-gamma/delta subset[13-15]. This is why we can also refer to the autoimmunity Age as the "AEA Age".

In the AEA Age, attention was focused on the particular relationship between CD and autoimmunity, which was initially thought to represent the clustering of different autoimmune disorders due to the sharing of the same HLA variants. More recently a multicenter study from the SIGEP suggested that in genetically predisposed subjects, the longer the exposure to gluten the higher the risk of developing autoimmune disorders[16]. In this picture, the risk of developing autoimmunity in CD could be higher in cases without the typical gastroenterological symptoms of the disease in patients who were more likely to be diagnosed later, and likely to remain exposed to gluten longer. It is noteworthy that postponing gluten intake in the first year of life could make the gastrointestinal symptoms less intense, thus delaying the diagnosis, and possibly increasing the risk of developing autoimmunity[17].

A milder gastroenterological presentation, because of a different environmental setting, could also be the cause of underestimating the prevalence of CD in the United States. However, the existence of AEA allowed for new screening and testing, which eventually demonstrated a similar prevalence of the disease in the United States, compared to many other countries, in the range of about 1%[18]. A good outcome of large screenings has been the increase in awareness of the disease in the population, making easier the clinical diagnosis and diet-based treatment.

The AEA Age ended with the idea that much still remained to be understood regarding CD, with the simile of the "celiac iceberg": while the tip is represented by cases with typical symptoms, the majority of individuals with gluten intolerance are under the water, and are difficult to identify because of atypical or even absent symptoms and/or due to apparently normal mucosa[19]. The iceberg idea was intriguing, as it suggested that a percentage of normal people exist, who can respond to gluten with different pathological reactions and that different diagnostic tools could unravel the disease. As a matter of fact, the AEA Age marked a major change in the knowledge of CD, from a rare gut disorder due to gluten and expressed with gastrointestinal complaints (just the tip of the iceberg), to a common autoimmune disorder triggered by gluten in the gut but expressed with a wide variety of clinical symptoms involving different systems. It is noteworthy that this submerged part of the iceberg is much bigger compared to the tip and, in the same way, clinical symptoms other than gastrointestinal are much more common than typical symptoms, where the disease itself is much more common than what was previously considered. Indeed, CD could be suspected in patients with a variety of autoimmune disorders such as diabetes, thyroiditis, dermatitis herpetiformis, autoimmune ataxia, alopecia, as well as symptoms directly due to malabsorption (Table 2).

THE AGE OF TRANSGLUTAMINASE: FROM TARGET TO DIAGNOSTIC TOOL

HLA variants DQ2 and DQ8 were the genetic factors most closely associated with CD. The isolation in duodenal biopsies of T cell clones recognizing gluten peptides in association with these HLA molecules further confirmed the pathogenic role of these genetic variants[20]. Furthermore, anti-gluten CD4 T cells produced large amounts of interferon gamma, which seemed to account for the typical mucosal damage seen in CD-affected mucosa. However, even this knowledge failed to explain why a HLA DQ2/DQ8 patient can present CD, yet another, not. In this scenario, the identification of the single antigen targeted in the Endomysial staining reaction was expected to permit a better knowledge of CD pathogenesis, and a better understanding of the origins of CD[21].

Thus, the search for the "endomysial antigen" represented an amazing adventure for most researchers involved in CD in the 1990s. In 1997, Dieterich and col-

Table 2 Old and new celiac disease before and after the identification of age of anti-endomysium anti-bodies

	Old CD, Pre-AEA age	New CD, Post-AEA age
Pathogenesis	Immune, intestinal	Autoimmune, systemic
Diagnosis	AGA + 3 biopsies	AEA + 1 biopsy
Prevalence	Rare 1:500-1:5000	Frequent 1:100 worldwide
Clinical picture	Malabsorption syndrome	Autoimmune disorders Malabsorption syndrome
Intestinal pathology	Severe villous atrophy and criptae hypertrophy	Severe villous atrophy and criptae hypertrophy or increased mucosal lymphocytes (latent celiac disease)

CD: Celiac disease; AGA: Age of gliadin antibodies; AEA: Age of anti-endomysium anti-bodies.

leagues found that the endomysial antigen involved in the autoimmune response in CD was the enzyme tissue transglutaminase or Type 2 transglutaminase (tTG or TG2)[22]. Indeed, tTG is present in the endomysial net, where it stabilizes the connective tissue by catalyzing the link between glutamine and lysine of different structural proteins. This activity is very important in tissue repair processes and an increased activity of the enzyme can be evidenced in damaged tissues, including the mucosa in CD. Furthermore, tTG plays another important role, in the packaging of debris after cell apoptosis, which allows for the correct removal of apoptotic bodies containing inflammatory response materials.

Ludwig Sollid was the first to publicly hypothesize a model linking gluten to tTG and to anti-tTG autoantibodies. Briefly, when large amounts of gluten enter the mucosa because of increased epithelial permeability (may be favored by other factors, such as infections), the anti-gluten response causes mucosal damage, causing the release and activation of tTG. Gluten itself, due to its high content in glutamine, can be a target of tTG and can be cross-linked with other proteins, including tTG. As a consequence, macromolecular complexes containing both gluten peptides and tTG can be recognized by AGA-producing B cells, as well as by AEA-producing B cells. According to the "Sollid hypothesis", B cells recognizing these macro-complexes, regardless of their antibody specificity will present gluten peptides to gluten-specific T cells. As a consequence, a single antigen can drive an immune response to many targets, overcoming the tolerance of the immune system, in a process also known as "antigen spreading".

Another finding connecting tTG and gluten relies on the capacity of the enzyme to deaminate gluten-derived peptides increasing their affinity to the DQ2 and DQ8 HLA, thus worsening the consequences of anti-gluten immunity[23,24]. Recently, measurement of the immune response to deaminated gliadin peptides (DGP)[25] has been utilized to increase the performances of the AGA assay[26]. This model could partly explain the role of environment, with gastrointestinal infections, in precipitating the pathogenic mechanisms of CD with a vicious circle of tissue damage, activation of tTG, entry and deamination of gluten, anti-gluten response and the spreading of autoantibodies. Hyper-production of IL-15 is associated with these mucosal changes, which could affect the production of the immunoregulatory cytokine TGF-beta[27,28]. Even if this model does not illuminate the specific relationship between CD and other autoimmune disorders, it describes a dysregulated mucosal immunity, which is likely to interfere with the normal mechanisms of immune tolerance.

Apart from contributing to pathogenic knowledge, the identification of the main CD autoantigen allowed for a further improvement of diagnostics for CD by using ELISA assays based on human recombinant tTG (htTG). Using htTG, population screening has been performed starting from finger puncture producing as little as a few drops of blood in children from primary schools[27], and more recently rapid tests have been produced for the consumer market. Due to the reliability of htTG assays, CD diagnosis can now be confirmed with just one jejunal biopsy without any need for repeating bioptic examinations after the start of the diet. In some cases, it is even thought that a confirmation by means of jejunal biopsy may not be necessary. In fact, considering that a strong correlation has been demonstrated between high levels of tTG antibodies and a higher grade of mucosal damage (Marsh score)[29,30], the ESPGHAN is currently evaluating the possibility of making the diagnosis without a confirmatory jejunal biopsy in patients who have symptoms that can be referred to CD, if IgA-tTG antibodies are > 10x the upper normal limit, AEA and HLA DQ2 and/or DQ8 are positive.

THE FUTURE AGE: WILL NEW TOOLS IDENTIFY NEW DISEASES?

Cut-off values generated for a quantitative tTG assay assume a semi-Gaussian distribution of the values in the healthy population, with a tail of high values representing true celiac patients. This means that positive results represent a statistical correlate of the disorder and are not to be confused with the disease itself. Even if these assays are very useful and reliable in assisting the diagnosis of CD, they just represent our best for today, not the confidence of identifying all individuals in whom a gluten-free diet could give measurable advantages. While it is almost certain that very high tTG antibody titers indicate the presence of the disease[29,30], it is less easy to give significance to low titers and border-line results. In fact, there are several lines of evidence that gluten-dependent pathology can develop even in some patients with negative tTG antibodies, albeit rarely. On the other hand, even some patients with positive tTG might not develop symptoms on a gluten-containing diet, the gluten-free diets should still be prescribed, as we are not able to predict the risk of developing pathology in individuals.

Indeed, CD is a multifactorial disorder. It just might be that, in considering the picture of the "celiac ice-

berg", there are different levels of intolerance to gluten, and exposure to gluten could have different consequences in each patient. In other words, we still know just a part of what made the iceberg. The forthcoming Age will clarify if we will be able to identify a single definite disorder by advances in molecular diagnostics, or if we will be faced with different forms of gluten intolerance (see Figure 1). Recently, it has been argued that intramucosal production of anti-tTG antibodies may precede their appearance in serum and could represent a specific indicator of gluten intolerance as well. An immunofluorescence technique on jejunal biopsies allows the detection of IgA deposits that co-localize with tTG in the villus connective tissue, which are considered *bona fide* tTG antibodies. These antibodies could be detected in patients with latent CD, regardless of their presence in serum, and have been shown to predict the development of villous atrophy and to disappear during a gluten-free diet[31]. Analysis of phage display antibody libraries confirmed that anti-tTG antibodies are indeed produced by mucosal lymphocytes and provided a further tool to identify latent CD, where tTG antibodies were produced in mucosa before that they can be found increased in serum[32]. These techniques have a role not only in research. In clinical practice, patients with a potential risk of developing gluten-related diseases, such as relatives of those diagnosed with CD, or with autoimmune disorders, may be positive for DQ2 HLA, but have normal levels of serum tTG antibodies. It was of particular interest to find that some of these patients did have mucosal tTG antibodies that behaved as gluten-dependent[33]. The characterization of such individuals, affected by intermediate or latent forms of CD is one of the goals of modern diagnostics and, a new key to better unravel knowledge on the disease. Further studies will address how gluten may interact with other environmental and genetic factors to condition the risk of developing different types of associated disorders.

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TOPIC HIGHLIGHT

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Risk factors for neoplastic progression in Barrett's esophagus

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Abstract

Barrett's esophagus (BE) confers a significant increased risk for development of esophageal adenocarcinoma (EAC), with the pathogenesis appearing to progress through a "metaplasia-dysplasia-carcinoma" (MDC) sequence. Many of the genetic insults driving this MDC sequence have recently been characterized, providing targets for candidate biomarkers with potential clinical utility to stratify risk in individual patients. Many clinical risk factors have been investigated, and associations with a variety of genetic, specific gastrointestinal and other modifiable factors have been proposed in the literature. This review summarizes the current understanding of the mechanisms involved in neoplastic progression of BE to EAC and critically appraises the relative roles and contributions of these putative risk factors from the published evidence currently available.

Key words: Barrett's esophagus; Esophageal adenocarcinoma; Metaplasia-dysplasia-carcinoma; Neoplastic progression; Risk factors

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INTRODUCTION

Barrett's esophagus (BE) describes a condition where native esophageal stratified squamous epithelium is replaced by metaplastic columnar epithelium, with cephalad displacement of the squamocolumnar junction. BE represents the only identified precursor lesion and most important risk factor for esophageal adenocarcinoma (EAC)[1]. Patients with BE have an estimated 30- to 125-fold greater risk of developing EAC than the general population[2]. A systematic review of 27 studies suggested annual progression rates of 0.5%[3], whereas a review of 8 UK studies by Jankowski *et al*[4] showed cancer risk of 1.0% per year.

BE PATHOGENESIS AND MECHANISMS OF NEOPLASTIC PROGRESSION

BE is an acquired condition where healing from esophageal mucosal injury [typically triggered by gastro-esophageal reflux disease (GERD)] is metaplastic, with replacement of damaged squamous cells by columnar epithelium. Ordinarily, esophageal healing involves regeneration of squamous cells; it remains unclear why the response is metaplastic in some individuals, since only a minority of patients with GERD develop BE. Progression of BE to EAC occurs by a metaplasia-dysplasia-carcinoma (MDC) sequence. Metaplastic columnar epithelial cells are predisposed to genetic damage with potential for developing

dysplasia[5]. Dysplasia represents a histological spectrum from low- to high-grade, defined by degree of cytological and architectural disruption present, with genetic instability resulting in progressive acquisition of genetic abnormalities towards a frankly neoplastic phenotype. These can be considered within the framework of Hanahan and Weinberg's[6] model of "cancer hallmarks" necessary for carcinogenesis, whereby cancer cells must acquire growth self-sufficiency, insensitivity to anti-growth signals, avoidance of apoptosis, limitless replicative potential, sustained angiogenesis, and invasive and metastatic potential[7].

Many genetic insults conferring these advantages in the BE MDC sequence have been characterized. Initiating events probably involve genes regulating cell cycle progression, notably *p16*. Mutations, loss of heterozygosity (LOH) or promoter hypermethylation (i.e. silencing) of *p16* have been identified in 80% of BE, whilst *p16* hypermethylation correlated with the degree of dysplasia in some studies[8]. Additional changes identified include upregulation of cyclins D1 and E, transforming growth factor-α and epidermal growth factor (EGF), each contributing towards growth autonomy[9,10]. These mutations should trigger apoptosis *via p53*-dependent pathways. However, subsequent accrual of *p53* lesions confers resistance to apoptosis, and has been identified in 52%-93% of EACs (compared with 1%-5% non-malignant BE cell lines)[11]. Inactivation of *p53* increases clonal genomic instability, predisposing to widespread DNA changes and evolution of ploidy lesions, late events in cancer progression. Many other genetic and molecular alterations have been described[8,9,12-64] (Table 1).

The concept of a linear, stepwise evolution of tumor suppressor gene mutations in which clonal expansion of a solitary mutated clone expands to fill the entire Barrett's segment has been termed the "selective sweep to fixation" model. However, an alternative model has been proposed by Leedham *et al*[65], who performed genetic analysis of individual crypts rather than a flow purified whole biopsy specimen. This technique permitted identification of certain mutations masked by whole biopsy segment analysis (attributed to dilution effect of the normal stroma on whole biopsy analysis), whilst also revealing a greater degree of genotypical and phenotypical heterogeneity within the same biopsy sample than previously appreciated. The demonstrated lack of a single founder mutation present in every crypt suggested that the clonal expansion arose from multiple independent clones rather than a single common founder mutation[65,66] (Figure 1).

This enhanced understanding prompted research into > 200 candidate novel biomarkers of disease progression in BE/EAC. Several, including 17p LOH, cyclin D1, tetraploidy and aneuploidy, have undergone phase 3/4 validation and in future might have clinical/prognostic utility as intermediate markers of progression[67]. However, Leedham's recent findings call into question the reliability of "surveillance" biomarker identification *via* genetic analysis of whole biopsy specimens, since minority clones within the sample (harboring neoplastic potential) might not be detected[65].

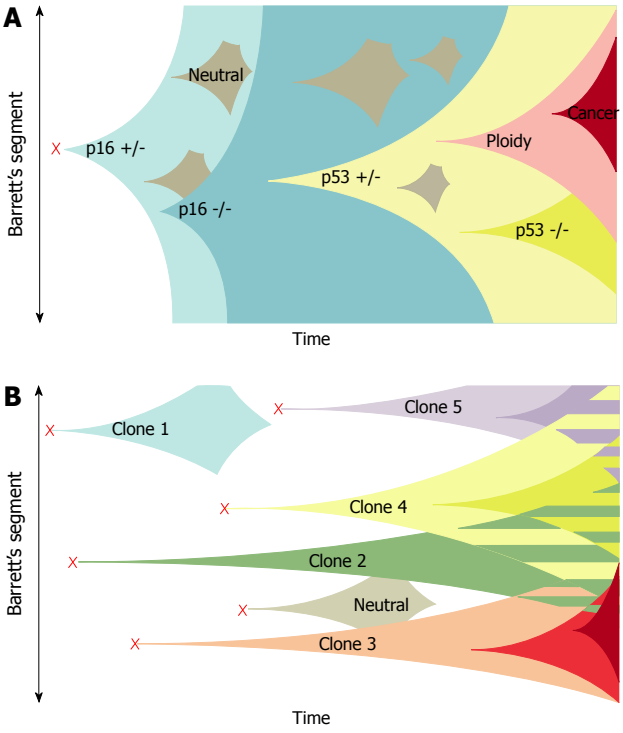

Figure 1 Clonal evolution models in Barrett's esophagus. A: The current model of clonal evolution adapted from Maley *et al*[66]. Founder mutation (red cross) occurs in a single progenitor and provides a growth advantage that predisposes to a selective sweep. Successive selective sweeps result in progression along the metaplasia dysplasia pathway. Clone bifurcation is responsible for the genetic heterogeneity in this model; B: The newly proposed model of evolution based on the mutation of multiple progenitor cells situated in esophageal gland squamous ducts located throughout the length of the esophagus (red crosses). Multiple independent clones then arise and evolve separately. The presence of multiple different clones gives rise to a mosaic interdigitating clonal pattern of the Barrett's segment represented as the striped areas[65].

Currently, dysplasia remains the only validated marker for identifying BE patients at risk, and forms the basis of EAC surveillance. However, this is imperfect. The tempo of progression towards EAC is highly variable and it remains unclear whether relentless progression through the MDC sequence is inevitable; some evidence suggests that high-grade dysplasia may remain stable for years or even regress[68]. Patients with BE may develop EAC during surveillance without detection of earlier MDC stages. This might relate to pace of progression, sampling error or lesions skipping directly from non-dysplastic disease to cancer. Other limitations of dysplasia as a prognostic marker include inter-observer variability in histological interpretation, and that inflammation may mimic dysplastic changes[69].

RISK FACTORS FOR NEOPLASTIC PROGRESSION

Until molecular biomarkers enter clinical practice it remains important to identify other clinical risk factors for malignant progression to effectively allocate resources and individualize surveillance programs, targeting those at highest risk. Identifying modifiable risk factors will also

Table 1 Published evidence from selected studies investigating genetic and epigenetic changes implicated in the metaplasia-dysplasia-carcinoma sequence of Barrett's esophagus

Factor	Summary of major findings/conclusions	Ref.
Growth self-sufficiency		
Cyclin D1	↑ nuclear cyclin D1 immunostaining in 46% BE specimens: -?cyclin D1 overexpression early event in MDC sequence	Arber *et al*[9]
	↑ nuclear cyclin D1 immunostaining in 64% EAC specimens	Arber *et al*[13]
	Cyclin D1 expression correlates with degree of dysplasia in BE	Coppola *et al*[14]
	Cyclin D1 expression 43% BE mucosa (*vs* 0% normal mucosa)	Umansky *et al*[15]
	Polyphenon E inhibits growth of BE and EAC cells *via* downregulation of cyclin D1 expression	Song *et al*[16]
Cyclin E	↑ cyclin E expression in neoplastic cells in BE	Coppola *et al*[14]
	Cyclin E expression 37% BE mucosa (*vs* 0% normal mucosa)	Umansky *et al*[15]
$p27^{Kip-1}$	83% EAC specimens displayed low p27 protein levels (despite high p27 mRNA): -p27 inactivated in most BE-associated EAC (post-transcriptional modification)→loss of cell cycle inhibition	Singh *et al*[17]
	Experimentally-induced BE and EAC development in mouse model significantly enhanced by p27 gene knockout	Ellis *et al*[18]
EGF (and EGF-R)	↑ EGF in cytoplasm of BE epithelial cells (*vs* gastric mucosa)	Jankowski *et al*[19]
	EGF-R expression area in inflamed mucosa (43.1%) significantly > normal mucosa (29.5%); all BE showed positive EGF-R staining	Jankowski *et al*[20]
	EGF/EGF-R expression significantly ↑ in BE and EAC mucosa (*vs* normal mucosa) by flow cytometry ($P < 0.01$)	Jankowski *et al*[21]
	EGF-R expression positive in 64% of BE-associated EAC; ↑ staining associated with poorer survival ($P = 0.004$)	Yacoub *et al*[22]
	EGF A61G G/G genotype associated with >double EAC risk in BE pts (*vs* A/A or A/G) (OR 2.2)	Lanuti *et al*[23]
TGF-α	↑ TGF-α expression in cells from BE and EAC mucosa (*vs* normal gastric mucosa) by flow cytometry ($P < 0.01$)	Jankowski *et al*[21]
	TGF-α expression positive in 100% of BE-associated EAC	Yacoub *et al*[22]
HGF (and HGF-R)	HGF expression significantly ↑ in BE specimens (*vs* normal esophageal mucosa)	Konturek *et al*[24]
	Intense HGF-R immunostaining in 100% EAC and dysplastic BE specimens (*vs* minimal staining in non-dysplastic BE or normal mucosa); HGF-R mRNA and protein levels ↑ in EAC cell lines	Herrera *et al*[25]
Erb family tyrosine kinases	Membranous c-erbB2 overexpressed in 26% EAC (*vs* 0% BE with dysplasia): -?later event in MDC sequence	Hardwick *et al*[26]
	c-erbB-2 gene amplification in 14% EAC *vs* 11% HG-dysplasia *vs* 0% metaplasia/LG-dysplasia specimens	Geddert *et al*[27]
FGF	Immunostaining intensity for FGF sequentially ↑ from metaplasia/LG-dysplasia (negligible)→HG-dysplasia (weak/moderate)→EAC (moderate/strong)	Soslow *et al*[28]
	FGF-1 mRNA and protein expression sequentially ↑ in HG-dysplasia/EAC (*vs* metaplasia/LG-dysplasia/controls)	Soslow *et al*[29]
Src family tyrosine kinases	Src-specific activity 3-4-fold ↑ in BE and 6-fold ↑ in EAC (*vs* controls): -?Src activation early event in MDC sequence	Kumble *et al*[30]
	Strong Src expression in 85% EAC *vs* 93% BE HG-dysplasia *vs* 72% BE LG-dysplasia *vs* 27% BE specimens	Iravani *et al*[31]
Insensitivity to anti-growth signals		
p16	9p21 (p16) LOH observed in 89% EAC specimens (*vs* 0% non-dysplastic BE); homozygous p16 deletion in only 25%	González *et al*[32]
	p16 promoter hypermethylation (inactivation) in 75% BE with HG-dysplasia *vs* 56% LG-dysplasia (*vs* 3% non-dysplastic BE)	Klump *et al*[8]
APC	5q (APC) LOH seen in 80% EAC specimens (and surrounding mucosa)	Barrett *et al*[33]
	APC gene LOH observed in 60% EAC specimens (*vs* 0% non-dysplastic BE)	González *et al*[32]
	APC promoter hypermethylation in 92% EAC *vs* 40% BE (*vs* 0% normal esophageal tissues)	Kawakami *et al*[34]
Avoidance of apoptosis		
p53	Positive p53 immunostaining in 87% EAC *vs* 55% BE with HG-dysplasia *vs* 9% LG-dysplasia *vs* 0% non-dysplastic BE	Younes *et al*[35]
	17p (p53) LOH found in 91% BE pts who developed aneuploid cell populations: -17p allelic losses precede aneuploidy	Blount *et al*[36]
	p53 overexpression in 64% EAC *vs* 31% dysplastic BE *vs* 0% non-dysplastic BE; trend of ↑ p53 expression with ↑tumour grade: -?p53 mutation early event in malignant progression	Symmans *et al*[37]
	p53 immunoreactivity only in EAC/BE with HG-dysplasia (not in BE with LG-/no dysplasia); mutated p53 in 69%: -?late event in MDC sequence (during transition to HG-dysplasia)	Rice *et al*[38]
	p53 protein expression in 85% EAC specimens *vs* 60% BE with HG-dysplasia *vs* 7% LG-dysplasia ($P < 0.001$)	Rioux-Leclercq *et al*[39]
	p53 mutations identified in 75% EAC specimens; p53 overexpression in 58% EAC *vs* 60% BE with HG-dysplasia *vs* 12% LG-dysplasia *vs* 0% non-dysplastic BE	Chung *et al*[40]
Fas (CD95)	↓ surface expression of Fas observed in EAC specimens; impaired translocation of Fas to membrane wild-type Fas protein retained in cytoplasm in EAC cell line: -?potential mechanism by which EAC cells evade Fas-mediated apoptosis	Hughes *et al*[41]
	↓ surface expression of Fas and resistance to Fas-mediated apoptosis observed in EAC cell lines	Mahidhara *et al*[42]
Bcl-xl/Bax/Bcl-2	Bcl-xl positive in all dysplasia and EAC cells, but negative in 47% non-dysplastic BE: -?switch to anti-apoptotic phenotype in transformation from metaplasia to EAC	van der Woude *et al*[43]

	Bcl-2 expression in 84% LG-dysplasia *vs* 0% HG-dysplasia or EAC	Rioux-Leclercq *et al*[39]
	Cytoplasmic Bcl-xl immunostaining in 59% EAC *vs* 71% BE/HG-dysplasia *vs* 60% LG-dysplasia *vs* 27% non-dysplastic	Iravani *et al*[31]
COX-2	↑ COX-2 mRNA levels in 80% BE and 100% EAC specimens (*vs* normal gastric controls) ($P < 0.001$); COX-2 immunostaining strongly positive in 100% BE samples (> gastric controls)	Wilson *et al*[44]
	COX-2 immunopositivity in 91% non-dysplastic BE *vs* 94% dysplastic *vs* 97% EAC	Lagorce *et al*[45]
	Natural/synthetic COX-2 inhibitors suppressed proliferation, induced apoptosis and blocked cell cycle in EAC cell lines	Cheong *et al*[46]
	Cox-2 mRNA strongly upregulated in experimentally-induced BE epithelium in rat model (*vs* absent in control animals); COX-2 overexpression observed in human BE patients with dysplasia	Majka *et al*[47]
Limitless replicative potential		
Telomerase	Telomerase RNA positive in 100% EAC/BE with HG-dysplasia *vs* 90% LG-dysplasia *vs* 70% non-dysplastic BE: marked ↑ telomerase RNA accompanies transition along MDC sequence	Morales *et al*[48]
	human telomerase reverse transcriptase (catalytic subunit of telomerase) expression ↑ at all stages of BE *vs* normal controls, and in EAC ($P = 0.003$) and dysplastic BE ($P = 0.056$) *vs* non-dysplastic BE	Lord *et al*[49]
	Telomerase activity (by telomeric repeat amplification protocol assay) ↑ in EAC samples *vs* adjacent mucosa ($P = 0.0002$) and in EAC *vs* BE ($P = 0.001$); no difference BE *vs* adjacent mucosa	Barclay *et al*[50]
	Telomerase inhibition (by small interference RNAs) induced senescence in 40% and apoptosis in 86% in BE cell lines	Shammas *et al*[51]
Sustained angiogenesis		
VEGF (and VEGF-R)	VEGF expression correlated with higher vascularisation in BE and EAC specimens	Couvelard *et al*[52]
	VEGF-A expressed in BE epithelium; VEGFR-2 strongly expressed in immature endothelial cells feeding BE epithelium; ↑ VEGF-C expression in BE (*vs* absent in normal epithelium); ↑ VEGFR-3 in EAC: ?aberrant neovasculature early in MDC sequence	Auvinen *et al*[53]
	VEGF expressed in 64% EAC specimens; significantly correlated with angiolymphatic invasion/survival	Saad *et al*[54]
	VEGF expression significantly ↑ in EAC (> dysplastic BE > BE > normal epithelium)	Griffiths *et al*[55]
Invasive/metastatic potential		
CAMs	↓ expression in EAC specimens of E-cadherin (in 74%), α-catenin (60%) and β-catenin (72%)	Krishnadath *et al*[56]
	Abnormal expression of β-catenin ($P = 0.022$), α-catenin ($P < 0.01$) and E-cadherin ($P = 0.049$) significantly associated with higher degrees of BE-related dysplasia	Washington *et al*[57]
	↓ expression of E-cadherin with progression along MDC sequence ($P < 0.01$); in contrast P-cadherin absent from BE (± dysplasia) but expressed in 67% EAC specimens	Bailey *et al*[58]
	Slug (E-cadherin repressor) immunostaining and mRNA levels overexpressed in EAC *vs* BE metaplasia specimens: -?Slug upregulation represents mechanism of E-cadherin silencing	Jethwa *et al*[59]
Cathepsins	Detected amplicon at chromosome 8p22-23 resulting in cathepsin B overexpression (observed in 73% EAC samples)	Hughes *et al*[60]
	↑ cathepsin C expression in EAC (*vs* BE *vs* normal) in rat model	Cheng *et al*[61]
CD44	Stepwise ↑ cathepsin D mRNA levels in GERD→BE→EAC tissue	Breton *et al*[62]
	CD44-H and -V6 variant frequently expressed in BE; differing expression patterns along spectrum normal→dysplastic BE→EAC: -?CD44H and V6 involved in carcinogenesis of BE mucosa	Lagorce-Pages *et al*[63]
	↓ CD44 expression in EAC/HG-dysplasia (*vs* BE/LG-dysplasia)	Darlavoix *et al*[64]

BE: Barrett's esophagus; MDC: Metaplasia-dysplasia-carcinoma; EAC: Esophageal adenocarcinoma; EGF: Epidermal growth factor; EGF-R: EGF receptor; pts: Patients; OR: Odds ratio; TGF: Transforming growth factor; HGF: Hepatocyte growth factor; HGF-R: HGF receptor; mRNA: Messenger RNA; FGF: Fibroblast growth factor; HG: High grade; LG: Low grade; LOH: Loss of heterozygosity; APC: Adenomatous polyposis coli; COX-2: Cyclooxygenase-2; VEGF: Vascular endothelial growth factor; VEGF-R: VEGF receptor; CAM: Cell adhesion molecule; GERD: Gastro-esophageal reflux disease.

Table 2 Clinical and demographic risk factors for neoplastic progression of Barrett's esophagus

Innate factors	Gastrointestinal factors	Other modifiable factors
Age	Bile and acid reflux	Obesity
Gender	Anti-reflux surgery	Diet
Ethnicity	Proton pump inhibition	Alcohol
	Pharmacological lower esophageal sphincter relaxation	Smoking
	Salivary nitrates	Socioeconomic status
	Barrett's segment length	Pharmacological COX-2 inhibition

COX-2: Cyclooxygenase-2.

inform disease prevention strategies. Epidemiological studies of EAC have described a "birth cohort effect", with higher incidence rates observed in recent cohorts suggesting exposure to an exogenous risk factor in early life contributing increased risk in all ages of the cohort[70] (Figure 2). Multiple risk factors for neoplastic progression of BE have been investigated (Table 2).

INNATE HOST FACTORS

Age is a well-recognized risk for both BE and EAC. Corley *et al*[71] reported an incidence of BE of 2/100 000 for 21-30-year-old and 31/100 000 for 61-70-year-old, whilst El-Serag *et al*[70] calculated the risk of EAC to increase by 6.6% for each 5-year age increase. Evidence specifically linking age to risk of neoplastic progression within BE is lacking, but it seems intuitive to propose advancing age as an independent risk factor.

BE displays a male preponderance of approximately 2:1, rising to 4:1 for BE-associated EAC, suggesting an independent influence of gender on risk of neoplastic pro-

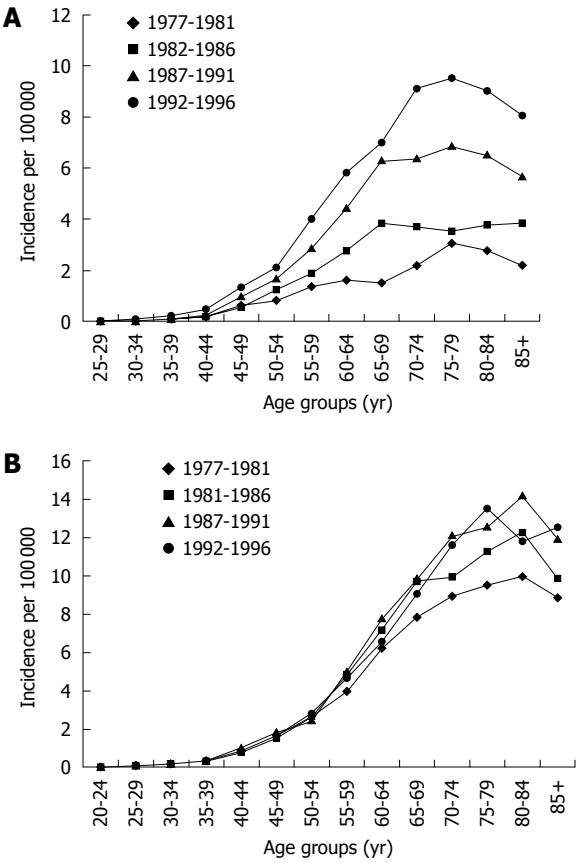

Figure 2 Age distribution of cases diagnosed with oesophageal adenocarcinoma (A) and gastric cardia adenocarcinoma (B) in the USA between 1977-1996, displaying the "birth cohort effect". Each individual curve represents the age-specific incidence rates in a five year period (from El-Serag *et al*[70]).

gression[71,72]. Why male gender should confer additional risk is unknown; some have speculated that male propensity toward visceral pattern of obesity might be relevant[73].

A higher prevalence of BE in Caucasians has long been recognized[74]; again, this association strengthens with development of BE-associated EAC[75]. Analysis of the US Surveillance, Epidemiology and End Results registry found that the annual incidence of EAC for Caucasian males was double that for Hispanic males and four times higher than Black, Asian, Pacific Island and Native American males[76]. Although selection bias and differing endoscopy uptakes between ethnic groups might partially explain this, other factors seem to be involved. Whilst environmental influences are probably important, hitherto-unidentified genetic variations influencing protection against reflux-induced mucosal damage seem likely. A US study found similar GERD prevalence in Caucasian and Black Americans from the same geographical population, yet the latter displayed significantly less esophagitis and almost no BE[77].

GASTROINTESTINAL FACTORS

Bile/acid reflux

The relationship between GERD and BE is well established, and whilst reflux of gastric acid is known to induce chronic mucosal esophageal injury the contribution of bile salts and acids (from duodenal refluxate) is increasingly recognized. Vaezi and Richter demonstrated patients with complicated BE (dysplasia/stricture/ulceration) reflux significantly greater amounts of both gastric and bile acids than those with uncomplicated BE, and postulated that complications might result from synergism between the two[78]. Bile salts induce esophageal injury over a wide pH range, and patients with BE display significantly more bile salts in aspiration studies than patients with mild reflux only[79]. Menges *et al*[80] observed a strong correlation between duration of esophageal exposure to acid and bile with severity of pathological change in BE. Furthermore, proton pump inhibitor (PPI) therapy predisposes to upper gastrointestinal bacterial colonization and consequent bile salt-deconjugation, which, in this high pH environment, has been linked to chronic inflammation[81].

Refluxate-mediated inflammation might promote carcinogenesis *via* both the arachidonic acid (AA) pathway and induction of oxidative stress. Low pH and bile salts promote expression of cyclooxygenase-2 (COX-2), catalyzing conversion of AA into various prostaglandins, including PGE_2. PGE_2 increases proliferation of BE epithelial cells and inhibits tumor surveillance through suppressing natural killer cell function. Consequently, abnormal cells displaying genomic instability may accumulate. COX-2 expression has been shown to increase with neoplastic progression of BE, supporting a role for the AA pathway in EAC carcinogenesis[44]. Chronic mucosal injury also induces production of reactive oxygen species (ROS), depletes antioxidants and increases expression of oxidative stress-related genes. High levels of oxygen radicals and lipid peroxidation products have been demonstrated in BE epithelial cells, with reduced levels of vitamin C and glutathione, indicating compromised oxidant defences[82]. ROS have well-established mutagenic capacity, whilst subsequent apoptosis of mutated cells is additionally suppressed by capacity of bile salts to induce proteasomal degradation of *p53*[83].

The Factors Influencing the Barrett's Adenocarcinoma Relationship (FINBAR) study suggested GERD symptom chronicity and frequency appeared better predictors for neoplastic progression than severity[84]. However, a significant proportion of EAC patients (40%-50%) do not recall ever having prior reflux[85]. Furthermore, reflux of gastroduodenal contents correlates poorly with heartburn symptoms, BE is frequently asymptomatic and development of less sensitive Barrett's epithelium may ameliorate symptoms. Thus, symptom-based risk scores for assessing progression risk have so far not proved useful in clinical practice.

PPIs

PPIs increase pH of gastric refluxate, attenuating acid-induced damage. Ouatu-Lascar *et al*[86] showed "normalization" of intraesophageal pH with acid suppression favors differentiation and reduces cellular proliferation in BE biopsy specimens. However, PPIs have not prevented

recent increases in EAC, and the observation of EAC with PPI administration in animal models raises concern they might actually favor progression of BE[87]. This might be mediated *via* interaction of gastrin with its cholecystokinin receptor, CCK_2R. PPIs elevate serum gastrin levels, which on binding to CCK_2R, stimulate expression of EGF and trefoil peptide, inducing COX-2 expression. Gastrin exposure increases proliferation in esophageal cell culture, and BE mucosa expresses more CCK_2R than normal squamous mucosa. CCK_2R stimulation also inactivates pro-apoptotic factors[88].

Despite this, the clinical relevance in humans remains unproven. Three large studies have examined PPI usage and EAC risk in BE patients, each reporting a strong inverse correlation. Two observed a decreased risk with longer duration of PPI, and one showed an increased risk with delayed PPI use[89]. Obszynska *et al*[90] investigated effects of hypergastrinemia induced by different PPI doses in cell models and BE patients. Despite increased cell proliferation *in vitro*, COX2 induction and enhanced epithelial restitution, they found no evidence of longer-term harm using surrogate biomarkers of proliferation or apoptosis *in vivo*. The Aspirin Esomeprazole Chemoprevention Trial (AspECT) is currently investigating effects of different PPI doses in combination with aspirin on EAC risk.

Anti-reflux surgery

Theoretically, anti-reflux surgery should prevent reflux of duodenal contents, against which PPIs have no effect, potentially mitigating against progression of BE. Unfortunately this is not supported by the available evidence. Two large cohort studies failed to show cancer protection in GERD patients[91,92], whilst a meta-analysis by Corey *et al*[93] concluded no reduction in progression risk for BE. However, different surgical procedures were employed and effectiveness of reflux control was not always assessed.

Lower esophageal sphincter-relaxing drugs

Pharmacological lower esophageal sphincter (LES) relaxation might promote development/progression of BE by increasing reflux, suggested by the observation that drugs with these effects (e.g. tricyclic antidepressants) have increased in use alongside the rise in EAC. A Swedish population-based study by Lagergren *et al*[94] reported a positive association between EAC and long-term use of LES-relaxing drugs, with the strongest association for anticholinergics; this association disappeared after adjustment for reflux symptoms.

Helicobacter pylori infection

An increase in BE-associated EAC alongside falling rates of *Helicobacter pylori* (*H. pylori*) infection has led some to propose a protective effect of *H. pylori*, mediated by its influence in reducing gastric acidity. The virulent cagA strain is particularly associated with high-grade gastric inflammation and atrophy[95]. A meta-analysis by Rokkas *et al*[96] reported statistically significant inverse relationships between *H. pylori* infection and both EAC and BE [odds ratio (OR), 0.52% and 0.64%, respectively]. Furthermore, a large prospective study of BE patients and GERD controls found less *H. pylori* infection with increasing "severity" of disease: 44% in GERD; 35% in uncomplicated BE; 14%-15% in BE with high-grade dysplasia/EAC[97].

However, another study, controlling for demographic and lifestyle factors, failed to demonstrate reduced EAC with cagA+ infection[98]. A confounding factor might be the degree of bile acid reflux, since excessive bile reflux may prevent *H. pylori* colonization and contribute to chronic mucosal injury[88]. The protective role for *H. pylori* is debatable and since *H. pylori* is a World Health Orgaisation class 1 mutagen for gastric adenocarcinoma it is difficult to argue against its eradication whenever it is detected.

Salivary nitrates

Dietary nitrate, concentrated in saliva and reduced to nitrites by oral flora, produces intraesophageal nitric oxide (NO) during reflux. Achlorhydria induced by PPI or atrophic gastritis may cause overgrowth of nitrate-reducing bacteria in the upper gut, providing another source of nitrite[88]. Clemons demonstrated the capacity of NO to induce double-strand DNA breaks in esophageal BE cells *in vitro*, which could promote neoplastic progression[99]. Increasing agricultural nitrate use in the latter 20th century caused significant increases in nitrate content of leafy vegetables and drinking water[100] and could have partially contributed to the increase in EAC incidence.

Barrett's segment length

Although EAC can develop in BE segments of any length, several observational studies support the intuitive notion that longer segments confer greater risk[101]. However, a meta-analysis by Thomas *et al*[102] showed only a non-significant trend towards reduced progression with shorter BE segments, and evidence remains insufficient to advocate surveillance strategies based on segment length alone.

OTHER MODIFIABLE RISK FACTORS

Obesity

Increasing obesity has also paralleled increased rates of BE and EAC. Strong links between obesity and both GERD and erosive esophagitis have been established[103]. It is logical that this might predispose to BE, but a meta-analysis specifically comparing body mass index (BMI) in BE cases with population controls showed only a modest risk increase[104]. However, elevated BMI is a strong risk factor for EAC (OR, 1.8 and 2.4 for BMI > 25 and BMI > 30, respectively)[105]. Increased risk may relate more to distribution of body fat than BMI alone, with visceral (abdominal) obesity conferring greater risk[106]. Other studies noted an association between obesity in early life and EAC risk, suggesting adiposity may act early in the disease process[84,107].

Although a small prospective study by Oberg and colleagues failed to identify any association between BMI

Table 3 Selected published evidence linking adipokines (and ghrelin) with Barrett's esophagus and progression to esophageal adenocarcinoma

Adipokine	Evidence in BE and EAC	
	Relevant study findings	Ref.
Adiponectin	↓ adiponectin receptors in Barrett's mucosa compared with normal mucosa from controls	Konturek *et al*[110]
(↓ in obesity)	↑ Bax (pro-apoptotic), ↓ Bcl-2 (anti-apoptotic) and ↑ apoptosis of EAC cell lines on incubation with adiponectin	Konturek *et al*[110]
	Plasma adiponectin levels inversely associated with BE risk in 50 matched cases (OR 4.7 for each 10 μg/mL ↓ in level) (independent of BMI)	Rubenstein *et al*[111]
	No difference in adiponectin levels between 51 BE patients and 67 controls	Kendall *et al*[112]
Leptin	Leptin receptors expressed in esophagus	Francois *et al*[113]
(↑ in obesity)	↑ proliferation and ↓ apoptosis (*via* various signalling pathways) in EAC cell lines	Ogunwobi *et al*[114]
	Leptin levels strongly associated with ↑ risk of BE in males (no association in females)	Kendall *et al*[112]
	Gastric (fundic) leptin levels positively associated with BE (no association with serum leptin)	Francois *et al*[113]
Ghrelin	↑ gastric emptying (so may ↓ gastric reflux)	Dornonville *et al*[115]
(↓ in obesity)	↓ TNF-α-induced COX-2 and interleukin-1-β expression in BE cell line	Konturek *et al*[110]
	Ghrelin expression negligible in archived EAC cell specimens (*vs* rich expression in normal mucosa)	Mottershead *et al*[116]
	↑ serum ghrelin associated with ↓ EAC risk (in overweight subjects)	de Martel *et al*[117]

BE: Barrett's esophagus; EAC: Esophageal adenocarcinoma; OR: Odds ratio; BMI: Body mass index; COX-2: Cyclooxygenase-2; TNF: Tumor necrosis factor.

and progression from BE to low- or high-grade dysplasia[108], it had limited power, and a larger study from the Seattle Barrett's Esophagus Program revealed strong correlations between waist-to-hip ratio and intermediate biomarkers of progression[109]; again, associations were less apparent for elevated BMI *per se*.

Obesity causes GERD through several mechanical and physiological mechanisms. However, part of the association between obesity and EAC is independent of GERD, suggesting a role for reflux-independent mechanisms, probably linked to important endocrine actions of adipose tissue. Many recent studies have linked several adipokines (metabolically active factors) to plausible actions in the MDC process[110-117] (Table 3).

Kristal *et al*[118] investigated whether weight loss (alongside other dietary measures) impacted upon measured biomarkers of cellular proliferation in BE. Despite weight loss (mean 3.6 kg) at 18 mo no differences in biomarkers were observed. This study was relatively small, and the lack of response might relate to the relatively modest weight loss, and/or choice of proliferation markers employed.

Diet

Several studies have shown an association between a diet high in fruit and vegetables and reduced EAC. A large population-based Swedish study found individuals in the highest exposure quartile of fruit and vegetable intake to have approximately 50% less EAC compared to the lowest quartile[119]. However, Kristal *et al*'s study observed no effect on biomarkers of BE cell proliferation despite a net increase in fruit and vegetable consumption[118], whilst the FINBAR study observed a reduction in EAC with increased fruit, but not vegetable, consumption[84]. A protective effect for the natural anti-oxidants in fruit was proposed. A well-controlled, prospective study by Dong *et al*[120] showed patients who took multivitamin pills had significantly decreased risk of tetraploidy [hazard ratio (HR), 0.19] and frank EAC (HR, 0.38). Significant inverse associations with EAC were also observed for supplemental vitamins C (HR, 0.25) and E (HR, 0.25), both well-recognized antioxidants.

