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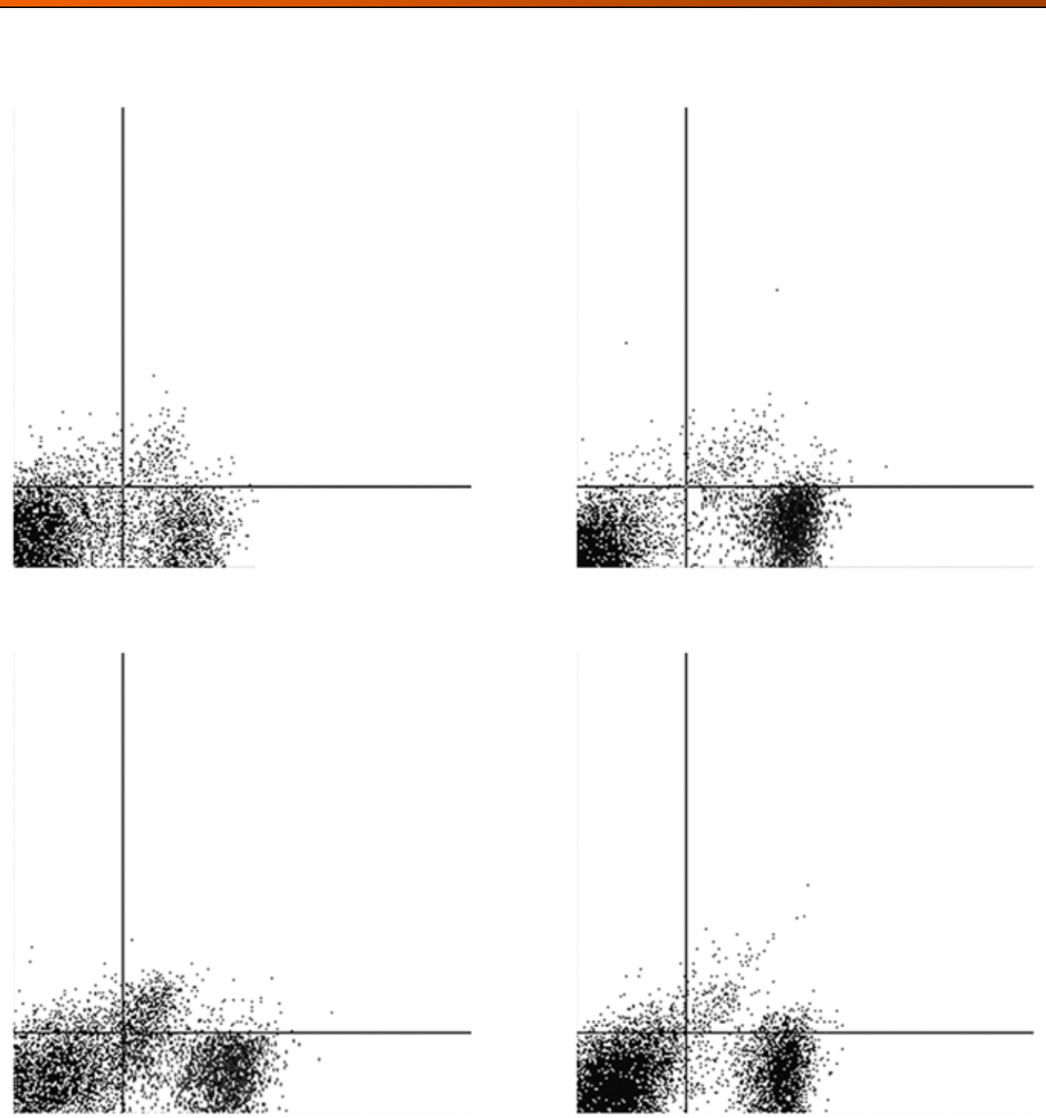

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EDITORIAL

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.

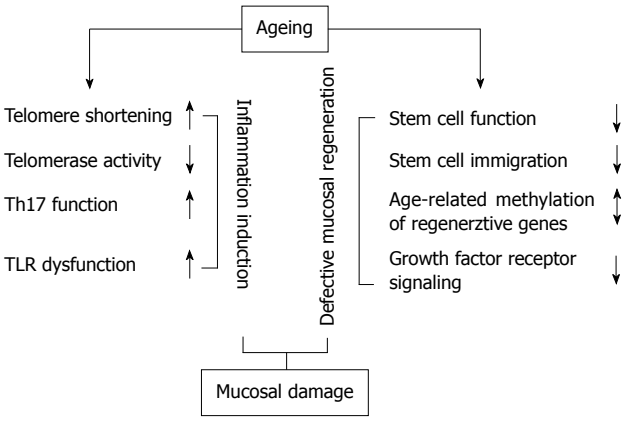

Figure 1 The effect of ageing on factors resulting in colonic mucosal damage.

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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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.

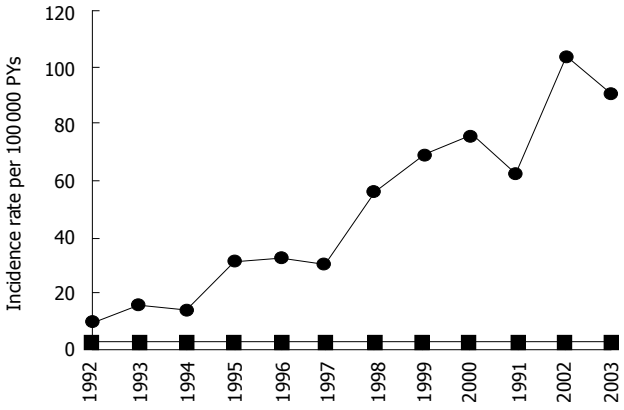

Figure 1 Annual incidence rates of anal cancer among HIV-infected persons (circles) and the general population (squares), USA 1992-2003.

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

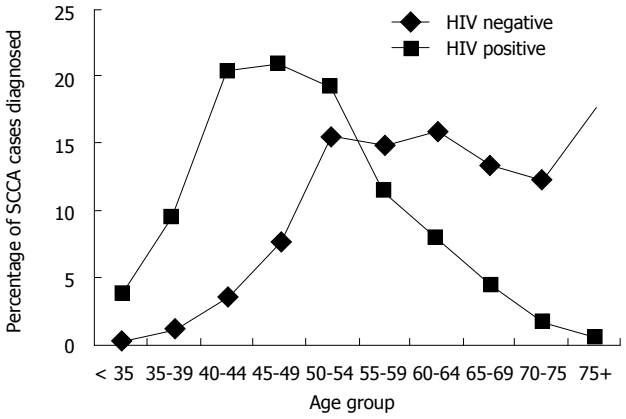

Figure 2 Percentage of anal cancer diagnosed among us veterans by age group (1998-2004). HIV: Human immunodeficiency virus.

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