Chen *et al*[121] observed a significant inverse association between zinc intake and EAC risk compared with controls (OR, 0.5); inverse associations were also noted for vitamin A, β-cryptoxanthin, riboflavin, folate, fiber, protein and carbohydrate, whilst saturated fat intake was positively associated with EAC. Rudolph *et al*[122] investigated selenium levels in 396 BE patients: those with levels in the upper three quartiles were less likely to display high-grade dysplasia (OR, 0.5), aneuploidy (OR, 0.4) or 17p LOH (OR, 0.5) than the lowest quartile. No association was observed with *p16* LOH (an early event in the MDC sequence), indicating selenium's protective effects might occur late in progression to EAC.

Alcohol

Data supporting links between alcohol and BE/EAC are sparse. The UK BE registry found no association between alcohol consumption in patients with BE compared with reflux esophagitis[123]. Although at least eleven studies have investigated the relationship between alcohol and EAC only six have shown a positive association, and in most it was weak[124-134]. One study even seemed to suggest wine to be protective[133].

Smoking

Studies of smoking and BE/EAC are contradictory. An Australian population-based case-control study found smoking was associated with 2- to 3-fold increased risk of BE and BE with dysplasia[135]. However, there was no dose-response effect. Other small studies found no clear association[131]. Whilst smoking is a strong risk factor for esophageal squamous cell carcinoma, studies of EAC have been inconsistent, yielding conclusions ranging from complete absence of association[132-134] to a significant OR of 3.4 for current smokers[128]. Problems with study methodology occur and certainly smoking has rarely been a primary endpoint for studies of BE/EAC.

Socioeconomic status

There are no clear associations between socioeconomic status and neoplastic progression of BE. Some studies suggest increased EAC risk in higher socioeconomic groups, others the reverse[72].

COX-2 inhibition

Given the role of the AA pathway in neoplastic progression, pharmacological inhibition of COX-2 might modify the natural history of BE. Various studies have investigated whether aspirin and non-steroidal anti-inflammatory drugs (NSAIDs) might confer protection against EAC. A meta-analysis by Corley *et al*[136] including 1813 EAC patients suggested a protective association (OR, 0.67). Both intermittent and frequent use appeared advantageous, with evidence of a dose-effect, whilst aspirin conferred greater protection than NSAIDs.

However the Chemoprevention for Barrett's Oesophagus Trial randomized 100 BE patients with dysplasia to either celecoxib 200 mg twice daily or placebo, with negative results[137]. A retrospective analysis of the UK BE registry with a total follow-up of 3683 patient-years also failed to demonstrate a protective effect of aspirin[138]. AspECT should provide further useful information.

CONCLUSION

The etiology of progression of BE is probably multi-factorial, with contributions from environmental risk factors interacting with genetically-determined characteristics. Obesity and ongoing bile and acid reflux are emerging as potentially modifiable risk factors, though designing practical interventions has so far proved difficult. Developments in understanding the MDC process in BE may provide future testable therapeutic targets.

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REVIEW

Future of liver transplantation: Non-human primates for patient-specific organs from induced pluripotent stem cells

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Abstract

Strategies to fill the huge gap in supply *versus* demand of human organs include bioartificial organs, growing humanized organs in animals, cell therapy, and implantable bioengineered constructs. Reproducing the complex relations between different cell types, generation of adequate vasculature, and immunological complications are road blocks in generation of bioengineered organs, while immunological complications limit the use of humanized organs produced in animals. Recent developments in induced pluripotent stem cell (iPSC) biology offer a possibility of generating human, patient-specific organs in non-human primates (NHP) using patient-derived iPSC and NHP-derived iPSC lacking the critical developmental genes for the organ of interest complementing a NHP tetraploid embryo. The organ derived in this way will have the same human leukocyte antigen (HLA) profile as the patient. This approach can be curative in genetic disorders as this offers the possibility of gene manipulation and correction of the patient's genome at the iPSC stage before tetraploid complementation. The process of generation of patient-specific organs such as the liver in this way has the great advantage of making use of the natural signaling cascades in the natural milieu probably resulting in organs of great quality for transplantation. However, the inexorable scientific developments in this direction involve several social issues and hence we need to educate and prepare society in advance to accept the revolutionary consequences, good, bad and ugly.

Key words: Induced pluripotent stem cells; Hepatocytes; Tetraploid; Non-human primates; Anencephaly; Chimpanzee; Fumaryl acetoacetate hydrolase deficient; Hhex

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INTRODUCTION

There is a huge gap in supply *versus* demand of human organs for transplantation. Currently 108 614 patients in United States are waiting for an organ transplant according to United Network for Organ Sharing (UNOS)[1] against 7136 donors. There is a need to bridge this gap. Either we have to motivate more people to allow organ donation, or rely on alternative methods such as improved artificial organ support systems (dialysis machines, bioartificial liver, etc) or search for better ways to circumvent the problems, mainly immunological, with xenografts.

This includes improved methods for suppressing host immunity and growing "humanized organs" in animals. Recent developments with induced pluripotent stem cells (iPSC) have yielded a new option-growing organs from pluripotent stem cells derived from the patient's own tissues. Attempts have been made to grow

organs *in vitro* with mixtures of different stem cells and biocompatible scaffolds, but the development of an organ cannot be replicated *in vitro* due to its complexity. This is a major obstacle in the generation of organs, including those attempts to make organs derived from a patient's iPSCs, the ultimate goal in regenerative medicine. The straightforward method is to generate a fetus from iPSC in a surrogate mother for the sole purpose of organ harvesting, but obviously a host of ethical issues precludes this line of thought. Here, I will make an attempt to review the latest developments and discuss their prospects, taking the liver as a model organ.

Currently, more than 17 000 people in the United States are waiting for liver transplants. According to UNOS, about 5300 liver transplantations were performed in the United States in 2002.

BIOARTIFICIAL ORGANS-THE BIOARTIFICIAL LIVER: A WRONG ROAD?

The bio-artificial liver (BAL) is still in its infancy. BAL as a replacement for the normal liver is very unlikely. At most it is currently of use in bridging the gap between organ failure and transplantation or liver regeneration (as the liver has exceptional capacity to regenerate). BALs are largely unsuccessful because: (1) most of the liver cell lines are not functionally efficient and human iPSC-derived "hepatocytes" are not functional enough and difficult to obtain in sufficient quantities; (2) the special arrangement of hepatocytes into chords in sinusoidal spaces is important for their function; (3) the relationship and communication between hepatocytes themselves and between biliary epithelial cells, sinusoidal endothelial cells, etc, are quite important for functions of hepatocytes, such as active and passive transport of metabolites in the right direction and optimal gene regulation; (4) currently there is no source of functional hepatocytes in large quantities for bioreactors except from animals, which always pose a problem of infection, immune system activation, and functional incompatibility of essential proteins secreted by or possibly interacting with hepatocytes; (5) hepatocytes have a low life span under culture conditions, and it is difficult to maintain conditions close to that of the human microenvironment; and (6) difficulties in developing complex membranes which allow highly selective exchange of biologically important molecules.

The efficiency of bioartificial liver devices such as the "extracorporeal liver assist device" (ELAD) or non-biological devices such as the "molecular adsorbent recycling system" (MARS) and other models are not very different from dialysis alone. The current data show that only the MARS system reduces mortality in acute liver failure and in acute exacerbations of chronic liver failure, although this reduction is non-significant[2].

HUMANIZED LIVER IN ANIMALS

Pigs are the preferred animal for humanized organs, although primates like chimpanzees or gibbons would the ideal for the generation of "humanized" organs. We have made remarkable progress in the last 10 years in the field of xeno-immunology of pig-to-nonhuman primate transplantation, and we are expecting clinical trials in the near future. A common school of thought is for engineered animals lacking certain antigens so that their organs can be used for transplantation in human patients with a reduced chance of immune rejection[3]. Pigs can be genetically modified for xenotransplantation by alteration of immunologically important genes such as human decay-accelerating factor (hDAF), and CD46 (membrane cofactor protein), α-galactosyl transferase knockout (GT-KO), CD55 or CD46, CD59 transgenics, as well as human leukocyte antigen (HLA)-Ⅱ transgenics, including DP, DQ, and DR[3,4]. HLA-DR15+ transgenic pig skin pieces were grafted onto severe congenital immunodeficiency (SCID) mice reconstituted intraperitoneally with HLA-DR15+ human peripheral blood mononuclear cells. The dermal graft survived and was integrated[4]. Using GT-KO pigs and novel immunosuppressant agents, 2 to 6 months' survival of heterotopic heart xenotransplants has been achieved. The issue of hyper-acute rejection is more or less solved with hDAF and GT-KO pigs, but acute humoral xenograft rejection, injury to the endothelium leading to thrombotic microangiopathy and coagulation dysregulation, remains unsolved for a meaningful survival rate to be achieved. Baboons died following massive internal bleeding and profound thrombocytopenia post-transplantation of livers from GT-KO pigs transgenic for CD46[5,6].

STEM CELLS, IPSC AND SOPHISTICATED SCAFFOLDS MEET THE ANGIOGENESIS OBSTACLE FOR GENERATING ORGANS

The genesis of organs is a very complex process. Organs such as the brain, liver and kidney have extremely complicated architecture and contain several cell types. The relationship between cells, their specific orientation, and physical and chemical characteristics are of crucial functional importance. Thus, even if we generate genetically intact and fully functional hepatocytes, biliary epithelial cells, angiogenic precursor cells, sinusoidal endothelial cells, kupffer cells and so on, we are unlikely to regenerate (or generate *de novo*) a liver through co-culture of these cells, injecting these cells in a defined proportion into a damaged liver, or populating an appropriate scaffold or matrix. A highly sophisticated scaffold or matrix with spatial and temporal cues-chemical, mechanical, ionic, electric charge or surface properties-for homing of different cell types is unlikely to be successful in the near future, considering the complexity of the micro architecture of organs required for normal physiological function. One of the major barriers to successful generation of organs *in vitro* is our inability to generate the vascular architecture necessary for growth, development

and maintenance of any organ. Recently, attempts have been made to use natural scaffolds by decellularization of an entire organ, the liver in this example, and preserve its vascular network. Preliminary studies showed the possibility of being able to efficiently re-cellularize the bioscaffold using perfusion cell seeding with primary human fetal liver progenitor cells and endothelial cells in a bioreactor[7]. However, as noted above, numerous difficult technical issues remain to be addressed to efficiently deliver primary human liver progenitor cells to generate functional hepatic tissue. Availability of decellularized human liver scaffolds would be another problem.

Although iPSC technology offers wonderful possibilities for generating practically every cell type from adult somatic cells through a pluripotent stem cell intermediate, currently this has limited applications in, for example, regeneration of tissues of lesser complexity such as bone marrow and adipose tissue with a genetic modification [example: C-C chemokine receptor type 5 (CCR5) in the bone marrow stem cell gene therapy of acquired immunodeficiency syndrome (AIDS) or adipocyte gene therapy in inherited forms of diabetes or lipodystrophy][8] or without a genetic modification (as in the management of leukemia or degenerative disease, old age), or drug testing. For example, iPSC-derived hepatocyte-like cells, and proximal or distal renal tubular epithelium for hepatic or renal toxicity testing, respectively, are useful in new drug development or assessment of drug response to different human genotypes, a step towards personalized medicine.

Small organs or tissues can be engineered successfully using scaffolds, for example, blood vessels or urinary bladder. By culturing cells on a biodegradable scaffold such as polyglycolic acid, and later passing media in a pulsatile fashion under optimum pressure, was found useful in generating functional small-caliber arteries[9]. The pulsatile flow triggers collagen deposition and alignment of the fibers and this is critical for attaining mechanical maturity to withstand pressure met under natural conditions[10].

Growing larger organs is a major problem because oxygenation and metabolite exchange becomes difficult as the thickness increases. Self-assembly of cells, for example cardiomyocytes, can take place in thin sheets (< 80 μm), and increasing the thickness by sequential deposition of multiple cardiac sheets has to be slow enough to allow the host vasculature to sprout into and vascularize each layer before the next layer is deposited[11]. However this method is very impractical in humans because of the necessity of multiple surgeries. Furthermore, this approach is unlikely to be successful for more complicated organs like the liver, not only because the liver has different types of cells in a highly ordered manner, but it also has a complicated dual vasculature forming the sinusoids. Following a nature mimetic approach, a vascular tree should have a capillary network (10 μm-20 μm) which can be generated by induction of sprouting by cytokines and co-culture with related cells; the intermediate microvessels (50-500 μm) may be obtained by microfabrication-microfluidic techniques and finally the microvasculature (about 2 mm) is produced by a combination of tissue engineering methods[12]. Unfortunately, achieving vascularization in a tissue by assembling all these and finally generating a fully vascularized organ which is functional is a very complicated process making this approach undesirable.

MAKING GENETICALLY HUMAN ORGANS IN ANIMALS

It is an ingenious idea to generate genetically human organs in animals. With the recent advancement in iPSC technology, transgenic technology and embryo manipulation, it is possible to generate organs of one animal species in another one. The best achievement in this direction is reported by Kobayashi *et al*[13] in Cell 2010. Mouse wild-type iPSCs injected into Pdx1 -/- rat blastocysts (Pdx is a critical gene for genesis of the pancreas and hence Pdx1 -/- rats are pancreatogenesis-disabled) developmentally compensated for the vacancy of the pancreatic "developmental niche", generating almost an entirely iPSC-derived rat pancreas inside the mouse, and mouse iPSC-derived pancreas inside the Pdx-/- mouse. Similarly it could be possible to generate a human pancreas (and other organs) in animals, for example in monkey, pig or sheep, which are genetically modified to support implantation and development of an embryo containing cell clusters/organ of human origin.

Production of a chimeric embryo/fetus[14] was performed largely to study organogenesis, cell migration, cell lineages, cell destination, development and function of the immune system, rather than with the aim of generating live chimeric animals for organ/tissue harvesting. However, efforts to make live intergeneric chimeric animals (for example rat-mouse chimera) were unsuccessful because of incompatibility between the fetal parts of the placenta and the uterus[15,16]. The only exceptions we know are hybrids like geep (a sheep and a goat)[15-17]. Thus it is one of the major achievements of 2010 to produce a rat-mouse intergeneric chimera by injecting mouse pluripotent stem cells into rat blastocysts.

NEW WORLD MONKEYS COULD BE USED TO GENERATE GENETICALLY HUMAN ORGANS

Rats and mice belong to same family (Muridae) and subfamily (Murinae), but of a different genus, while human beings and chimpanzees belong to the same genus and there are seven species in the sub-family "Hominini" which contains man, chimpanzees, gorillas and orangutans. Man is closer to the chimpanzee than the rat is to the mouse (Table 1). Modern molecular studies have spectacularly confirmed this prediction and have refined the relationships, showing that the common chimpanzee

Table 1 Comparison between mouse and rat *versus* chimpanzee and human

	Mouse	Rat	Chimpanzee	Human
Size	20 g-40 g	250 g-520 g	35 kg-75 kg	45 kg-100 kg
Chromosomes	20 pairs	21 pairs	24 pairs	23 pairs
Genome similarity	96.50%		98%	
Gestation period	20 d	22 d	9 mon	9 mon
Birth weight	0.5 g-1.5 g	5 g-6 g	1.5 kg-2 kg	2 kg-4 kg
Liver	4-5 distinct lobes	4 distinct lobes	3 lobes which are not separate	3 lobes which are not separate
Gallbladder	Present	Absent	Present	Present

(*Pan troglodytes*) and bonobo (*Pan paniscus* or pygmy chimpanzee) are our closest living evolutionary relatives[18]. This opens an exciting possibility to generate and harvest human organs, genetically identical to the recipient, in new world monkeys. There is about 98% sequence similarity between human and chimpanzee genomes, and the global variation between humans at the single nucleotide level has been estimated at about 0.1%[19,20]. Chimpanzee body temperature, general blood biochemistry (glucose, sodium, potassium, calcium, phosphate, insulin, hemoglobin, urea, etc), red blood cell count, white cell count, platelet count, osmolarity, plasma protein composition, etc, falls within the range of human values[21]. It may be noted that rather than genetic differences, what makes humans unique are "aspects of human uniqueness which arose because of a primate evolutionary trend towards increasing and irreversible dependence on learned behaviors and culture"[20].

There are multiple possible approaches to generate a human organ in a chimpanzee or a higher primate. One approach is to make a true chimera by populating the chimpanzee donor blastocyst with patient specific human iPSC, which is modified to have genes for development of the liver but deficient in genes for brain development. This ensures that under no circumstances will a human brain develop inside an animal or grow with cells of animal origin. The chimpanzee donor blastocyst should be deficient in the genes which are critical and specific for the development of the organ in question (Pdx1 in pancreas and probably Hhex in liver). However, for the human liver we have yet to identify the most suitable liver-specific gene which can be knocked out without affecting general development of the fetus. Foxa1, 2 and 3, Gata-6, HNF-4a, HNF-1a, Hhex, Sox-9 are among key genes involved in foregut-liver development[22-26]. Alternatively, the blastocyst may be deficient in a protein which is metabolically important, and whose deficiency would result in selection pressure, such that only the iPSC-derived cells would survive. Taking the liver as a model organ, fumaryl acetoacetate hydrolase (FAH)-deficient chimpanzee blastocysts would be a good example which would be populated with human patient derived iPSC with the normal (wild type) FAH gene. During the development of the fetus, human liver cells expressing FAH would have a survival advantage over chimpanzee liver cells lacking FAH. Fumarylacetoacetate, a toxic metabolite, will accumulate in FAH deficient liver cells and kill them[27]. This selection process can be controlled at will using NTBC to facilitate a smooth and optimum rate of cell replacement without affecting the liver architecture. The introduction of 2-(2-nitro-4-trifluoromethylbenzoyl)-1,3-cyclohexanedione (NTBC) inhibits p-hydroxyphenylpyruvate dioxygenase (the second enzyme in tyrosine degradation) and stops the formation of the toxic metabolites[27]. Thus, giving animals NTBC and slowly weaning them off might lead to a liver which is exclusively composed of human liver cells inside a chimpanzee fetus. One major worry in creating human organs in animals is the formation of germ cells from human donor cells in the gonads of the recipient animal, although the possibility is remote. Using animal blastocysts as well as the surrogate mother animal where the critical genes for spermatogenesis/oogenesis are knocked out, the theoretical possibility of germline transmission of human genes can be ruled out. A practical approach would be to use a mixture of human patient-derived iPSC with nonhuman primate embryonic cells/iPSC, in which some specific genes for fertility (with no effect on implantation or the development of the embryo, e.g., an acrosomal protein for male infertility) are knocked out for introduction into a tetraploid embryo (tetraploid complementation technique)[28]. It may be noted that there are reports of efficient generation of iPSC from non human primates[29]. There exist several methods to generate genetically intact 'virus free' iPSC from adult primate cells[30-34].

The proposal is very attractive but we can anticipate the following problems: (1) a chimpanzee-human mosaic fetus may not survive (though unlikely) because: (a) growth factors and transcription factors and/or signaling pathways may be incompatible; (b) cell adhesion molecules or response to directional molecule gradients may be different; and (c) the developing fetus may abort due to unforeseen reasons (e.g., failure of the tetraploid complementation technique or implantation and development); (2) possible immune rejection on transplantation even after perfusion washes and immune cell depletion due to small quantities of antigens, for example glycoproteins, adhering to the vessel walls, interstitial spaces, growth of some animal blood vessels into the "human" organ, etc; (3) a possibility that iPSC-derived organs are more prone to tumors; and (4) ethical issues involved in making human-chimpanzee mosaic embryos which might survive to near full term, even if it is ensured that human brain (or certain types of human neurons important in cerebral cortex for human identity) will not be present in the fetus by using human iPSC knockout for genes specific for brain development.

Despite these problems success is very likely because the genetic difference between mouse and rat is greater than that between human and chimpanzee and it was proved by Kobayashi *et al* that it is possible to generate a rat pancreas in a mouse. The immunological rejection

is less likely to be a major problem at least in the case of the liver because: (1) liver is a very immune tolerant organ compared with several other organs such as the kidneys[35,36]; (2) the patients own cells will be used to generate the new liver, ensuring 100% HLA matching; (3) better and less toxic immune-suppressants and immune-modulators are currently available; and (4) one can perform immunodepletion on the liver prior to harvest, first by treating with an immunocyte-specific mitogen and then treating with cyclophosphamide; this will push the immunocytes into mitosis which would then be preferentially killed by cyclophosphamide. There are more than a few ways to overcome the obstacle presented by the immune system in this setting, including the induction of immunological tolerance in the host[37]. Novel methods such as inhibition of leukocyte costimulatory molecules may offer a way to suppresses T cell activation resulting in immune suppression[38]. Several studies have found increased abnormal epigenetic changes, mutations in coding regions, and copy number variations in induced pluripotent cells compared with normal in a small proportion of cells[39-42].

It may be noted that iPSC is a relatively new technology and it might take another decade for the technology to mature. Similarly, newer screening methods which would facilitate selection of genetically intact cells, such as faster methods for whole genome scanning for mutations and epigenetic abnormalities are expected to resolve these issues.

Any research involving implantation of human embryos into the uterus after *in vitro* manipulation at any stage of development in humans or primates is illegal. However using a non-human primates (NHP) embryo to develop a human organ inside a NHP fetus inside the uterus of a NHP may not be illegal in many countries. The National Academy of Sciences (United States) Guidelines, recommends that human-nonhuman chimeras will not be allowed to breed, but this recommendation is only voluntary[43-45].

MAKING HUMAN ORGANS IN PARTLY *IN VITRO* SYSTEMS-ETHICAL ISSUES

Many ideas which are quite logical cannot be put into practice because of ethical concerns. One great example is therapeutic cloning. Commoditization of human oocytes and human sperm or human embryos and human organs is considered unethical in many countries. Any *in vitro* or *in utero* culture of intact human embryos, regardless of the method of its derivation, beyond 14 d or formation of the primitive streak, whichever is earlier, is illegal. One of the main concerns is the identity of the embryo as a human. However, human identity is technically the development of the brain and the nervous system which defines and determines all emotions, pain, memories, self respect, ethics and self identification. Growing an embryo which is anencephalic (without brain) for organ harvest would be a solution. However carrying an anencephalic fetus may be emotionally devastating for the surrogate mother in some cases. This can be avoided in turn by having a "*in vitro*-uterus" /semi-artificial uterus system (or uterus with some of the supporting organs) to facilitate the growth of the anencephalic system containing the organ of interest. However, these futuristic concepts are well beyond the consideration of current society for ethical reasons and the unpredictable social and medical consequences.

SCANNING AND PRINTING AN ORGAN

Development of a fetus from a zygote is an example of directed self-assembly processes, in which, through chemical or physical gradients, or predetermined cell-cell and cell-extracellular matrix interactions, the developing organism gradually acquires its final shape. Thus it is logical to assume that if we could provide the appropriate gradients, position and neighbors, then cells will migrate, self-assemble, and establish the correct connections to form the organ. This is quite true for tissues or organs of low complexity such as cartilages, bone, skin, urinary bladder or heart valves, but is unlikely for complex organs such as the liver or brain. Thus the human cornea, urinary bladder, etc. may be ideal tissues/organs for bio-artificial/"engineered" organs rather than complex organs such as the liver.

Most organs are composed of several types of cells in a very specific order in 3-dimensional space which is critical for their function. The concept of inkjet printing opens up a solution to this problem because it allows precise delivery of multiple cell types and matrix components into pre-determined sites with high precision. Multiple cell types in suspension are placed, instead of ink, into different "ink" chambers of a sterilized cartridge and the printer is directed to arrange or "print" these cells in a specific order. It is also possible to use conventional 2-dimensional printing to generate cells of different phenotypes with differential coatings of cell adhesion molecules printed in a specific pattern on extremely thin films with differential cell adhesion properties, which would result in a final pattern formation through minimization of configuration energy, the driving force in cell rearrangement. If we could use a suitable matrix, a chemical gradient also can be printed[46,47].

There is concern that bioprinting would result in non-functional tissues. However, in an elegant experiment by Jacob *et al*[48], synchronous macroscopic beating was demonstrated throughout a sheet obtained by the fusion of chick cardiac cell spheroids through bioprinting.

Imagine a scanner that can scan in 3 dimensions in sub-nanometer resolution and store an enormous amount of data with spatial coordinates of each molecule in the scanned object! Similarly, imagine a 3-dimensional printer that can print at sub-nanometer resolution. If such a scanner and printer could exist, one could scan an entire organ no matter how complex it is, if not an entire human body and reconstruct (clone) it, perhaps so perfectly that it includes the memories! The printer

would be using all molecules which constitute the human body as its ink equivalent! This is science fiction today but tomorrow this may become a reality!

CONCLUSION

The development of iPSC technology has enabled us to generate cells which are very similar to pluripotent stem cells from adult cells. Improvements in this technology will have radical consequences in regenerative medicine, transplantation medicine, therapeutic cloning, and generation of patient-specific whole organs. Generation of iPSC-derived whole organs inside the uterus, making use of the natural developmental signals and environment may result in more natural and high quality organs for transplantation. In future, non-human primates or an "*in vitro*-uterus" may be useful for producing patient-specific organs such as the liver for transplantation. The society needs to be prepared in advance to accept the revolutionary consequences, good, bad and ugly, of these ongoing scientific developments.

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ORIGINAL ARTICLE

Induction of CD69 expression by *cag*PAI-positive *Helicobacter pylori* infection

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Abstract

AIM: To investigate and elucidate the molecular mechanism that regulates inducible expression of CD69 by *Helicobacter pylori* (*H. pylori*) infection.

METHODS: The expression levels of CD69 in a T-cell line, Jurkat, primary human peripheral blood mononuclear cells (PBMCs), and $CD4^+$ T cells, were assessed by immunohistochemistry, reverse transcription polymerase chain reaction, and flow cytometry. Activation of CD69 promoter was detected by reporter gene. Nuclear factor (NF)-κB activation in Jurkat cells infected with *H. pylori* was evaluated by electrophoretic mobility shift assay. The role of NF-κB signaling in *H. pylori*-induced CD69 expression was analyzed using inhibitors of NF-κB and dominant-negative mutants. The isogenic mutants with disrupted *cag* pathogenicity island (*cag*PAI) and *virD4* were used to elucidate the role of *cag*PAI-encoding type Ⅳ secretion system and CagA in CD69 expression.

RESULTS: CD69 staining was detected in mucosal lymphocytes and macrophages in specimens of patients with *H. pylori*-positive gastritis. Although *cag*PAI-positive *H. pylori* and an isogenic mutant of *virD4* induced CD69 expression, an isogenic mutant of *cag*-PAI failed to induce this in Jurkat cells. *H. pylori* also induced CD69 expression in PBMCs and $CD4^+$ T cells. The activation of the CD69 promoter by *H. pylori* was mediated through NF-κB. Transfection of dominant-negative mutants of IκBs, IκB kinases, and NF-κB-inducing kinase inhibited *H. pylori*-induced CD69 activation. Inhibitors of NF-κB suppressed *H. pylori*-induced CD69 mRNA expression.

CONCLUSION: The results suggest that *H. pylori* induces CD69 expression through the activation of NF-κB. *cag*PAI might be relevant in the induction of CD69 expression in T cells. CD69 in T cells may play a role in *H. pylori*-induced gastritis.

Key words: CD69; T cells; *Helicobacter pylori*; *cag* pathogenicity island; Nuclear factor-κB

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INTRODUCTION

The leukocyte receptor CD69 is a C-type lectin, disulfide-linked homodimer, type Ⅱ protein that can be induced after activation[1,2]. In healthy subjects, CD69

is not detected in peripheral blood lymphocytes, but is expressed on small subsets of T and B cells in peripheral lymphoid tissues[3]. In addition, CD69 is selectively expressed in chronic inflammatory infiltrates at the sites of active immune responses *in vivo*[4,5]. However, the biological significance of CD69-induced cell activation is poorly understood.

Helicobacter pylori (H. pylori) is a Gram-negative bacterium that colonizes the human stomach, as well as areas of gastric metaplasia in the duodenal bulb[6]. The precise role of *H. pylori* in gastric pathology, especially the mechanism responsible for the transition of chronic active gastritis to gastric carcinoma, has been studied by many researchers. The infection triggers a local cellular immune response resulting in chronic cellular infiltration with or without an active component of neutrophils, as well as the development of lymphoid follicles in the lamina propria[7]. Although the exact mechanisms of the induction of various diseases by *H. pylori* infection have not been elucidated, one factor strongly associated with *H. pylori* virulence and the development of peptic ulcers and gastric cancer is the *cag* pathogenicity island (PAI), which constitutes a gene cluster encoding a type Ⅳ secretion system (T4SS)[8].

Enarsson *et al*[9] examined the transendothelial migration of human lymphocytes in response to *H. pylori* with the use of the Transwell system, employing a monolayer of human umbilical vein endothelial cells. *H. pylori* induced a significant T-cell migration and the presence of the *H. pylori cag*PAI increased T-cell transendothelial migration. Overexpression of CD69 was noted on migrating T cells[9]. These results suggest that *H. pylori* infection induces the expression of CD69 on T cells.

The present study was designed to test the hypothesis that *H. pylori* can induce both the surface expression of CD69 antigen and the promoter activity of the CD69 gene in human T cells, and to investigate whether such induction involves the *cag*PAI-coding T4SS and the nuclear factor (NF)-κB pathway. The presence of NF-κB motifs within the proximal promoter region of the CD69 gene may account for the *H. pylori*-inducible promoter activity.

MATERIALS AND METHODS

Reagents and bacterial strains

N-acetyl-L-leucyl-L-leucyl-L-norleucinal (LLnL) and Bay 11-7082 were purchased from Sigma-Aldrich (St Louis, MO) and Calbiochem (La Jolla, CA), respectively. *H. pylori* ATCC 49503 (American Type Culture Collection, Rockville, MD) was used in most experiments described in this study. An isogenic *H. pylori* mutant lacking the *cag*PAI[9] or *virD4* also was employed, together with the parental wild-type strain (26695). *H. pylori* strains were plated on blood agar plates and incubated at 37 ℃ for 2 d under microaerophilic conditions. Using inoculating needles, bacteria harvested from the plates were suspended in 50 mL of brucella broth containing 5% fetal bovine serum (FBS) and then cultured in a liquid medium at 37 ℃ for 1 d in a controlled microaerophilic environment. Bacteria were harvested from the broth culture by centrifugation and then resuspended at the concentrations indicated below in antibiotic-free medium. All procedures were performed with the approval of the appropriate institutional biosafety review committee and in compliance with the guidelines for biohazards.

Cell culture

The human T-cell line, Jurkat, was maintained in RPMI 1640 medium containing 10% FBS, 100 U/mL penicillin G, and 100 μg/mL streptomycin. Human peripheral blood mononuclear cells (PBMCs) were isolated from the peripheral blood of a healthy donor using Ficoll-Hypaque gradients. PBMCs then were further purified using positive selection with immunomagnetic beads specific for CD4 (Miltenyi Biotec, Auburn, CA). On the day of the experiment, cells were refed with fresh antibiotic-free medium and cocultured with *H. pylori* for the time intervals indicated below.

Tissue samples

Stomach biopsy specimens from ten patients with *H. pylori* gastritis were examined histopathologically for CD69. The presence of *H. pylori* infection was confirmed by culture, serological analysis (with anti-*H. pylori* immunoglobulin G antibody), rapid urease test, and histological examination with Giemsa staining. Patients with *H. pylori* gastritis showed polymorphonuclear neutrophil infiltration in the gastric epithelium in conjunction with the presence of bacterial forms, which is consistent with *H. pylori* infection. All samples were collected after obtaining informed consent from each patient.

Reverse transcription-polymerase chain reaction

Total RNA was extracted with Trizol (Invitrogen, Carlsbad, CA) according to the protocol provided by the manufacturer. First-strand complementary DNA was synthesized from 1 μg total cellular RNA using a RNA-polymerase chain reaction (PCR) kit (Takara Bio, Otsu, Japan) with random primers. The specific primers used were as follows: for CD69, 5'-CATAGCTCTCATTGCCTTATCAGT-3'(forward primer) and 5'-CCTCTCTACCTGCGTATCGTTT-3'(reverse primer); for β-actin, 5'-GTGGGGCGCCCCAGGCACCA-3'(forward primer) and 5'-CTCCTTAATGTCACGCACGATTTC-3'(reverse primer). Thereafter, cDNA was amplified using 30 and 28 cycles for CD69 and β-actin, respectively. The product sizes were 254 bp for CD69 and 548 bp for β-actin. The PCR products were fractionated on 2% agarose gels and visualized by ethidium bromide staining.

Transfection and luciferase assay

The IκBαΔN- and IκBβΔN-dominant-negative mutants are IκBα and IκBβ deletion mutants lacking the N-terminal 36 and 23 amino acids, respectively[10,11]. The dominant-negative mutants of IκB kinase (IKK)α, IKKα (K44M), IKKβ, IKKβ (K44A), IKKγ, IKKγ(1-305), and

NF-κB-inducing kinase (NIK), NIK (KK429/430AA) have been described previously[12,13]. The CD69 promoter pXP2 luciferase reporter plasmid containing the wild-type sequence (position -255 to position +16), pAIM255-LUC, was described previously[14]. The internal deletion mutants of the NF-κB sites were constructed by deletion of the NF-κB sites of pAIM255-LUC. Jurkat cells were transfected with the appropriate reporter and effector plasmids by electroporation. After 24 h, *H. pylori* was added and incubated for 6 h. The cells were washed in phosphate buffered saline and lysed in reporter lysis buffer (Promega, Madison, WI). Lysates were assayed for reporter gene activity with the dual-luciferase assay system (Promega). Luciferase activities were normalized relative to the Renilla luciferase activity from phRL-TK.

Preparation of nuclear extracts and electrophoretic mobility shift assay

Nuclear proteins were extracted and transcription factors bound to specific DNA sequences were examined by electrophoretic mobility shift assay (EMSA) as described previously[15]. The top strand sequence of the oligonucleotide probes or competitors are as follows: for the NF-κB element (κB1) of the CD69 gene, 5'-GATCCAGACAACAGGGAAAACCCATACTTC-3'; for the NF-κB element (κB2) of the CD69 gene, 5'-GATCCAGAGTCTGGGAAAATCCCACTTTCC-3'; for the NF-κB element of the interleukin-2 receptor α chain (IL-2Rα) gene, 5'-GATCCGGCAGGGGAATCTCCCTCTC-3'; and for the AP-1 element of the IL-8 gene, 5'-GATCGTGATGACTCAGGTT-3'. The oligonucleotide 5'-GATCTGTCGAATGCAAATCACTAGAA-3', containing the consensus sequence of the octamer binding motif, was used to identify specific binding of the transcription factor Oct-1. The above underlined sequences are the NF-κB, AP-1, and Oct-1 binding sites, respectively. To identify NF-κB proteins in the DNA-protein complex shown by EMSA, we used antibodies specific for various NF-κB family proteins, including p50, p65, c-Rel, p52, and RelB (Santa Cruz Biotechnology, Santa Cruz, CA).

Immunohistochemical analysis

CD69 immunohistochemistry was performed using a mouse monoclonal antibody (clone FN50) to CD69 (BioLegend, San Diego, CA) after pretreatment of the deparaffinized tissue sections with ready-to-use proteinase K (Dako, Carpinteria, CA). The sections were counterstained with methyl green for 10 min, hydrated in ethanol, cleaned in xylene, and mounted. The stained cells were examined under a light microscope (Axioskop 2plus; Zeiss, Jena, Germany) with an Achroplan × 40/0.65 lens (Zeiss). Images were acquired with an AxioCam MRC camera and AxioVision 3.1 software (Zeiss). Gastric lymphocytes and macrophages were identified based on their morphological features.

Flow cytometry

Cells were washed with cell WASH (Becton Dickinson Immunocytometry Systems, San Jose, CA) and incubated for 30 min with phycoerythrin-labeled mouse monoclonal antibody against CD69 (clone TP1.55.3) or control mouse IgG2b, which were purchased from Beckman Coulter (Fullerton, CA). Cells were analyzed on an Epics XL flow cytometer.

RESULTS

Overexpression of CD69 in gastric lymphocytes and macrophages in H. pylori gastritis

We investigated the expression of CD69 by immunostaining in *H. pylori*-positive gastric tissues (n = 10). CD69 staining was detected in mucosal lymphocytes and macrophages (Figure 1A). In contrast, only a faint staining for CD69 was detected in the normal mucosa, and the expression level was much weaker than in *H. pylori*-positive gastric tissues (data not shown).

H. pylori increases CD69 mRNA levels in CD4$^+$ T cells

Using reverse transcription (RT)-PCR, we next examined the effect of coculture of Jurkat T cells (a transformed human T-cell line) with *H. pylori* ATCC 49503 on the induction of CD69 mRNA. Coculture with ATCC 49503 significantly enhanced the steady-state levels of CD69 mRNA in Jurkat cells. CD69 transcript levels clearly increased 1 h after the addition of ATCC 49503 to Jurkat cells (Figure 1B). In another series of experiments, in which Jurkat cells were infected with ATCC 49503 at different concentrations [i.e., the multiplicity of infection (MOI)] for 2 h (Figure 1B), *H. pylori* induced dose-dependent expression of CD69 mRNA. To characterize the effect of *H. pylori* infection on human T cells, we employed RT-PCR to examine CD69 mRNA expression in PBMCs and CD4$^+$ T cells in response to ATCC 49503. After 2-h infection, *H. pylori* induced CD69 mRNA expression in PBMCs and CD4$^+$ T cells, similar to the observation with Jurkat cells (Figure 1C).

To analyze whether the increase of mRNA synthesis results in elevated expression on the cell surface, direct immunofluorescent staining and flow cytometry were performed. Consistent with the RT-PCR analysis, the expression was upregulated in a dose-dependent manner (Figure 2B). The peak expression level of cell surface CD69 was noted at 8 h after infection (Figure 2A). *H. pylori* ATCC 49503 also enhanced cell surface CD69 expression on PBMCs (Figure 2C).

H. pylori-induced CD69 expression is cagPAI-dependent

The *cag*PAI, a cluster of about 28 genes, is one of the best known virulence factors; it encodes a T4SS that transports CagA protein, peptidoglycan, and possibly other molecules into host epithelial cells[16]. The *cag*PAI also encodes a homologue of the coupling protein *virD4*, which in *Agrobacterium tumefaciens* and conjugation systems is thought to deliver the T4SS substrates to the secretion machinery[17]. In *H. pylori*, *virD4* is necessary for CagA translocation but dispensable for the induction of

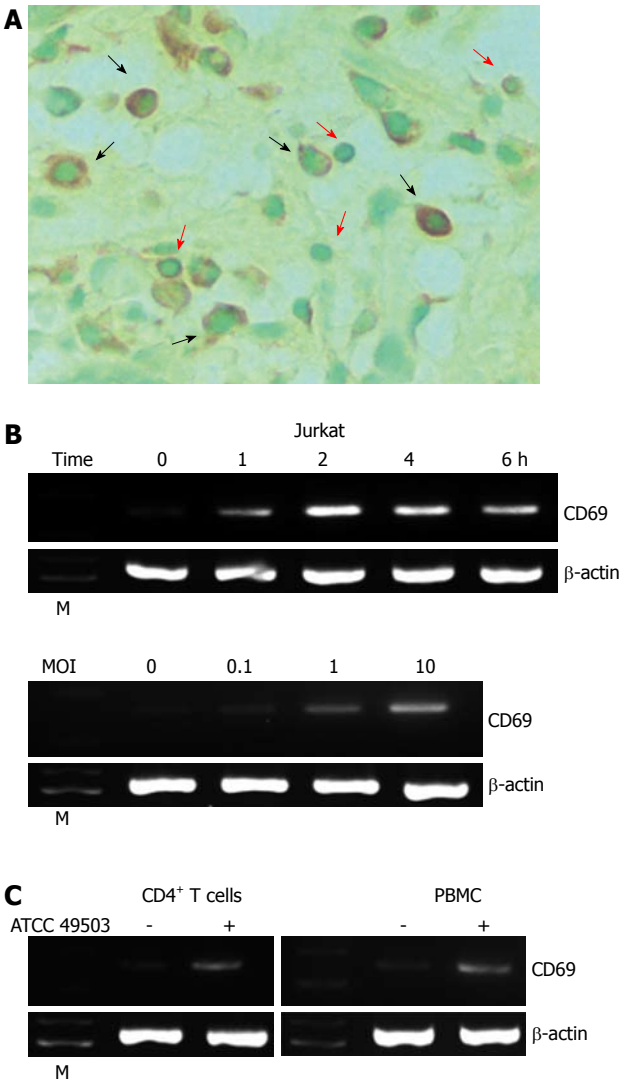

Figure 1 Expression of CD69 in *Helicobacter pylori*-infected T cells. A: Immunohistochemical detection of CD69 in tissues of patients with *Helicobacter pylori (H. pylori)*-positive gastritis. Serial sections of gastric biopsy specimens were stained with a mouse monoclonal antibody to CD69 and counterstained with methyl green. Shown is a representative example of mucosa from a patient with *H. pylori*-positive gastritis. Note the positive staining for CD69 in lymphocytes as well as macrophages. Original magnification, × 800. The red and black arrows indicate the surfaces of lymphocytes and macrophages, respectively; B: *H. pylori*-induced CD69 mRNA expression in Jurkat cells. Total RNA was extracted from Jurkat cells infected with *H. pylori* strain ATCC 49503 [the multiplicity of infection (MOI) of 100] for the indicated time intervals and used for reverse transcription-polymerase chain reaction (RT-PCR) (top). Jurkat cells were infected with the indicated concentrations of ATCC 49503 for 2 h. Total RNA was extracted and used for RT-PCR (bottom); C: *H. pylori*-induced CD69 mRNA expression in peripheral blood mononuclear cells (PBMCs) and $CD4^+$ T cells. Total RNA was extracted from PBMCs and $CD4^+$ T cells infected with ATCC 49503 for 2 h and used for RT-PCR (MOI of 10). β-actin expression served as a control. Lane M: Markers.

IL-8[18,19]. Accordingly, we compared the abilities of the wild-type *H. pylori* strain 26695, an isogenic *cag*PAI mutant (Δ*cag*PAI), and a *virD4* mutant (Δ*virD4*), with regard to the induction of CD69 transcripts and expression of CD69 on the cell surface. Infection with wild-type strain 26695 induced CD69 mRNA expression in Jurkat cells, while the isogenic mutant that lacked *cag*PAI expression did not induce CD69 mRNA expression (Figure 2D). In contrast, the *virD4* mutant induced CD69 mRNA expression in Jurkat cells (Figure 2D). These results were confirmed by the cell surface expression of CD69 analyzed by flow cytometry (Figures 2B and E).

Role of NF-κB in H. pylori-induced activation of the CD69 promoter

In the next series of experiments, we investigated whether the *H. pylori*-mediated upregulation of CD69 gene expression directly enhances the activity of its promoter. Jurkat cells were transiently transfected with a reporter gene construct containing a segment from position -255 to position +16 of the CD69 upstream regulatory sequences. Coculture of *H. pylori* strain ATCC 49503 resulted in a dose-dependent increase in the activity of this CD69-driven reporter construct (Figure 3B). The NF-κB signaling pathway is activated in epithelial cells infected with *cag*PAI-positive *H. pylori* but not in cells infected with *cag*PAI-negative strains of *H. pylori*[20-22]. Two potential NF-κB binding sequences were identified at positions -160 (κB1) and -223 (κB2) (Figure 3A). κB1 and κB2 were identical to those found in the gene promoters of c-myc and IL-6, respectively[23]. To test the relative contribution of the NF-κB binding sites to the *H. pylori*-mediated activation of CD69, plasmids with internal deletion mutants of these sites of the CD69 promoter were transfected (Figure 3C). After *H. pylori* infection, single deletion of the κB2 site resulted in marked reduction of the inducible activity. Single deletion of the κB1 site and the combination of double deletions abolished *H. pylori*-mediated activation of this reporter construct. These data clearly indicate that the two NF-κB binding sites in the CD69 promoter regulate CD69-enhanced expression after infection with *H. pylori*.

H. pylori infection induces binding of NF-κB family proteins to the κB1 and κB2 motifs of the CD69 promoter in T cells

The data presented above indicate that *H. pylori*-induced CD69 expression is mediated by the κB1 and κB2 sites. To analyze whether these two putative NF-κB binding sites of the CD69 promoter could bind NF-κB family members, gel retardation assays were performed using as probes two double-stranded oligonucleotides (CD69 κB1 and CD69 κB2) containing these motifs. To characterize the NF-κB-related proteins that bind to the NF-κB sites of the CD69 promoter in CD69-expressing cells, the two oligonucleotide probes were incubated with nuclear extracts prepared from untreated Jurkat cells and from Jurkat cells infected with *H. pylori*. Jurkat cells were infected with *H. pylori* at different times after challenge, and nuclear protein extracts were prepared and analyzed to determine NF-κB DNA binding activity. As shown in Figure 4A, complexes were induced in these cells within 30 min after infection with *H. pylori* and were detected at 180 min after infection with both oligonucleotide probes. The amounts of these inducible DNA-protein complexes were *H. pylori* dose-dependent.

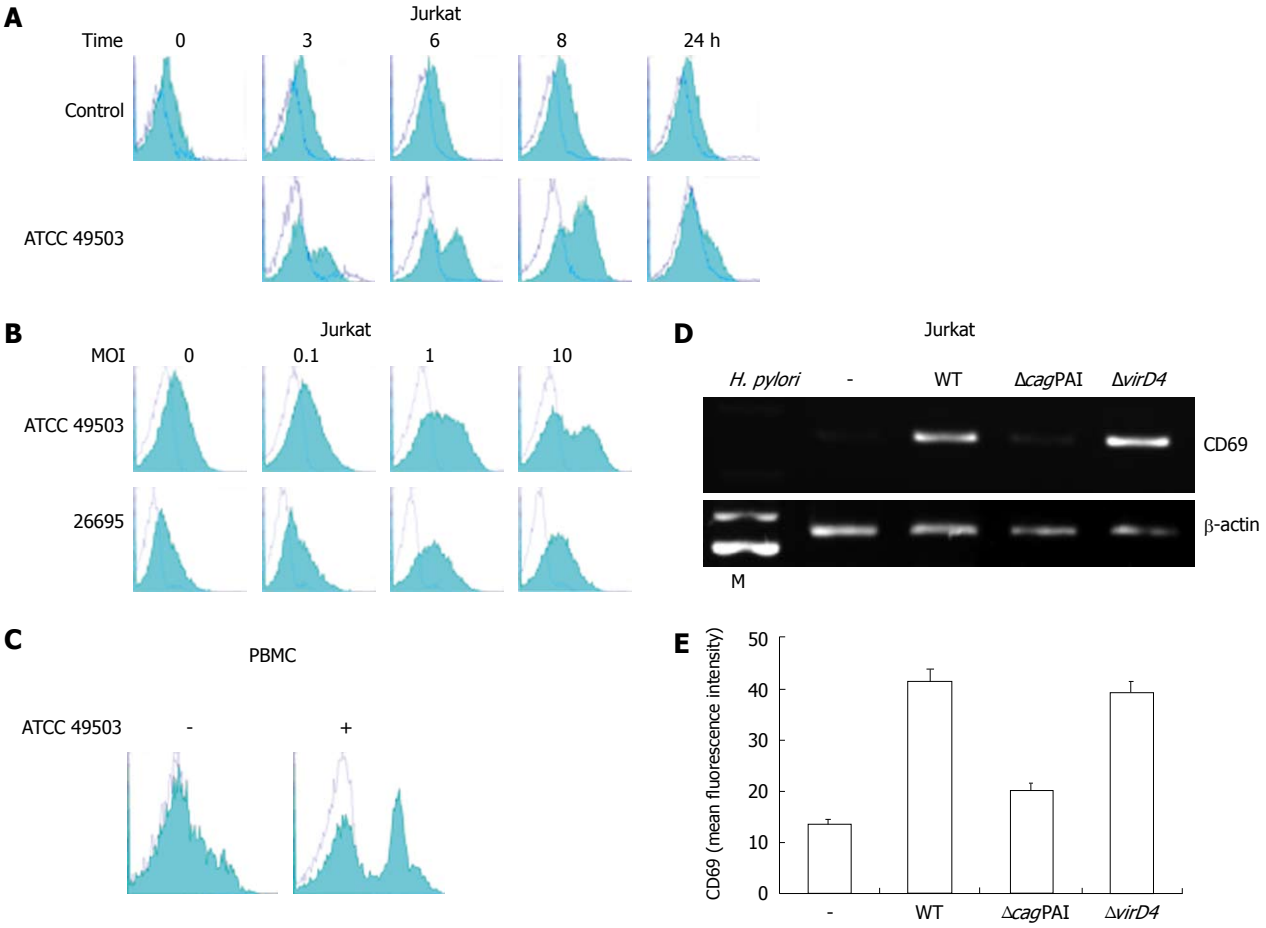

Figure 2 CD69 expression on Jurkat cells. A: Time course of cell surface expression of CD69 on Jurkat cells exposed to *Helicobacter pylori (H. pylori)*. Jurkat cells were cultured for the indicated times in culture medium (control) or in the presence of ATCC 49503 [the multiplicity of infection (MOI) of 10]. After cell harvest, CD69 expression on the cells was determined by flow cytometry; B: *H. pylori* infection increases cell surface expression of CD69 on Jurkat cells in a dose-dependent fashion. Jurkat cells were infected with different concentrations of *H. pylori* strains, ATCC 49503 and 26695, and CD69 levels were measured by flow cytometry on cells harvested after 8 h; C: *H. pylori* infection increases cell surface expression of CD69 on peripheral blood mononuclear cells (PBMCs). PBMCs were infected with ATCC 49503 (MOI of 10), and CD69 levels were measured on cells harvested after 8 h; D: *cag* pathogenicity island (*cag*PAI) products of *H. pylori* are required for the induction of CD69 mRNA expression. Total RNA was extracted from Jurkat cells that had been infected with the wild-type strain 26695 (WT) or the isogenic mutants Δ*cag*PAI and Δ*virD4* (MOI of 10) for 2 h and used for reverse transcription-polymerase chain reaction. Lane M: Markers; E: Flow cytometric analysis was carried out for the surface expression of CD69 in Jurkat cells infected with the wild-type strain 26695 (WT) or the isogenic mutants Δ*cag*PAI and Δ*virD4*. Jurkat cells were infected for 8 h with various *H. pylori* strains (MOI of 10). Cells were stained with phycoerythrin-labeled monoclonal antibody. Datas are mean ± SD of three experiments.

In both probes, the addition of an excess of unlabeled κB1 and κB2 oligonucleotides to the binding reaction completely abolished the formation of the inducible DNA-protein complexes (Figure 4B, lanes 3 and 4). Similarly, an equal amount of the oligonucleotide IL-2R κB, which contained the NF-κB motif of the IL-2R α chain gene, efficiently competed with the specific complexes (Figure 4B, lane 5). In contrast, the formation of these DNA-protein complexes was not blocked by the addition of an excess of the unrelated oligonucleotide AP-1 (Figure 4B, lane 6).

To identify the NF-κB family members that bind to the NF-κB motifs of the CD69 gene promoter, the binding reactions were preincubated with antibodies specific to p50, p65, c-Rel, p52, and RelB (Figure 4B). The anti-p50 antibody induced the supershifted bands or reduced the intensity of complexes κB1 and κB2 (Figure 4B, lane 7). The anti-p65 antibody induced supershifted bands or blocked the formation of complexes κB1 and κB2 (Figure 4B, lane 8). The anti-c-Rel antibody induced the supershifted band and reduced the intensity of only complex κB1 (Figure 4B, lane 9). In contrast, the anti-p52 or anti- RelB antibody did not interfere with the formation of any of these complexes (Figure 4B, lanes 10 and 11). These results indicate that the complexes κB1 and κB2 correspond to p50/p65/c-Rel and p50/p65, respectively. These results suggest that *H. pylori* infection seems to induce CD69 gene expression at least in part through the induced binding of NF-κB family members to the NF-κB sites in the CD69 promoter region.

NF-κB signal is essential for induction of CD69 expression by H. pylori in T cells

We also examined whether the *H. pylori*-mediated up-regulation of CD69 gene expression involves signal transduction components in NF-κB activation. Activation of NF-κB requires the phosphorylation of two conserved serine residues of IκBα (Ser-32 and Ser-36)

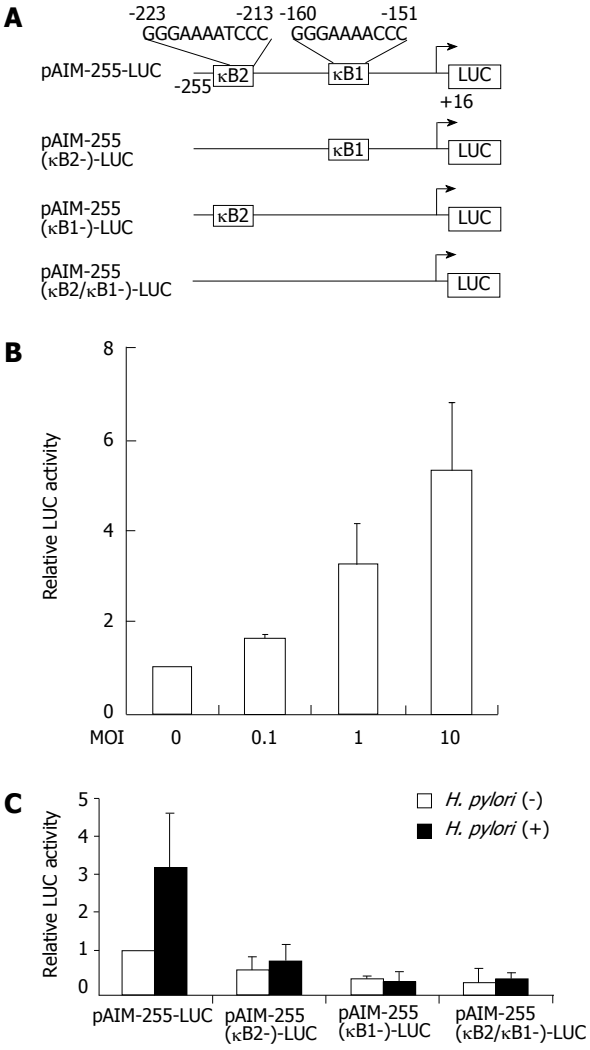

Figure 3 *Helicobacter pylori* activates the CD69 promoter through two nuclear factor-κB binding sites. A: Schematic diagram of the CD69 reporter constructs containing the wild-type (pAIM-255-LUC) and internal deletion mutants of κB1 and/or κB2 motifs. LUC: Luciferase; B: *Helicobacter pylori* (*H. pylori*) infection increases CD69 promoter activity in a dose-dependent fashion. pAIM-255-LUC was transfected into Jurkat cells, and the cells were subsequently infected with *H. pylori* ATCC 49503 for 6 h; C: The indicated CD69 reporter constructs were transfected into Jurkat cells, and subsequently the cells were infected with ATCC 49503 for 6 h (the multiplicity of infection of 10). The activity is expressed relative to that of cells transfected with pAIM-255-LUC without further *H. pylori* infection, which was defined as 1. Datas are mean ± SD of three experiments.

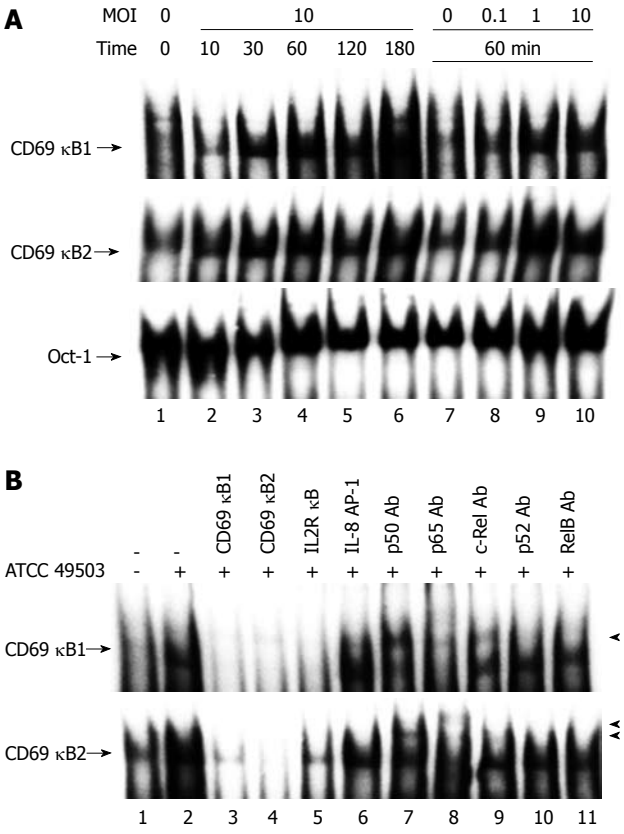

Figure 4 *Helicobacter pylori* infection induces nuclear factor-κB binding activity. A: Nuclear factor (NF)-κB activation in Jurkat cells infected with *Helicobacter pylori (H. pylori)*, as evaluated by electrophoretic mobility shift assay (Oct-1). Nuclear extracts from Jurkat cells infected with different densities [the multiplicity of infection (MOI)] of *H. pylori* ATCC 49503 (lanes 7 to 10) for the indicated times (lanes 1 to 6) were mixed with oligonucleotide probes CD69 κB1 (top) and CD69 κB2 (middle), which contained the putative NF-κB motifs located at positions -160 and -223, respectively; B: Competition assays were performed with nuclear extracts from Jurkat cells infected with ATCC 49503 (MOI of 10) for 180 min. Where indicated, the excess amounts of each specific competitor oligonucleotide were added to the reaction mixture with each oligonucleotide probe (lanes 3 to 6). A supershift assay of NF-κB DNA binding complexes in the same nuclear extracts was also performed. Where indicated, appropriate antibodies (Ab) were added to the reaction mixture before the addition of probe CD69 κB1 (top) or CD69 κB2 (bottom). Arrows indicate the specific complexes, while arrowheads indicate the DNA binding complexes supershifted by the antibodies.

and IκBβ (Ser-19 and Ser-23) within the N-terminal domain[24]. Phosphorylation leads to the ubiquitination and 26S proteasome-mediated degradation of IκBs, thereby releasing NF-κB from the complex and its translocation to the nucleus and activation of various genes[24]. The IKK complex, which is composed of two catalytic subunits (IKKα and IKKβ) and a regulatory subunit (IKKγ), phosphorylates IκBs[24]. Previous studies indicated that members of the mitogen-activated protein kinase kinase kinase family mediate the physiologic activation of IKK[25]. These kinases include NIK[26]. IκBα-, IκBβ-, and IKKγ-dominant-interfering mutants and IKKα, IKKβ, and NIK kinase-deficient mutants were tested to determine their abilities to inhibit the *H. pylori*-mediated activation of the CD69-driven reporter gene. The expression of these various inhibitory mutants abolished *H. pylori*-induced CD69 expression (Figure 5A). These results emphasize the importance of signaling components involved in the activation of NF-κB in *H. pylori*-induced activation of the CD69 promoter.

Because activation of the CD69 promoter by *H. pylori* infection requires the activation of NF-κB, we blocked NF-κB activation with Bay 11-7082, an inhibitor of IκBα phosphorylation[27], or LLnL, a proteasome inhibitor[28]. The latter is known to inhibit the activation of NF-κB by blocking the degradation of the IκBα protein. Both Bay 11-7082 and LLnL markedly inhibited the *H. pylori*-induced expression of CD69 mRNA (Figure 5B).

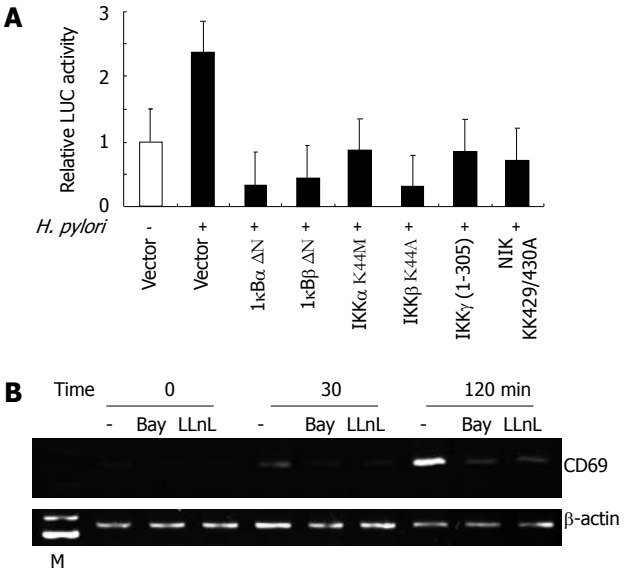

Figure 5 Nuclear factor-κB signal is essential for the activation of CD69 expression by *Helicobacter pylori* in T cells. A: Functional effects of IκBα-, IκBβ-, and IKKγ-dominant-interfering mutants and kinase-deficient IKKα, IKKβ, and NIK mutants on *Helicobacter pylori (H. pylori)*-induced activation of the CD69 promoter. Jurkat cells were transfected with the CD69 reporter construct (pAIM-255-LUC) and the indicated mutant plasmids or empty vector (pCMV4) and then infected with *H. pylori* ATCC 49503 for 6 h. Open bar: Luciferase (LUC) activity of the CD69 reporter construct and pCMV4 without *H. pylori* infection. All values were calculated as the change (n-fold) in induction values relative to the basal level measured in uninfected cells. Data are mean ± SD of three independent experiments. B: Bay 11-7082 and N-acetyl-L-leucyl-L-leucyl-L-norleucinal (LLnL) inhibit CD69 mRNA expression induced by *H. pylori*. Jurkat cells were pretreated with Bay 11-7082 (20 μmol/L) or LLnL (20 μmol/L) for 2 h prior to *H. pylori* infection and subsequently infected with *H. pylori* ATCC 49503 for 30 or 120 min. CD69 mRNA expression on harvested cells was analyzed by reverse transcription-polymerase chain reaction. Lane M: Markers.

DISCUSSION

Early studies showed that CD69 regulates the immune response by modulating the expression of various cytokines; CD69-deficient mice show increased anti-tumor and autoimmune responses caused at least in part by increased production of proinflammatory cytokines and chemokines[29,30]. Although the functions of CD69 have been studied extensively, there is little or no information on its role in the immune response against microbial pathogens. Recently, CD69 was reported to be a critical negative regulator of immune activation during intracellular bacterial infection, protecting infected mice against lethal tissue damage[31]. The present study explores the way in which *H. pylori* infection controls the expression of CD69 gene in T cells.

The main findings of the study were: (1) *H. pylori* deregulated the expression of CD69 in T cells; (2) CD69 protein was upregulated in gastric lymphocytes of patients with *H. pylori* gastritis; (3) the importance of *H. pylori cag*PAI in the induction of CD69 expression in T cells; and (4) *H. pylori* stimulates the NF-κB signaling pathway to activate CD69 gene expression and also to activate the CD69 promoter.

This is the first report to demonstrate that CD69 gene expression is regulated by *H. pylori*. Despite the development of immune responses against *H. pylori* infection, the bacteria are rarely eliminated, and colonization generally is persistent. Factors that contribute to the failure of the immune response to clear the organism remain elusive[31]. Recent studies have suggested that CD69 may downregulate the immune response through the production of the pleiotropic cytokine, transforming growth factor-β[32]. Thus, CD69 expressed on T cells may regulate the immune responses against *H. pylori* infection.

It has been reported that the inducible expression of CD69 gene is tightly regulated by transcription factors of the NF-κB, AP-1, EGR, and ATF/CREB families, which are rapidly activated through different signaling pathways[14,33]. However, nothing is known about the regulation of CD69 expression in T cells infected with *H. pylori*. We demonstrate herewith that *cag*PAI-positive *H. pylori* can induce the expression of the CD69 antigen and that this induction is mediated by an increase in the CD69 promoter activity. Deletion of the sequences that contain the κB1 and/or κB2 motifs abolished the response to *H. pylori*. Pharmacologic inhibition of NF-κB, as well as IκBα-, IκBβ-, IKKγ-dominant-interfering mutants and kinase-deficient IKKα, IKKβ, and NIK mutants, determined the role of NF-κB signaling molecules targeted by *H. pylori* to activate CD69 gene expression. Thus, our results suggest that NF-κB is essential for *H. pylori cag*PAI-mediated CD69 induction in T cells, and the two NF-κB sites (κB1 and κB2) appear to play an important role in this process.

Our results also demonstrated that the two NF-κB motifs of the CD69 promoter bind *H. pylori*-inducible NF-κB-related complexes. Antibodies directed against the different NF-κB proteins were used to identify the family members present in the DNA-protein complexes detected with the NF-κB motif-derived probes. These experiments demonstrated that the DNA-binding activities consisted of p50/p65/c-Rel and p50/p65 complexes binding to the κB1 and κB2 motifs, respectively. Although NF-κB clearly plays an important role in *H. pylori*-mediated induction of CD69, the role of CD69 in the control of immune responses against *H. pylori* infection needs to be further clarified. We are planning further studies using CD69-deficient mice to investigate the role of CD69 in the regulation of immune responses against *H. pylori* infection.

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COMMENTS

Background

Helicobacter pylori (*H. pylori*) is regarded as the major cause of various gastric diseases. Despite the development of immune responses against *H. pylori* infection, the bacteria are rarely eliminated. Factors that contribute to the failure of the immune response to clear the organism remain elusive. Recently, the leukocyte receptor CD69 was reported to be a critical negative regulator of immune activation during bacterial infection.

Research frontiers

CD69 is selectively expressed in chronic inflammatory infiltrates at the sites of active immune responses. However, whether and how *H. pylori* can induce the expression of CD69 has not been addressed in T cells. In this study, the authors demonstrate that *H. pylori* can induce CD69 expression through the activation of nuclear factor-κB and show that *cag* pathogenicity island (*cag*PAI) might be relevant in the induction of CD69 expression in T cells.

Innovations and breakthroughs

CD69 staining was detected in mucosal lymphocytes and macrophages in specimens of patients with *H. pylori*-positive gastritis. *H. pylori* also induced CD69 expression in peripheral blood mononuclear cells and $CD4^+$ T cells *in vitro*. This is the first study to report the regulation of intracellular events leading to CD69 expression by *H. pylori* infection in T cells. The results also demonstrate that the two nuclear factor-κB motifs of the CD69 promoter are important in *H. pylori*-mediated induction of CD69.

Applications

By understanding how CD69 is induced and the role of CD69 in the control of immune responses against *H. pylori* infection, and by blocking its expression, this study may indicate a future strategy for elimination of *H. pylori*.

Terminology

CD69 is a C-type lectin, disulfide-linked homodimer, type Ⅱ protein that can be induced after lymphocyte activation. The *cag*PAI is responsible for the secretion of the CagA effector through a type Ⅳ secretion system apparatus as well as transport of peptidoglycan.

Peer review

This article by Mori *et al* described induction of CD69 expression by *cag*PAI-positive *H. pylori* infection. According to my literature review, this might be the first study reporting to demonstrate that CD69 gene expression is regulated by *H. pylori*. This manuscript is scientific and well-written.

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ORIGINAL ARTICLE

RNAi knockdown of PIK3CA preferentially inhibits invasion of mutant PIK3CA cells

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Abstract

AIM: To explore the effects of siRNA silencing of PIK3CA on proliferation, migration and invasion of gastric cancer cells and to investigate the underlying mechanisms.

METHODS: The mutation of PIK3CA in exons 9 and 20 of gastric cancer cell lines HGC-27, SGC-7901, BGC-823, MGC-803 and MKN-45 was screened by polymerase chain reaction (PCR) followed by sequencing. BGC-823 cells harboring no mutations in either of the exons, and HGC-27 cells containing PIK3CA mutations were employed in the current study. siRNA targeting PIK3CA was chemically synthesized and was transfected into these two cell lines *in vitro*. mRNA and protein expression of PIK3CA were detected by real-time PCR and Western blotting, respectively. We also measured phosphorylation of a serine/threonine protein kinase (Akt) using Western blotting. The proliferation, migration and invasion of these cells were examined separately by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), wound healing and Transwell chambers assay.

RESULTS: The siRNA directed against PIK3CA effectively led to inhibition of both endogenous mRNA and protein expression of PIK3CA, and thus significantly down-regulated phosphorylation of Akt ($P < 0.05$). Furthermore, simultaneous silencing of PIK3CA resulted in an obvious reduction in tumor cell proliferation activity, migration and invasion potential ($P < 0.01$). Intriguing, mutant HGC-27 cells exhibited stronger invasion ability than that shown by wild-type BGC-823 cells. Knockdown of PIK3CA in mutant HGC-27 cells contributed to a reduction in cell invasion to a greater extent than in non-mutant BGC-823 cells.

CONCLUSION: siRNA mediated targeting of PIK3CA may specifically knockdown the expression of PIK3CA in gastric cancer cells, providing a potential implication for therapy of gastric cancer.

Key words: Gastric cancer; Metastasis; PIK3CA; PI3K/Akt pathway; RNAi

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INTRODUCTION

Gastric cancer is one of the most frequent cancers and is the second leading cause of cancer-related death worldwide[1-2]. Although diagnostic and surgical techniques as well as combined chemotherapy and radiotherapy for the treatment of gastric cancer have advanced in recent years, the overall 5-year survival rate is still less than 20%. Thus, it is necessary to further explore and investigate the tumorigenesis of gastric cancer and its novel therapy targets.

RNA interference (RNAi) refers to the inhibition of gene expression by small double-stranded RNA (dsRNA) molecules targeting specific mRNAs for degradation[3]. The discovery of RNAi has revolutionized our understanding of gene regulation, led to the development of new strategies for blocking gene function, and may yield RNA-based drugs to treat human disease[4]. To date, a great number of studies have demonstrated that RNAi-mediated gene silencing has promising therapeutic potential for cancer therapy[5].

PIK3CA encodes the key enzymatic subunit p110α of phosphatidylinositol 3-kinase (PI3K) and is located at 3q26.3[6]. Few studies have addressed PIK3CA expression in malignancies, although its mutation has been found in many human solid cancers, including breast, gastric and pituitary cancer[7-9] and it plays an essential role as an oncogene in tumor development and progression. Our previous studies have demonstrated that increased expression of PIK3CA in the cytoplasm of gastric cancer tissues was likely associated with lymph node metastasis[10]. However, the relationship between down-regulation of PIK3CA and proliferation as well as metastatic ability or invasion of gastric cancer cells and the mechanism underlying any such relationship remains largely unknown.

In this study, we investigated the effects of down-regulation of PIK3CA by small interfering RNA (siRNA) on proliferation, migration and invasion of two gastric cancer cell lines (BGC-823 and HGC-27) as well as p-Akt expression, with the aim of evaluating whether the expression of PIK3CA may be linked to tumor progression, and explored the underlying mechanisms.

MATERIALS AND METHODS

Detection of PIK3CA mutation in gastric cancer cell lines

Detection of PIK3CA mutation in exons 9 and 20 was performed in 5 gastric cancer cell lines (HGC-27, SGC-7901, BGC-823, MGC-803 and MKN-45), covering the majority of hot spots of PIK3CA gene mutation. The polymerase chain reaction (PCR) amplification primers were designed according to the study published by Lin *et al*[9]. PCR products were electrophoresed on 1.5% agarose gels to ensure their integrity before purification and DNA sequencing on an ABI 3730 XL DNA Analyzer (Applied Biosystems, Foster City, CA, United States) by Biosune Ltd (Shanghai, China).

siRNA synthesis and transfection

The siRNAs were designed and synthesized by GenePharma (Shanghai, China). PIK3CA siRNA: sense 5'-GGC UAA AGA AAG CCU UUA UTT-3', antisense 5'-AUA AAG GCU UUC UUU AGC CTT-3'; Negative control siRNA: sense 5'-UUC UCC GAA CGU GUC ACG UTT-3', antisense 5'-ACG UGA CAC GUU CGG AGA ATT-3'. All sequences were submitted to National Institutes of Health Blast program to ensure gene specificity. Human gastric carcinoma BGC-823 cells were conventionally cultured in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen Inc., Carlsbad, CA, United States) supplemented with 2 mmol/L of L-Glutamine and 10% FBS at 5% CO_2 and 37 ℃. The cells were divided into 3 groups: control group (containing only transfection reagent), negative control group (transfected with negative control siRNA) and experimental group (transfected with PIK3CA-siRNA). When cells reached 80-90% confluency, siRNA transfections were conducted using Lipofectamine 2000 (Invitrogen) according to the manufacturer's recommendations. Total RNAs and proteins were prepared from samples collected before transfection and at 24 h and 48 h post transfection and used for real-time quantitative PCR or Western blotting analysis.

Real time quantitative PCR

Transcript abundance of PIK3CA and β-actin (internal control) was relatively quantified by quantitative real time PCR (qRT-PCR) on total RNA isolated from the three cell groups. Briefly, 1 μg of total RNA was reverse transcribed in a 25 μL reaction volume using oligo dT (15) primers and M-MLV reverse transcriptase (Promega'Madison, WI, United States). The PCR amplifications and fluorescence detections were carried out using the ABI Prism 7500 Sequence Detection System following the manufacturer's instructions. For each sample, a relative quantity was calculated using the $2^{-\Delta\Delta C(T)}$ method[11]. Nucleotide sequences of specific primers for the selected genes were as follows: PIK3CA forward primer (5'-TGCTAAAGAGGAACACTGTCCA-3'), reverse primer: (5'-GGTACTGGCCAAAGATTCAAAG-3'); β-actin forward primer (5'-CTGAGCAGATCATGAAGAC-3'), reverse primer (5'-CTTGGTGGACGCATCCTGAG-3').

Western blotting

Cell lysates were prepared in a buffer containing 0.5 mmol/L Tris•HCl (pH 7.0), 0.1% beta-mercaptoethanol, 0.5 mmol/L ethylenediaminetetraacetic acid (pH 7.0), 0.5 mmol/L ethyleneglycol-bis (2-aminoethylether)-N,N,N',N'-tetraacetic acid (pH 7.0), 2 mmol/L leupeptin, 1 mmol/L phenylmethylsulfonyl fluoride, 2.5 mg/mL

Aprotinin, 1 mmol/L dithiothreitol and 0.5% Triton X-100. After protein quantitation using the Branford assay, 30 μg of proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes (Amersham Life Sciences, Piscataway, NJ, United States). The membranes were blocked using phosphate buffered saline (PBS) (pH 7.4) containing 5% nonfat milk for 1 h, probed with primary antibody (anti-PIK3CA) (Cell Signaling Technology, Beverly, MA, United States) overnight at 4 ℃. The membrane was then washed with PBST (PBS + 0.1% Tween-20) and incubated with a peroxidase-conjugated secondary antibody (goat anti-mouse IgG, Santa Cruz Biotechnology, Santa Cruz, CA, United States) for 1 h. Immunoreactive proteins were detected using an enhanced chemiluminescence detection reagent from BestBio (Shanghai, China). The membrane was stripped and reprobed with anti-phosphorylated Akt (Ser473), anti-Akt and anti-β-actin antibodies (Cell Signaling Technology).

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay

The proliferation of BGC-823 and HGC-27 cells was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. This assay measures the dehydrogenase enzyme activity in metabolically active tumor cells, as reflected by the conversion of MTT to formazan, whose absorbance can be quantified by measuring at the wavelength of 570 nm. The production of formazan is proportional to the number of living cells, with the intensity of the produced color serving as an indicator of cell viability. Briefly, approximately 5×10^3 cells/well from the three groups were respectively seeded in a 96-well microtiter plate, each group had six parallel wells. At 24, 36 and 48 h post-transfection, 20 μL of MTT (Sigma Chemical Co, MO, United States) (5 g/L) labeling reagent was added to the designated wells and cells were incubated at 37 ℃ for 4 h and centrifuged at 1000 rpm for 5 min. The supernatant was removed, and 150 μL dimethyl sulfoxide (DMSO) (Sigma) was then added to each well. After shaking the plate for 15 min, the absorbance (A) at 570 nm was measured using Wellscan MK3 Automatic Microplate Reader. The blank control wells with medium only were set as zero absorbance. All experiments were performed at least three times.

Cell wound healing assay

To measure cell motility, 4×10^5 cells were seeded in 6-well plates. A central linear wound was created by scraping the cell monolayer with a 200 μL sterile pipette tip. The media were carefully changed to remove any floating cells and cultured at 5% CO_2 and 37 ℃. Migration of cells into the denuded areas in the scraped region was calculated at 24 h and 48 h, respectively. The wound at 0 h was considered 100% of the average gap.

Cell invasion assay

Cell invasion was assessed using Transwell chambers (Corning, NY, United States) with 50 μL sera-free DMEM containing 1 μg/μL Matrigel (BD, NJ, United States) in the upper chamber. Cells (4×10^4) were suspended with 200 μL DMEM without fetal bovine serum and placed onto the Matrigel. The lower chamber was filled with DMEM 500 μL containing 0.1 μg/μL fibronectin (Sigma). After 24 h incubation at 5% CO_2 and 37 ℃, the number of cells with Hematoxylin and Eosin (H and E) staining on the undersurface of the polycarbonate membranes (pore size 8 mm) was scored visually in 8 random fields using a light microscope.

Table 1 PIK3CA mutation in gastric cancer cell lines

Cell line	Nucleotide substitution	Amino acid change	Exon	Domain
HGC-27	G1633A	E545K	9	Helical
MKN-45	A3140G	H1047R	20	Kinase

Detection of PIK3CA mutation in exons 9 and 20 was screened by PCR followed by sequencing in 5 gastric cancer cell lines (HGC-27, SGC-7901, BGC-823, MGC-803 and MKN-45). Only two cell lines carried mutations of PIK3CA. HGC-27 cells contained the G1633A (E545K) mutation in exon 9 and MKN-45 contained the A3140G (H1047R) mutation in exon 20.

Statistical analysis

Data were analyzed by GraphPad Prism 4.0 and SigmaPlot 8.0 software. The results were expressed as mean ± SD. Two or multiple comparisons were performed with Student's *t*-test or a one-way analysis of variance (ANOVA), respectively. A value of $P < 0.05$ was considered statistically significant.

RESULTS

Mutation analysis of PIK3CA

Among the 5 gastric cancer cell lines (HGC-27, SGC-7901, BGC-823, MGC-803 and MKN-45) analyzed, PIK3CA mutations in exon 9 or 20 were found in 2 of the 5 (40%) cell lines. HGC-27 cells harbored the G1633A (E545K) mutation in exon 9 and MKN-45 harbored the A3140G (H1047R) mutation in exon 20 (Table 1), which were consistent with a previous study in gastric cancer tissues[8].

To gain insight into the outcome through functional knockdown of PIK3CA, two gastric cancer cell lines (BGC-823 and HGC-27) harboring non-mutant and mutant PIK3CA, respectively, were selected to fulfill this task. The selection was based on the fact that in these three gastric cancer cell lines, the higher the expression of PIK3CA both at the mRNA and protein level, the more invasive the cells are[12].

RNAi decreases PIK3CA and p-Akt expression

We analyzed the expression of PIK3CA mRNA in BGC-823 cells transfected with siRNA by qRT-PCR. Before transfection, PIK3CA mRNA was abundantly expressed among the three groups of cells, with no statistical significance between them ($P < 0.05$) (data not shown). However, PIK3CA mRNA expression was

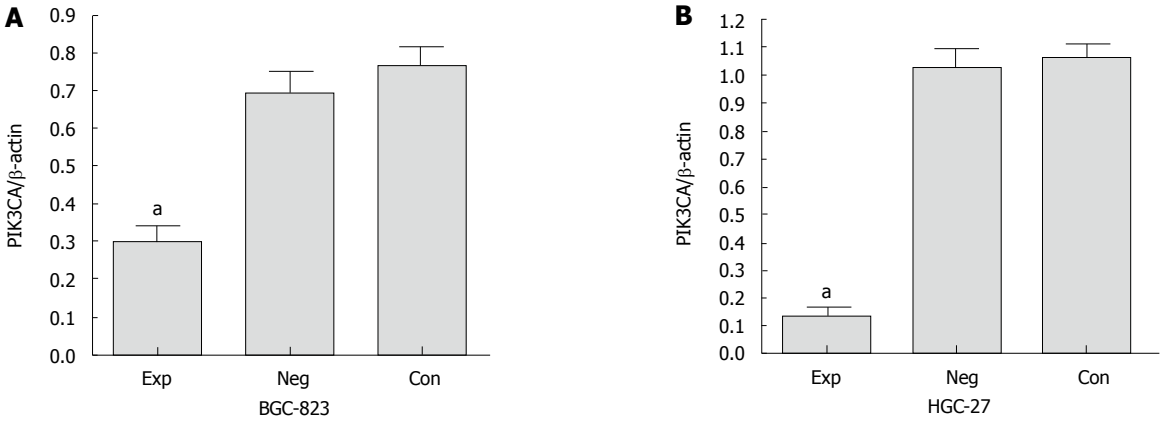

Figure 1 Expression analyses of PIK3CA mRNA determined by quantitative real time polymerase chain reaction at 24 h post-transfection. Values are shown as mean ± SD. Exp: Experimental group; Neg: Negative control group; Con: Control group. [a]$P < 0.05$ *vs* controls, $n = 3$.

markedly decreased by about 70% in the experimental group compared with the two control groups (Figure 1). Similarly, densitometric analysis showed that PIK3CA protein expression in the experimental group was about 2.5- and 2-fold lower than those in the control group and negative control group, respectively ($P < 0.05$), while no statistical difference in PIK3CA protein expression was found between these two control groups ($P > 0.05$) (Figure 2A and B). Interestingly, the level of p-Akt protein in the experimental group was also dramatically down-regulated compared with the two control groups as shown in Figure 2A and C. Additionally, the levels of PIK3CA mRNA and protein were dramatically reduced by about 85% and 80% in the experimental HGC-27 cells in comparison with the two control groups (Figure 1, Figure 2A and B). A similar difference was also observed when the HGC-27 cells were assessed for protein expression of p-Akt (Figure 2A and C). Interestingly, no significantly statistical difference in PIK3CA protein levels was found in experimental wild-type BGC-823 and mutant HGC-27 cells (Figure 2B). These data indicated that siRNA silencing of PIK3CA led to obvious inhibition of mRNA and protein expression of PIK3CA in these two gastric cell lines, and decreased activation of Akt was probably due to constitutive inactivation of PIK3CA rather than changes in its protein levels.

Effects of PIK3CA down-regulation on cell proliferation, migration and invasion

Because PIK3CA-siRNA was able to impair activation of the PI3K/Akt pathway, it was of significant interest and a priority to assess whether this siRNA had a functional effect on the biological properties of gastric cancer cells. To address this, we performed a MTT assay on gastric cancer cells transfected with siRNA against PIK3CA. As shown in Figure 3, transfection with PIK3CA-siRNA significantly decreased the proliferation of BGC-823 cells as compared with the controls ($P < 0.01$), while the proliferation of BGC-823 cells between the control group and the negative control group showed no statistical significance ($P > 0.05$), implying that knockdown of PIK3CA had an obvious inhibitory impact on the proliferation of BGC-823 cells. To examine the effect of PIK3CA-siRNA on cell motility, an *in vitro* wound-healing assay was performed. The results showed that the cells transfected with PIK3CA-siRNA had a reduced migration rate compared with the control groups at 24 h ($P < 0.05$) and 48 h ($P < 0.01$) (Figure 4). To further investigate the effect of PIK3CA-siRNA on cell invasion, we determined the invasion ability of the three groups of cells using the Transwell chambers assay. After incubation for 24 h, the number of control group and negative control group cells which had invaded the polycarbonate membrane of the Matrigel chamber was approximately 3.3- and 2.8-fold greater than that of the experimental group, respectively [(23.35 ± 1.37) and (20.24 ± 1.16) *vs* (6.98 ± 0.56)]($P < 0.01$) (Figure 5). The results of this experiment support the suggestion that PIK3CA-siRNA reduces invasion ability of gastric cancer BGC-823 cells. As expected, silencing of PIK3CA in mutant HGC-27 cells led to reduced cell proliferation and invasion to a greater extent than that in non-mutant BGC-823 cells (Figures 3-5), implying that PIK3CA knockdown may preferentially inhibit proliferation and invasion of mutant PIK3CA cells.

DISCUSSION

It is currently thought that gastric cancer develops through a complex process, such as the activation of oncogenes and/or the inactivation of tumor suppressor genes[13]. However, the critical underlying molecular mechanism of its progression is largely unclear. In recent years, many researchers have focused on signaling pathway deregulation in cancers. Among them, dysregulation of the PI3K/Akt pathway in a wide spectrum of human cancers has become a research hotspot.

The PI3Ks are heterodimers consisting of p110 catalytic and p85 regulatory subunits and have been linked to an extraordinarily diverse group of cellular functions, including differentiation, cell adhesion, apoptosis and tumor invasion[14]. Many of these functions relate to the

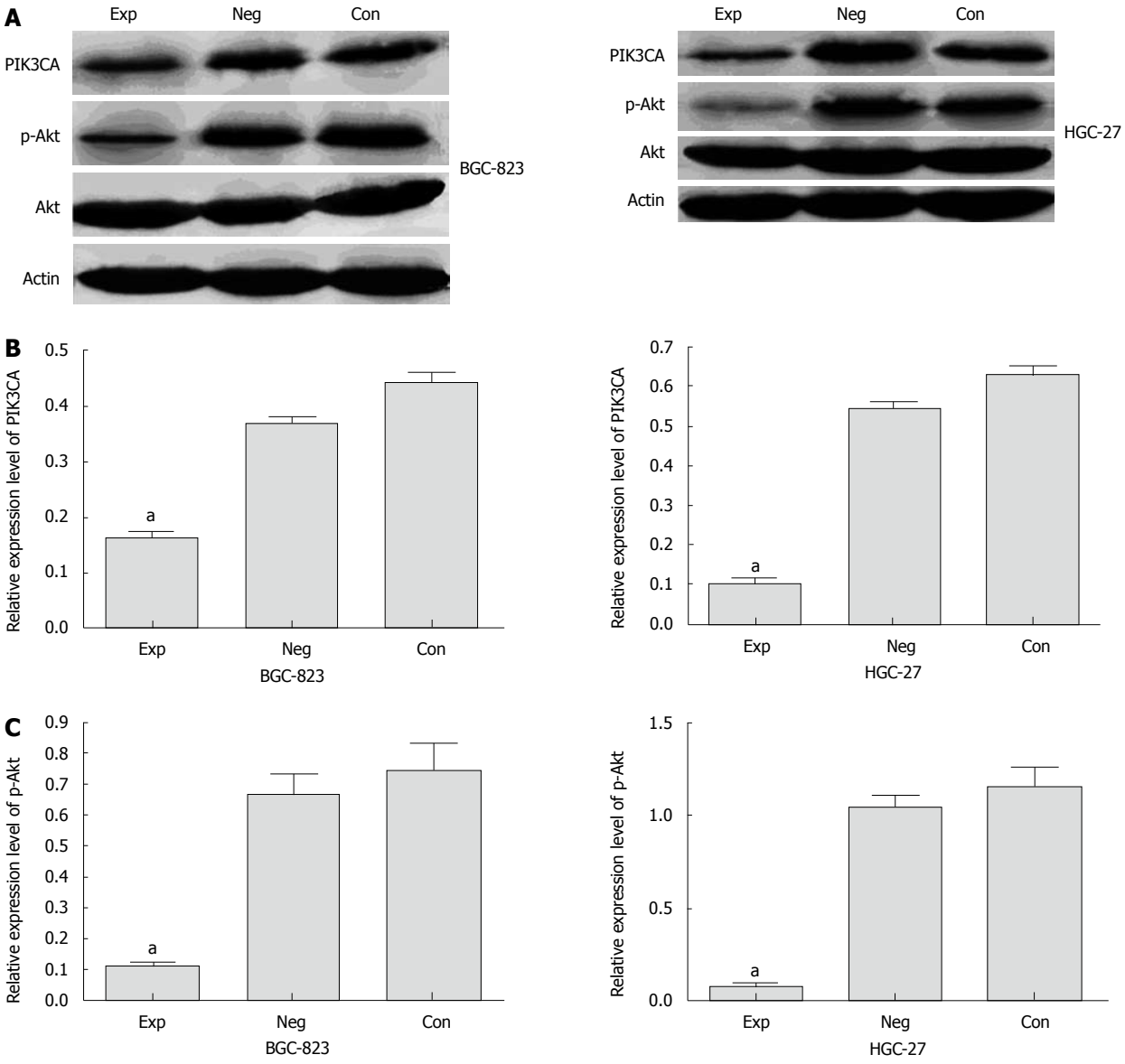

Figure 2 Protein expression of PIK3CA and p-Akt in gastric cancer cells. A: Expression analyses of PIK3CA and p-Akt protein in cells by Western blotting assay. The representative data are shown in triplicate experiments. β-actin: internal control protein; B and C: Statistical evaluation of relative expression of PIK3CA and p-Akt protein quantified by grey analyses with SigmaPlot 8.0 software. Exp: Experimental group; Neg: Negative control group; Con: Control group. [a]$P < 0.05$ *vs* controls, $n = 3$.

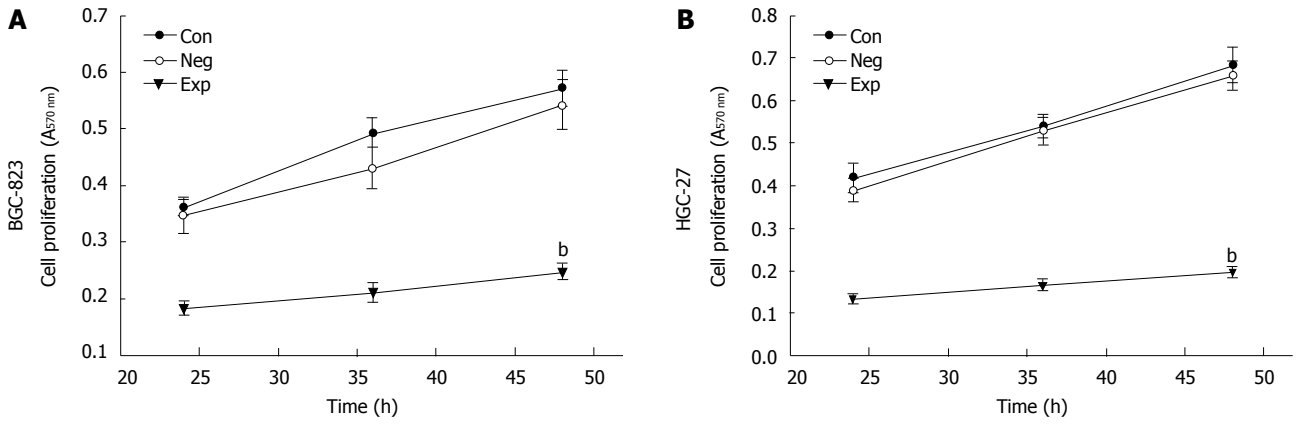

Figure 3 Cell proliferation assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Values are mean ± SD. Con: Control group; Neg: Negative control group; Exp: Experimental group. [b]$P < 0.01$ *vs* controls, $n = 3$.

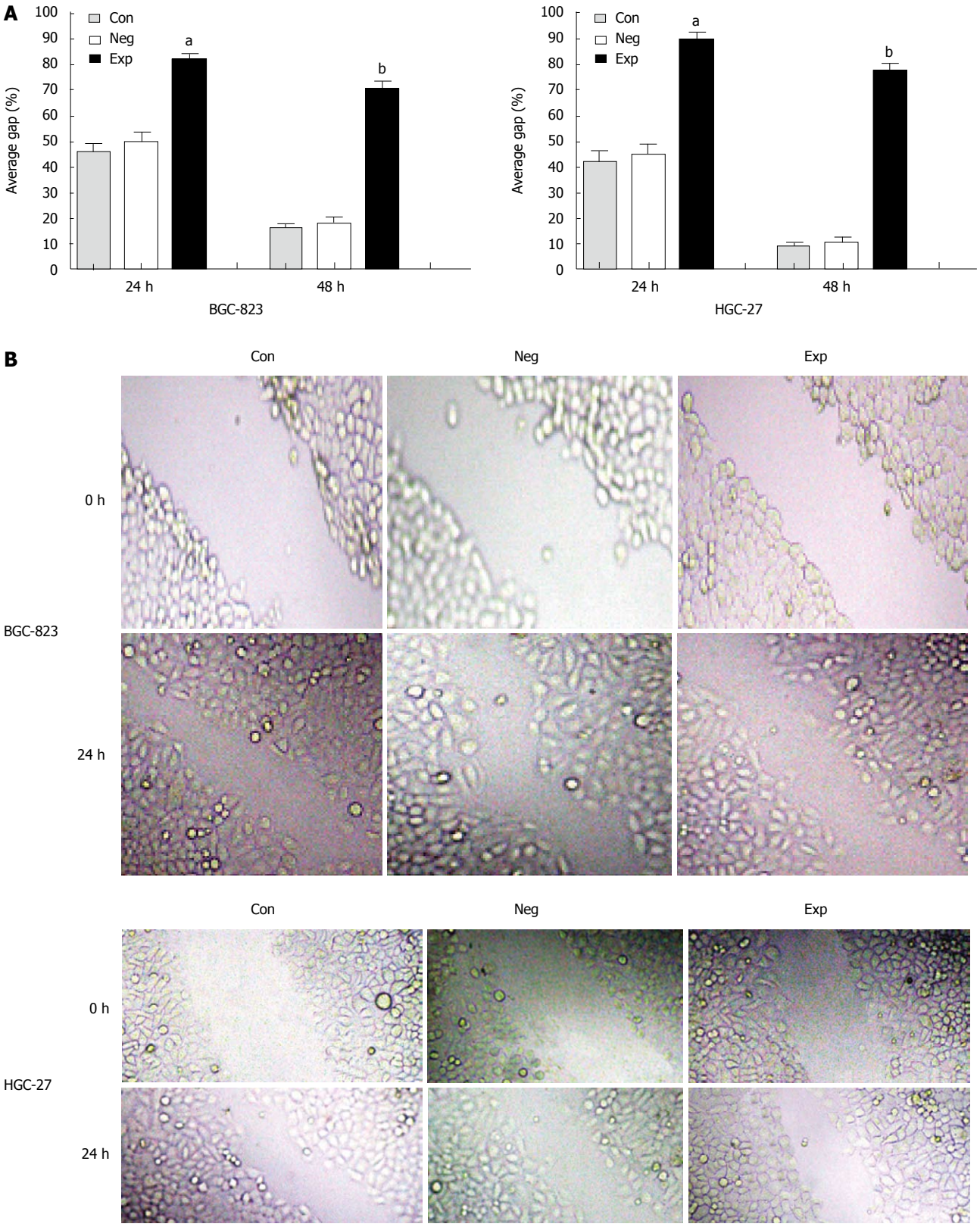

Figure 4 PIK3CA-siRNA reduced migration of BGC-823 and HGC-27 cells using an *in vitro* wound healing assay. A: The results were expressed as average gap and compared with the two control groups. The distance of the wound was measured at five reference points along the scratch wound. [a]$P < 0.05$ *vs* controls, [b]$P < 0.01$ *vs* controls, $n = 3$; B: A representative result of cell migration at 0 h and 24 h (original magnification, × 150). Con: Control group; Neg: Negative control group; Exp: Experimental group.

ability of PI3K to activate its key downstream effector Akt[15,16]. Many studies have shown that Akt activity is detectable in a variety of tumors[17-19], including gastric cancer shown by our group[10]. Elevated phosphorylated

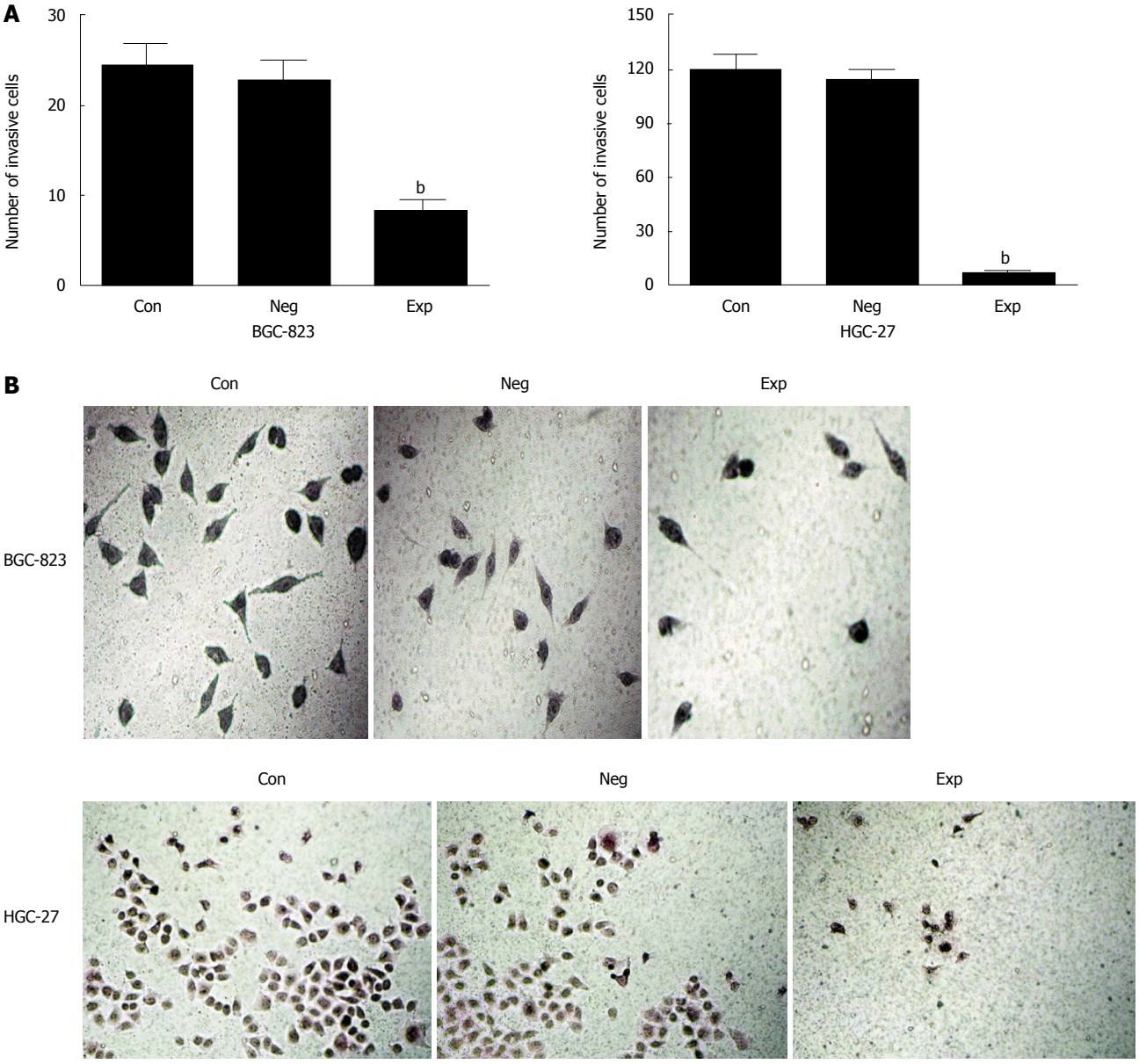

Figure 5 Effect of PIK3CA silencing on tumor cell invasion. A: PIK3CA knockdown reduces invasion ability of gastric cancer cells; B: A representative result of cell invasiveness among the three groups of cells (original magnification of BGC-823 and HGC-27 cells, × 200, × 150, respectively). Con: Control group; Neg: Negative control group; Exp: Experimental group. $^{b}P < 0.01$ *vs* controls, $n = 3$.

Akt (p-Akt), the activated form, has been demonstrated in multiple malignancies[20] and is often functionally linked to tumor progression, such as in thyroid cancer[21], and metastasis, such as in gastric cancer[10]. As reported by Grille *et al*[16], Akt activation in cancer cells increased the motility required for tissue invasion and metastases and was consequently associated with poor prognosis in many cancers. Our previous study[12] also demonstrated that different gastric cancer cell lines (HGC-27, BGC-823 and SGC-7901) varied in their invasiveness which was associated with their expression level of PIK3CA.

In the present study, our results revealed that both PIK3CA mRNA and protein were markedly inhibited in two cell lines transfected with PIK3CA-siRNA, which is consistent with many studies showing that the introduction of a 21 nt dsRNA into cancer cells strongly suppressed the expression of specific mRNAs[22,23]. Furthermore, RNAi-directed targeting of PIK3CA in these cells could reduce the capability of cell proliferation, migration and invasion. More importantly, a low level of p-Akt in the experimental group was detected compared with the two control groups. The above evidence indicates that a robust knockdown of PIK3CA by siRNA may result in decreased catalytic activity of PI3K, subsequent de-phosphorylation of the downstream effector Akt, and thus low activity or aberrant inactivation of the PI3K/Akt pathway in these cells. Our data are in agreement with previous observations that PI3-kinase activity is solely caused by gene-dependent expression of the catalytic subunit p110α (PIK3CA)[24,25].

Interestingly, the Transwell chambers assay showed that PIK3CA mutant HGC-27 cells had an approximate-

ly 5-fold increased ability to invade the Matrigel (Figure 5) compared to PIK3CA non-mutant BGC-823 cells, suggesting that PIK3CA mutation contributed to cell invasion, which is consistent with a previous study in which PIK3CA mutations occur late in glioma progression[26].

Taken together, siRNA targeting PIK3CA effectively inhibits the proliferation and invasion of gastric cancer cells *via* aberrant inactivation of the PI3K/Akt pathway, and would be expected to become a new strategy for the therapy of gastric cancer regardless of PIK3CA mutation. However, like all other newly developed therapeutic methods, applying RNAi *via* siRNAs to living animals, especially humans, poses many challenges such as their poor stability and different effectiveness in different cell types[27,28]. Further studies will be required to develop efficient approaches for the delivery of siRNA into target cells.

ACKNOWLEDGMENTS

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COMMENTS

Background

Gastric cancer is the second leading cause of cancer-related death worldwide, and no ideal approach is available to treat this disease. Thus, it is necessary to further explore and investigate the tumorigenesis of gastric cancer and its novel therapy targets. PIK3CA encoding the key enzymatic subunit p110α of phosphatidylinositol 3-kinase (PI3K), plays a vital role as an oncogene in tumor development and progression. Few studies have addressed PIK3CA expression in malignancies, although its mutation has been found in various human solid cancers. In addition, the relationship between PIK3CA expression and invasion of gastric cancer cells and the mechanism underlying any such relationship remains largely unknown.

Research frontiers

It is currently thought that gastric cancer develops through a complex process, such as the activation of oncogenes and/or the inactivation of tumor suppressor genes. However, the critical underlying molecular mechanism of its progression is largely unclear. In recent years, many researchers have focused on signaling pathway deregulation in cancers. Among them, dysregulation of the PI3K/Akt pathway in a wide spectrum of human cancers has become a research hotspot.

Innovations and breakthroughs

Previous studies have mainly focused on PIK3CA mutations in many human solid cancers. In the present study, the authors investigated the effects of the knockdown of PIK3CA by small interfering RNA on proliferation, migration and invasion of two gastric cancer cell lines with or without mutation of PIK3CA, with the aim of evaluating whether the expression of PIK3CA may be linked to tumor progression and may ultimately benefit from gastric cancer therapy regardless of the presence of PIK3CA mutations.

Applications

Functional knockdown of PIK3CA mediated by siRNA effectively inhibits the proliferation and invasion of gastric cancer cells with or without mutation of PIK3CA *via* aberrant inactivation of the PI3K/Akt pathway, and would be expected to become a new strategy for gastric cancer therapy. Further studies will be required to develop efficient approaches for the delivery of siRNA into target cells.

Terminology

PIK3CA gene, encoding the key catalytic subunit p110α of PI3K, is located on chromosome 3q26.3. AKT, a serine/threonine kinase, serving as the major downstream effector of PI3K, regulates many biological processes, such as proliferation, apoptosis and growth.

Peer review

The paper is well written and executed. The results are correctly described and commented.

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BRIEF ARTICLE

Advantage of autologous blood transfusion in surgery for hepatocellular carcinoma

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Abstract

AIM: To evaluate the significance of autologous blood transfusion (AT) in reducing homologous blood transfusion (HT) in surgery for hepatocellular carcinoma (HCC).

METHODS: The proportion of patients who received HT was compared between two groups determined by the time of AT introduction; period A (1991-1994, n = 93) and period B (1995-2000, n = 201). Multivariate logistic regression analysis was performed in order to identify independent significant predictors of the need for HT. We also investigated the impact of AT and HT on long-term postoperative outcome after curative surgery for HCC.

RESULTS: The proportion of patients with HT was significantly lower in period B than period A (18.9% *vs* 60.2%, $P < 0.0001$). Multivariate logistic regression analysis identified AT administration as a significant independent predictor of the need for HT ($P < 0.0001$). Disease-free survival in patients with AT was comparable to that without any transfusion. Multivariate analysis identified HT administration as an independent significant factor for poorer disease-free survival ($P = 0.0380$).

CONCLUSION: AT administration significantly decreased the need for HT. Considering the postoperative survival disadvantage of HT, AT administration could improve the long-term outcome of HCC patients.

Key words: Hepatocellular carcinoma; Surgery; Autologous blood transfusion; Homologous blood transfusion

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INTRODUCTION

Surgical resection is a safe and effective treatment for hepatocellular carcinoma (HCC). Because HCC usually develops in patients with liver cirrhosis, most of such patients present with bleeding tendencies related to

chronic liver dysfunction[1-3]. Therefore, surgery for HCC frequently requires intraoperative transfusion. Homologous blood transfusion (HT) is necessary for patients with excessive intraoperative bleeding, though this is still associated with risks of infections and/or immunological complications[4,5]. Moreover, evidence suggests that HT may be adversely associated with tumor recurrence and poor postoperative survival in various kinds of cancers[6-13]. Autologous transfusion (AT), which represents collection and reinfusion of the patient's own blood or blood components before surgery, and has been developed as a strategy to reduce the need for HT, is currently used for patients scheduled for surgery for various diseases including HCC[11,14-17]. It has been the policy in our hospital since 1995 to prepare for AT for patients scheduled for HCC surgery. To date, several investigators have examined the significance of AT in terms of reducing the need for HT and of postoperative outcome, but only a few were conducted with proper statistical analyses to identify the significance of AT[16,17].

In the present study, we reviewed the frequency of HT and AT administration in patients undergoing surgery for HCC, and statistically analyzed the significant factors that could predict the need for HT. We also compared the difference between the effects of AT and HT on long-term postoperative outcome after curative surgery for HCC.

MATERIALS AND METHODS

The present study included 294 patients with HCC who underwent hepatic resection at the Department of Surgery, Osaka University Hospital between January 1991 and December 2000. In 93 patients between 1991 and 1994 (period A), AT was not administered, and, when blood was needed, HT was administered. Between 1995 and 2000 (period B), AT was carried out preoperatively in the remaining 201 patients provided: (1) they agreed to the storage; (2) their hemoglobin (Hb) level was $\geqslant$ 11.0 g/dL before storage; and (3) they were free of severe cardiopulmonary and/or cerebrovascular diseases, or infection. Autologous blood was collected 1 to 3 times, with 200-400 mL of blood at a time. The blood was stored in a liquid state without freezing. Iron supplements were given daily to the patients who deposited the autologous blood in the post-storage period. In addition, if the total volume of the collected blood was $\geqslant$ 800 mL, recombinant human erythropoietin was administered. All through the study period, during hepatic resection, blood transfusion was carried out when the Hb level fell to < 8.0 g/dL in patients with normal cardiopulmonary function or < 9.0 g/dL in patients with severe cardiopulmonary or cerebrovascular diseases. In patients who had previously deposited autologous blood, autologous blood was first used prior to homologous blood. In this study, patients who required HT were defined as the HT group, irrespective of prior AT, and the remaining patients without HT were defined as the non-HT group. Furthermore, patients in whom only AT was performed were defined as the AT group, and patients without AT or HT were as defined as the non-transfusion group.

Hospital records were collected retrospectively to gather clinical information including clinical factors, tumor-related factors and surgery-related factors. In patients with autologous blood storage, preoperative Hb was indicated as Hb before the storage. The surgical procedure was selected based on the extent of the tumor and residual liver function. The indication for surgery and selection of surgical procedure were not different between period A and period B. The histological grade of differentiation of HCC was determined according to the Edmondson-Steiner classification, and was based on the areas of the tumor with the highest grade[18]. Data were expressed as mean ± standard deviation. Differences between groups were assessed by the chi-square test, Fisher's exact test or the Mann-Whitney *U* test. Survival rates were calculated according to the Kaplan-Meier method, and compared using the log-rank test. Multivariate logistic regression analysis was performed for the selection of significant variables. Statistical analysis was performed using StatView (version 5.0; SAS Institute Inc., Cary, NC). A P value < 0.05 was considered significant. The study protocol was approved by the Human Ethics Review Committee of Osaka University Hospital and a signed consent form was obtained from each patient.

RESULTS

Table 1 shows the clinicopathological characteristics of patients in period A (n = 93) and in period B (n = 201). The clinical features, tumor-related features, and surgery-related factors were not significantly different between patients of the 2 groups. HT was administered in 56 of the 93 patients (60.2%) in period A. In period B, HT was administered in 38 patients (18.9%) (HT group), AT in 134 patients (66.7%), and neither AT nor HT in 45 patients (22.4%) (non-transfusion group). In 134 AT patients, the amount of transfused autologous blood was 200 mL in 3 patients, 400 mL in 63 patients, 600 mL in 2 patients, 800 mL in 62 patients, 1000 mL in 1 patient, and 1200 mL in 3 patients. Among the 134 patients with AT, only AT was administered in 118 patients (58.7%) (AT group), and both AT and HT in the remaining 16 patients (8.0%). Figure 1 shows the distribution of patients according to blood transfusion. Thus, the proportion of patients who received HT was significantly lower in period B than in period A (P < 0.0001). With regard to disease-free survival examined only in patients with curative surgery for HCC, there were no significant differences between period A and period B; the 1-, 3-, 5-, and 10-year disease-free survival rates were 73.9%, 39.5%, 24.7%, and 7.2% for patients in period A, and 65.9%, 34.8%, 21.9%, and 7.2% for patients in period B (P = 0.5688), respectively. The 1-, 3-, 5- and 10-year overall survival rates were 85.7%, 75.6%, 63.1%, and 28.5% for patients in period A, and 92.9%, 70.6%,

Table 1 Clinicopathological characteristics of patients of periods A and B with hepatocellular carcinoma

	Period A (1991-1994) (*n* = 93)	Period B (1995-2000) (*n* = 201)	*P*-value
Clinical factors			
Gender (male/female)	81/12	161/40	0.144
Age (yr)[1]	61 ± 9	62 ± 9	0.102
HBs-Ag (±)	73/20	169/32	0.243
Anti-HCV Ab (±/unknown)	29/62/2	71/125/5	0.471
Child-Pugh classification (A/B)	79/14	160/41	0.275
Preoperative Hb (g/dL)[1]	13.6 ± 1.5	13.3 ± 1.6	0.213
Tumor-related factors			
Number of tumors (single/multiple)	70/23	146/55	0.635
Maximum tumor size (cm)[1]	3.8 ± 2.7	4.1 ± 3.1	0.450
Vascular invasion (±)	83/10	172/29	0.388
Histological grade (Ⅰ, Ⅱ/Ⅲ, Ⅳ/unknown)	40/41/12	89/92/20	0.975
Surgery-related factors			
Procedure (nonanatomical/anatomical)	45/48	101/100	0.767
Operation time (min)[1]	291 ± 144	295 ± 151	0.853
Resected liver volume (g)[1]	218 ± 406	214 ± 289	0.925
Intraoperative blood loss (mL)[1]	2190 ± 5689	1621 ± 2209	0.219

[1]Data are expressed as number of patients and mean ± standard deviation. HBs-Ag: Hepatitis B surface antigen; Anti-HCV Ab: Anti-hepatic C virus antibody; Hb: Hemoglobin.

Table 2 Clinicopathological characteristics of patients with hepatocellular carcinoma according to homologous blood transfusion

	Non-HT group (*n* = 200)	HT group (*n* = 94)	*P*-value
Clinical factors			
Gender (male/female)	162/38	80/14	0.390
Age (yr)[1]	62 ± 9	60 ± 9	0.084
HBs-Ag (±)	168/32	74/20	0.269
Anti-HCV Ab (±/unknown)	65/130/5	35/57/2	0.437
Child-Pugh classification (A/B)	167/33	72/22	0.157
Preoperative Hb (g/dL)[1]	13.5 ± 1.6	13.2 ± 1.7	0.171
AT administration (±)	82/118	78/16	< 0.0001
Tumor-related factors			
Number of tumors (single/multiple)	149/51	67/27	0.559
Maximum tumor size (cm)[1]	3.6 ± 2.4	4.9 ± 3.7	0.000
Vascular invasion (±)	177/23	78/16	0.193
Histological grade (Ⅰ, Ⅱ/Ⅲ, Ⅳ/unknown)	91/88/21	38/45/11	0.446
Surgery-related factors			
Procedure (nonanatomical/anatomical)	111/89	35/59	0.004
Operation time (min)[1]	264 ± 130	356 ± 166	< 0.0001
Resected liver volume (g)[1]	159 ± 196	336 ± 490	< 0.0001
Intraoperative blood loss (mL)[1]	993 ± 707	3522 ± 6104	< 0.0001

[1]Data are expressed as number of patients and mean ± standard deviation. HBs-Ag: Hepatitis B surface antigen; Anti-HCV Ab: Anti-hepatic C virus antibody; Hb: Hemoglobin; AT: Autologous transfusion; HT: Homologous transfusion.

58.2%, and 40.3% for patients in period B ($P = 0.3202$).

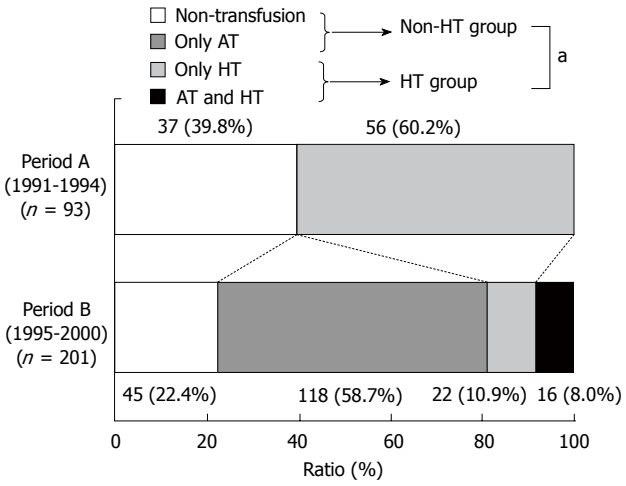

Figure 1 Distribution of patients according to transfusion status during periods A and B. The proportion of patients who received HT was significantly lower in period B than period A ([a]$P < 0.0001$). AT: Autologous transfusion; HT: Homologous transfusion.

In order to identify the factors that can predict the need for HT, various clinical parameters, tumor-related factors, and surgery-related factors were compared between the non-HT group and the HT group (Table 2). The preoperative Hb level was not significantly different between the 2 groups (13.5 ± 1.6 g/dL *vs* 13.2 ± 1.7 g/dL, $P = 0.1708$). The proportion of patients who received AT was significantly lower in the HT group than the non-HT group [59.0% (118/200) *vs* 17.0% (16/94), $P < 0.0001$]. The maximum tumor size was significantly larger in the HT group than in the non-HT group (4.9 ± 3.7 cm *vs* 3.6 ± 2.4 cm, $P = 0.0003$). As for surgery-related factors, there were significant differences in surgical procedure ($P = 0.0035$), operation time ($P < 0.0001$), resected liver volume ($P < 0.0001$), and intraoperative blood loss ($P < 0.0001$), suggesting that surgery in the HT group was major compared to that in the non-HT group.

To identify significant factors that could predict the need for HT, multivariate logistic regression analysis was performed (Table 3). The analysis was carried out using the 6 significant factors identified in the comparison of the non-HT group and the HT group. The analysis identified AT administration, intraoperative blood loss, and resected liver volume as significant independent predictors for the need of HT ($P < 0.0001$, $P < 0.0001$, $P = 0.0362$, respectively). Long-term postoperative outcome after surgery for HCC was examined. In this analysis, patients were limited to those with curative resection, which was defined as complete removal of all macroscopically evident tumors [non-HT group: 193 patients (non-transfusion group: 78 patients; AT group: 115 patients), HT group: 83 patients]. Among the 276 patients, 37 patients (13.4%) were followed-up for more than 10 years. The clinicopathological features of the groups are shown in Table 4. First, we compared the long-term postoperative outcome between the non-transfusion group and the AT group. The preoperative Hb level was significantly higher in the AT group than in the non-

Table 3 Results of multivariate logistic regression analysis for the need for homologous blood transfusion

		OR	95% CI	*P*-value
AT administration	±	28.571	9.615-83.333	< 0.0001
Maximum tumor size (cm)	< 5/≥ 5	1.126	0.500-2.538	0.774
Procedure	Nonanatomical/anatomical	1.016	0.449-2.202	0.967
Operation time (min)	< 300/≥ 300	0.986	0.435-2.242	0.974
Resected liver volume (g)	< 200/≥ 200	2.532	1.062-6.061	0.036
Intraoperative blood loss (mL)	< 2000/≥ 2000	30.303	9.346-100.000	< 0.0001

OR: Odds ratio; CI: Confidence interval; AT: Autologous transfusion.

Table 4 Clinicopathological characteristics of patients who underwent curative surgery for hepatocellular carcinoma

	Non-HT group (*n* = 193)	HT group (*n* = 83)	*P*-value (Non-HT *vs* HT)	Non-HT group		
				Non-transfusion group (*n* = 78)	AT group (*n* = 115)	*P*-value (Non-transfusion *vs* AT)
Clinical factors						
Gender (male/female)	156/37	70/13	0.488	63/15	93/22	0.986
Age (yr)[1]	62 ± 8	61 ± 9	0.115	62 ± 8	61 ± 9	0.878
HBs-Ag (±)	163/30	67/16	0.445	Nov-67	96/19	0.649
Anti-HCV Ab (±/unknown)	62/127/4	31/51/1	0.426	21/54/3	41/73/1	0.254
Child-Pugh classification (A/B)	161/32	65/18	0.262	66/12	95/20	0.833
Preoperative Hb (g/dL)[1]	13.5 ± 1.5	13.4 ± 1.6	0.425	12.8 ± 1.8	14.1 ± 1.1	< 0.0001
Tumor-related factors						
Number of tumors (single/multiple)	147/46	64/19	0.866	62/16	85/30	0.372
Maximum tumor size (cm)[1]	3.5 ± 2.4	4.8 ± 3.7	0.000	3.3 ± 2.2	3.6 ± 2.4	0.287
Vascular invasion (±)	172/21	70/13	0.268	73/5	99/16	0.101
Histological grade (Ⅰ, Ⅱ/Ⅲ, Ⅳ/unknown)	89/83/21	33/41/9	0.304	36/34/8	53/49/13	0.412

[1]Data are expressed as number of patients and mean ± standard deviation. HBs-Ag: Hepatitis B surface antigen; Anti-HCV Ab: Anti-hepatic C virus antibody; Hb: Hemoglobin; AT: Autologous transfusion; HT: Homologous transfusion.

transfusion group (14.1 ± 1.1 g/dL *vs* 12.8 ± 1.8 g/dL, $P < 0.0001$). Tumor-related factors were similar in the 2 groups. There were no significant differences in the disease-free survival rates between the AT group (1-, 3-, 5-, and 10-year: 70.6%, 37.1%, 22.3%, and 11.2%, respectively) and the non-transfusion group (73.1%, 41.3%, 30.7%, and 9.6%, respectively) ($P = 0.3874$) (Figure 2A). Next, we compared the long-term survival rates of the non-HT group and the HT group. Although the cumulative disease-free survival rate of the non-HT group was significantly better than that of the HT group ($P = 0.0305$) (Figure 2B), since the maximum tumor size was significantly different in the comparison (3.5 ± 2.4 cm *vs* 4.8 ± 3.7 cm, $P = 0.0004$), additional comparison was also performed based on the tumor size. The disease-free survival rates for the non-HT group (1-, 3-, 5-, and 10-year: 75.6%, 42.6%, 29.4%, and 10.8%, respectively) was significantly better than those of the HT group (69.0%, 31.6%, 16.7%, and 4.5%, respectively) of the subgroup with tumor size 5.0 cm or smaller than 5.0 cm ($P = 0.0452$) (Figure 2C), but not in patients with tumor size larger than 5.0 cm (1-, 3-, 5-, and 10-year: 56.4%, 24.1%, 10.8%, and 5.4% in the non-HT group, and 39.4%, 26.3%, 13.1%, and 0.0% in the HT group, respectively, $P = 0.7391$) (Figure 2D). Furthermore, multivariate analyses using significant factors identified in the univariate analyses demonstrated that transfusion status (non-HT/HT) was one of the independent significant factors for disease-free survival ($P = 0.0380$) (Table 5), suggesting disadvantages of HT on postoperative prognosis.

DISCUSSION

The results of the present study demonstrated a reduction in HT administration in surgery for HCC after the introduction of AT. Our results are in agreement with those of previous reports which emphasized the significance of AT in reducing the need for HT in surgery for HCC[16,17]. However, in these previous reports, only 20-30 patients were included in the AT group. Furthermore, although the Hb level immediately before surgery was reported in the AT group, the Hb level before storage was not indicated, suggesting a different clinical background of patients who received HT and those of other groups. On the other hand, in the present study, despite its retrospective design, the clinicopathological background, including the Hb level, was similar in the 2 groups as shown in Table 1. In this regard, the present study is significant as it identified the benefits of AT in the reduction of HT administration.

In the present study, we analyzed the data for significant predictors of HT use. The results showed that AT administration was an independent significant predictor of the need for HT, and support the significance of AT in reducing the need for HT. In the analysis, preoperative Hb, which is reported to be significantly associated with the need for HT[19,20], was not an independent significant factor. While the reason for this difference in the results

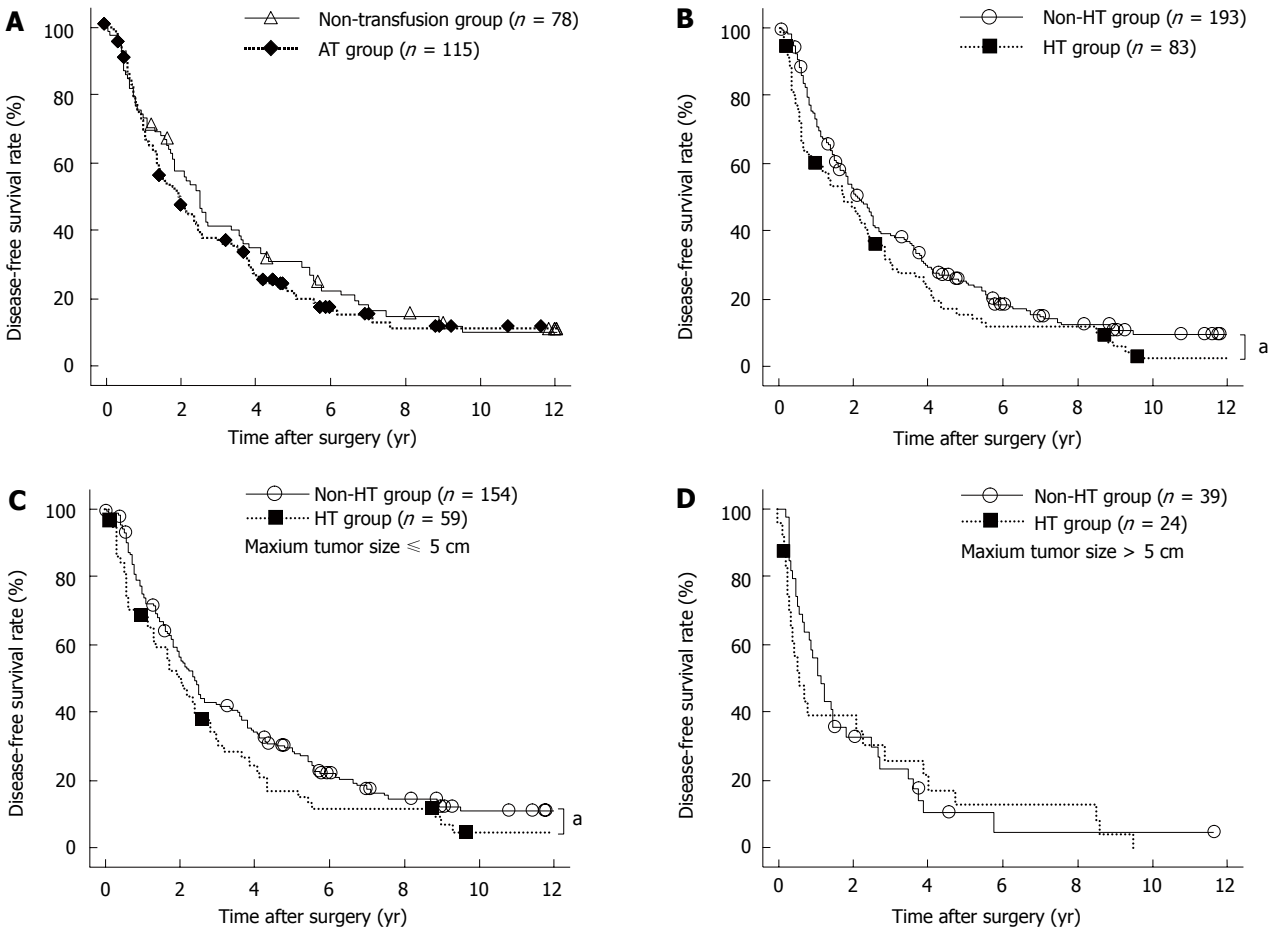

Figure 2 Disease-free survival after curative surgery for hepatocellular carcinoma. A: There were no significant differences between the non-transfusion group (solid line) and the Autologous transfusion (AT) group (dotted line) (P = 0.3874); B: The cumulative disease-free survival in the non-Homologous transfusion (HT) group (solid line) was significantly better than in the HT group (dotted line) (^aP = 0.0305); C: The disease-free survival in the non-HT group (solid line) was significantly better in than the HT group (dotted line) in patients with maximum tumor size of ≤ 5.0 cm (^aP = 0.0452); D: No significant differences were noted between the non-HT group (solid line) and the HT group (dotted line) in patients with the maximum tumor size > 5.0 cm (P = 0.7391).

Table 5 Statistical analysis of disease-free survival of patients with curative resection for hepatocellular carcinoma

	Univariate		Multivariate	
	P-value	OR	95% CI	*P*-value
Clinical factors				
Gender (male/female)	0.840			
Age (yr) (≤ 63/> 63)	0.402			
HBs-Ag (±)	0.279			
Anti-HCV Ab (±)	0.045	1.401	1.032-1.901	0.031
Child-Pugh classification (A/B)	0.079			
Preoperative Hb (g/dL) (≤ 12/> 12)	0.824			
Transfusion (non-HT group/HT group)	0.031	1.372	1.018-1.849	0.038
Tumor-related factors				
Number of tumors (single/multiple)	0.000	1.819	1.290-2.564	0.001
Maximum tumor size (cm) (≤ 5/> 5)	0.001	1.07	0.750-1.525	0.709
Vascular invasion (±)	< 0.0001	2.473	1.606-3.806	< 0.0001
Histological grade (Ⅰ, Ⅱ/Ⅲ, Ⅳ)	0.017	1.188	0.898-1.570	0.227

OR: Odds ratio; CI: Confidence interval; HBs-Ag: Hepatitis B surface antigen; Anti-HCV Ab: Anti-hepatic C virus antibody; Hb: Hemoglobin; HT: Homologous transfusion.

remains unclear, it could be due to the effect of recombinant human erythropoietin administered after the storage of autologous blood. Alternatively, it is possible that, since the subjects of the above previous studies did not receive AT, the significance of preoperative Hb is overestimated. Thus, the present study is significant in terms of identifying the effect of AT in reducing HT using appropriate statistical analysis.

We also investigated the effects of AT and HT on postoperative outcome after curative surgery for HCC. The study revealed that the disease-free survival rates were comparable between the non-transfusion group and the AT group when the clinical background was similar. Furthermore, the disease-free survival rates of the HT group were significantly worse than those of the non-HT group, based on the results of univariate analysis. Since there was a significant difference in the maximum tumor size between the 2 groups, which suggests the possibility of different tumor biology and recurrences between the HT group and the non-HT group, the survival rate was compared in subgroups based on tumor size, and showed significant differences in the pa-

tients with tumor size ≤ 5.0 cm. In addition, the difference was confirmed to be independently significant by multivariate analyses.

Since the report of a survival advantage of HT in patients undergoing colectomy for colon cancer[21], some investigators have indicated that HT triggers recurrence in various kinds of cancers[6-8]. This HT-induced disadvantage is speculated to be derived from transfusion-associated immunomodulation. Actually, several investigators suggested that HT induces downregulation of natural killer cell activity and cytotoxic T-cell function, resulting in a subclinical state of anergy or tolerance[22-24].

The correlation has been reported also in patients with HCC[9-14,25]. Although the results of the present study were comparable to these previous reports, we think that the present study reports a new finding based on the inclusion of patients who were followed-up for more than 10 years. With regard to the long-term survival advantages, to our knowledge, there are only a few reports describing the survival advantage of HT on long-term prognosis (> 10 years). Hirano *et al*[14] investigated the long-term (> 10 years) survival disadvantage of HT over AT, but their reports did not include the clinical background of patients and described the results of only univariate analysis, suggesting inadequate analysis. Also in this regard, the present study provides significant data.

Thus, the present study revealed that AT is significant in reducing the need for HT, which is associated with a long-term postoperative survival disadvantage after HCC surgery. In this study, however, in order to investigate the long-term postoperative outcome for more than 10 years, we limited inclusion in the study to patients who underwent surgery between 1991 and 2000. Based on this limitation, it is possible that the selected time period does not reflect recent advances in both surgical and anesthetic techniques, which could explain the recent decrease in intraoperative blood loss. Considering such recent advances affecting intraoperative blood loss, one can speculate that there are increasingly more patients with HCC who do not need AT. It was also reported recently that the practice of using autologous blood requires more administrative work and laborious collection procedures, and is not without disadvantages[26-29]. Taken together, AT actually has advantage an over HT, but currently, it may be necessary to deliberate on the need for AT itself during surgery for HCC.

In summary, the present study showed that AT administration significantly decreased the need for HT in surgery for HCC, and that AT was one of the significant independent predictor of the need for HT. Considering that HT was disadvantageous with regard to long-term postoperative survival, one can assume that AT administration can lead to improvement in the long-term postoperative outcome of patients with HCC.

COMMENTS

Background

Some evidences suggest that homologous blood transfusion (HT) may be adversely associated with tumor recurrence and poor survival in various kinds of cancers, and autologous blood transfusion (AT) is currently used for patients scheduled for surgery. To date, several investigators have examined the significance of AT in terms of reducing the need for HT and postoperative outcome, but few were conducted with proper statistical analyses to identify the significance of AT in surgery for hepatocellular carcinoma (HCC).

Research frontiers

The authors compared the proportion of patients who received HT between 2 groups determined by the time of AT introduction; period A (1991-1994, *n* = 93) and period B (1995-2000, *n* = 201), and performed multivariate logistic regression analysis for identification of independent significant predictors of the need for HT. Furthermore, they investigated the impact of AT and HT on long-term postoperative outcome after curative surgery for HCC.

Innovations and breakthroughs

The present study showed that the proportion of patients having HT was decreased after AT introduction, that AT administration was a significant independent predictor of the need for HT, and identified HT administration as an independent significant factor for poorer disease-free survival.

Applications

Considering the results of the present study, it could be suggested that AT administration could improve the long-term outcome of patients with HCC.

Peer review

This is a large series of patients treated in several ways with respect to the need for blood transfusion during their surgery for HCC. Unfortunately the authors have a mix of numbers that they have used in different ways to make the conclusion they wanted to make.

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BRIEF ARTICLE

Potential risk factors for nonalcoholic steatohepatitis related to pancreatic secretions following pancreaticoduodenectomy

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Abstract

AIM: To identify risk factors for nonalcoholic steatohepatitis following pancreaticoduodenectomy, with a focus on factors related to pancreatic secretions.

METHODS: The medical records of 228 patients who had a pancreaticoduodenectomy over a 16-mo period were reviewed retrospectively. The 193 patients who did not have fatty liver disease preoperatively were included in the final analysis. Hepatic steatosis was diagnosed using the differences between splenic and hepatic attenuation and liver-to-spleen attenuation as measured by non-enhanced computed tomography.

RESULTS: Fifteen patients (7.8%) who showed postoperative hepatic fatty changes were assigned to Group A, and the remaining patients were assigned to Group B. Patient demographics, preoperative laboratory findings (including levels of C-peptide, glucagon, insulin and glucose tolerance test results), operation types, and final pathological findings did not differ significantly between the two groups; however, the frequency of pancreatic fistula (P = 0.020) and the method of pancreatic duct stenting (P = 0.005) showed significant differences between the groups. A multivariate analysis identified pancreatic fistula (HR = 3.332, P = 0.037) and external pancreatic duct stenting (HR = 4.530, P = 0.017) as independent risk factors for the development of postoperative steatohepatitis.

CONCLUSION: Pancreatic fistula and external pancreatic duct stenting were identified as independent risk factors for the development of steatohepatitis following pancreaticoduodenectomy.

Key words: Nonalcoholic fatty liver diseases; Nonalcoholic steatohepatitis; Pancreatic duct stenting; Pancreatic fistula; Pancreatic surgery

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INTRODUCTION

Steatohepatitis refers to a spectrum of nonalcoholic fatty liver diseases (NAFLD) ranging from simple triglyceride

deposition and accumulation with or without fibrosis to the development of cirrhosis, end-stage liver failure, and even hepatocellular carcinoma[1-6]. Nonalcoholic steatohepatitis (NASH), first described by Ludwig *et al*[7] at the Mayo Clinic in 1980, refers to hepatic lobular or portal inflammation and focal necrosis with fatty changes in patients without a history of alcohol abuse[4,8-12]. Steatohepatitis is associated with lipodystrophy, metabolic syndrome (dyslipidemia, insulin resistance, and diabetes mellitus), genetic susceptibility, environmental factors, and hepatocyte apoptosis associated with mitochondrial dysfunction and the production of reactive oxygen species, which can lead to hepatic fibrogenesis and inflammation[10,13-15].

Fatty liver disease refers to either the accumulation of fat in hepatocytes in excess of 5% of the total liver weight or the fatty degeneration of more than one-third of the total number of cells in the liver[16]. In the general population, the prevalence of NAFLD is 6%-40% among asymptomatic patients, and the incidence of fatty liver disease is 60%-75% in obese patients and 84%-96% in morbidly obese patients who undergo bariatric surgery, with 25%-55% of these patients also having NASH[1,3-5,9,17].

Glucose intolerance in response to insulin resistance induces an elevation in blood levels of glucose and insulin and results in increased synthesis of hepatic free fatty acids[5]. The oxidation of free fatty acids can lead to the production of reactive oxygen free radicals that can be cytotoxic to DNA, mitochondria, and other cellular structures and can lead to the production of pro-inflammatory cytokines. Steatohepatitis reportedly develops after a "first hit" involving triglyceride accumulation and a "second hit" involving oxidative stress, lipid peroxidation, pro-inflammatory cytokines, and mitochondrial dysfunction[4,18].

Previous studies in humans or murine models have identified independent risk factors for hepatic fibrosis, including advanced age, obesity, hypertension, Type Ⅱ diabetes, insulin resistance, dyslipidemia, an aspartate transaminase (AST)/alanine transaminase (ALT) ratio greater than 1, hyperinsulinemia, altered lipid homeostasis, and pancreatic steatosis[4,7-9,17-20]. Additional risk factors that might contribute to disease progression include increased transferrin saturation, long-term total parenteral nutrition leading to choline deficiency, jejunoileal bypass surgery for morbid obesity, environmental toxins, and drugs such as chemotherapeutic agents or glucocorticoids[3,5,18].

Ductal adenocarcinoma of the pancreatic head is the most predominant tumor in the pancreas, and pancreaticoduodenectomy is the treatment of choice[11]. Pancreaticoduodenectomy is also used to treat various other malignancies of the periampullary region, the bile duct, and the duodenum or the borderline diseases of the pancreas[21]. Side-effects associated with pancreaticoduodenectomy include weight loss, abdominal pain, fatigue, and exocrine and endocrine insufficiencies. Pancreaticoduodenectomy also has a high rate of morbidity and mortality, including the postoperative development of steatohepatitis. Only a few reports have explored the relationship between pancreaticoduodenectomy and the development of steatohepatitis[22]. Therefore, we aimed to identify the risk factors for steatohepatitis after pancreaticoduodenectomy, with a particular focus on factors related to pancreatic secretions.

MATERIALS AND METHODS

Patient demographics and clinical variables

All study procedures were approved by the Institutional Review Board (No. 2010-09-082, Samsung Medical Center). The study included 228 patients who had pylorus-preserving pancreaticoduodenectomy (PPPD), Whipple' s procedure, or hepato-pancreato-duodenectomy (HPD) between January 2009 and April 2010. Electronic medical records and data were retrospectively reviewed. Exclusion criteria were: (1) patients without non-enhanced computed tomography (CT) findings (n = 12); (2) a preoperative diagnosis of fatty liver disease by non-enhanced CT (n = 19); and (3) mortalities resulting from postoperative pseudoaneurysm (n = 4). Thirty-five patients who consumed more than 150 g of alcohol per week were not excluded from the study because they did not have a diagnosis of preoperative fatty liver disease by non-enhanced CT. The final study group therefore consisted of 193 patients that were divided into two groups: Group A consisted of 15 patients who developed postoperative steatohepatitis, and Group B consisted of 178 patients who did not develop postoperative steatohepatitis.

Data were collected on patient demographics, operative procedures, pathologies, and perioperative clinical variables, including levels of insulin, C-peptide, and glucagon, and results from an oral glucose tolerance test conducted preoperatively. Data were also collected on postoperative liver function and the postoperative attenuation ratios for the liver and spleen.

Data on pancreatic enzyme levels in serum on postoperative day 7, pancreatic duct size, pancreatic fistula, pancreatic duct stenting, and type of stenting were collected and considered as potential parameters associated with pancreatic secretions. Pancreatic fistula was diagnosed according to the International study group pancreatic fistula (ISGPF) definition[23]. External pancreatic duct stenting was usually placed during the first postoperative month. Post-discharge pancreatic enzyme supplementation was administered routinely to all patients who had a pancreaticoduodenectomy.

Evaluation of steatohepatitis

Fatty liver disease was defined according to the difference between the splenic and hepatic attenuation ratios (CT_{S-L}) and the liver-to-spleen attenuation ratio ($CT_{L/S}$). To minimize sampling error, we used two CT images from the liver, one from the right lobe and one from the left lobe, and we excluded images from the periphery of the liver. Perioperative steatohepatitis was presumed when CT_{S-L}

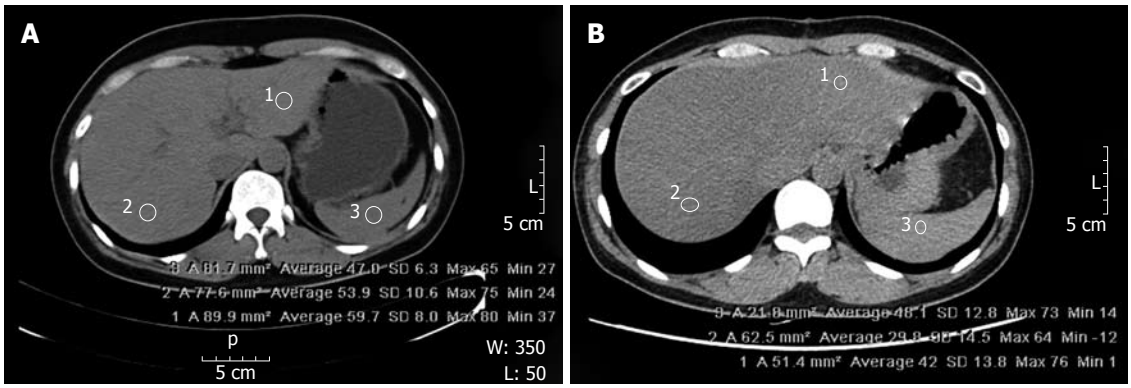

Figure 1 Preoperative and postoperative non-enhanced computed tomography images of a representative patient demonstrating different attenuation values for the spleen and liver. The inclusion criteria were: (1) $CT_{S-L} \geqslant 10$ Housefield unit (HU); or (2) $CT_{L/S} \leqslant 0.9$ HU [L: Mean attenuation value for two random points (1, 2) of the liver; S: Attenuation value for one random point (3) of the spleen]. A: Preoperative; B: Postoperative.

Table 1 Preoperative characteristics and laboratory findings for the two groups

	Group A(n = 15)	Group B (n = 178)	P
Gender (M:F), n	7:8	112:66	0.270
Age in years, mean (range)	58.7 (40-74)	61.8 (15-81)	0.295
BMI (kg/m^2) ± SD	20.6 ± 2.9	22.0 ± 3.3	0.082
Hepatitis B viral infection (+), n (%)	0 (0)	6 (3.4)	1.000
Type Ⅱ diabetes, n (%)	2 (13.3)	34 (19.1)	0.582
Alcohol consumption > 150 g/wk, n (%)	3 (20.0)	32 (18.0)	0.738
Biliary drainage, n (%)	4 (26.7)	74 (41.6)	0.245
Albumin/globulin ratio (range)	1.79 (1.2-4.1)	1.53 (0.8-2.4)	0.234
Total cholesterol (mg/dL), mean (range)	209.3 (136-309)	200.6 (73-470)	0.641
Serum amylase (U/L), mean (range)	91.3 (22-263)	129.8 (15-1361)	0.388
Serum lipase (U/L), mean (range)	170.4 (24-707)	336.7 (7-13562)	0.593
AST (U/L), mean (range)	134.3 (11-547)	111.7 (12-1230)	0.621
ALT (U/L), mean (range)	156.1 (11-551)	144.0 (9-1371)	0.826
ALP (U/L), mean (range)	315.9 (64-913)	267.3 (34-2236)	0.518
INR, mean (range)	0.99 (0.85-1.11)	1.07 (0.81-8.78)	0.585
Total bilirubin (mg/dL), mean (range)	5.7 (0.3-18.8)	6.2 (0.2-44.3)	0.799
Fasting glucose (mg/dL), mean (range)	140 (93-150)	135 (47-458)	0.735

BMI: Body mass index; SD: Standard deviation; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; ALP: Alkaline phosphatase; INR: International normalized ratio.

was equal to or greater than 10 Housefield Units (HU) or when $CT_{L/S}$ was equal to or less than 0.9 HU (Figure 1).

CT images were obtained with a 64-channel, 4-multidetector, CT scanner (General Electric®, NY, United States). The parameters for non-enhanced CT were: 100-300 mAs; rotation speed of 0.6 s; table speed of 3 mm; noise index of 11.57; detector coverage of 40 mm; pitch-to-speed ratio (mm/rot) of 0.984:1; and helical thickness of 5 mm. CT images were reviewed on a Picture Archiving Communication System workstation (General Electric®).

Statistical analysis

For continuous variables, the paired-sample Student's t test was used to compare the two patient groups. For categorical variables, Chi Square analysis or Fisher's exact test was used to compare the two groups. Two-way Analysis of Variance was used to analyze group differences in repeated measures of the levels of glucose, insulin, C-peptide, and glucagon. Pearson's correlation coefficient test was used to determine the correlation between the postoperative difference of CT_{S-L} and the postoperative liver function test results. Multivariate analysis of risk factors was conducted using multivariate Cox proportional hazards modeling. Statistical analyses were performed using SPSS, version 16.0 (SPSS Inc., Chicago, IL, United States), and P values < 0.05 were considered statistically significant.

RESULTS

Perioperative clinical characteristics

The mean period between the operation and the patient's postoperative follow-up appointment was 3.2 ± 2.0 mo (range: 1-11). For each group, the average period was 2.4 mo in Group A and 3.3 mo in Group B (P = 0.106). Fifteen patients (7.8%) who showed postoperative hepatic fatty changes were included in Group A,

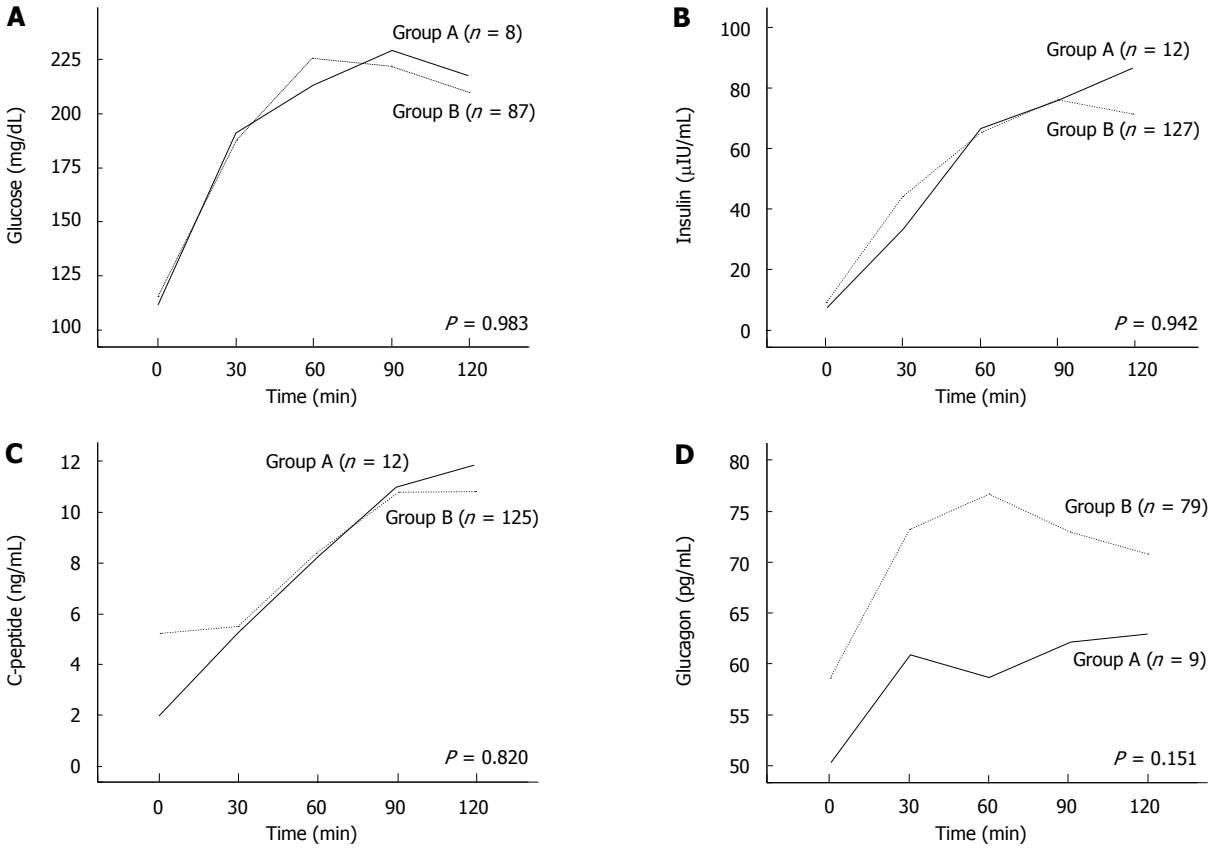

Figure 2 Preoperative oral glucose tolerance test results, continuous stimulation test results for insulin, C-peptide, and glucagon levels. A: Glucose levels; B: Insulin levels; C: C-peptide levels; D: Glucagon levels.

Table 2 Operative treatments and final pathological findings for the two groups

	Group A (n = 15)	Group B (n = 178)	P
PPPD	12	126	
Whipple's procedure	3	47	0.482
HPD	0	5	
Pancreatic cancer	7	50	
Common bile duct cancer	3	45	
Ampulla of vater cancer	5	27	
Duodenal cancer	0	11	
IPMN of pancreas	0	23	
NET of pancreas or duodenum	0	3	0.665
MCN or SPT of pancreas	0	5	
Duodenal GIST	0	2	
Hilar cholangiocarcinoma	0	4	
Colon cancer with duodenal invasion	0	4	
Gallbladder cancer with duodenal invasion	0	1	
Pancreatitis	0	3	

PPPD: Pylorus-preserving pancreaticoduodenectomy; HPD: Hepato-pancreato-duodenectomy; IPMN: Intraductal papillary mucinous neoplasm; NET: Neuroendocrine tumor; MCN: Mucinous cystic neoplasm; SPT: Solid pseudopapillary tumor; GIST: Gastrointestinal stromal tumor.

and the remaining 178 patients (92.2%) were included in Group B (Table 1). Seventy-eight patients (40.4%) had preoperative biliary drainage, including percutaneous transhepatic biliary drainage, endoscopic retrograde biliary drainage, endoscopic nasobiliary drainage, and biliary stenting. None of the patients had undergone bariatric surgery (data not shown).

Preoperative patient characteristics and laboratory findings, including liver function test results and levels of pancreatic enzymes, were similar in the two groups (Table 1). PPPD or Whipple's procedure was performed on 15 patients (100%) in Group A and 173 patients (97.2%) in Group B (P = 0.842) (Table 2). HPD was performed on 5 patients (2.8%) in Group B. Based on final pathologic reports, all patients (100%) in Group A were diagnosed with malignant disease, whereas patients in Group B were diagnosed with a variety of diseases (Table 2). Preoperative oral glucose tolerance test results, continuous stimulation test results for insulin, C-peptide and glucagon levels were not significantly different between the two groups (Figure 2). For patients with malignant disease, the two groups were similar in terms of cancer stage (P = 0.190), perineural invasion (P = 0.259), and vessel invasion (P = 1.000). The liver function test for all patients showed that postoperative $CT_{L/S}$ values correlated with postoperative ALT levels (γ = -0.149, P = 0.039) but not with postoperative AST or ALP levels (Table 3).

Factors associated with pancreatic secretions

Serum levels of pancreatic enzymes on postoperative day 7, pancreatic duct size, and the proportion of patients that received a pancreatic duct stent were similar in the two groups (Table 4); however, the proportion

Table 3 Pearson's correlation coefficients for the correlation between postoperative liver function and the difference in the postoperative attenuation values between the spleen and liver (CT_{S-L}) in all patients

Postoperative liver function test result	γ	*P*
AST	-0.138	0.056
ALT	-0.149	0.039
ALP	-0.023	0.755

AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; ALP: Alkaline phosphatase.

of patients that developed a pancreatic fistula postoperatively ($P = 0.020$) or had an external pancreatic duct stent placed ($P = 0.005$) was higher in Group A than in Group B (Table 4). A multivariate analysis of risk factors identified the postoperative development of a pancreatic fistula (HR = 3.332, $P = 0.037$) and the postoperative placement of an external pancreatic duct stent (HR = 4.530, $P = 0.017$) as independent risk factors for postoperative steatohepatitis (Table 5). Among the 41 patients who showed postoperative pancreatic fistula in the two groups, 32 and 9 patients showed grade A and B fistulas guided by ISGPF definition, respectively. Grade A fistula was observed in 6 patients (85.7%) in group A, and in 26 patients (76.5%) in group B ($P = 1.000$).

DISCUSSION

Steatohepatitis associated with pancreatic secretions

Duct-to-mucosal anastomosis and pancreatic duct stenting are used to prevent pancreatic leakage. In our institution, pancreatic stenting is usually performed when the diameter of the pancreatic duct is 2-5 mm, although there is some variability according to the attending surgeon's preferences. The methods chosen for pancreatic duct stenting in association with steatohepatitis involve several considerations. The remnant pancreas secretes several enzymes that are associated with digestion, including enterokinase, trypsinogen, chymotrypsin, amylase, lipase, cellulose, phospholipase, and esterase[24-26]. External pancreatic duct stenting induces an earlier impairment in the secretion of pancreatic enzymes into the bowel lumen. Phospholipases A1 and A2 cleave fatty acids from phospholipids, and esterase hydrolyzes cholesterol esters. We hypothesize that hepatic fibrosis can be prevented by inhibiting the entry of free fatty acids into hepatocytes. Furthermore, lipid absorption by bile acid denaturation, which can lead to fat accumulation in hepatocytes, might be prevented by impairing bicarbonate secretion within the acidic gastric environment.

Adipokines such as leptin, resistin, adiponectin, and tumor necrosis factor (TNF)-α are known to regulate hepatic and peripheral glucose levels and lipid metabolism[27]. Decreased serum levels of adiponectin and increased serum levels of leptin or TNF-α are associated with NASH. Also, the hydrolysis of starches and glycogen into disaccharides or trisaccharides by amylolytic pancreatic enzymes can be impaired by external pancreatic duct drainage. These endocrine abnormalities are accelerated by the loss of insulin and glucagon, and they can induce insulin insensitivity and abnormal glucose metabolism.

Reduced motilities of the stomach and duodenum after pancreaticoduodenectomy can lead to the development of diabetes (20%-40%) with hyperglycemia and delayed gastric emptying (15%-40%), and a reduction in the release of pancreas-stimulating hormones from the duodenum (100%)[28]. These effects can aggravate insulin resistance. Moreover, motility dysfunction can be induced by intestinal bacterial overgrowth as well as anatomic alterations that may result from anastomotic procedures. Wu *et al*[29] found that decreased small intestinal motility was associated with delayed intestinal transit followed by bacterial overgrowth (*Escherichia coli*) in a rat model of NASH. Furthermore, patients who have undergone a pancreaticoduodenectomy are at increased risk of developing an ascending infection through hepaticojejunostomy and jejunojejunostomy. The effectiveness of antibiotics for decreasing elevated liver enzymes in NASH needs to be further investigated, however.

A previous study[23] identified the duration of untreated jaundice, malignancy, small pancreatic duct size, and soft pancreatic texture as risk factors for pancreatic fistula. The relationship between pancreatic fistula and steatohepatitis is still questionable, but the use of long-term total parenteral nutrition or the development of a secondary infection or sepsis after a pancreatic fistula might influence hepatic function.

Other factors related to steatohepatitis

High levels of low density lipoprotein-cholesterol (LDL-C) and low levels of high density lipoprotein-cholesterol (HDL-C) are established risk factors for atherogenesis in patients with diabetic dyslipidemia[30]. Insulin resistance is a key factor in the development of metabolic syndrome involving dyslipidemia and Type Ⅱ diabetes. Dyslipidemia is associated primarily with low levels of HDL-C, high levels of LDL-C, and hepatic overproduction of triglyceride-rich very low density lipoprotein-cholesterol (VLDL-C)[31]. In the present study, LDL-C levels tended to be higher in Group A than in Group B, but this apparent difference was not significant (data not shown); therefore, the relationship between LDL-C and steatohepatitis remains to be determined.

Bariatric surgery is often considered for patients who are morbidly obese. Roux-en-Y gastric bypass, gastroplasty, or adjustable gastric banding are commonly performed, and jejunoileal or ileoileal bypass surgeries are no longer preferred[4,5]. Biliopancreatic diversion that involves a small bowel bypass procedure to form a short common channel from the ileocecal valve can induce metabolic derangement and is associated with a high incidence of postoperative hepatic steatosis[3,32]. This is caused by a combination of malnutrition and malabsorption of vitamins, iron, ferritin, and calcium. Pancreaticoduodenectomy involving a Roux-en-Y jejunojejunal bypass appears

Table 4 Perioperative clinical variables related to pancreatic secretions

	Group A (n = 15)	Group B (n = 178)	P
Serum amylase[1], mean (range)	44.5 (9.0-119.0)	51.4 (6.0-266.0)	0.558
Serum lipase[1], mean (range)	31.9 (6.0-266.0)	30.0 (1.0-215.0)	0.793
Pancreatic duct size, mean (range) (mm)	4.5 (2-18)	3.5 (1-11)	0.401
Pancreatic fistula[2], n	7	34	0.020
Placement of pancreatic duct stent, n	13	131	0.363
Internal, n	4	95	0.005
External, n	9	36	

[1]Postoperative day 7; [2]Diagnosed according to International study group pancreatic fistula criteria[23].

Table 5 Multivariate analysis of risk factors for the development of steatohepatitis after pancreaticoduodenectomy[1]

Variables	HR	95% confidence interval	P
Serum amylase[2]	0.990	0.973-1.007	0.262
Serum lipase[2]	1.014	0.986-1.041	0.332
Pancreatic duct size[3]	0.882	0.635-1.224	0.452
Pancreatic fistula[4] (-)	1.000	-	-
Pancreatic fistula[4] (+)	3.332	1.075-10.321	0.037
Internal pancreatic duct stenting	1.000	-	-
External pancreatic duct stenting	4.530	1.312-15.643	0.017

[1]Analyzed by logistic regression; [2]Postoperative day 7; [3]Dichotomized for categorical variables using a median split; [4]Diagnosed according to International study group pancreatic fistula criteria[23]. HR: Hazard ratio.

to be associated with the same side-effects as small bowel bypass surgery. We also found that all patients with postoperative steatohepatitis had malignant pathological findings, but the pathogenesis remains uncertain.

Diagnostic methods to identify fatty liver disease

Histological evaluation through liver biopsy remains the gold-standard method for distinguishing NASH from simple fatty liver disease and for estimating intrahepatic fat content, the extent of necroinflammatory lesions and fibrosis. However, liver biopsy is associated with sampling errors and the risk of bleeding, infection, and biliary leakage[1,6,8,20,33]. Kleiner *et al*[15] proposed a semi-quantitative scoring system (the NAFLD activity score) to assess the histological features of NAFLD and to discriminate between NASH and non-NASH fatty liver disease. Five features-steatosis, hepatocellular ballooning, lobular inflammation, fibrosis, and the absence of lipogranulomas-were independently associated with the accurate diagnosis of NASH using adult liver biopsies.

Ultrasonography, non-enhanced CT, magnetic resonance imaging, and proton magnetic resonance spectroscopy (^{1}H MRS) are radiological, non-invasive methods to diagnose hepatic steatosis[1,4,16], but these methods cannot accurately distinguish NASH from simple fatty liver disease or objectively quantify fat content[1,6]. Recently, fatty infiltration of the liver was detected using chemical-shift imaging and a selective fat-suppression technique, acquired by the percentage of relative signal intensity loss on magnetic resonance T1- or T2-weighted images, the ratio of peak lipid to water by ^{1}H MRS, dual-energy multi-slice spiral CT, and non-enhanced CT measuring tissue density as a radiographic attenuation that can be objectively measured in Housefield units[16,34]. In a study by Nomura *et al*[22], non-enhanced CT was found to be useful for diagnosing steatohepatitis with established accuracy and for evaluating $CT_{L/S}$ and CT_{S-L}. Other studies identified a correlation between $CT_{L/S}$ and CT_{S-L} and histological findings of steatohepatitis, and some reports defined steatohepatitis as $CT_{L/S} < 0.9$ HU or $CT_{S-L} \geqslant 10$ HU[16,35]. Unlike the study by Nomura *et al*[22], which identified a relationship between postoperative AST and CT_{S-L}, our study found that CT_{S-L} correlated significantly with postoperative ALT levels, not AST levels. Kato *et al*[36] proposed a NAFLD scoring system that was based on the development of pancreatic adenocarcinoma, the pancreatic resection line, and postoperative diarrhea. This group diagnosed NASH by percutaneous liver biopsy after pancreaticoduodenectomy and revealed a significant correlation between their scoring system and CT findings.

Limitations

The present study has several limitations that should be considered when interpreting the results. First, the study was retrospective, and the period between the operation and the postoperative follow-up CT was not uniform and averaged 3.2 mo, which is short. Nevertheless, if we consider the prevalent period for the development of steatohepatitis postoperatively, the results could present useful information.

Second, there were only 15 patients (7.8%) who developed steatohepatitis after surgery in our study, whereas in the study by Nomura *et al*[22] 33% of asymptomatic patients without severe obesity had decreased hepatic attenuation meeting the criteria for steatohepatitis after pancreaticoduodenectomy. The reason for the difference in the incidence between our study and that of Nomura *et al* is not clear, but it might reflect differences in the rates of obesity and the timing between the operation and the follow-up CT scan. Our study had a very small number of obese patients (24 patients with a BMI $\geqslant$ 25 kg/m^2 and 2 patients with a BMI $\geqslant$ 30 kg/m^2).

Third, we used a radiological method to diagnose steatohepatitis or NAFLD without histopathological evidence. Non-enhanced CT was reported to have a sensitivity of 73%-100% and a specificity of 95%-100% for the detection of moderate or severe steatohepatitis, al-

though hepatic iron overload might alter these rates[1,37].

Finally, the location and the extent of the pancreatic resection and postoperative patient-related factors such as steroid use, weight loss, and exercise were not included in our statistical analyses. In addition, the effect of adjuvant chemoradiotherapy, which was administered to 7 patients (46.7%) in Group A and 80 patients (44.9%) in Group B, was not included in our analysis.

Future prospective controlled studies with a larger sample size based on histopathological findings are needed to verify the relationships identified in the present study. Postoperative steatohepatitis might not be a significant problem, especially in late-stage malignant patients. Nevertheless, this preliminary report provides evidence for operation-related causes of steatohepatitis following pancreaticoduodenectomy, ruling out other factors causing hepatic fatty change or injury.

COMMENTS

Background

Only a limited number of reports have examined operation-related causes of postoperative steatohepatitis following pancreaticoduodenectomy.

Research Frontiers

To identify the risk factors for steatohepatitis after pancreaticoduodenectomy, with a particular focus on factors related to pancreatic secretions.

Innovations and breakthroughs

This preliminary report helps to identify operation-related causes of steatohepatitis following pancreaticoduodenectomy, and it is the first study to identify potential risk factors related to pancreatic secretions.

Applications

In this study, pancreatic fistula and external pancreatic duct stenting significantly influenced the development of steatohepatitis following pancreaticoduodenectomy. These findings have clinical implications and could be used to design future clinical trials.

Peer review

This is very interesting clinical research about the mechanism of post-operative steatohepatitis development following pancreaticoduodenectomy.

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BRIEF ARTICLE

Characteristics and outcomes of acute upper gastrointestinal bleeding after therapeutic endoscopy in the elderly

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Abstract

AIM: To characterize the effects of age on clinical presentations and endoscopic diagnoses and to determine outcomes after endoscopic therapy among patients aged ⩾ 65 years admitted for acute upper gastrointestinal bleeding (UGIB) compared with those aged < 65 years.

METHODS: Medical records and an endoscopy database of 526 consecutive patients with overt UGIB admitted during 2007-2009 were reviewed. The initial presentations and clinical course within 30 d after endoscopy were obtained.

RESULTS: A total of 235 patients aged ⩾ 65 years constituted the elderly population (mean age of 74.2 ± 6.7 years, 63% male). Compared to young patients, the elderly patients were more likely to present with melena (53% *vs* 30%, respectively; $P < 0.001$), have comorbidities (69% *vs* 54%, respectively; $P < 0.001$), and receive antiplatelet agents (39% *vs* 10%, respectively; $P < 0.001$). Interestingly, hemodynamic instability was observed less in this group (49% *vs* 68%, respectively; $P < 0.001$). Peptic ulcer was the leading cause of UGIB in the elderly patients, followed by varices and gastropathy. The elderly and young patients had a similar clinical course with regard to the utilization of endoscopic therapy, requirement for transfusion, duration of hospital stay, need for surgery [relative risk (RR), 0.31; 95% confidence interval (CI), 0.03-2.75; $P = 0.26$], rebleeding (RR, 1.44; 95% CI, 0.92-2.25; $P = 0.11$), and mortality (RR, 1.10; 95% CI, 0.57-2.11; $P = 0.77$). In Cox's regression analysis, hemodynamic instability at presentation, background of liver cirrhosis or disseminated malignancy, transfusion requirement, and development of rebleeding were significantly associated with 30-d mortality.

CONCLUSION: Despite multiple comorbidities and the concomitant use of antiplatelets in the elderly patients, advanced age does not appear to influence adverse outcomes of acute UGIB after therapeutic endoscopy.

Key words: Adverse outcomes; Elderly; Therapeutic end-oscopy; Upper gastrointestinal bleeding

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INTRODUCTION

The 2003 World Health Report highlighted the accelerated aging of the global population, as the number of elderly people will double in the next few decades[1]. Upper gastrointestinal bleeding (UGIB) affects a substantial number of elderly people and is a potentially life-threatening clinical event. Age has been considered as a significant prognostic factor for adverse outcomes, including rebleeding and mortality, from acute UGIB in numerous clinical risk models[2-6]. However, it is unclear if the role of age in UGIB is due to a more severe disease or differences in the treatment received. Generally, the elderly have often been treated less aggressively than younger patients because of an assumption of increased risk of any therapeutic procedures, secondary to comorbid conditions. However, recent studies of gastrointestinal endoscopy conducted in elderly patients reported an overall procedural success and morbidity similar to that reported for the general population, even in patients undergoing upper endoscopy for the evaluation of acute gastrointestinal bleeding[7,8].

Over the last decades, clinical considerations related to the diagnosis and treatment of UGIB has changed dramatically. Paramount to these changes have been the increased involvement of acute care specialists during resuscitation, advances in diagnostic and therapeutic endoscopy, the use of powerful acid suppressive and vasoactive agents, and more selective and less invasive surgical approaches that may offer a promising outcome for patients. Hence, outcome studies on the appropriate approach to UGIB in the elderly are needed. We therefore conducted the current study to characterize the effects of age on clinical presentations and endoscopic diagnoses and to determine outcomes after pharmacologic and endoscopic therapy with regard to the transfusion requirement, duration of hospital stay, need for surgical intervention, rate of rebleeding, and 30-d mortality among a large cohort of patients aged $\geq$ 65 years who were hospitalized for acute UGIB compared with those aged < 65 years.

MATERIALS AND METHODS

Patient population

This retrospective study was approved by the Institutional Review Board of the Hospital and was conducted at Siriraj Hospital, a tertiary academic medical center for the Bangkok metropolitan area and surrounding communities. All consecutive adult patients who were hospitalized for acute UGIB (e.g., hematochezia, melena, coffee-ground vomiting or hematemesis with or without hypotension) and who underwent endoscopy between January 2007 and December 2009 were included in the study. For the purposes of this study, we defined "elderly" as those older than 65 years of age.

Management of Acute UGIB

At our institution, patients who presented with acute UGIB were given appropriate initial resuscitation followed by diagnostic and therapeutic measures. Empiric therapy using either an intravenous proton pump inhibitor (PPI) or somatostatin analogue infusion was given before the endoscopy for suspicion of peptic ulcer and varices, respectively. Urgent endoscopy within the first 12 h after admission was performed in patients with signs of ongoing bleeding as determined by gastroenterologists. Endoscopic treatment was given in the form of injection therapy with epinephrine, coaptive thermocoagulation, hemostatic clip or combination therapy in patients with active bleeding, nonbleeding visible vessels or adherent clots. High-dose PPI was administered by infusion for 72 h after endoscopy in patients who required endoscopic intervention. Bleeding esophageal and gastric varices were treated with band ligation and cyanoacrylate injection, respectively, in addition to the use of vasoactive drugs and intravenous antibiotics. After the procedure, the patients were subsequently transferred to a medical ward for monitoring. Endoscopy was repeated in the event of rebleeding. Patients underwent surgery if bleeding persisted or if rebleeding occurred after two therapeutic endoscopies.

Clinical, Endoscopic, and Laboratory Data

Medical records and an endoscopy database of all patients were reviewed. Patient demographics, clinical presentations, initial vital signs, the presence of comorbid conditions, drugs taken at the time of admission and initial laboratory tests were obtained. We abstracted data describing the endoscopic management, including endoscopic diagnosis and the presence of stigmata of recent bleeding, endoscopic hemostasis, and medication use following endoscopy. Outcome data describing the overall course within 30 d after the initial endoscopic treatment with specific attention to rebleeding, the need for surgery, a requirement for blood transfusion, the length of hospital stay, and mortality were gathered.

The presence of hemodynamic instability was defined as systolic blood pressure < 100 mmHg, a heart rate > 100 beats/min and/or orthostatic changes in systolic blood pressure (a decrease of > 10%) or heart rate (an increase of > 10%) between a supine and seated position. Rebleeding was defined by the presence of hematemesis or melena with signs of hemodynamic instability or a decrease in hemoglobin level > 2 g/dL in a previously stable patient. Endoscopic grading of ulcer lesions was categorized according to the Forrest's classification[9]. The stigmata of recent bleeding included arterial spurting or pulsatile bleeding from the ulcer base, a non-bleeding visible vessel, and an adherent clot covering the base of an ulcer. Grading of varices was carried out using the classification of the Italian Liver Cirrhosis Project[10].

Statistical analysis

Data were summarized using descriptive statistics. Continuous variables were compared using the *t* test or the

Table 1 Characteristics of patients for the entire group and for each cohort

	Total (n = 526)	Patients aged ≥ 65 yr (n = 235)	Patients aged < 65 yr (n = 291)	P value
Age (yr)	60 ± 15.9	74.2 ± 6.7	48.4 ± 11.1	< 0.001
Male: n (%)	370 (70)	148 (63)	222 (76)	< 0.001
Presenting symptoms: [1]n (%)				
Hematemesis	207 (39)	65 (28)	142 (49)	< 0.001
Melena	214 (41)	126 (53)	88 (30)	< 0.001
"Coffee ground" vomiting	86 (16)	36 (15)	50 (17)	0.57
Hematochezia	19 (4)	8 (4)	11 (4)	0.82
Clinical findings: n (%)				
Red blood on nasogastric lavage	97 (18)	41 (17)	56 (19)	0.60
Systolic blood pressure < 100 mmHg	163 (31)	62 (26)	101 (35)	0.04
Heart rate > 100 beats/min	192 (37)	63 (27)	129 (44)	< 0.001
Presence of hemodynamic instability	313 (60)	114 (49)	199 (68)	< 0.001
Comorbid illness: [1]n (%)				
Cardiovascular disease	109 (21)	81 (34)	28 (10)	< 0.001
Cerebrovascular disease	49 (9)	34 (14)	15 (5)	< 0.001
Chronic renal failure	37 (7)	17 (7)	20 (7)	0.87
Liver cirrhosis	140 (27)	45 (19)	95 (33)	< 0.001
Cancer	64 (12)	33 (14)	31 (11)	0.24
Diabetes mellitus	126 (24)	87 (37)	39 (1)	< 0.001
Hypertension	186 (35)	125 (53)	61 (21)	< 0.001
Alcohol drinking: n (%)	246 (47)	66 (28)	180 (62)	< 0.001
Previous use of medications: [1]n (%)				
Low-dose aspirin	111 (21)	82 (35)	29 (10)	< 0.001
Clopidogrel	33 (6)	30 (13)	3 (1)	< 0.001
Warfarin	38 (7)	22 (9)	16 (6)	0.09
NSAID other than aspirin	123 (23)	57 (24)	66 (23)	0.67
Laboratory features at presentation:				
Hemoglobin (g/dL)	8.7 ± 4.7	8.4 ± 2.3	9.0 ± 6.0	0.19
White blood count (10^3/μL)	11.8 ± 8.1	11.2 ± 5.3	12.3 ± 9.7	0.15
Platelets (10^3/μL)	232 ± 125	242 ± 116	223 ± 131	0.09
Prothrombin time (s)	13.4 (11.4-248)	13.2 (11.4-160)	13.9 (11.4-248)	0.84
Creatinine (mg/dL)	1.1 (0.2-11.8)	1.3 (0.4-10.8)	0.9 (0.2-11.8)	0.44

Categorical variables are presented as number and percentage, and continuous variables are presented as the mean ± SD or median and range when appropriate. [1]Some patients presented with more than 1 symptom or comorbid illness and used more than 1 drug. NSAID: Non-steroidal anti-inflammatory drug.

Mann-Whitney test. Categorical variables were compared using the χ^2 or Fisher exact test. The Kaplan-Meier method with the log-rank test was used to compare differences in the rates of rebleeding and death within 30 d after primary endoscopic treatment. Cox's regression analysis was used to detect possible prognostic variables on recurrent bleeding and survival. All statistical testing was performed at the conventional 2-tailed α level of 0.05.

RESULTS

Patient population

During a three-year period, a total of 526 patients (370 men, 156 women) with acute UGIB were identified. The age distribution at presentation is shown in Figure 1. Acute UGIB occurred in patients aged 18-40 years (13%), 41-50 years (15%), 51-60 years (20%), 61-70 years (24%), 71-80 years (19%) and > 80 years (9%). Two hundred thirty-five patients were at least 65 years of age and constituted the elderly population, with a mean age of 74.2 ± 6.7 years. Two hundred ninety-one patients were < 65 years old and constituted the young population, with a mean age of 48.4 ± 11.1 years. The patient demographics and clinical characteristics of the entire group and each of the cohorts are shown in Table 1.

Clinical characteristics

Patients ≥ 65 years of age were more likely to present with melena, receive antiplatelet agents, and have comorbid conditions including cardiovascular disease, cerebrovascular disease, cirrhosis, diabetes, and hypertension compared with the young population (Table 1). The rates of antiplatelet use were increased with older age (P < 0.001). In contrast, patients < 65 years old presented with hematemesis (49% *vs* 28%, respectively; P < 0.001) and hemodynamic instability (68% *vs* 49%, respectively; P < 0.001) more commonly than the elderly. There were no differences in terms of 'coffee ground' vomiting, hematochezia, red blood on the initial nasogastric lavage, the use of anticoagulants and non-steroidal anti-inflammatory drugs (NSAIDs), and laboratory indices at presentation between the two groups (Table 1).

Endoscopic findings

The endoscopic findings for the entire patient group and each of the cohorts are shown in Table 2. Distributions of peptic ulcer and varices as the source of bleeding among each age range at presentation are summarized in Figure 1. Bleeding peptic ulcers were identified more

Table 2 Endoscopic findings for the entire group and for each cohort

	Total (n = 526)	Patients aged ⩾ 65 yr (n = 235)	Patients aged < 65 yr (n = 291)	P value
Peptic ulcer as source of bleeding: [1]n (%)				
Active bleeding	19 (4)	11 (5)	8 (3)	0.20
Non-bleeding visible vessel	59 (11)	25 (11)	34 (12)	0.71
Clot with underlying vessel	16 (3)	8 (3)	8 (3)	0.66
Flat, pigmented spot	41 (8)	23 (10)	18 (6)	0.13
Clean base	224 (46)	112 (48)	112 (38)	0.03
Portal hypertensive related-lesions as source of bleeding: [1]n (%)				
Esophageal varices	137 (26)	41 (17)	96 (33)	< 0.001
Gastric and duodenal varices	17 (3)	4 (2)	14 (5)	0.051
Portal hypertensive gastropathy	58 (11)	17 (7)	41 (14)	0.01
Other endoscopic findings: [1]n (%)				
Esophageal ulcer	19 (4)	9 (4)	10 (3)	0.81
Esophagitis	36 (7)	17 (7)	19 (7)	0.75
Gastropathy, duodenitis, or erosions	129 (25)	61 (26)	68 (23)	0.49
Mallory-Weiss tear	26 (5)	5 (2)	21 (7)	0.007
Gastric cancer	15 (3)	6 (3)	9 (3)	0.71
Dieulafoy's lesion	11 (2)	6 (3)	5 (2)	0.51
Angiodysplasia	2 (0.004)	1 (0.4)	1 (0.3)	0.88
No clinically significant finding	11 (2)	3 (1)	8 (3)	0.24

[1]Some patients presented with more than 1 endoscopic finding.

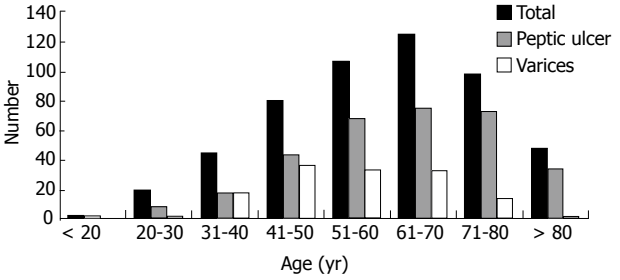

Figure 1 Age distribution of patients with acute upper gastrointestinal bleeding.

frequently in patients aged 60-80 years, suggesting that peptic ulcers are a more common source of bleeding in the elderly than in patients aged < 65 years (68% *vs* 56%, respectively; P = 0.006). Among those with peptic ulcer bleeding, clean base ulcers were seen more frequently in the elderly compared with young patients (48% *vs* 38%, respectively; P = 0.03). The numbers of active bleeding, non-bleeding visible vessels, clots, and flat pigmented spots did not differ significantly between the two groups (Table 2). Of these, 45 (28%) elderly patients required therapeutic endoscopy compared with 55 (34%) young patients (relative risk for the elderly, 0.84; 95% confidence interval (CI), 0.60 to 1.16; P = 0.29) (Table 3).

Variceal bleeding tended to decrease after the fourth decade of life. As a result of higher alcohol consumption, esophageal varices (33% *vs* 17%, respectively; P < 0.001) and Mallory-Weiss tears (7% *vs* 2%, respectively; P = 0.007) were noted more frequently in patients < 65 than in those > 65 years of age. Furthermore, young patients had a trend toward higher numbers of bleeding from gastric and duodenal varices compared to the elderly population (Table 2). Of those with variceal bleeding, 27 (66%) elderly patients received therapeutic endoscopy compared with 75 (75%) young patients (relative risk for the elderly, 0.89; 95% CI, 0.69 to 1.14; P = 0.34) (Table 3).

Treatment outcomes

Urgent endoscopy was performed in 47 patients aged ⩾ 65 years and in 64 patients aged < 65 years. Hemodynamic instability at presentation was less frequent in the elderly patients compared to the young patients (49% *vs* 68%, respectively; P < 0.001). One of the elderly patients and four of the young patients underwent emergency surgery because of failure to achieve hemostasis during endoscopy. The mean number of units of packed erythrocytes transfused prior to endoscopy and during hospitalization was similar in both groups. In addition, the length of hospital stay was not significantly different between the two groups.

Rebleeding occurred within 30 d after the initial endoscopic therapy in 36 patients (15%) aged ⩾ 65 years and in 31 patients (11%) aged < 65 years (P = 0.1) (Figure 2A). When we analyzed the rebleeding rate according to the source of bleeding, the probability of rebleeding within 30 d after endoscopic therapy among patients with peptic ulcer bleeding was similar in both groups (P = 0.35) (Figure 2B), but the rebleeding rate among patients who had ulcers with stigmata of recent bleeding was higher in the elderly patients (P = 0.02) (Figure 2C). The rate of recurrent variceal bleeding was also higher in the elderly patients than in the young patients, although the difference was not statistically significant (P = 0.08) (Figure 2D).

Sixteen patients (7%) aged ⩾ 65 years and 18 patients (6%) aged < 65 years died within 30 d after the initial endoscopic treatment. The observed survival was virtually identical for both groups (P = 0.8) (Figure 3A). However, all deaths tended to occur in a greater proportion of elderly patients who had ulcers with stigmata of recent bleeding (P = 0.3) (Figure 3C) and varices (P = 0.1)

Table 3 Clinical outcomes after pharmacologic and endoscopic therapy

	Patients aged ⩾ 65 yr (*n* = 235)	Patients aged < 65 yr (*n* = 291)	*P* value	Relative risk (95% CI)
Urgent Endoscopy: *n* (%)	47 (20)	64 (22)	0.58	0.91 (0.65-1.27)
For peptic ulcers bleeding	22 (14)	29 (18)	0.33	0.78 (0.47-1.29)
For variceal bleeding	16 (46)	30 (37)	0.36	1.25 (0.79-1.98)
Endoscopic therapy for bleeding peptic ulcers: [1]*n* (%)	45 (28)	55 (34)	0.29	0.84 (0.60-1.16)
Epinephrine injection	36 (80)	50 (91)	0.12	
Coaptive thermocoagulation	28 (62)	39 (71)	0.36	
Hemostatic clip	11 (24)	14 (25)	0.91	
Combined therapy	29 (64)	42 (76)	0.19	
Endoscopic therapy for variceal bleeding: [1]*n* (%)	27 (66)	72 (75)	0.34	0.89 (0.69-1.14)
Esophageal band ligation	25 (93)	64 (89)	0.59	
Cyanoacrylate injection	3 (11)	10 (14)	0.72	
The 72-h infusion of PPI after endoscopic therapy for bleeding peptic ulcers: *n* (%)	47 (29)	49 (30)	0.84	0.97 (0.69-1.35)
The 3-5 d infusion of vasoactive agent after endoscopic therapy for variceal bleeding: *n* (%)	27 (66)	73 (76)	0.22	0.87 (0.68-1.11)
Units of blood transfused:				
Before endoscopy	1.8 ± 1.4	1.8 ± 1.6	0.88	
During hospitalization	2.7 ± 0.2	2.9 ± 0.2	0.33	
Hospital stay (d)	5 (1-14)	4 (1-13)	0.84	
Hospital stay < 3 d: *n* (%)	85 (36)	106 (36)	0.95	0.99 (0.79-1.25)
Recurrent bleeding: *n* (%)				
Within 3 d	11 (5)	11 (4)	0.61	1.24 (0.55-2.81)
Within 7 d	22 (9)	17 (6)	0.13	1.60 (0.87-2.95)
Within 30 d	36 (15)	31 (11)	0.11	1.44 (0.92-2.25)
Emergency surgery: *n* (%)	1 (0.4)	4 (1.4)	0.26	0.31 (0.03-2.75)
Death within 30 d: *n* (%)	16 (7)	18 (6)	0.77	1.10 (0.57-2.11)

Categorical variables are presented as number and percentage, and continuous variables are presented as the mean ± SD or median and range when appropriate. CI: Confidence interval. [1]Some patients were treated with more than 1 endoscopic modality.

(Figure 3D). The cause of death in the elderly patients was profound shock at presentation caused by a spurting hemorrhage from the ulcer that failed endoscopic therapy (two) and refractory variceal bleeding after endoscopic band ligation (one). The deaths in the remaining 13 patients were related to their comorbid illnesses: nosocomial pneumonia (five), septicemia (two), spontaneous bacterial peritonitis (one), myocardial infarction (one), congestive heart failure (one), liver failure (two), and primary liver cancer (one). The causes of death in the young patients were profound shock at presentation caused by active variceal bleeding (five) and ulcer bleeding (one), which failed endoscopic intervention. Twelve patients died of their comorbid illnesses: nosocomial pneumonia (four), septicemia (three), renal failure as a consequence of massive gastrointestinal bleeding (four), and metastatic biliary cancer (one).

Prognostic Factors for Rebleeding and Death

By univariate analysis, an increased risk of rebleeding after endoscopic hemostasis was associated with the presence of endoscopic stigmata of recent bleeding ($P < 0.001$) and high blood transfusion requirement before the endoscopy ($P = 0.003$). The risk of rebleeding was not associated with age ⩾ 65 years ($P = 0.11$) or even with age ⩾ 85 years ($P = 0.53$), male gender ($P = 0.75$), the presence of comorbid illness ($P = 0.23$), the use of antiplatelet agents ($P = 0.78$), the presence of hemodynamic instability at presentation ($P = 0.19$), hematemesis ($P = 0.27$), hematochezia ($P = 0.75$), peptic ulcer bleeding ($P = 0.33$), variceal bleeding ($P = 0.14$), and medium to large variceal size ($P = 0.57$). In the multivariable Cox regression model, the number of blood transfusions before endoscopy and stigmata of recent bleeding remained significantly associated with rebleeding (Table 4).

In the univariate analysis, the following variables had a significant influence on patient survival within 30 d: the presence of hemodynamic instability at presentation ($P < 0.001$), liver cirrhosis ($P < 0.001$), disseminated malignancy ($P < 0.001$), variceal bleeding ($P < 0.001$), the total number of blood transfusions ($P = 0.02$), and the occurrence of rebleeding ($P < 0.001$). The risk of death was not significantly associated with age ⩾ 65 years ($P = 0.80$) or even with age ⩾ 85 years ($P = 0.13$), male gender ($P = 0.74$), hematemesis ($P = 0.09$), hematochezia ($P = 0.51$), and the endoscopic stigmata of recent bleeding ($P = 0.89$). In the multivariate analysis, the presence of hemodynamic instability at presentation, a background of liver cirrhosis or disseminated malignancy, a transfusion requirement during admission, and the development of rebleeding remained significantly associated with 30-d mortality (Table 4).

DISCUSSION

The current study shows that age is associated with a steep rise in the incidence of acute UGIB. The elderly patients had different sources and clinical presentations

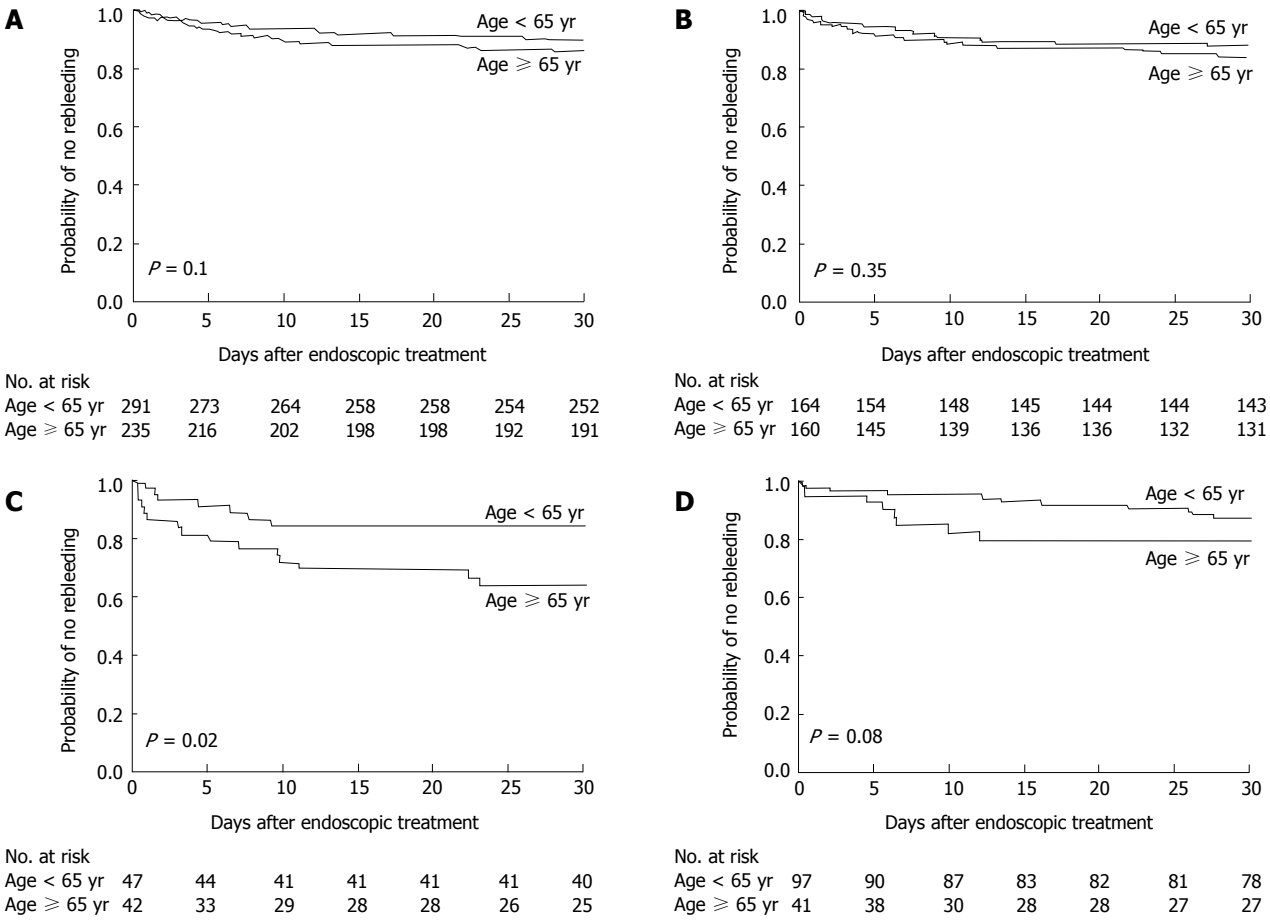

Figure 2 Kaplan-meier estimates of the likelihood that bleeding would not recur within 30 d after endoscopic treatment. A: Among all causes for bleeding; B: Among peptic ulcers as the source of bleeding; C: Among peptic ulcers with stigmata of recent bleeding; D: Among varices as the source of bleeding.

Table 4 Prognostic factors for rebleeding and mortality in acute upper gastrointestinal bleeding

Variable	Parameter estimate	Standard error	Hazard ratio (95% CI)	*P* value
Prediction for Recurrent bleeding				
Number of blood transfusions before endoscopy	0.22	0.07	1.25 (1.08-1.42)	0.003
Stigmata of recent bleeding	0.99	0.26	2.68 (1.58-4.41)	< 0.001
Prediction for Mortality				
Hemodynamic instability	0.95	0.50	2.57 (1.05-7.76)	0.04
Liver cirrhosis	1.33	0.62	3.77 (1.10-12.2)	0.03
Disseminated malignancy	1.8	0.65	6.06 (1.37-18.8)	0.02
Number of blood transfusions	0.11	0.03	1.12 (1.03-1.19)	0.004
Recurrent bleeding	1.62	0.39	5.07 (2.30-10.9)	< 0.001
Variceal bleeding	0.51	0.61	1.66 (0.55-5.79)	0.39

CI: Confidence interval.

of acute UGIB compared with the young patients. The risk for rebleeding correlated with endoscopic stigmata of recent bleeding and the severity of bleeding, as reflected by blood transfusion requirement, but not with advanced age. Furthermore, the elderly patients did not show a significant difference in clinical course from the young patients with regard to the utilization of endoscopic therapy, transfusion requirement, the duration of hospital stay, the need for surgery, the rate of rebleeding, and mortality.

Aging may result in various physiologic changes in the gastrointestinal tract[11], which may increase the risk for the development of acid-related disorders. Our study confirms that the incidence of acid-related bleeding increases with increasing age[12-14]. Consistent with previous studies, we found that approximately 80% of UGIB occurring in patients aged ⩾ 65 years is derived from acid-related disorders[12-15]. One of the major factors that

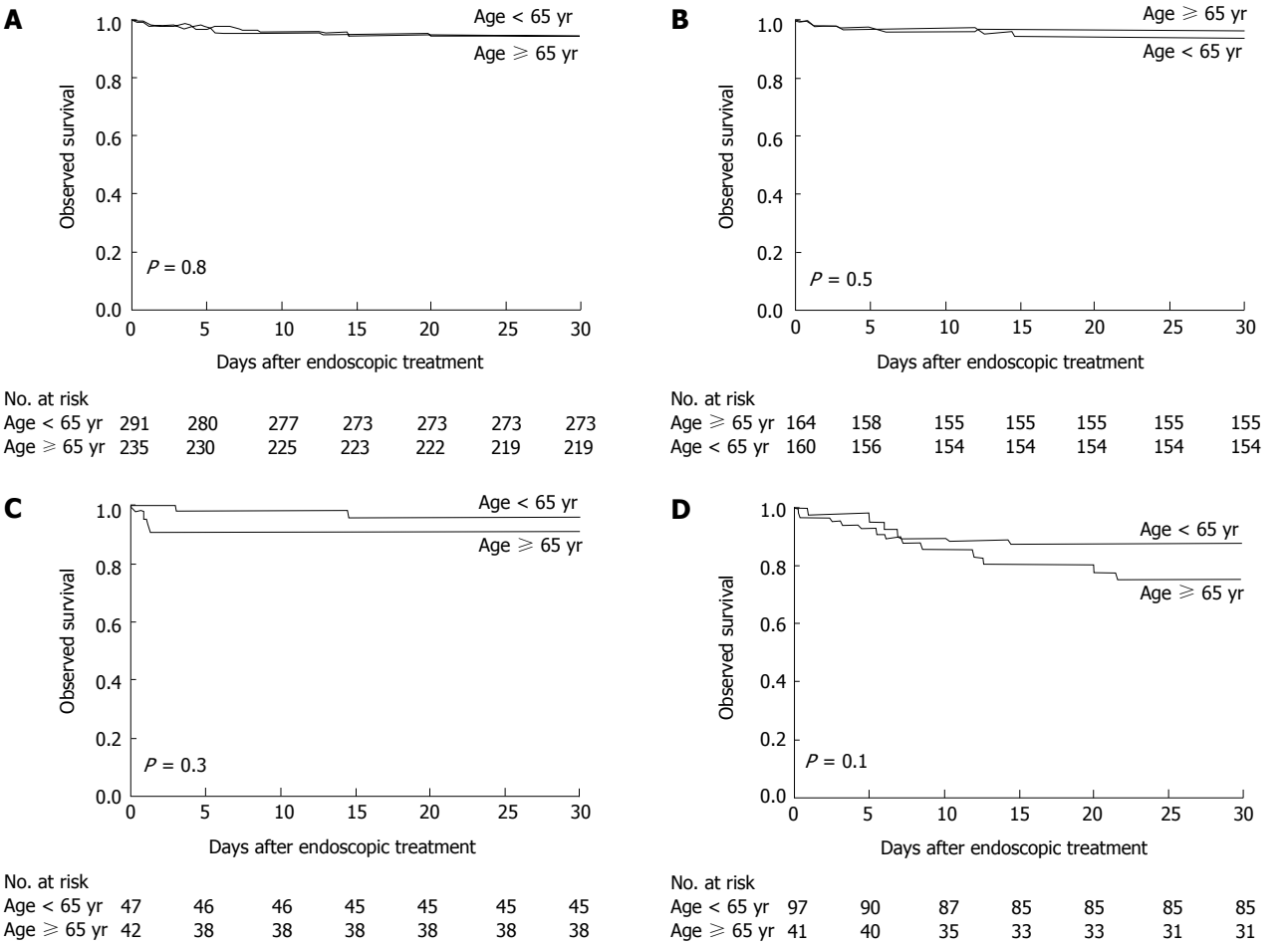

Figure 3 Kaplan-meier estimates of observed survival within 30 d after endoscopic treatment. A: Among all causes for bleeding; B: Among peptic ulcers as the source of bleeding; C: Among peptic ulcers with stigmata of recent bleeding; D: Among varices as the source of bleeding.

might explain this feature in the elderly population is the increased prescribing of gastroduodenal-damaging drugs, including aspirin, clopidogrel, and NSAIDs. As expected, 60% of our elderly population used either antiplatelet agents or a prescription NSAID, which was 2 times greater than that seen in the young population. Furthermore, some investigators have hypothesized that a mechanism underlying various manifestations of gastrointestinal bleeding in the elderly may involve ischemic damage to gastrointestinal mucosa[16,17]. In the current study, while direct measurements of visceral atherosclerosis were not available, we examined whether clinically recognized cardiovascular or cerebrovascular diseases were associated with the risk of ulcer-related bleeding. We found no association between clinical atherosclerotic disease and the risk of ulcer-related bleeding (data not shown).

Patients with acute UGIB typically present with vomiting of fresh blood or coffee ground-like material or the rectal passage of blood. Compared with young individuals, our elderly patients commonly presented with mild symptoms or subtle bleeding. One possible explanation could be that these patients were more likely to be hospitalized for non-life-threatening bleeding for close medical attention given their vulnerability and multiple comorbid diseases. Therefore, it is possible that patient comorbidity may result in the early recognition or management of UGIB rather than reflecting the etiology of the bleeding. Our experiences are in agreement with prior evidence that suggests an association between hospitalized gastrointestinal bleeding and poor health[16,18,19]. Moreover, we cannot exclude the possibility that the incidence of hemodynamic instability from UGIB was underestimated in this study because the use of beta blockers, which is common in the elderly who have multiple comorbid conditions, can mask tachycardia in patients with UGIB.

Patient risk stratification can be performed based on predictive factors for rebleeding, and resources can be allocated accordingly. There are numerous studies that have reported the predictive factors for rebleeding[2-6]. The current study confirms that endoscopic stigmata of recent bleeding is the most important predictor of rebleeding and influences other important end points such as transfusion requirement[20-23]. In contrast to prior studies, our data do not show a significant association between older age and the risk of rebleeding. It is possible that the current study included all patients with UGIB regardless of etiology in the analysis, and thus, the effect of stigmata of recent bleeding in elderly patients with peptic ulcers could have been diluted by the inclusion of a large number of patients with clean-based ulcers that

are at low risk of rebleeding. When we restricted our analysis according to the endoscopic findings, we found that age ⩾ 65 years was associated with an increase in the risk of ulcer rebleeding among those with high-risk ulcer stigmata. Although the reason for this observation was unclear, we hypothesize that it may be related to impaired hemostasis caused by platelet dysfunction because elderly patients are more likely to have received antiplatelet therapy or NSAIDs before admission. The late rebleeding seen in our elderly patients also suggests that there may be an unknown pathogenic process that adversely affects the healing of peptic ulcers (Figure 2C). In addition, there was a trend toward a higher rate of variceal rebleeding in elderly patients. A larger study size may be required to clarify the possible prognostic factor of older age for variceal rebleeding.

Despite advances in the management of UGIB during the past decade, the reported mortality for patients over 60 years of age with UGIB is 12%-25% and nearly 35% in those over 80 years of age[24,25]. The lack of change is probably explained by the associated comorbidities with increasing age. These patients are also more vulnerable to a physiological challenge from an acute bleeding episode. However, the mortality of our elderly cohort for acute UGIB was 7%, which was lower than those of previous reports. The leading cause of death in the elderly is sepsis followed by multiorgan failure. This study also reports a low rate of surgical intervention, which was seen in approximately 1% of all patients. The decrease in surgical requirement and mortality in our patients could reflect the increasing use of endoscopic hemostasis and likely underlines the systematic use of potent antisecretory agents for acid-related bleeding and vasoactive agents for variceal bleeding after therapeutic endoscopy. These therapeutic measures have been reported to improve the outcome of patients with UGIB[26,27].

Several bleeding scoring systems have been developed to predict the outcomes for patients with UGIB[2-6] and have shown that the risk for adverse outcomes increases when the patients are older. However, our study showed that age ⩾ 65 years did not influence the transfusion requirement, duration of hospital stay, need for surgery, and mortality. Furthermore, multivariate analysis showed that comorbid illnesses with liver cirrhosis or disseminated malignancy, severe bleeding represented by significant hemodynamic change requiring multiple blood transfusions and the development of rebleeding were significant predictive factors for mortality. These findings are consistent with the reported predictive models on mortality for UGIB in the literature[2-6].

Some factors may limit the generalizability of our findings. First, our patients awaiting endoscopy, who were suspected to have a high risk of ulcer or variceal rebleeding, received the preemptive use of high-dose intravenous PPI or vasoactive agents, respectively. It is possible that this management could influence endoscopic findings and the course of UGIB. Second, we cannot exclude referral bias, which may select patients with severe diseases. However, we would not expect the age-related differences in clinical presentation and the source of UGIB to be a large artifact of this bias. The similar outcome in young and elderly patients after endoscopic therapy indicates that a selection bias for severe disease does not have an adverse impact on treatment outcome.

In conclusion, the etiology of UGIB in the elderly has changed little in recent years. Despite multiple comorbidities and the concomitant use of antiplatelet therapy in elderly patients, advanced age does not appear to influence adverse outcomes of acute UGIB after therapeutic endoscopy. Morbidity and mortality from UGIB in the elderly are determined by the nature of the bleeding lesions and the presence of comorbid conditions. With the growth of older populations, a coordinated approach to diagnosis and management of acute UGIB should optimize favorable outcomes in this vulnerable patient population similar to those in younger people.

COMMENTS

Background

Upper gastrointestinal bleeding (UGIB) affects a substantial number of elderly individuals and is a potentially life-threatening clinical event. Advanced age has been considered a significant prognostic factor for adverse outcomes from acute UGIB; however, it remains unclear if this is due to the severity of disease or differences in the treatment received. A better understanding of the prognostic significance of age should enhance the accuracy during triage and could lead to the more efficient use of critical care resources for the management of acute UGIB.

Research frontiers

The aim of the research was to characterize the effects of age on clinical presentations and endoscopic diagnoses and to determine outcomes after pharmacologic and endoscopic therapy with regard to transfusion requirement, the duration of hospital stay, the need for surgical intervention, the rate of rebleeding, and 30-d mortality among a large cohort of patients aged ⩾ 65 years who were hospitalized for acute UGIB compared with those aged < 65 years.

Innovations and breakthroughs

In the current study, the authors demonstrated that the elderly patients had different sources and clinical presentations of acute UGIB compared with the young patients. The risk for rebleeding correlated significantly with endoscopic stigmata of recent bleeding and the severity of bleeding but not with advanced age. Furthermore, the clinical course did not significantly differ between the elderly and young patients with regard to the utilization of endoscopic therapy, transfusion requirement, the duration of hospital stay, the need for surgery, the rate of rebleeding, and mortality.

Applications

In conclusion, advanced age does not appear to influence adverse outcomes of acute UGIB after therapeutic endoscopy. The promising outcomes of acute UGIB in the elderly may be due in part to the increased involvement of acute care specialists during resuscitation, advances in diagnostic and therapeutic endoscopy, the use of powerful acid suppressive and vasoactive agents, and more selective and less invasive surgical approaches. Therefore, the authors recommend a coordinated approach to manage acute UGIB, which should serve to optimize favorable outcomes in this vulnerable patient population similar to those in young people.

Peer review

The authors did not discuss beta blockers usages in the patients. Beta blocker usages are common in the elderly who have multiple comorbid conditions. They select heart rate as one of a predictor of hemodynamic instability. Use of beta blockers can mask tachycardia in the patients with UGIB. This would underestimate the incidence of hemodynamic instability from UGIB in the study.

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BRIEF ARTICLE

Moxibustion activates mast cell degranulation at the ST25 in rats with colitis

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Abstract

AIM: To investigate the effects of moxibustion on the morphology and function of mast cells (MC) at Tianshu (ST25) in rats with trinitro-benzene-sulfonic acid (TNBS)-induced colitis.

METHODS: A total of 53 male Sprague-Dawley rats were randomly divided into a normal group and experimental group. In the experimental group, a rat model of TNBS-induced colitis was established, and the rats were then randomly divided into a model group, moxibustion group, moxibustion plus disodium cromoglycate (M + DC) group and moxibustion plus normal saline (M + NS) group. Rats in the moxibustion group received suspended moxibustion at bilateral ST25 for 10 min, once a day for 7 d. Rats in the M + DC and M + NS groups were pretreated with disodium cromoglycate and normal saline at bilateral ST25, respectively, and were then concurrently subjected to the same treatment as rats in the moxibustion group. The hematoxylin-eosin staining method was used to observe histology of the colon and the toluidine blue-improved method was used to observe mast cells at ST25 acupoint areas.

RESULTS: An improvement in colonic injury in the moxibustion group was observed and the degranulation ratio of MC at ST25 acupoint was markedly higher in the moxibustion group than in the model group (45.91 ± 11.41 *vs* 32.58 ± 8.28, $P < 0.05$). After inhibition of degranulation of MC at ST25 by disodium cromoglycate, no improvement in colon tissue injury was observed.

CONCLUSION: Moxibustion exerted its effect on healing impaired colonic mucosa in rats with TNBS-induced colitis by increasing the degranulation ratio of local MC, but had little effect on the morphology of MC at ST25 acupoint.

Key words: Disodium cromoglycate; Colitis; Mast cell; Moxibustion; ST25 acupoint

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INTRODUCTION

Acupuncture, one of the alternative or complementary therapies, is receiving increasing acceptance in Western medicine for the treatment of certain medical conditions[1-6]. Moxibustion, involving warm stimulation by moxa combustion at acupoint areas, is one of the acupuncture-moxibustion therapies for treating certain disorders in the clinic[7-10]. It was shown that the effective mechanism of acupuncture stimulation is closely related to the activation of mast cells (MC) at acupoint areas[11]. Acupoints have complex structures and are composed of nerve endings, plexuses, blood vessels, lymphatic vessels and connective tissues[11-14]. However, the correlation between the effect of moxibustion and the response of MC at acupoint areas is still unclear and merits further study.

MC are common effector cells and are widespread in connective tissues, especially in subcutaneous and submucosal loose connective tissues. MC act in several ways including changes in degranulation and the release of various bioactive mediators [5-hydroxytryptamine (5-HT), P substance (SP), heparin, and leukotriene][15-17]. It was reported that acupuncture stimulation markedly increased the density of local MC and activated MC degranulation at needle acupoints, which led to downstream effects in activating certain cellular pathways[11-14,18]. Pretreating the acupuncture point with disodium cromoglycate not only counteracted degranulation, it also reduced the effect of acupuncture[18].

Previous studies by our research team have indicated that moxibustion has a beneficial effect on inflammatory bowel disease (IBD)[8-10]. Moreover, we found that moxibustion at ST25 can heal impaired colonic mucosa in a rat model of colitis created by an immunological method associated with local stimulation. ST25 is the primary large intestinal meridian point of hand Yangming, which regulates the function of the large intestine, spleen, and stomach. ST25 is an efficacious point in the clinical treatment of patients with IBD.

In this study, we established a colitis rat model induced by trinitro-benzene-sulfonic acid (TNBS). The hematoxylin-eosin staining method was adopted for histological assessment of colonic mucosal injuries after moxibustion intervention and the toluidine blue-improved method was used to observe morphology and degranulation of MC at ST25 acupoint areas.

MATERIALS AND METHODS

Animals

Fifty-three male Sprague-Dawley rats (SPF class), weighing 100-140 g, were supplied by the Experimental Animal Center of Shanghai University of Traditional Chinese Medicine (TCM), and randomly divided into a normal group (n = 11) and an experimental group (n = 42). All rats were housed at a constant temperature and humidity with free access to food and water. All studies were performed in accordance with the proposals of the Committee for Research and Ethical Issues of the International Association and approved by the Committee on the Use of Human and Animal Subjects in Teaching and Research, Shanghai University of TCM.

Establishment of the colitis rat model

An experimental colitis rat model was established according to the TNBS-induced method reported by Morris[19]. After weighing and administering anesthesia (1% sodium pentobarbital; i.p., 45 mg/kg), the 42 experimental rats were injected with TNBS/ethanol (100 mg/kg TNBS + 50% ethanol 0.25 mL) into the anus *via* a rubber tube; the solution was retained in the gut cavity at a depth of 6 cm-8 cm. Rats in the normal control group were given an enema with 0.9% NaCl of the same volume as given to the experimental rats. The rats were subsequently held upside down before removing the enema apparatus, and were kept in this position for 1 min to prevent the solution from flowing out.

After colitis was induced, one rat from the normal group and two rats from the model group were dissected to remove colon tissue. The tissue was stained with hematoxylin-eosin to confirm the establishment of the experimental colitis model. The remaining rats in the experimental group were then randomly divided into four groups: a model group, moxibustion group, moxibustion plus pretreated disodium cromoglycate (M + DC) group and moxibustion plus pretreated normal saline (M + NS) group.

Treatment

Location of ST25 acupoints in the rats were based on an anatomic method referenced in the "Map of Animal Acupoints" from Shi Yan Zhen Jiu Xue written by Lin WZ. In the moxibustion group, moxibustion was administered at bilateral ST25 acupoints using a fine moxa stick with the smoldering end 2 cm away from the acupoints for 10 min once daily for 7 d in total. In the M + DC group, bilateral ST25 acupoints were injected with disodium cromoglycate (55 mg/kg 0.2 mL; 0.1 mL for each acupoint) before moxibustion. In the M + NS group, bilateral ST25 acupoints were injected with normal saline (0.2 mL; 0.1 mL for each acupoint) before moxibustion.

Observation of colonic mucosa by hematoxylin eosine method

Following sacrifice of the animal and laparotomy, the inflamed segment of colon approximately 8 cm from the anus was removed, washed with iced saline, fixed in 10% formalin, embedded in paraffin, sectioned, stained with hematoxylin and eosin, dehydrated in 95%, 90% and 70% ethanol, cleared in xylene, mounted in Permount or Histoclad, and observed under a microscope.

Observation of MC at ST25 acupoints by the toluidine blue-improved method

Sequential paraffin slices 4-μm thick were prepared after 48 h of fixation at 4 ℃ in fixing solution (10% formalin).

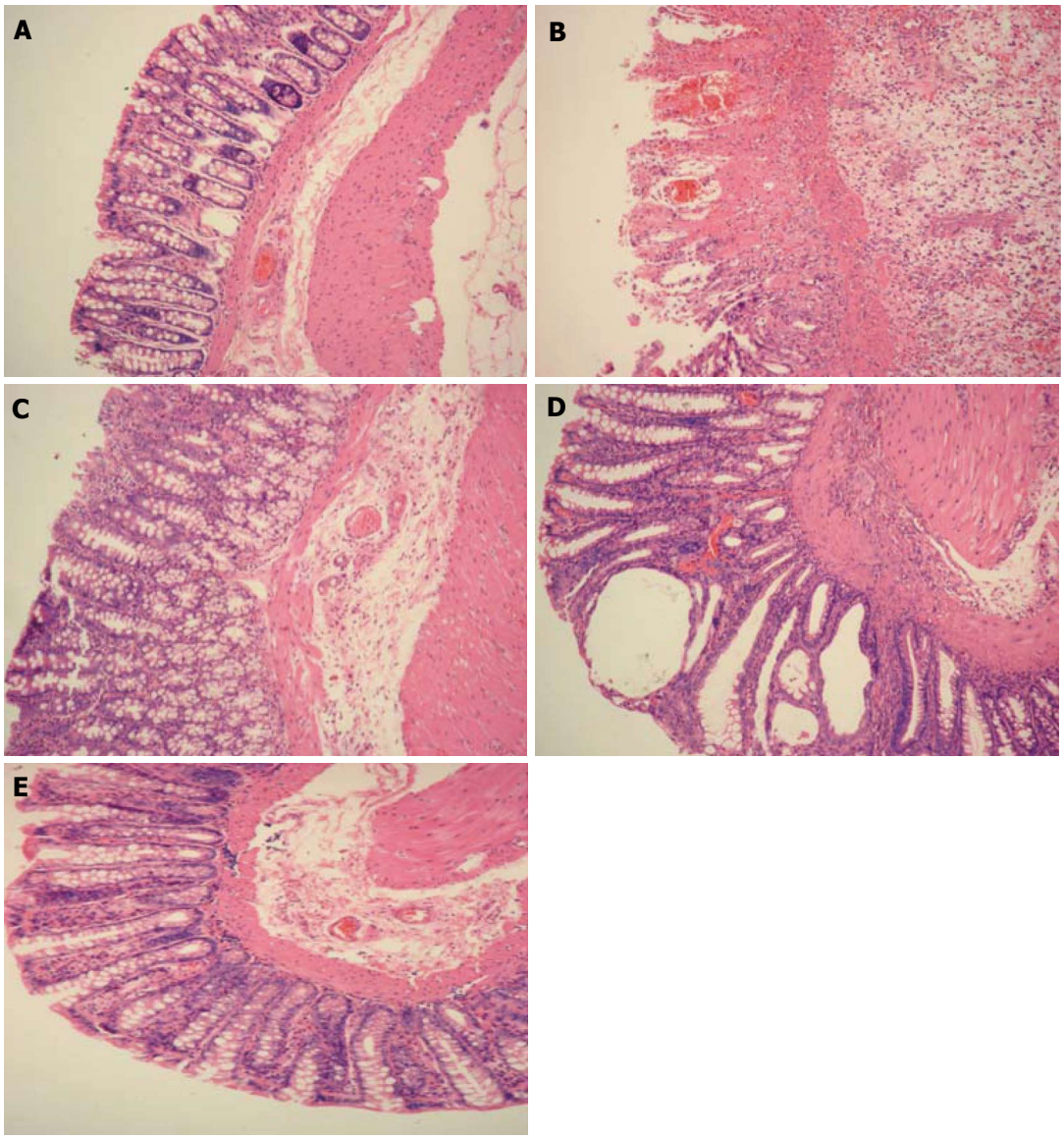

Figure 1 Results of hematoxylin eosine staining of rat colonic tissue (× 100). A: Normal group: the colonic mucosa was complete and the colonic gland was regularly arranged, with no apparent inflammatory cell infiltration; B: Model group: damage to colonic mucosa was found and there were monocytes and a large number of inflammatory cells infiltrating the mucosa or submucosa; C: Moxibustion group: the colonic gland was regularly arranged compared with the model group and ulceration was covered by regenerated epithelium; D: Moxibustion plus disodium cromoglycate group: slight congestion of colonic mucosa and fibroplasia of submucosa were found and a large number of infiltrating inflammatory cells, however, this was not as serious as in the model group; E: Moxibustion plus normal saline group: the colonic gland was regularly arranged and inflammatory cell infiltration of the submucosa was noted.

The subcutaneous tissue samples were stained with 0.5% toluidine blue. The numbers of MC per microscopic view (0.16 mm^2 at × 200 magnification) were counted at 4 areas per slice and then averaged. Mast cells with more than 3 granules outside the cell shape or with empty cavities in the cytoplasm were considered to be degranulated. The ratios of degranulated to total MC were calculated. Representative photomicrographs were obtained at a magnification of × 400 for morphological evaluation.

Statistical analysis

Experimental data were expressed as mean ± SD. Statistical analyses were performed using SPSS 13.0 (SPSS Inc., United States). Differences in mean were compared by one way ANOVA. $P < 0.05$ was considered statistically significant.

RESULTS

Improvement of colonic ulceration in rats treated with moxibustion

In the stained colon tissue slices, colonic glands and caliciform cells were observed by light microscopy (Figure 1). In the normal group, the colonic mucosa epithelium was complete and the colonic gland was regularly arranged, with no apparent inflammatory cell infiltration (Figure 1A). In the model group, damage to colonic mucosa was observed, mucosal villi were damaged or missing, there was congestion and edema in the submucosa, the gland was damaged or missing, caliciform cells were reduced, monocytes or mast cells were present, a large number of infiltrating inflammatory cells was present in the mucosa

Table 1 Average diameter and size of mast cells at ST25 acupoint areas (mean ± SD)

Group	*n*	MC average diameter	MC average size
Normal	10	14.0266 ± 2.1240	163.9111 ± 51.3831
Model	10	14.6944 ± 2.8082	182.1338 ± 79.6975
Moxibustion	10	12.8357 ± 3.5726	148.2694 ± 88.3540
M + DC	10	15.2929 ± 1.5578	194.111 ± 35.6967
M + NS	10	14.2835 ± 1.5379	162.4508 ± 28.2383

MC: Mast cells; M + DC: Moxibustion plus disodium cromoglycate; M + NS: Moxibustion plus normal saline.

Table 2 Degranulation ratio of mast cells at ST25 acupoint areas (mean ± SD)

Group	*n*	MC total number	MC degranulation number	Degranulation ratio (%)
Normal	10	7.16 ± 1.27	1.22 ± 0.29	17.01 ± 4.11
Model	10	10.38 ± 2.52	3.44 ± 1.27[a]	32.58 ± 8.28[a]
Moxibustion	10	13.22 ± 4.40[a]	5.78 ± 1.97[a,b]	45.91 ± 11.41[a,b]
M + DC	10	10.14 ± 4.26	3.36 ± 1.64[a,c]	33.41 ± 9.56[a,c]
M + NS	10	12.24 ± 4.34[a]	5.12 ± 2.26[a,b]	42.41 ± 7.71[a,b]

MC: Mast cells; M + DC: Moxibustion plus disodium cromoglycate; M + NS: Moxibustion plus normal saline.. [a]$P < 0.05$ *vs* normal group; [b]$P < 0.05$ *vs* model group; [c]$P < 0.05$ *vs* moxibution group.

or submucosa, and ulceration was noted (Figure 1B). In the moxibustion group, the colonic gland was regularly arranged, ulceration was covered by regenerated epithelium, submucosal edema was found, and a small number of infiltrating inflammatory cells was observed (Figure 1C). In the M + DC group, the colonic gland was distended and irregularly arranged, slight congestion of colonic mucosa and fibroplasia of the submucosa were found, and a large number of infiltrating inflammatory cells was noted, however, this was not as serious as in the model group (Figure 1D). In the M + NS group, the colonic gland was regularly arranged, there was slight congestion of the colonic mucosa, and edema and inflammatory cells had infiltrated the submucosa (Figure 1E).

Effects of moxibustion on the morphology and function of MC

There were no significant differences among the groups in the average diameter and size of MC at ST25 acupoint areas (Table 1). The number of degranulated MC and the ratio of MC in the normal group were lower than those in the other groups ($P < 0.05$); the degranulation ratio of MC in the moxibustion group was greater than those in the model group and the M + DC group ($P < 0.05$); no significant difference was found in the degranulation ratio of MC between the moxibustion group and the M + NS group (Table 2, Figure 2).

DISCUSSION

It has been suggested that MC could play a primary role in the effective mechanism of moxibustion at acupoint areas. An improvement in colonic injury in the moxibustion group was demonstrated and the degranulation ratio of MC at ST25 acupoint areas was remarkably higher in the moxibustion group than in the model group, which indicated a correlation between MC and moxibustion effects. Following inhibition of the degranulation of MC at ST25 acupoints by injection of the MC stabilizer, disodium cromoglycate, we found that not only did this treatment counteract degranulation of MC, but there was no improvement in colon tissue injury after moxibustion. In addition, it was found that moxibustion had no obvious influence on the size of MC at ST25 acupoints, which further suggested that the degranulation of MC at ST25 acupoints could participate in the mechanisms of moxibustion therapy.

The degranulation of MC at acupoint areas has been confirmed to participate in the analgesic activity of acupuncture[20-22]. These studies revealed that there are many more MC degranulated at acupoints following acupuncture intervention. The shapes of the degranulated MC were irregular with a vague boundary and the granules in the cytoplasm were scattered and small in size, and some granules released by MC spread over the entire tissue space. It has been shown that the analgesic effect of acupuncture could be significantly attenuated by repression of the degranulation of MC at acupoint areas using sodium cromoglycate[23]. Kimura found that the region of moxibustion treatment instantly received a large number of immunocyte infiltrations, which consisted of lymphocytes, monocytes, some granulocytes and MC[24]. Menjo showed that there was immediate degeneration of the epidermal cell layer and increased amounts of MC were observed after moxibustion treatment[25]. In this study, we also demonstrated a correlation between the activation of MC at moxibustion acupoint areas and the effects of moxibustion in TNBS-induced colitis rats.

MC, as resident cells in human loose connective tissue, are usually found gathered around small vessels and collaterals, and are particularly rich at nerve endings and nerve plexuses, forming a complex system of intercellular communications[26-30]. The cytoplasts of MC are filled with metachromatic basophilic granules in which various bioactive mediators are resident. Under acupuncture and moxibustion stimulation, large quantities of these bioactive mediators are released by activated MC to interact with surrounding tissues，producing the original so-called Qi sensation[20,22,30-32]. These bioactive substances (histamine, SP and 5-HT) in the granules penetrate into the tissue spaces, and on the one hand, transmit to other MC through the tissue fluid in a direction flowing along the meridian line, which induces further degranulation of MC[33]; on the other hand, these bioactive substances directly stimulate peripheral nerve receptors or nerve endings causing neuraxial reflection, releasing substance P, which can induce MC degranulation, stimulating the adjacent nerve endings further. Moxibustion signals are integrated and modification occurs in different stages from acupoint areas to the center and target organs, by which the moxibustion effect is achieved and target organs adjust.

It has also been reported that there was a significant-

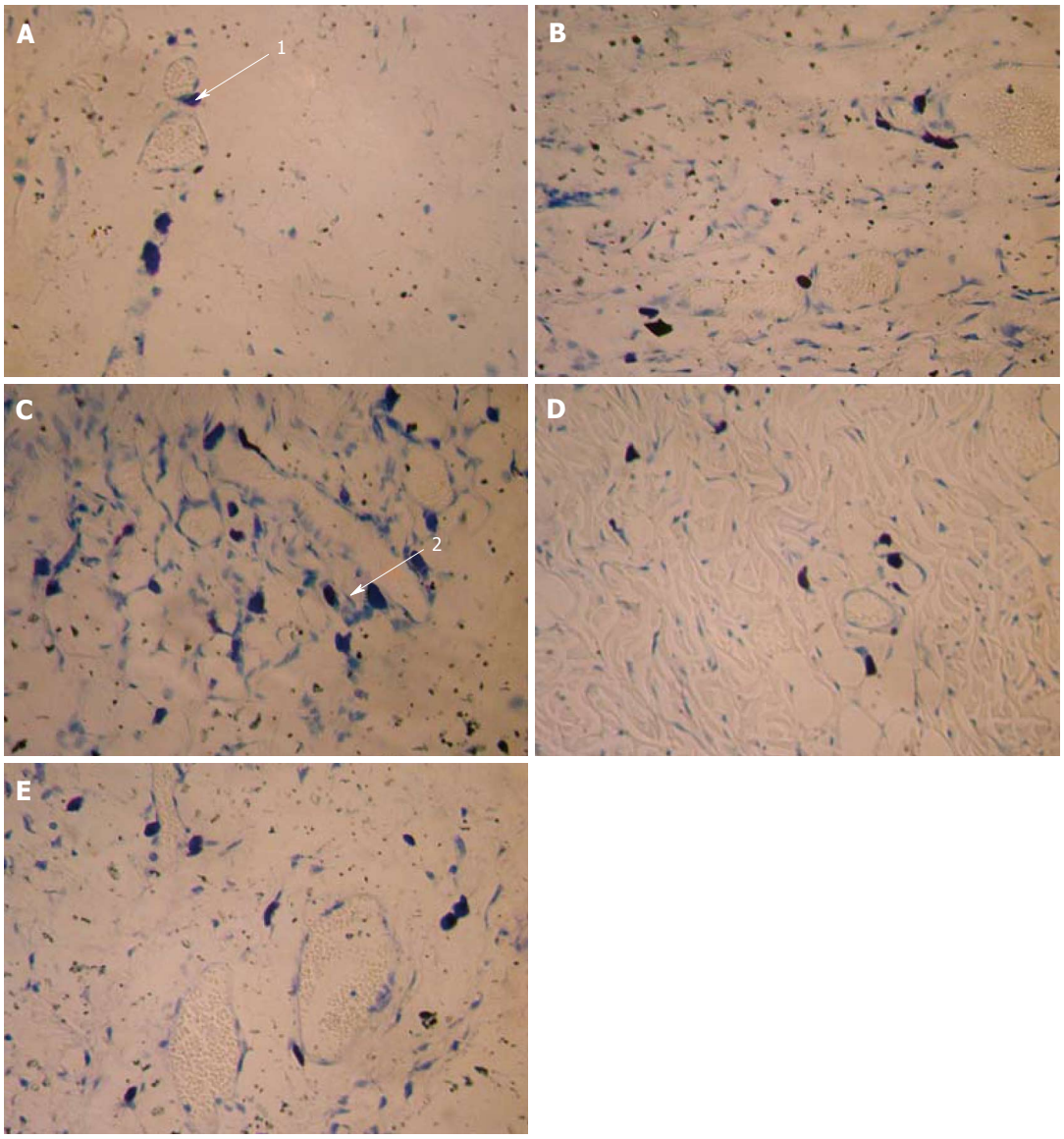

Figure 2 Results of the mast cells toluidine blue-improved method at ST25 (× 400). Normal group (A), model group (B), moxibustion group (C), moxibustion plus disodium cromoglycate group (D) and moxibustion plus normal saline group (E). Arrow 1: Intact mast cells (MC); Arrow 2: Degranulated MC. MC plasma stains purple, and nucleus is shown as dark blue, scattering in subcutaneous loose connective tissues, or gathering in a group or lining up; cell shape appears round, oval, shuttle-like, erose; small cells had little plasma and a clear shape, large cells had more plasma and an unclear shape.

ly different effect between acupuncture and moxibustion on the quantities of degranulated cells and the distribution of MC at acupoint areas. Furthermore, the effect of moxibustion is stronger than that of acupuncture, and this may be attributed to heat radiation and some chemical substances released from the burning of moxa, which possibly stimulates the MC at acupoint areas by the penetrating effect of moxibustion heat.

In conclusion, moxibustion stimulation may exert its effect on TNBS-induced colitis rats by triggering the degranulation of local MC at ST25 acupoints.

COMMENTS

Background

Previous studies on the effective mechanism of acupuncture stimulation show that it is closely related to the degranulation of mast cells (MC) at acupoint areas. The research has indicated that moxibustion stimulation has a beneficial effect on inflammatory bowel disease, and ST25 is an efficacious point in the clinical treatment of patients with inflammatory bowel disease. However, the correlation between the effect of moxibustion and the response of MC at acupoint areas is still unclear.

Research frontiers

With further study on the mechanism of moxibustion, more and more data show that MC at acupoints play an important role in bridging acupoint areas and target organs, which had become a the hot topic of study.

Innovations and breakthroughs

The results of the authors' study have proved that moxibustion is effective in TNBS-induced colitis rats. Moxibustion therapy exerts its effect on healing impaired colonic mucosa by triggering degranulation of local MC at ST25 acupoints.

Applications

The experimental data has important clinical significance and can be used in the further study of moxibustion therapy in the treatment of inflammatory bowel disease.

Peer review

This study investigated the relationship between the mast cell degranulation at the Tianshu (ST25) acupoint of moxibustion and the development of trinitrobenzene-sulfonic acid-induced colitis in rats. This is a study supported by The National Basic Research Program of China (973 program), etc, attempting to explore the mechanism of moxibustion. This kind of research would have important clinical significance and should certainly be encouraged. In general, this paper was well written.

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BRIEF ARTICLE

Sleeve gastrectomy prevents lipoprotein receptor-1 expression in aortas of obese rats

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Abstract

AIM: To investigate the effects of sleeve gastrectomy on adipose tissue infiltration and lectin-like oxidized low density lipoprotein receptor-1 (LOX-1) expression in rat aortas.

METHODS: Twenty-four rats were randomized into three groups: normal chow (control), high fat diet (HD) and high fat diet with sleeve gastrectomy (SG). After surgery, the HD and SG groups were fed a high fat diet. Animals were sacrificed and plasma high density lipoprotein (HDL) and low density lipoprotein (LDL) levels were determined. LOX-1 protein and LOX-1 mRNA expression was also measured. Aortas were stained with Nile red to visualize adipose tissue.

RESULT: Body weights were higher in the HD group compared to the other groups. HDL levels in control, HD, and SG groups were 32.9 ± 6.2 mg/dL, 43.4 ± 4.0 mg/dL and 37.5 ± 4.3 mg/dL, respectively. LDL levels in control, HD, and SG groups were 31.8 ± 4.5 mg/dL, 53.3 ± 5.1 mg/dL and 40.5 ± 3.7 mg/dL, respectively. LOX-1 protein and LOX-1 mRNA expression was greater in the HD group *versus* the other groups. Staining for adipose tissue in aortas was greater in the HD group in comparison to the other groups. Thus, a high fat diet elevates LOX-1 protein and mRNA expression in aorta.

CONCLUSION: Sleeve gastrectomy decreases plasma LDL levels, and downregulates LOX-1 protein and mRNA expression.

Key words: Sleeve gastrectomy; Morbid obesity; High fat diet; Aorta; Lipoprotein receptor-1 expression

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INTRODUCTION

Morbid obesity is a serious health problem worldwide. The incidence of diet-induced obesity in the United States has risen to 32%[1]. Approximately 127 million individuals are overweight, of which 60 million are obese and 8-10 million have morbid obesity with serious medical comorbidities, such as increased disability, morbidity

and early mortality[1-3].

Atherosclerosis is an important comorbidity of obesity that accounts for over 500 000 deaths annually in the United States. Diseases associated with atherosclerosis, such as myocardial infarction and stroke, account for the majority of deaths in industrialized countries. Atherosclerosis is a complex, multifactorial disease with both genetic and environmental determinants.

In clinical trials on atherosclerosis and hypertension, researchers found a direct association between the amount of weight loss and blood pressure reduction following a 36 mo weight loss intervention[4,5]. Prospective cohort studies have also found that the prevalence of atherosclerosis and hypertension decreases with weight loss[6-8]. Additionally, several researchers have found a positive relationship between weight gain and atherosclerosis[6,9-12].

Lectin-like oxidized low density lipoprotein receptor-1 (LOX-1) is a type of oxidized low density lipoprotein (OX-LDL) receptor[13]. After binding with OX-LDL, LOX-1 can induce vascular smooth muscle cell migration to the tunica intima via extracellular signal-regulated kinase (ERK) activation. It can also promote vascular smooth muscle cell proliferation and increase lipid intake, and thereby pathological vascular changes that significantly affect the formation and progress of atherosclerotic disease[14].

Thus, we hypothesized that sleeve gastrectomy would result in weight loss, and thereby prevent LOX-1 protein and LOX-1 mRNA expression, as well as adipose tissue infiltration in the aorta.

MATERIALS AND METHODS

Twenty-four male, 8-week-old, Wistar rats weighing 180 g-200 g (Beijing Laboratory Animal Research Center, China) were acclimatized for 7 d, and then randomized into three groups: normal chow (control), high fat diet (HD) and high fat diet with sleeve gastrectomy (SG). The normal diet consisted of 10% kcal of fat (D12450B diet, Research Diets Inc, New Brunswick, NJ), whereas the high fat diet consisted of 60% kcal of fat (D12492 diet, Research Diets Inc, New Brunswick, NJ). Throughout the study, rats were kept in individual metabolic cages with a natural light/dark cycle, at a temperature of 18 ℃ ± 2 ℃ and humidity of 50% ± 2%.

Rats were anesthetized with an intraperitoneal injection of 300 mg/kg chloral hydrate and placed in the supine position on a surgical board with their extremities immobilized. An epigastric incision of approximately 1.5 cm-2 cm in length was made. The incision was kept open with a blade retractor, and the gastric omentum dissociated to reveal the gastric cardium. The gastric cavity was then closed with vascular clamps and cut off with a cauterizer, which also induced hemostasis. A gastric tube was made from the distal antrum (1.5 mm-2 mm from the pylorus) to the Hiss angle using an 8-0 unabsorbable suture. The fundus was completely removed (i.e., 70%-80% of total stomach). After the gastric tube was constructed, the peritoneal cavity was cleaned with saline and closed with a 6-0 silk suture. In the control group, a sham operation was performed as described above with the exception of the stomach incisions. All animals were given 5 mL of sterile, warmed saline subcutaneously to avoid dehydration, and allowed to recover from anesthesia and surgery. Rats were then returned to their home cages, and provided with food and water *ad libitum* 24 h after the surgery.

Following the surgery, rats in the HD and SG groups received a high fat diet for 30 d, whereas rats in the control group received normal chow. Body mass was checked in all rats prior to the operation and sacrifice. Thirty days after surgery, all rats were sacrificed and blood samples were collected to measure high-density lipoprotein (HDL) and low-density lipoprotein (LDL) using fast-phase liquid chromatography (FPLC) and their respective colorimetric assay kits.

Aortas were homogenized and centrifuged at 15 000 rpm at 4 ℃ for 15 min. Protein concentrations were determined with a protein assay (Thermo Fisher Scientific Inc., IL, United States). Forty micrograms of protein were separated by electrophoresis *via* a 12.5% sodium dodecyl sulfate polyacrylamide gel electrophoresis gel. Gels were then blotted onto nitrocellulose membranes, which were blocked with 5% skimmed milk for 1 h and then blotted overnight at 4 ℃ with Rbt polyclonal primary antibody (ab60178, Abcam, Unit 225A and 225B, 2/F Core Building 2, No. 1 Science Park West Avenue, Hong Kong Science Park, Shatin, N.T., Hong Kong). After blotting with goat anti-Rbt secondary antibody, immune-complexes were visualized using an electrochemiluminescence Western blotting analysis system (FUJI film, United States).

Real-time quantitative polymerase chain reaction (PCR) analysis was carried out using an iQ5 Real-Time PCR Detection System (Bio-rad, CA, United States). The total amount of RNA used in reverse transcription was 1 μg. The following steps were performed to synthesize cDNA: samples were placed at 25 ℃ for 10 min, then 42 ℃ for 50 min, then 85 ℃ for 5 min, then chilled on ice, then 1 μL of *Escherichia coli* RNase H was added, and lastly the samples were incubated at 37 ℃ for 20 min. LOX-1 primers were as follows: sense: 5'-GACTGGATCTGGCATAAAGA-3'; antisense: 5'-CCTTCTTCTGACATATGCTG-3'.

GAPDH sequences were as follows: sense 5'-CACCCTGTGCTGCTCACCGAGGCC-3'; antisense 5'-CCACACAGATGACTTGCGCTCAGG-3'.

Real-time PCR parameters were as follows: 2 min at 50 ℃, 2 min at 95 ℃, followed by 40 cycles of 15 s at 95 ℃, 30 s at 55 ℃ and 30 s at 60 ℃. All measurements were performed in triplicate and each series of experiments was repeated twice. All quantifications were standardized to the amount of GAPDH amplification.

Aortas were taken out and frozen (Leica CM1850, Germany). A frozen slide (5 μm) was made, and stained immediately with 0.5 mL of Nile red (1 mg/mL) (Sigma

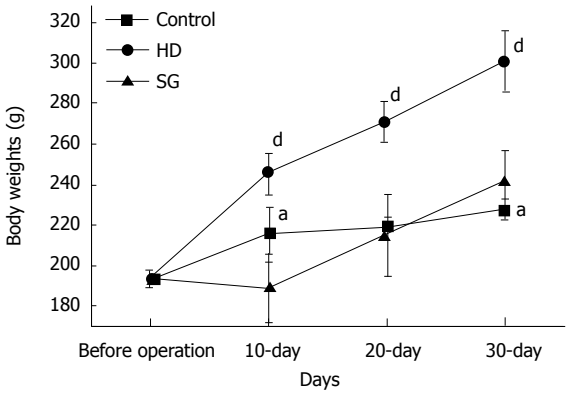

Figure 1 Body weights (g) of control, high fat diet, and high fat diet plus sleeve gastrectomy groups. $^{a}P < 0.05$ *vs* SG group; $^{d}P < 0.01$ *vs* other groups. SG: Sleeve gastrectomy; HD: High fat diet.

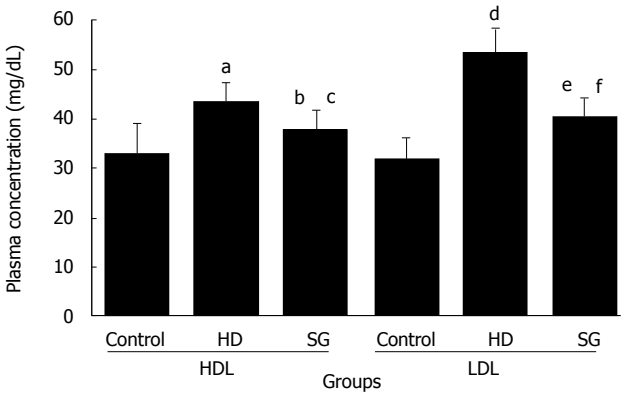

Figure 2 Plasma high-density lipoprotein and low-density lipoprotein concentration (mg/dL). $^{a}P < 0.01$ *vs* control; $^{b}P < 0.05$ *vs* control; $^{c}P > 0.05$ *vs* HD; $^{d}P < 0.01$ *vs* control; $^{e}P < 0.05$ *vs* control; $^{f}P < 0.01$ *vs* HD. HD: High fat diet; SG: Sleeve gastrectomy; HDL: High density lipoprotein; LDL: low density lipoprotein.

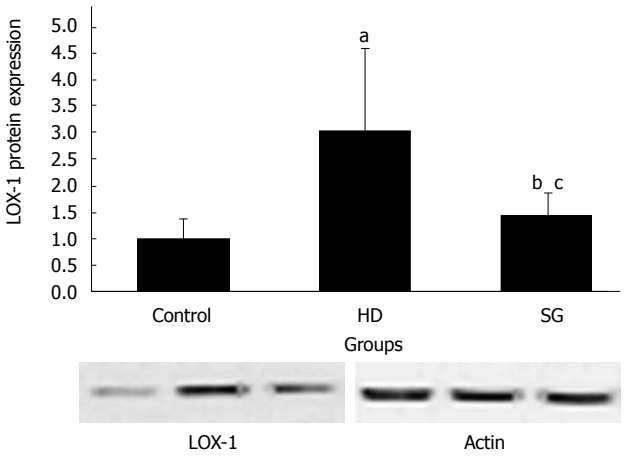

Figure 3 Lipoprotein receptor-1 protein expression compared to control levels. $^{a}P < 0.01$ *vs* control; $^{b}P < 0.05$ *vs* control; $^{c}P < 0.05$ *vs* HD. HD: High fat diet; SG: Sleeve gastrectomy; LOX-1: Lipoprotein receptor-1.

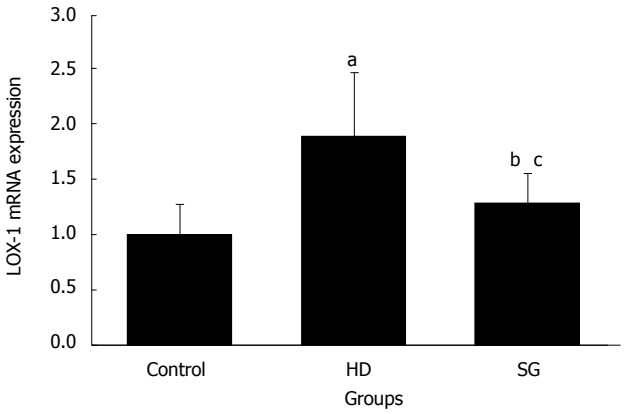

Figure 4 Lipoprotein receptor-1 mRNA expression compared to control levels. $^{a}P < 0.01$ *vs* control; $^{b}P > 0.05$ *vs* control; $^{c}P < 0.05$ *vs* HD. HD: High fat diet; SG: Sleeve gastrectomy; LOX-1: Lipoprotein receptor-1.

Co, St Louis, MO, United States) for 5 min in the dark[15]. Fluorescence microscopy (Olympus IX51 10X10, Japan) was used to visualize adipose tissue in the slides.

RESULTS

All of the rats survived and recovered from the gastrectomy. Body weights were significantly higher in the HD group compared to the control and SG groups ($P < 0.05$) (Figure 1). HDL levels in control, HD and SG groups were 32.9 ± 6.2 mg/dL, 43.4 ± 4.0 mg/dL and 37.5 ± 4.3 mg/dL, respectively. However, there were no statistical differences between the HD and SG groups ($P > 0.05$). LDL levels in the control, HD and SG groups were 31.8 ± 4.5 mg/dL, 53.3 ± 5.1 mg/dL and 40.5 ± 3.7 mg/dL, respectively (Figure 2). There was a statistically significant difference in LDL between the HD and SG groups ($P < 0.01$). LOX-1 protein expression in the HD and SG groups was 3.0 ± 1.6-fold and 1.5 ± 0.4-fold higher compared to control (Figure 3). There was a statistically significant difference between the HD and SG groups ($P < 0.05$). Furthermore, LOX-1 mRNA expression was 1.9 ± 0.6-fold and 1.3 ± 0.3-fold greater in the HD and SG groups versus control (Figure 4). There was a statistically significant difference between the SG and HD groups ($P < 0.05$). Nile red staining of control, HD and SG aortas is illustrated in Figures 5.

DISCUSSION

Obesity is associated with low-grade inflammation[16,17], which has been shown to be an initiating factor in endothelial dysfunction and atherosclerosis, and thus may cause arterial stiffness[17,18].

A direct association between the amount of weight loss and blood pressure reduction has been reported in some clinical trials[4,5]. Prospective cohort studies have also found that the prevalence of atherosclerosis and hypertension decreases with weight loss[6-8]. Additionally, several researchers have found a positive association between weight gain and obesity with atherosclerosis and hypertension[6,9-12].

Pro-inflammatory cytokines may play a role in the development of insulin resistance, which can be reversed by anti-inflammatory agents. These findings suggest that inflammation may be directly involved in the pathogenic

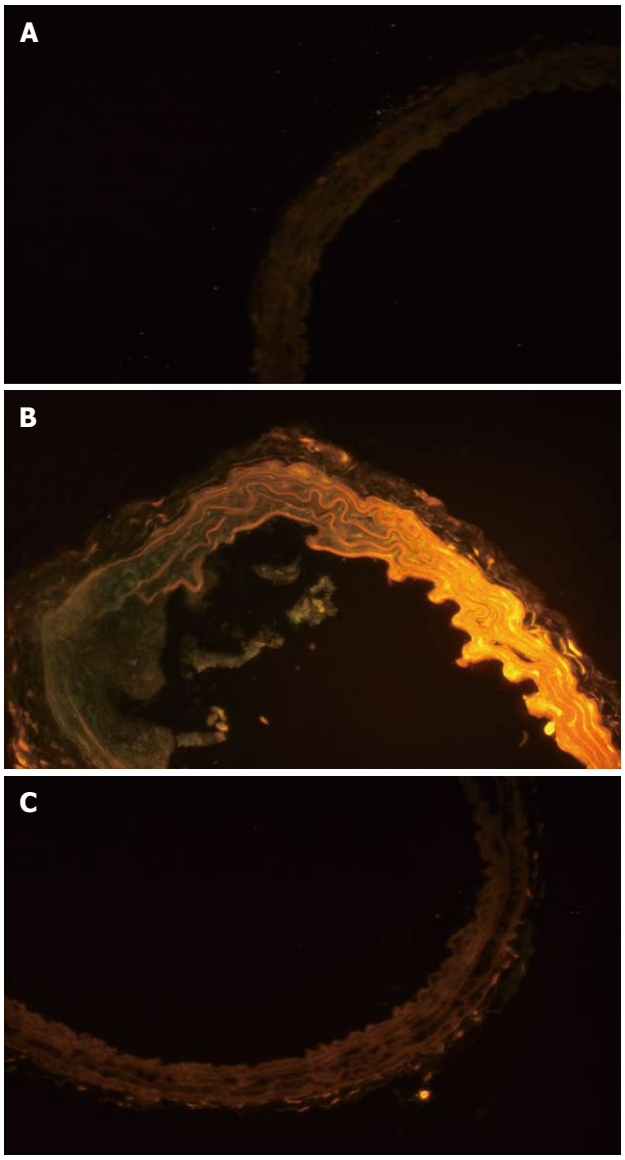

Figure 5 Nile red staining of an aorta from the three groups (× 100). A: Nile red staining of an aorta from the control group; B: Nile red staining of an aorta from the high fat diet group; C: Nile red staining of an aorta from the sleeve gastrectomy group.

properties of cytokines. Evidence suggests that both macronutrient intake and obesity may activate inflammatory signaling pathways in cells[19].

In the present study, LOX-1 protein expression in the aorta was upregulated in the HD group, and was prevented by gastrectomy in the SG group. Furthermore, LOX-1 mRNA expression was downregulated in the SG group *versus* the HD group. LOX-1 is a major receptor of ox-LDL and LDL in the vascular endothelium. The role of LOX-1 in atherogenesis is supported by several lines of evidence. LOX-1 demonstrates a strong affinity for binding, internalizing and degrading OX-LDL[20]. The oxidized form of LDL (OX-LDL) is thought to be more important in atherogenesis than the native LDL form[21]. OX-LDL injures the endothelium and is an important mediator in atherogenesis[22]. OX-LDL activates LOX-1 and induces endothelial dysfunction and apoptosis[23,24]. There are other mediators of atherosclerosis, such as angiotensin Ⅱ, cytokines, sheer stress and advanced glycation end-products, that upregulate LOX-1. Furthermore, LOX-1 is dynamically upregulated by pro-atherogenic conditions, such as diabetes, hypertension and dyslipidemia. LOX-1 is present in atheroma-derived cells, and in human and animal atherosclerotic lesions[25-28].

To date, surgery has been proven to be the only effective method for treating morbid obesity[29,30]. Observational studies suggest that weight-loss surgery is associated with a 60% to 80% diabetes remission rate in severely obese individuals, and that earlier interventions are more likely to provide remission[31]. Additionally, there are concerns regarding the lack of evidence, as well as the safety, invasiveness, and cost-effectiveness of such surgical weight-loss procedures. Providing appropriate evidence has been problematic due to the invasive nature of the surgery, which makes recruitment difficult. However, with the advent of safer, less invasive surgical weight-loss procedures, randomized clinical trials are now feasible.

Sleeve gastrectomy, a type of bariatric surgery, was performed in this experiment. In the SG group, body weights were significantly lower than those of the HD group. As a result, LOX-1 protein and mRNA expression levels, as well as LDL levels, were significantly lower in the SG group *versus* the HD group. SG is a type of purely restrictive surgery, where a moderate restriction is created, while the integrity of the duodenum, pylorus, antrum, lesser curvature and vagal nerve, and a relatively normal eating behavior, are maintained. Recent findings also suggest that SG might be a safe, beneficial, and effective stand-alone approach[32-34]. Moon *et al* reported that SG resolves all comorbidities of obesity in over 90% of subjects over a 12-mo period, with the exception of dyslipidemia, which is resolved in 65% of subjects[33]. Moreover, there was a dramatic loss of appetite in more than half of the patients postoperatively[33]. Karamanakos *et al*[35] found that SG preserved the integrity of the pylorus and did not induce an intestinal bypass. Furthermore, LDL levels, as well as liver enzymes, were decreased significantly in SG patients.

In summary, a high fat diet elevates LOX-1 protein and mRNA expression in the aorta. Sleeve gastrectomy can prevent increases in plasma LDL levels, as well as an upregulation in LOX-1 protein and mRNA expression associated with a high fat diet.

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COMMENTS

Background

Morbid obesity is a serious health problem worldwide. Atherosclerosis is an important comorbidity of obesity. Diseases associated with atherosclerosis, such as myocardial infarction and stroke, account for the majority of deaths in industrialized countries. Studies have found a direct association between the amount of weight loss and the prevalence of atherosclerosis. Lectin-like

oxidized low density lipoprotein receptor-1 (LOX-1) is a type of oxidized low density lipoprotein (OX-LDL) receptor. After binding with OX-LDL, LOX-1 can affect the formation and progress of atherosclerotic disease. Thus, the authors hypothesized that sleeve gastrectomy would result in weight loss, and thereby prevent LOX-1 protein and LOX-1 mRNA expression, as well as adipose tissue infiltration in the aorta.

Research frontiers

The hotspot about this paper is the treatment of atherosclerosis in obese animals after bariatric surgery.

Innovations and breakthroughs

It was difficult to control morbid obesity and its comorbidities, such as atherosclerosis and non-alcoholic steatohepatitis, before bariatric surgery was used in the clinic. This kind of surgery can decrease body weight and reverse many comorbidities caused by obesity.

Applications

These results could expand the indication of bariatric surgery in the clinic and many obese patients with atherosclerosis could undergo surgery in order to decrease body weight and cure atherosclerosis.

Terminology

Bariatric surgery: Bariatric surgery, or weight loss surgery, includes a variety of procedures performed on people who are obese. Weight loss is achieved by reducing the size of the stomach with an implanted medical device (gastric banding) or through removal of a portion of the stomach (sleeve gastrectomy or biliopancreatic diversion with duodenal switch) or by resecting and re-routing the small intestines to a small stomach pouch (gastric bypass surgery); Atherosclerosis: Atherosclerosis (also known as arteriosclerotic vascular disease) is a condition in which an artery wall thickens as the result of a build-up of fatty materials such as cholesterol. It is a syndrome affecting arterial blood vessels; a chronic inflammatory response in the walls of arteries, in large part due to the accumulation of macrophage white blood cells and promoted by low-density lipoproteins (plasma proteins that carry cholesterol and triglycerides) without adequate removal of fats and cholesterol from the macrophages by functional high density lipoproteins. It is commonly referred to as a hardening or furring of the arteries.

Peer review

With great interest I have read the article entitled: "Sleeve gastrectomy prevents LOX-1 expression of aortas in obese rats". This is a well-performed study with some interesting findings concerning the influence of obesity surgery on atherosclerosis.

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BRIEF ARTICLE

Overexpression of TLR3, TLR4, TLR7 and TLR9 in esophageal squamous cell carcinoma

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Abstract

AIM: To investigate the expression of Toll-like receptor (TLR) 3, TLR4, TLR7 and TLR9 in esophageal squamous cell carcinoma (ESCC).

METHODS: Reverse transcription-polymerase chain reaction and immunohistochemistry were used to analyze the expression of TLR3, TLR4, TLR7 and TLR9 mRNA and protein in samples from 87 esophageal cancer patients consisting of both tumor and normal tissue.

RESULTS: A significant increase in TLR3, TLR4, TLR7 and TLR9 mRNA levels was detected in ESCC samples. Tumors exhibited high TLR protein expression, (70.1%, 72.4%, 66.7% and 78.2% for TLR3, TLR4, TLR7 and TLR9, respectively, $P < 0.05$). Nevertheless, a significant percentage of tumors also exhibited TLR4 expression in mononuclear inflammatory cells (48.3%) and TLR9 expression in fibroblast-like cells (60.9%). Tumors with high TLR3 expression in tumor cells or high TLR4 expression in mononuclear inflammatory cells were significantly associated with a higher probability of lymph node metastasis and increased depth of invasion. However, tumors with high TLR9 expression in fibroblast-like cells were associated with low probabilities of invasion and metastasis. There was no significant variation between the expression of TLR3, TLR4, TLR7 and TLR9 among different ethnic groups.

CONCLUSION: TLR3, TLR4, TLR7 and TLR9 expression appears important to the biological pathogenesis of ESCC. TLRs may represent therapeutic targets for ESCC.

Key words: Esophageal squamous cell carcinoma; Invasion; Metastasis; Prognosis; Toll-like receptor

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INTRODUCTION

Esophageal carcinoma (EC) remains a major threat to health worldwide, with a 5-year survival rate below 10%, and in China, EC is characterized by its distinct geographic distribution and differences in ethnic preva-

lence[1]. Xinjiang, in Western China, has one of the highest prevalences of esophageal squamous cell carcinoma (ESCC) in the world, and the ratio in ESCC incidence between different ethnic groups is as large as 13.4:1. ESCC has become the main cause of tumor-related deaths in the Kazak ethnic group in Xinjiang[2]. Despite advances in clinical treatment, ESCC prognosis remains poor due to its relapse and metastasis characteristics. For these reasons, prognostic factors are essential to improve the classic risk classification in ESCC.

Chronic infection and inflammation can induce cancer formation *via* cytokines and chemokines, which play vital roles in promoting angiogenesis and metastasis, the most important factors contributing to cancer development and growth. Toll-like receptors (TLRs) comprise an important family of pattern recognition receptors that allow immune cells to recognize pathogens and trigger inflammatory responses, as they are expressed not only in a variety of immune cells but also in non-immune cells such as fibroblasts and epithelial cells. These responses include the secretion of cytokines that increase the resistance of infected cells as well as the release of chemokines that recruit immune cells to necrotic cells. Chronic inflammation can promote carcinogenesis by inducing gene mutations, inhibiting apoptosis, or stimulating angiogenesis and cell proliferation. Research has demonstrated that basement membrane changes induced by chronic inflammation are correlated with the aberrant proliferation of esophageal epithelia[3]. The TLR signaling pathway activates several different signaling elements, including nuclear factor kappa B (NF-κB), extracellular signal regulated kinase, and Jun-NH-kinase/p38, which regulate many immunologically related proteins[4]. Several researchers also found that MyD88 (the TLR-mediated signaling adapter protein) plays an important role not only in the pathway of TLR-mediated inflammation but also in Ras-MAPK signaling, cell-cycle control, and cell transformation, which promote carcinogenesis[5,6]. Evidence indicates that TLR expression in tumor cells can promote inflammation and cell survival in the tumor microenvironment[7-9]. These results suggested that TLR stimulation could lead to tumor progression. These findings may be useful in elucidating potential prognostic markers.

The purpose of the present study was to investigate the expression of TLR3, TLR4, TLR7 and TLR9 in ESCC as well as their association with the clinicopathologic characteristics of ESCC. To this aim, we analyzed the protein levels of TLR3, TLR4, TLR7 and TLR9 by immunohistochemical techniques and their mRNA levels by reverse transcription-polymerase chain reaction (RT-PCR).

MATERIALS AND METHODS

Clinical samples

A total of 87 formalin-fixed and paraffin-embedded tissue blocks were obtained from esophageal carcinoma patients who had not received pre-operative radiotherapy or chemotherapy; all patients were treated at the Department of Thoracic Surgery of the First Affiliated Hospital in Medical University of Xinjiang from June 2007 to March 2009, and the borderline tumor tissues were used as controls. Patient ethnicity was as follows: Han, 30 patients; Uyghur, 25 patients; and Kazak, 32 patients. The mean age of the patients was 50.5 years; the youngest patient was 39 years old, and the oldest patient was 73 years old at the time of surgery. Each specimen was histologically examined, and the tumor was graded by at least two experienced pathologists. The main characteristics of ESCC patients, including tumor grade, stage, and lymph node status of the tumor, were categorized according to the TNM (American Joint Committee on Cancer, 4th edition) as follows: (1) 20 cases; (2) 34 cases; and (3) 33 cases. Among the 87 tumors were 30 well-differentiated tumors, 29 moderately differentiated tumors, and 28 poorly differentiated tumors. Fifty-three patients had lymph node metastases. In addition, 40 frozen biopsies that included 20 normal esophageal epithelia and 20 ESCC samples were subjected to RT-PCR for the detection of TLR3, TLR4, TLR7 and TLR9 mRNA expression. All patients were enrolled by written informed consent, and the study was approved by the Ethical Committee of the Medical University of Xinjiang.

Immunohistochemical studies

Sections of 3-μm-thick paraffin-embedded tissue were deparaffinized in xylene and then rehydrated in a graded ethyl alcohol series (100%, 95%, 80% and 70%). For increased specificity and sensitivity, tissues were microwave-treated at 95 ℃ for 15 min to retrieve the antigen. After cooling and rinsing in distilled water, endogenous peroxide activity was blocked with 3% H_2O_2 for 15 min, after which samples were rinsed in 0.01 mol/L phosphate-buffered saline [phosphate buffered saline (PBS), pH 7.4] for 10 min and then preincubated with a protein blocking solution for 10 min. Primary antibodies (mouse monoclonal anti-human TLR3, TLR4, TLR7 and TLR9 were obtained from Santa Cruz Biotechnology, Santa Cruz, CA, United States). Antibodies were diluted at 1:200 in PBS and applied at 4 ℃ overnight in a humid chamber. Slides were washed three times in PBS and then incubated with secondary biotinylated antibody for 15 min at room temperature. Antigen-antibody complexes were detected using the streptavidin-peroxidase method (15-min exposure) with diaminobenzidine [diaminobenzidine (DAB)] as the chromogen substrate (Vectastain Elite ABC kit, Vector Laboratories, Burlingame, CA, United States). The peroxidase signal was visualized by treatment with a DAB substrate-chromogen system for 8 min. Finally, the sections were stained lightly with hematoxylin, and PBS was used in place of the primary antibody as a negative control. All immunostained sections were coded and independently examined by two investigators using light microscopy. The results were scored on a scale from 0 to 4 for the percentage of positive cells and from 0 to 3 for the intensity of positive cells. The percentage of positive cells was scored as follows:

Table 1 Polymerase chain reaction primers used for the detection of Toll-like receptors

TLR	Forward	Reverse
TLR3	AGTGCCGTCTATTTGCCACACA	AACAGTGCACTTGGTGGTGGAG
TLR4	TCTTCAACCAGACCTCTACATTCCA	GGAACATCCAGAGTGACATCACAG
TLR7	CCGTGACAATTACCTGGCCTTC	CAGGGCCTTCAGCTGGTTTC
TLR9	AGGATGATGCCAGGATGATGTC	TCAGGTCCAGGTTCTTGGTTGAG
β-actin	GGCACCCAGCACAATGAAG	CCGATCCACACGGAGTACTTG

TLR: Toll-like receptor.

≤ 10%, 11%-25%, 26%-50%, 51%-75%, and ≥ 76%. The intensity of staining was scored as follows: absent, weak staining, moderate staining, and intense staining. The overall score (percentage of stained cells ×intensity of staining) was then used to identify the mean score by using an Excel spreadsheet [≥ mean score for positive (+); < mean score for negative (-)], in line with a previous study[10].

RNA extraction and RT-PCR

Total RNA was extracted from fresh frozen tissue using TRIzol (Invitrogen, Carlsbad, CA, United States) as described by the manufacturer. mRNA was reverse-transcribed with RevertAid (MBI Fermentas, Burlington, Ontario, Canada) at 42 ℃ for 60 min, and the synthesized cDNA (20 ng) was subjected to polymerase chain reaction (PCR) (95 ℃ for 1 min, 25 or 30 cycles of 95 ℃ for 3 s, 60 ℃ for 30 s, and 68 ℃ for 1 min, and a single extension at 68 ℃ for 10 min). PCR products were separated on a 4% agarose gel and visualized with ethidium bromide. Each analysis was repeated at least twice to ensure reproducibility. mRNA for β-actin was used as a normalization control in RT-PCR and as a loading control in conventional PCR. Forward and reverse primer pairs are listed in Table 1, and their products were 181 bp, 198 bp, 172 bp and 97 bp.

Statistical analysis

All statistical analyses were performed with the SPSS statistical software package (version 15.0). The chi-square test was used to compare the differences in cumulative TLR3, TLR4, TLR7 and TLR9 expression between normal and ESCC groups, and to determine whether the clinicopathologic variables were associated with the levels of TLR3, TLR4, TLR7 and TLR9 as well as compare the mRNA expression in fresh frozen ESCC tissues with that of normal samples as determined by RT-PCR. P values < 0.05 were considered statistically significant.

RESULTS

TLR protein expression in ESCC and their association with ESCC clinicopathologic characteristics

Immunohistochemistry (IHC) staining of 87 primary ESCC lesions and normal esophageal tissues was performed using anti-TLRs antibodies (Table 2). Representative staining patterns for TLRs are shown in Figure 1. IHC staining demonstrated that TLRs were localized in the cytoplasm, but TLR3 was also expressed in the cell membrane. Positive staining for TLR4 and TLR9 was generally observed within normal esophageal surface epithelium, but weak or no TLR4 and TLR9 staining was detected in stromal cells. However, in ESCC, TLRs were strongly expressed not only in cancer cells, but also in some stromal cells, such as fibroblast-like cells and mononuclear inflammatory cells. The positive rates of TLR3, TLR4, TLR7 and TLR9 expression in the normal esophageal surface epithelium were 8.0%, 5.7%, 9.2% and 4.6%, respectively. These values sharply increased to 70.1%, 66.7%, 72.4% and 78.2%, respectively, in ESCC lesions (P < 0.05 compared with the positive rate in healthy tissue). Nevertheless, a significant percentage of tumors also exhibited TLR4 expression in mononuclear inflammatory cells (48.3%) and TLR9 expression in fibroblast-like cells (60.9%). Table 3 summarizes the percentages of TLR staining in each cellular type. TLR3 and TLR7 were mainly expressed in esophageal tumor cells, and there was a statistically significant difference compared with the expression in the control group.

We also evaluated the possible relationship between the expression of TLRs in tumor cells and the clinicopathologic characteristics of ESCC including tumor stage, histological grade, lymph node metastasis, and depth of invasion. TLR3 expression in tumor cells was significantly associated with depth of invasion and lymph node metastasis. TLR4 expression in tumor cells was significantly associated with lymph node metastasis. TLR7 expression in tumor cells was significantly associated with tumor grade. TLR9 expression was found to gradually increase with worsening histopathological grade (P < 0.005, Table 2). However, the TLR9 IHC staining scores did not correlate with the depth of invasion and lymph node metastasis.

We analyzed the association between the expression of TLR4 and TLR9 in tumor stromal cells and poor prognostic indicators because a significant percentage of tumors also exhibited TLR4 expression in mononuclear inflammatory cells and TLR9 expression in fibroblast-like cells. We found that carcinoma patients with higher TLR4 expression in the stromal compartment had a significantly higher risk of disease progression. TLR4 expression in mononuclear inflammatory cells (48.3%) was significantly associated with the depth of invasion and lymph node metastasis. Conversely, TLR9 expression in fibroblast-like cells (60.9%) was significantly associated with reduced depth of invasion and lymph node metas-

Table 2 Statistical analysis of Toll-like receptor expression and clinicopathologic factors in esophageal carcinoma

Characteristics	TLR3 Positive (%)	*P*	TLR4 Positive (%)	*P*	TLR7 Positive (%)	*P*	TLR9 Positive (%)	*P*
Normal control (*n* = 87)	7 (8)		5 (5.7)		8 (9.2)		4 (4.6)	
Tumor differentiation	61 (70.1)		63 (72.4)		58 (66.7)		68 (78.2)	
W (*n* = 30)	22 (75.3)	0.889	22 (75.3)	0.539	14 (46.7)	0.003	18 (60.0)	0.004
M (*n* = 29)	20 (69.1)		19 (65.6)		19 (65.6)		23 (79.3)	
P (*n* = 28)	19 (67.9)		22 (78.6)		25 (89.3)		27 (96.4)	
Depth of invasion								
≤ muscularis (*n* = 37)	18 (48.6)	< 0.001	27 (73.1)	0.92	23 (62.2)	0.002	27 (73.1)	0.314
≥ adventitia (*n* = 50)	43 (86.0)		36 (72.0)		35 (66.0)		41 (82.0)	
LN metastasis								
Negative (*n* = 34)	18 (52.9)	0.005	20 (58.8)	0.023	26 (76.5)	0.12	29 (85.3)	0.197
Positive (*n* = 53)	43 (81.1)		43 (81.1)		32 (60.3)		39 (73.6)	
Ethnic groups								
Han (*n* = 30)	23 (76.7)	> 0.05	22 (73.3)	> 0.05	17 (56.7)	> 0.05	23 (76.7)	> 0.05
Uyghur (*n* = 25)	18 (72.0)		20 (80.0)		16 (64.0)		20 (80.0)	
Kazak (*n* = 32)	20 (62.5)		21 (65.6)		25 (78.1)		25 (78.1)	

All values are presented as the number of cases, with percentages in parentheses. W: Well differentiated; M: Moderately differentiated; P: Poorly differentiated; LN: Lymph node; TLR: Toll-like receptor.

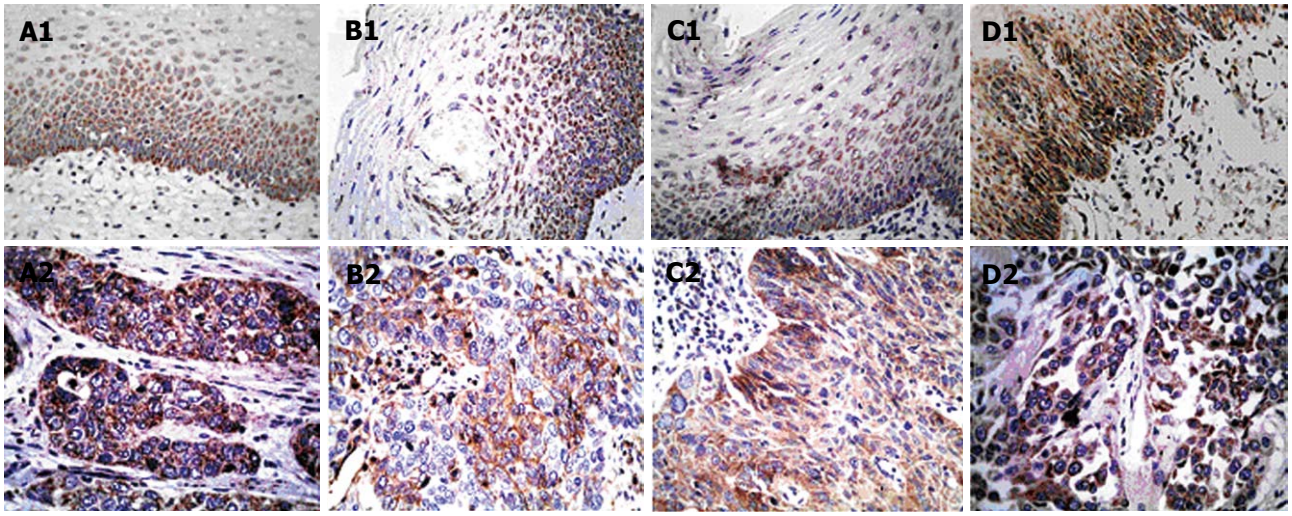

Figure 1 Immunohistochemistry staining of esophageal lesions with Toll-like receptor-specific mAbs. A1 to D1 show the expression of Toll-like receptor (TLR) 3, TLR4, TLR7 and TLR9 in normal esophageal epithelium, respectively. A2 and C2 show positive staining for TLR3 and TLR7 in esophageal squamous cell carcinoma cells. B2 shows positive TLR4 staining in tumor cells and mononuclear inflammatory cells, and D2 shows positive TLR9 staining in tumor cells and fibroblast-like cells (Original magnification, × 400).

Table 3 The percentage expression of Toll-like receptors in each cellular type within esophageal squamous cell carcinoma tissues

Factors	Tumor cells Positive cases (%)	Fibroblast Positive cases (%)	MICs Positive cases (%)
TLR3	61 (70.1)	3 (3.4)	4 (4.6)
TLR4	63 (72.4)	8 (9.2)	47 (48.3)
TLR7	58 (66.7)	5 (5.7)	2 (2.3)
TLR9	68 (78.2)	53 (60.9)	12 (13.8)

All values are presented as the number of cases, with percentages in parentheses. MICs: Mononuclear inflammatory cells; TLR: Toll-like receptor.

tasis (Table 4). We also observed variations between the expression of TLR3, TLR4, TLR7 and TLR9 in tumor cells among different ethnic groups in Xinjiang, although the differences were not statistically significant.

TLR mRNA expression in ESCC and normal controls

To confirm the IHC results, TLR3, TLR4, TLR7 and TLR9 mRNA expression in esophageal biopsies was detected by RT-PCR (Figure 2). Similar to the IHC results, TLR3, TLR4, TLR7 and TLR9 mRNA expression was increased in ESCC tissues. TLR3 and TLR7 gene expression was quantified in 15 ESCC and 3 normal esophageal tissues. TLR4 mRNA expression was higher in ESCC samples than in normal controls after normalization to β-actin expression. The percentages of TLR9 mRNA positivity in ESCC and normal tissues were 55% and 15%, respectively. Although the sample size was limited,

Table 4 Analysis of the relationship between the expression of Toll-like receptors in each cellular type with the clinicopathologic characteristics of esophageal squamous cell carcinoma *n* (%)

Factors	Tumor Grade			Depth of invasion		LN metastasis	
	W	M	P	⩽ muscularis	⩾ adventitia	Negative	Positive
Number of cases	30	29	28	37	50	34	53
TLR4 MICs (+)	19 (63.3)	16 (55.2)	12(42.9)	12(32.4)	35(70)	14(41.2)	33(62.3)
P		0.245		0.001		0.018	
TLR9 fibroblast (+)	20 (66.7)	18 (62.1)	17(60.7)	30(81.1)	23(43.4)	29(85.3)	24(45.3)
P		0.885		0.001		0.006	

All values are presented as the number of cases, with percentages in parentheses. TLR: Toll-like receptor; LN: Lymph node; MICs: Mononuclear inflammatory cells; W: Well differentiated; M: Moderately differentiated; P: Poorly differentiated.

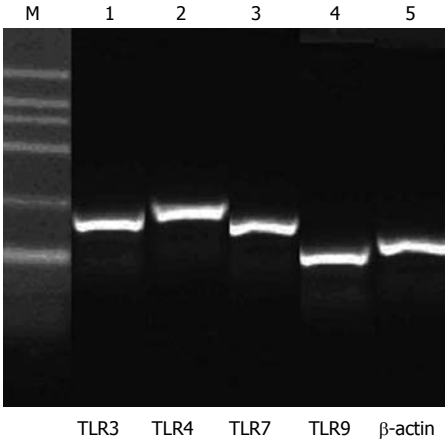

Figure 2 mRNA expression patterns of Toll-like receptor 3, Toll-like receptor 4, Toll-like receptor 7 and Toll-like receptor 9. M: 100-600 bp marker ladder. Lanes 1 to 4 show the expression of Toll-like receptors, and lane 5 shows the expression of β-actin.

the differences in TLR3, TLR4, TLR7 and TLR9 mRNA expression levels between normal esophageal epithelia and ESCC were statistically significant ($P < 0.05$).

DISCUSSION

This study demonstrated that samples of recurrent EC exhibited significantly higher mRNA levels of TLR3, TLR4, TLR7 and TLR9 than normal tissue. ESCC tumors exhibited high TLR protein expression in cancer cells. Nevertheless, a significant percentage of tumors also exhibited TLR4 expression in mononuclear inflammatory cells and TLR9 expression in fibroblast-like cells. Tumors with high TLR3 expression in tumor cells or high TLR4 expression in mononuclear inflammatory cells were significantly associated with poor prognosis. However, tumors with high TLR9 expression in fibroblast-like cells were associated with a low probability of metastasis.

In this study, high TLR3 expression in esophageal cancer cells was associated with a high probability of lymph node metastasis. Similar observations were made in different cancer types. Studies on breast and prostate carcinomas demonstrated that high TLR3 expression was significantly associated with higher probabilities of metastasis and biochemical recurrence[10,11], which is in agreement with previous studies indicating that TLR3 expression is related to tumor aggressiveness[12,13]. Although the precise effect of increased TLR3 expression requires further investigation, our work suggests that TLR3 plays a vital role in esophageal carcinogenesis. Therefore, TLR3 may represent a good therapeutic target in esophageal cancer.

This study also demonstrated that the expression level of TLR4 in tumor cells was significantly associated with depth of invasion. TLR7 and TLR9 expression was positively associated with tumor grade in ESCC. Moreover, studies on TLR4 and TLR7 expression in gastric and lung cancer cells have suggested that high TLR expression results in increased tumor progression[14,15] and stimulation with TLR7 agonists lead to NF-κB activation, upregulated expression of the antiapoptotic protein Bcl-2, increased tumor cell survival, and chemoresistance[16,17]. It has also been reported that TLR9 expression gradually increased during the progression from normal cervical squamous epithelial tissues to cervical intraepithelial neoplasia and invasive cervical cancer[18]. These findings indicated that increased TLR protein expression may interfere with normal TLR signaling pathways and function and may represent useful markers of the malignant transformation of cancer cells. In addition, cancer cells activated by TLR signals may release cytokines and chemokines that in turn recruit and stimulate immune cells to release additional cytokines and chemokines. This process results in immune tolerance, cancer progression, and propagation of the tumor microenvironment.

In this study, we also observed the expression of TLR3, TLR4, TLR7 and TLR9 in tumor stromal cells as well as their association with the clinicopathologic characteristics of ESCC. Tumor stromal cells such as fibroblast-like cells, mononuclear inflammatory cells, and numerous intracellular mediators comprise the tumor microenvironment. These factors actively participate in tumor progression and infiltration, where the tumor microenvironment not only responds to and supports carcinogenesis but also contributes to tumor initiation, progression, and metastasis. The interaction between transformed cells and the microenvironment determines the fate of the tumor. Another interesting finding was TLR4 expression

in mononuclear inflammatory cells and TLR9 expression in fibroblast-like cells, which are associated with ESCC prognostic factors. TLR4 expression in mononuclear inflammatory cells was associated with an increased incidence of lymph node metastasis and depth of invasion, and TLR9 expression in fibroblast-like cells was associated with a low rate of lymph node metastasis. These findings are supported by research that demonstrated the importance of tumor stromal cells in tumor behavior through the release of various growth factors, proteases, and extracellular matrix proteins, which induce gene mutations, inhibit apoptosis, and stimulate angiogenesis and cell proliferation[19-21].

Metastatic relapse attributable to the presence of tumor cells within lymph nodes is the most frequent cause of cancer-related death in patients with esophageal tumors[22]. In the current study, the high expression of TLR4 by mononuclear inflammatory cells was associated with an increased incidence of lymph node metastasis and depth of invasion, suggesting that the regulation of the immune response within the tumor microenvironment might be another consequence of TLR activation. TLR9 expression by fibroblast-like cells was associated with good patient prognosis, suggesting that the surrounding connective tissue of the tumor is important for preventing tumor spread. Therefore, our results also suggest the existence of different phenotypes of stromal cells that influence prognosis depending upon the expression pattern of TLRs. In this study, TLR3, TLR4, TLR7 and TLR9 expression appeared important to the biological pathogenesis of esophageal cancer. However, different phenotypes of TLR expression in stromal cells can lead to different results and as a series of candidate prognostic factors, the function of these markers in ESCC should be further investigated.

COMMENTS

Background

The prognosis of esophageal carcinoma remains poor due to its relapse and metastasis characteristics. Toll-like receptors (TLRs) comprise an important family of pattern recognition receptors, and the TLR signaling pathway activates several different signaling elements, including nuclear factor kappa B, extracellular signal regulated kinase, and Jun-NH-kinase/p38, which regulate many immunologically related proteins, alter the microenvironment of tumors, and promote tumor progression and metastasis. Esophageal squamous cell carcinoma (ESCC) progression is associated with TLR stimulation, a crucial event in immune escape and metastasis.

Research frontiers

TLR expression is a common event in several cancers; this can promote carcinogenesis by inducing gene mutations, inhibiting apoptosis, or stimulating angiogenesis and cell proliferation. However, TLR expression has not been reported in esophageal carcinoma. In this study, the authors demonstrated that the overexpression of TLR3, TLR4, TLR7 and TLR9 in ESCC appears to be important in the biological pathogenesis of ESCC.

Innovations and breakthroughs

This is the first study to report that TLR3, TLR4, TLR7 and TLR9 overexpression appears important in the biological pathogenesis of ESCC. Furthermore, high TLR4 expression in mononuclear inflammatory cells was significantly associated with a higher probability of lymph node metastasis and depth of invasion, and high TLR9 expression in fibroblast-like cells was associated with low probabilities of invasion and metastasis.

Applications

TLR3, TLR4, TLR7 and TLR9 expression may represent potential prognostic markers and therapeutic targets for ESCC.

Terminology

TLRs, a family of pattern recognition receptors expressed in immune and non-immune cells, play a crucial role in the innate immune response and the subsequent induction of adaptive immune responses against microbial infection or tissue injury. Furthermore, TLR expression in cancer cells is associated with tumor proliferation and invasion.

Peer review

This manuscript by Dr. Sheyhidin *et al* investigated the expression of TLR3, TLR4, TLR7 and TLR9 in ESCC tissues from a good number of patients. The resulted data confirmed upregulation of TLRs in ESCC and its association with clinic pathological characteristics. This study is clinical significance because it included a good number of patients including patients from various ethnic groups. Nevertheless, the data generated from this study are confirmative in nature.

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BRIEF ARTICLE

Repeated anastomotic recurrence of colorectal tumors: Genetic analysis of two cases

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Abstract

AIM: To investigate genetics of two cases of colorectal tumor local recurrence and throw some light on the etiopathogenesis of anastomotic recurrence.

METHODS: Two cases are presented: a 65-year-old female receiving two colonic resections for primary anastomotic recurrences within 21 mo, and a 57-year-old female undergoing two local excisions of recurrent anastomotic adenomas within 26 mo. A loss of heterozygosity (LOH) study of 25 microsatellite markers and a mutational analysis of genes *BRAF*, *K-RAS* and *APC* were performed in samples of neoplastic and normal colonic mucosa collected over the years.

RESULTS: A diffuse genetic instability was present in all samples, including neoplastic and normal colonic mucosa. Two different patterns of genetic alterations (LOH at 5q21 and 18p11.23 in the first case, and LOH at 1p34 and 3p14 in the second) were found to be associated with carcinogenesis over the years. A role for the genes *MYC-L* (mapping at 1p34) and *FIHT* (mapping at 3p14.2) is suggested, whereas a role for *APC* (mapping at 5q21) is not shown.

CONCLUSION: The study challenges the most credited intraluminal implantation and metachronous carcinogenesis theories, and suggests a persistent, patient-specific alteration as the trigger of colorectal cancer anastomotic recurrence.

Key words: Anastomotic recurrence; Colorectal cancer; Allelic loss; Genetic alterations

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INTRODUCTION

Local recurrences (LRs) from colorectal cancer are often inoperable and have poor prognoses, with an estimated 5-year survival of 10 percent and a median survival of 16 mo[1]. LRs are defined as being perianastomotic (when

rising in the extramural tissue) or primitively anastomotic[2]. These latter may be due to implantation of exfoliated cancerous cells in the suture line[3,4] or to metachronous carcinogenesis[5].

We report two singular cases of patients repeatedly developing recurrent tumors (adenocarcinoma, adenoma) at the suture line and/or in the contiguous colonic mucosa within 21 and 26 mo of left hemicolectomy and anterior rectal resection for colorectal adenocarcinoma, respectively. To clarify the molecular mechanism(s) implicated in such a singular feature and, more in general, the development of anastomotic recurrence, we performed an extended genetic analysis of patients' tumor tissues and colonic mucosa obtained from surgical specimens and follow up endoscopy. The investigation was focused on the chromosomal alterations most frequently associated with colorectal cancer development, including mutational analysis of *BRAF*, *K-RAS* and *APC* genes and loss of heterozygosity (LOH) analysis of 25 chromosomal sites known to be involved in colonic carcinogenesis. This is the first genetic study performed on anastomotic recurrence of colorectal cancer.

MATERIALS AND METHODS

Case 1

In November 1998, a 65-year-old woman underwent a left hemicolectomy with a stapled colorectal anastomosis for a 4.5 cm × 3 cm fungating tumor of the sigmoid colon, 32 cm from the anal verge. Preoperative workup did not show any local infiltration or liver/pulmonary metastases. Histological examination showed a moderately differentiated adenocarcinoma infiltrating the whole colonic wall up to the pericolic fat tissue with uninvolved mucosa 23 cm proximal and 20 cm distal to the tumor edges, and 20 tumor-free lymph nodes (pT2N0M0).

In accordance with our follow-up policy[6], the patient underwent clinical and ultrasound evaluation and circulating carcino-embryonic antigen (CEA) determination every three months, as well as computed tomography (CT) scan and colonoscopy one year postoperatively. The latter procedure identified a recurrence involving half the circumference of the colorectal anastomosis. No local or distant metastases were disclosed by a CT scan, and CEA level was normal. A colorectal resection with mesorectal excision and stapled colorectal anastomosis by the double-stapling technique[7] was performed 6 cm from the anal verge. The resected specimen showed a moderately differentiated adenocarcinoma infiltrating the muscle layer with 12 tumor free lymph nodes (pT2N0M0) and normal mucosa, 9 cm proximal and 6 cm distal to the tumor.

The patient presented nine months later with rectal bleeding. At colonoscopy the anastomosis showed a circumferential tumor recurrence and five polyps. The CEA level was normal and a CT scan of the abdomen and thorax did not disclose distant metastases. A colorectal resection with double-stapled coloanal anastomosis 2 cm from the anal verge was performed with a defunctioning ileostomy which was later closed. The patient received adjuvant radiotherapy (45 G) to the pelvis three weeks later. The histopathologic examination showed a moderately differentiated adenocarcinoma infiltrating the muscle layer (pT2N0M0) and five adenomas (3 located proximal and 2 distal) within 3 cm of the suture line, < 1 cm in size, with severe dysplasia.

The regular yearly follow up revealed no further sign of local recurrence or distant metastases and the patient is in good health 11 years after the initial resection. In January 2010, the patient underwent endoscopic exploration with biopsy.

Case 2

In May 2006, a 57-year-old woman underwent anterior rectal resection with coloanal anastomosis and ileostomy for a 4 cm polypoid lesion of the lower rectum (5 cm from the anal verge); on histological examination of endoscopic biopsies, this proved to be an adenocarcinoma arising in a villous adenoma. Neither regional nor distant spread was present at preoperative CT scan. In the resected specimen the histological diagnosis of adenocarcinoma developing from a high grade villous adenoma was confirmed, with initial invasion of the submucosa (early colorectal cancer), and free lymph nodes (n = 27) and surgical margins (pT1N0M0). After ileostomy closure, an anastomotic substenosis was easily resolved by 2 mechanical dilatations.

The patient was submitted to regular follow-up[6]. Twenty-two months after surgery, a colonoscopy revealed an asymptomatic anastomotic 3 cm polyp, which was completely removed by transanal resection. Histological examination showed a tubulo-villous adenoma with high grade dysplasia.

At the subsequent colonoscopy, 4 mo later, a second anastomotic 2 cm polyp was removed by transanal resection, again revealing an adenoma with high grade dysplasia. Neither local recurrences nor distant metastasis were detected at further follow-up. In September 2009, the patient underwent endoscopic exploration with biopsy.

Tissue processing and genetic analysis (Table 1)

In case 1 the LOH study (see Table 1) and the mutational analysis for *BRAF*, *KRAS* and *APC* (see below) were performed on the following samples: (1) primary adenocarcinoma and the corresponding peritumoral, distal and proximal mucosa; (2) first recurrence and peritumoral and distant mucosa (12 mo postoperatively); (3) second recurrence and adenoma (21 mo after initial surgery); and (4) anastomotic and distant colorectal mucosa (134 mo after initial surgery).

In case 2 the LOH study (see Table 1) and the mutational analysis for *BRAF* and *KRAS* were performed on: (1) primary tumor and peritumoral mucosa; (2) villous adenoma (22 mo postoperatively); (3) recurrent anastomotic adenomas (26 mo after initial surgery); and (4) anastomotic mucosa (40 mo after initial surgery).

Table 1 Microsatellite markers used in the loss of heterozygosity study, with relevant cytogenetic locations, putative genes involved and their function, and references to papers describing a role for colonic carcinogenesis

Microsatellite Markers	Cytogenetic band	Gene	Function	Ref.
BAT40	1p13.1			[8]
MYC-L	1p34			[8]
BAT 26	2p16.3	*hMSH2*	Mismatch repair enzyme	[8]
D2S123	2p16			[8]
D3S1481	3p14	*FHIT*	Histidine triad gene family (purine metabolism)	[9]
D4S2397	4p15.2			[10]
D5S346	5q21	*APC*	Antagonist of the Wnt signaling pathway	[8]
D10S1671	10q25			
D10S169	10q26.3	*MGMT*	DNA defense *vs* O6-methylguanine	[11]
D10S1765	10q23.3	*PTEN*	Protein tyrosine phosphatase	[12]
D16S421	16q22	*CDH1*	Ca++ dependent cell-cell adhesion glycoprotein	
D16S402	16q23-q24			
D16S507	16q23.2			
D17S250	17q21			[8]
TP53ALU	17p13.1	*TP53*	Tumor protein "guardian of the genome"	
TP53	17p13	*TP53*	Tumor protein "guardian of the genome"	[13]
D18S452	18p11.23			[14]
D18S53	18p11.22-p11			[13]
D18S64	18q21	*DCC*	Receptor for netrin 1	[15]
D18S857	18q22.1	*DCC*	Receptor for netrin 1	
DXYS233	Xp22.32-Yp11.3			[16]
SHOX	Xp22.3			[16]
DXYS154	Xqter-Yqter			[16]
DXS8009	Xq25-q26			
DXS8098	Xq24-q25			

Using 5 μm haematoxylin stained sections of tissue specimens routinely formalin fixed and paraffin embedded, DNA was isolated by manual microdissection and extracted using the QIAamp Tissue Kit (Qiagen GmbH, Hilden, Germany). Only tumor samples containing more than 70% tumor cells were included in the study. All microdissection were conducted in close collaboration with the pathologist to ensure consistency with histological diagnoses and accurate dissection for tumor cell enrichment. For each patient DNA extracted from normal lymphocytes was used as reference DNA. DNA quality was assessed by polymerase chain reaction (PCR) amplification of the human beta-globin gene.

Polymerase chain reaction

The molecular analysis was performed with a panel of 25 polymorphic microsatellite markers located on chromosomal regions potentially involved in colorectal cancer development and progression and listed in Table 1. Primer sequences and amplification conditions were in accordance with the Genome Database information (http://www.ncbi.nlm.nih.gov/genemap99). Forward primers were synthesized with a fluorescent tag (WellRed dyes from Research Genetics, Huntsville, AL, United States).

The target sequences were amplified by PCR in a 25 μL reaction mixture containing 2 μL DNA sample, 10x buffer (10 mmol/L Tris-HCl pH 9.0, 50 mmol/L KCl, 0.1% Triton X-100), 1.5 mmol/L $MgCl_2$, 0.2 mmol/L of each dNTP (Promega, Madison, WI), 0.4 μmol/L of each primer and 1.25 U Taq Polymerase (Promega, Madison, WI). Microsatellites were submitted to 35-40 cycles of amplification at different annealing temperatures (range 57 ℃-61 ℃). The presence and correct size of amplimers were evaluated by 2% agarose gel electrophoresis. The fluorescently labelled PCR products were subjected to electrophoresis on an automated DNA sequencer CEQ 8000XL (Beckman Coulter Inc., Fullerton, CA), and the fluorescent signals from the different sized alleles were recorded and analyzed using CEQ 10000XL analysis software (Beckman Coulter).

Definition of LOH and allelic imbalance

The LOH was defined as the ratio of relative allelic peak height in the tumor DNA to relative allelic peak height in the corresponding normal DNA. The formula employed for the calculation was T2: T1/N2: N1, where T1 and N1 are the height values for the smaller allele and T2 and N2 are the height values for the larger allele of the tumor (T) and normal (N) samples respectively. For informative markers LOH was scored when the signal reduction for one allele was of 40%. This degree of allelic imbalance (AI) indicates that a substantial proportion of the cells within a sample contains the same DNA abnormality and likely represents the presence of a clonal population. Abnormal results were demonstrated at least twice with equivalent results. At certain loci AI probably reflects increased copy number rather than loss of an allele. Distinguishing between these possibilities is important conceptually, but would not change data analysis. Therefore, all AIs were labelled as LOH.

The same areas of chromosomal regions showing LOH were repeated in an independently microdissected sample from different paraffin blocks when sufficient tissue was available.

Microsatellite instability

The novel appearance in the tumor DNA of one or more alleles, i.e. new peaks in the electropherogram, not present in its paired normal DNA, was considered as an indicator of microsatellite instability (MSI). Samples were classified as microsatellite stable or unstable according the revised Bethesda Criteria[8].

BRAF, KRAS and APC mutation analysis

Direct sequencing was performed to identify *BRAF* V600E mutations, *KRAS* codon 12/13 mutations and *APC* exon 15. Primer sequences for *BRAF* and *KRAS* were: *BRAF-F* (5'TGCTTGCTCTGATAGGAAAATGA-3'), *BRAF-R* (5'-TGGATCCAGACAACTGTTCAAA-3'), *KRAS-F* (5'-GCCTGCTGAAAATGACTGAA-3') and *KRAS-R* (5'-AGAATGGTCCTGCACCAGTAA-3'), which generated fragment lengths of 165 and 167 bp respectively. *APC* mutation analysis was performed using three sets of primers, amplifying two overlapping portions of exon 15 in accordance with Su *et al*[17]: *APC-1F* (5'-CATCAGCTGAAGATGAAATAGGA-3') and *APC-1R* (5'-GCAATCGAACGACTCTCAAA-3'), codons 1281–1402, 364 bp; *APC-2F* (5'-ATGTTCAGGAGACCCCACTC-3') and *APC-2R* (5'-CACTCAGGCTGGATGAACAA-3'), codons 1376–1508, 396 bp; and *APC–3F* (5'-GGGTCCAGGTTCTTCCAGAT-3') and *APC-3R* (5'-TTGCCACAGGTGGAGGTAAT-3'), codons 1478-1607, 387 bp.

DNA sequencing was performed using EurofinsMWGOperon/M-Medical (Milano, Italy). Sequencing results were verified in our laboratory in both directions using DNA STAR PC software (Lasergene, Madison, WI, United States). The presence of mutations was determined through alignment with normal sequences as reported in NCBI/Blast Human Genome database (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

RESULTS

LOH analysis

The results of LOH analysis for all 25 chromosomal markers investigated are reported in Table 2.

Case 1

A generalised genetic instability at various sites in tumoral and non-tumoral, histologically normal mucosal samples was seen. Chromosomes 5q, 16q and 18 presented with the highest frequency of LOH. In particular, LOH at 5q21 and 18p11.23 loci was consistently found in all tumor samples, including primary and recurrent adenocarcinomas and the late occurring adenoma, but was not found in any sample of non-tumoral mucosa. In contrast, LOH at 16q23-24 was consistently present in all extratumoral mucosa samples (except one showing MSI), being absent in all tumor specimens. LOH at 10q26.3 and 18q21 loci was also found to be restricted to non-tumoral mucosal samples, but only in those collected at the time of the initial surgery. The primary tumor, but not the neoplasms observed at the time of recurrences, showed LOH at 1p13.1 and 1p34.

When investigated for MSI, all samples showed a stable phenotype (in accordance with the Bethesda revised criteria[8]) except for one individual (proximal) sample of normal mucosa at the initial surgery, which was characterized by MSI in 5 of the chromosomal markers analyzed (low-MSI). The mutational analysis of exon 15 of *APC* gene demonstrated the presence of a single nucleotide polymorphism in the codon 1493 ACG > ACA (T1493T) in all tumoral and non-tumoral samples.

Case 2

LOH at 3p14 was found to be a consistent, specific tumor change occurring in all neoplastic specimens but not in samples of non-neoplastic mucosa. Furthermore, allelic loss was seen at the locus 1p34 of tumor specimens (except for the third adenoma) but was also observed in the peritumoral non-neoplastic mucosa, whereas LOH at 10q23.3 was restricted to the primary tumor and corresponding peritumoral mucosa. Scattered LOH changes were also found in homologous pseudoautosomal regions (DXYS233, DXYS154, SHOX) of the sex chromosomes X-Y in the normal mucosa and the third adenoma. No evidence for MSI was yielded by any of the samples analyzed in this case.

Mutation analysis

The sequence analyses for *K-RAS* and *BRAF* mutation performed in tumor and normal tissues of both cases showed a wild type phenotype in all samples. Mutation analyses for exon 15 of *APC*, performed in Case 1, in which LOH was present at the gene locus in 5q21, yielded negative results.

DISCUSSION

Sixteen percent of patients undergoing colorectal resection for colon cancer present with a local recurrence[18], and, since in 12% of cases[19] the recurrences occur primarily at the site of the anastomosis, it may be estimated that roughly 2% of patients undergoing a colorectal resection for cancer will eventually develop an anastomotic recurrence. The mechanism(s) involved in the development of anastomotic recurrence are poorly understood. The present study has focused on genetic alterations occurring in primary and recurrent tumors as well as in the extra-tumoral colonic mucosa of two patients with repeated and rather early recurrence of anastomotic/perianastomotic neoplasms. To this end, a search was carried out for allelic losses at 25 chromosomal sites known to be involved in colonic carcinogenesis (Table 1) and for mutational events in three genes (*K-RAS*, *BRAF* and *APC*) commonly altered in colorectal cancer. This extensive genetic analysis included the normal mucosa at the time of the resection of the primary tumor ("genetic predisposition") and the potential changes in the genetic pattern of recurrent tumors and/or colonic mucosa pos-

Table 2 Results of loss of heterozygosity analysis with 25 microsatellite markers amplified in the present cases

	Case 1 (adenocarcinoma of the sigmoid colon – pT2N0M0)												Case 2 (adenocarcinoma of the rectum – pT1N0M0)					
	Initial surgery (left hemicolectomy)				Second procedure (colorectal resection) 12 mo after initial surgery (a.i.s.)			Third procedure (colorectal resection) 21 mo a.i.s.		Follow up (colonoscopy) 134 mo a.i.s.			Initial surgery (proctocolectomy)			Second procedure (endosc. resection) 22 mo a.i.s.	Third procedure (endosc. resection) 26 mo a.i.s.	Follow up (colonoscopy) 40 mo a.i.s.
	Prim. tumor	Peritu. mucosa	Distal mucosa	Prox. mucosa	First recurr.	Peritu. mucosa	Distant mucosa	Second recurr.	Aden	Anast. mucosa	Rectal mucosa	Colon mucosa	Prim. tumor	Peritu. mucosa	Aden.	Adenoma	Adenoma	Anast. mucosa
1p13.1	LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH
1p34	LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	LOH	LOH	LOH	LOH	NO LOH	NO LOH
2p16.3	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH
2p16	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI
3p14	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	LOH	NO LOH	LOH	LOH	LOH	NO LOH
4p15.2	NO LOH	NO LOH	LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	LOH	NI	NI	NI	NI	NI	NI
5q21	LOH	NO LOH	NO LOH	NO LOH	LOH	NO LOH	NO LOH	LOH	LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH
10q23.3	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI	LOH	LOH	NO LOH	NO LOH	NO LOH	NO LOH
10q25	NI	NI	NI	MSI	NI	NI	NI	NI	NI	NI	NI	NI	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH
10q26.3	NO LOH	NO LOH	LOH	LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NI	NI	NI	NI	NI	NI
16q22	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI
16q23.2	NO LOH	NO LOH	NO LOH	MSI	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH
16q23 -q24	NO LOH	LOH	LOH	MSI	NO LOH	LOH	LOH	NO LOH	NO LOH	LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	
17p13	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NI	NI	NI	NI	NI	NI
17p13.1	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH
17q21	NO LOH	NO LOH	NO LOH	MSI	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH
18p11.23	LOH	NO LOH	NO LOH	NO LOH	LOH	NO LOH	NO LOH	LOH	LOH	LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH
18p11.22 -p11	NO LOH	NO LOH	LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NI	NI	NI	NI	NI	NI
18q21	NO LOH	LOH	MSI	LOH	NO LOH	NO LOH	NO LOH	NO LOH	LOH	NO LOH	LOH	NO LOH	NI	NI	NI	NI	NI	NI
18q22.1	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH
Xp22.32-Yp11.3	NI	NI	NI	MSI	NI	NI	NI	NI	NI	NO LOH	NO LOH	NO LOH	NO LOH	LOH	NO LOH	NO LOH	NO LOH	NO LOH
Xp22.32-Yp11.3	NO LOH	NO LOH	LOH	NO LOH	NO LOH	LOH	NO LOH	NO LOH	NO LOH	LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	LOH	LOH
Xqter-Yqter	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI	NO LOH	LOH	NO LOH	NO LOH	NO LOH	NO LOH
Xq24-q25	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI
Xq25-q26	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NI	NI	NI	NI	NI	NI
FAL (%)	23.5	12.5	29.4	13.3	11.1	11.1	5.9	11.1	16.7	16	10.5	5.2	20	26.7	13.3	13.3	13.3	6.25

LOH: Loss of heterozygosity; NO LOH: Retention of heterozygosity; NI: Not informative; MSI: Microsatellite instability; FAL%: Fractional allelic loss (No. of markers with LOH/total No. of informative markers).

sibly involved in tumor progression and recurrences.

The LOH analysis (Table 1) showed in both cases a diffuse genetic instability at various sites both in tumor tissue and in extra-tumoral mucosa, although the affected loci largely differed between neoplastic and non-neoplastic samples as well as between non-neoplastic samples taken from different colonic regions. The fractional allelic loss did not significantly vary in the tumors

as compared to the normal mucosa or in the peritumoral as compared to distant mucosa (Table 2). In both cases a noticeable persistence of genetic changes both in the primary and recurrent tumors was found even though the affected chromosomal loci differed from one case to the other, being 5q21 and 18p11.23 in Case 1 and 1p34 and 3p14 in Case 2. Since these changes (with the exception of 3p14 in the first samples of Case 2) were consistently absent in the extra-tumoral mucosa, they may reasonably be considered as reflecting chromosomal alterations responsible for tumor development. Their consistent appearance in primary and recurrent tumors (with the exception of the third adenoma in Case 2) supports the supposition of an identical genetic mechanism for anastomotic recurrences. The potential involvement of the *APC* gene, which maps at 5q21, in colonic carcinogenesis of Case 1 is not supported by the lack of detectable mutations in the gene exon 15, which is more commonly altered in colo-rectal cancers. In Case 2, the LOH at 1p34 and 3p14.2 sites, the loci of mapping of the *MYC-L* and *fragile histidine triad* (*FHIT*) genes respectively suggest a key role of these two genes in this patient' s tumor development. In this regard it is worth noting that 1p34 LOH occurs in both tumoral and non-tumoral samples, whereas 3p14.2 LOH is absent in normal mucosa. This may suggest that the *MYC-L* alteration may reflect a "mark" of a proliferative instability leading to carcinogenesis, whereas the alteration of the *FHIT* gene may suggest its role in the events occurring at the early phase of carcinogenesis (i.e., transformation from normal mucosa to adenoma).

The two most credited theories to explain the occurrence of anastomotic recurrence are the intraluminal implantation of exfoliated cancerous cells[3,4] and the metachronous carcinogenesis[5], possibly triggered by modifications of the microenvironment around the suture depending on the surgical technique[20,21] or the materials used[22-24]. Both theories fail to satisfactorily explain our findings. Indeed, in both cases the intraluminal implantation theory, though supported by the presence of consistent genetic alterations in primary and recurrent tumors, is contradicted by other genetic alterations (such as LOH at 1p13.1 and 1p34 loci in case 1, and at 10q23.3 in case 2) that are present in the primary but not in the recurrent neoplasms. Moreover, the recurrence of perianastomotic benign tumors (adenomas) with overlapping genetic changes is also in contrast with the implantation theory. On the other hand, the metachronous carcinogenesis theory by itself cannot explain the short time needed to develop new tumors and their location at the suture line or within a short distance from it, since metachronous carcinogenesis *per se* implies the chance onset of a second adenoma/adenocarcinoma in any segment of the colonic remnant at an interval of years. Also the hypothesis that the anastomosis' surgical techniques[20,21] and/or the materials used[22-24] may be implicated in carcinogenesis by altering DNA at specific sites seems to be confounded by our findings, since the same genetic alterations found in recurrent neoplasms were present in the primary tumors, whose development obviously cannot be associated to previous surgical procedures. Moreover, such a hypothesis does not explain why recurrences occur in a very small minority of patients, in spite of the standardized surgical procedures performed (including the materials used) in all patients affected by colorectal cancer.

A genomic instability of DNA in tumor and adjacent tissues has already been described in breast cancer, where independent mutational events were observed[25-27]. Considering colon cancer, Ahlquist *et al*[28] found various epigenetic changes in mucosa surrounding colorectal neoplastic lesions, and hypothesised that the tumor itself may have caused a "field cancerization" of the contiguous mucosa. This phenomenon, in our opinion, is unlikely to have occurred in the present cases, owing to the large discrepancy in genetic changes between the tumors and the normal extratumoral mucosa in spite of a diffuse, tumor-independent genetic instability in the colonic mucosa of our patients. Independently of its aetiology, Umeto *et al*[29] suggested that colonic genetic instability associated with microenvironmental changes may "predispose" to metachronous carcinogenesis by altering several genes implicated in colon cancer development. In our cases, the consistency of genetic alterations among primary and recurrent tumors, even if separated by an interval of years, suggests a persistent, patient-specific alteration rather than a generic, diffuse DNA instability, as the trigger of recurrent carcinogenesis after radical resection of colorectal cancer.

In conclusion, although the mechanism of elective recurrent carcinogenesis in the anastomotic and/or perianastomotic areas still remains unsolved, since genetic alteration patterns differ in the two cases, our study supports a role for the genes found to be altered. Further studies in larger series of patients are warranted for assessing the potential role of these gene changes in the detection of patients at risk of developing an early anastomotic recurrence, and for confirming the patient-specificity of genetic alterations responsible of cancerogenesis, regardless of other genetic alterations occurring in colonic mucosa through years.

COMMENTS

Backgrounds

Local recurrences (LRs) from colorectal cancer are often inoperable and have poor prognoses, with an estimated 5-year survival of 10 percent and a median survival of 16 mo. LRs are defined as being perianastomoticor primitively anastomotic. These latter may be due to implantation of exfoliated cancerous cells in the suture line or to metachronous carcinogenesis.

Innovations and breakthroughs

This is the first genetic study performed on anastomotic recurrence of colorectal cancer.

Applications

Although the mechanism of elective recurrent carcinogenesis in the anastomotic and/or perianastomotic areas still remains unsolved, since genetic alteration patterns differ in the two cases in the study,it supports a role for the genes found to be altered.

Peer review

The conclusion needs to be modified given that the findings are based on a sample of two patients.

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Role of diaphragm in pancreaticopleural fistula

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Abstract

A pancreatic pleural effusion may result from a pancreatopleural fistula. We herein discuss two interesting issues in a similar case report of a pleural effusion caused after splenectomy, which was recently published in the *World Journal of Gastroenterology*. Pancreatic exudate passes directly through a natural hiatus in the diaphragm or by direct penetration through the dome of the diaphragm from a neighboring subdiaphragmatic collection. The diaphragmatic lymphatic "stomata" does not contribute to the formation of such a pleural effusion, as it is inaccurately mentioned in that report. A strictly conservative approach is recommended in that article as the management of choice. Although this may be an option in selected frail patients, there has been enough accumulative evidence that a pancreaticopleural fistula may be best managed by early endoscopy in order to avoid complications causing prolonged hospitalization.

Key words: Fistula; Pleural effusion; Pancreatic surgery

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TO THE EDITOR

We have read with great interest the article by Shu-Guang Jin *et al*[1] that presented a case of pancreatic pleural effusion caused after splenectomy. In their work, the authors support the notion that the leaking fluid from a pancreatic duct disruption may reach the thoracic cavity by the lymphatic system and stomata. They also conclude that an active conservative treatment should be carried out in the early period of this complication to reduce the need for endoscopy or surgery. We feel that both of these statements need further discussions.

Pancreaticopleural fistula is a rare occurrence. This internal pancreatic fistula is usually caused by a chronic pancreatitis or, more rarely, it is a traumatic consequence. A pancreatic pleural effusion develops due to a direct passage of pancreatic exudate through a natural hiatus in the diaphragm[2] or by direct penetration through the dome of the diaphragm[3] from a neighboring subdiaphragmatic collection.

The most common cause of pancreaticopleural fistula is a pseudocyst formed in the lesser sac from an anterior disruption of the pancreatic duct that erodes the overlying diaphragm.

Although studies of the pathways of peritoneal fluid absorption indicate that the peritoneal surface of the diaphragm is the main site of drainage, this does not nec-

essarily suggest that this mechanism is implicated in pancreatic fluid transportation into the pleural cavity. Pleural liquid is a filtrate from capillaries in the parietal pleura lining the chest wall. Drainage from pleural space occurs *via* the lymphatics in the parietal pleura[4]. Peritoneal fluid enters the lymphatic lacunae (a rich plexus of flattened terminal lymphatics) *via* special mesothelial openings, the so called "stomata"[5]. This fluid is further transported *via* the parasternal route to the mediastinal nodes and then to the terminal thoracic duct or the right lymphatic duct[6] and not to the pleural cavity. The "stomata" system provides a direct route between the peritoneal cavity and lymphatics[7].

In the presented case of pancreatic pleural effusion after splenectomy, a left subphrenic encapsulated fluid collection was clearly revealed by an abdominal computed tomography. A pancreatic pseudocyst such as the aforementioned is almost invariably implicated in these rare cases of pancreatopleural fistula[8]. This communication happens through normal orifices or diaphragmatic erosion.

The protein-rich fluid with an elevated amylase content drained by the thoracocentesis was a great indicator of the pancreatopleural fistula which, as speculated, was the result of a posterior pancreatic duct rupture due to an intraoperative injury. The authors proposed that a purely medical treatment was appropriate for their patient, in order to reduce the need for endoscopy or surgery. This policy was recommended in their conclusions. Although the medical treatment proved effective in their case, one has to bear in mind the adverse consequences that may be caused by such an approach. Such a notion has been extensively emphasized by many researchers in the field. This therapeutic option usually requires prolonged hospitalization which contributes substantially to morbidity and cost. On the contrary, an early instituted endoscopic retrograde cholangiopancreatography (ERCP)[9] combined with either a papillotomy, a stent or a nasopancreatic tube may be an optional initial treatment. The role of early therapeutic endoscopy is constantly expanding[10] as it has proved beneficial instead of long-term conservative treatment. Up to 90% of the patients with pancreatic fistulas can be successfully treated by this modality, with minimal morbidity and no mortality[11].

Although formal treatment recommendations have not been adopted, the first line of treatment supported by most of the authors in the field includes drainage of the effusion, inhibition of pancreatic secretion with octreotide and ERCP plus stenting of the pancreatic duct.

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Events Calendar 2011

January 14-15, 2011
AGA Clinical Congress of Gastroenterology and Hepatology: Best Practices in 2011 Miami, FL 33101, United States

January 20-22, 2011
Gastrointestinal Cancers Symposium 2011, San Francisco, CA 94143, United States

January 27-28, 2011
Falk Workshop, Liver and Immunology, Medical University, Franz-Josef-Strauss-Allee 11, 93053 Regensburg, Germany

January 28-29, 2011
9. Gastro Forum München, Munich, Germany

February 4-5, 2011
13th Duesseldorf International Endoscopy Symposium, Duesseldorf, Germany

February 13-27, 2011
Gastroenterology: New Zealand CME Cruise Conference, Sydney, NSW, Australia

February 17-20, 2011
APASL 2011-The 21st Conference of the Asian Pacific Association for the Study of the Liver
Bangkok, Thailand

February 22, 2011-March 04, 2011
Canadian Digestive Diseases Week 2011, Vancouver, BC, Canada

February 24-26, 2011
Inflammatory Bowel Diseases 2011-6th Congress of the European Crohn's and Colitis Organisation, Dublin, Ireland

February 24-26, 2011
2nd International Congress on Abdominal Obesity, Buenos Aires, Brazil

February 24-26, 2011
International Colorectal Disease Symposium 2011, Hong Kong, China

February 26-March 1, 2011
Canadian Digestive Diseases Week, Westin Bayshore, Vancouver, British Columbia, Canada

February 28-March 1, 2011
Childhood & Adolescent Obesity: A whole-system strategic approach, Abu Dhabi, United Arab Emirates

March 3-5, 2011
42nd Annual Topics in Internal Medicine, Gainesville, FL 32614, United States

March 7-11, 2011
Infectious Diseases: Adult Issues in the Outpatient and Inpatient Settings, Sarasota, FL 34234, United States

March 14-17, 2011
British Society of Gastroenterology Annual Meeting 2011, Birmingham, England, United Kingdom

March 17-19, 2011
41. Kongress der Deutschen Gesellschaft für Endoskopie und Bildgebende Verfahren e.V., Munich, Germany

March 17-20, 2011
Mayo Clinic Gastroenterology & Hepatology 2011, Jacksonville, FL 34234, United States

March 18, 2011
UC Davis Health Informatics: Change Management and Health Informatics, The Keys to Health Reform, Sacramento, CA 94143, United States

March 25-27, 2011
MedicReS IC 2011 Good Medical Research, Istanbul, Turkey

March 26-27, 2011
26th Annual New Treatments in Chronic Liver Disease, San Diego, CA 94143, United States

April 6-7, 2011
IBS-A Global Perspective, Pfister Hotel, 424 East Wisconsin Avenue, Milwaukee, WI 53202, United States

April 7-9, 2011
International and Interdisciplinary Conference Excellence in Female Surgery, Florence, Italy

April 15-16, 2011
Falk Symposium 177, Endoscopy Live Berlin 2011 Intestinal Disease Meeting, Stauffenbergstr. 26, 10785 Berlin, Germany

April 18-22, 2011
Pediatric Emergency Medicine: Detection, Diagnosis and Developing Treatment Plans, Sarasota, FL 34234, United States

April 20-23, 2011
9th International Gastric Cancer Congress, COEX, World Trade Center, Samseong-dong, Gangnam-gu, Seoul 135-731, South Korea

April 25-27, 2011
The Second International Conference of the Saudi Society of Pediatric Gastroenterology, Hepatology & Nutrition, Riyadh, Saudi Arabia

April 25-29, 2011
Neurology Updates for Primary Care, Sarasota, FL 34230-6947, United States

April 28-30, 2011
4th Central European Congress of Surgery, Budapest, Hungary

May 7-10, 2011
Digestive Disease Week, Chicago, IL 60446, United States

May 12-13, 2011
2nd National Conference Clinical Advances in Cystic Fibrosis, London, England, United Kingdom

May 19-22, 2011
1st World Congress on Controversies in the Management of Viral Hepatitis (C-Hep), Palau de Congressos de Catalunya, Av. Diagonal, 661-671 Barcelona 08028, Spain

May 21-24, 2011
22nd European Society of Gastrointestinal and Abdominal Radiology Annual Meeting and Postgraduate Course, Venise, Italy

May 25-28, 2011
4th Congress of the Gastroenterology Association of Bosnia and Herzegovina with international participation, Hotel Holiday Inn, Sarajevo, Bosnia and Herzegovina

June 11-12, 2011
The International Digestive Disease Forum 2011, Hong Kong, China

June 13-16, 2011
Surgery and Disillusion XXIV SPIGC, II ESYS, Napoli, Italy

June 14-16, 2011
International Scientific Conference on Probiotics and Prebiotics-IPC2011, Kosice, Slovakia

June 22-25, 2011
ESMO Conference: 13th World Congress on Gastrointestinal Cancer, Barcelona, Spain

June 29-2, 2011
XI Congreso Interamericano de Pediatria "Monterrey 2011", Monterrey, Mexico

September 2-3, 2011 Falk Symposium 178, Diverticular Disease, A Fresh Approach to a Neglected Disease, Gürzenich Cologne, Martinstr. 29-37, 50667 Cologne, Germany

September 10-11, 2011
New Advances in Inflammatory Bowel Disease, La Jolla, CA 92093, United States

September 10-14, 2011
ICE 2011-International Congress of Endoscopy, Los Angeles Convention Center, 1201 South Figueroa Street Los Angeles, CA 90015, United States

September 30-October 1, 2011
Falk Symposium 179, Revisiting IBD Management: Dogmas to be Challenged, Sheraton Brussels Hotel, Place Rogier 3, 1210 Brussels, Belgium

October 19-29, 2011
Cardiology & Gastroenterology | Tahiti 10 night CME Cruise, Papeete, French Polynesia

October 22-26, 2011
19th United European Gastroenterology Week, Stockholm, Sweden

October 28-November 2, 2011
ACG Annual Scientific Meeting & Postgraduate Course, Washington, DC 20001, United States

November 11-12, 2011
Falk Symposium 180, IBD 2011: Progress and Future for Lifelong Management, ANA Interconti Hotel, 1-12-33 Akasaka, Minato-ku, Tokyo 107-0052, Japan

December 1-4, 2011
2011 Advances in Inflammatory Bowel Diseases/Crohn's & Colitis Foundation's Clinical & Research Conference, Hollywood, FL 34234, United States

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Text

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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 DOI:10.1097/01.ju.0000067940.76090.73]

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6 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303 DOI:10.1136/bmj.325.7357.184]

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Patent (list all authors)

16 **Pagedas AC**, inventor; Ancel Surgical R&D Inc., assignee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 20020103498. 2002 Aug 1

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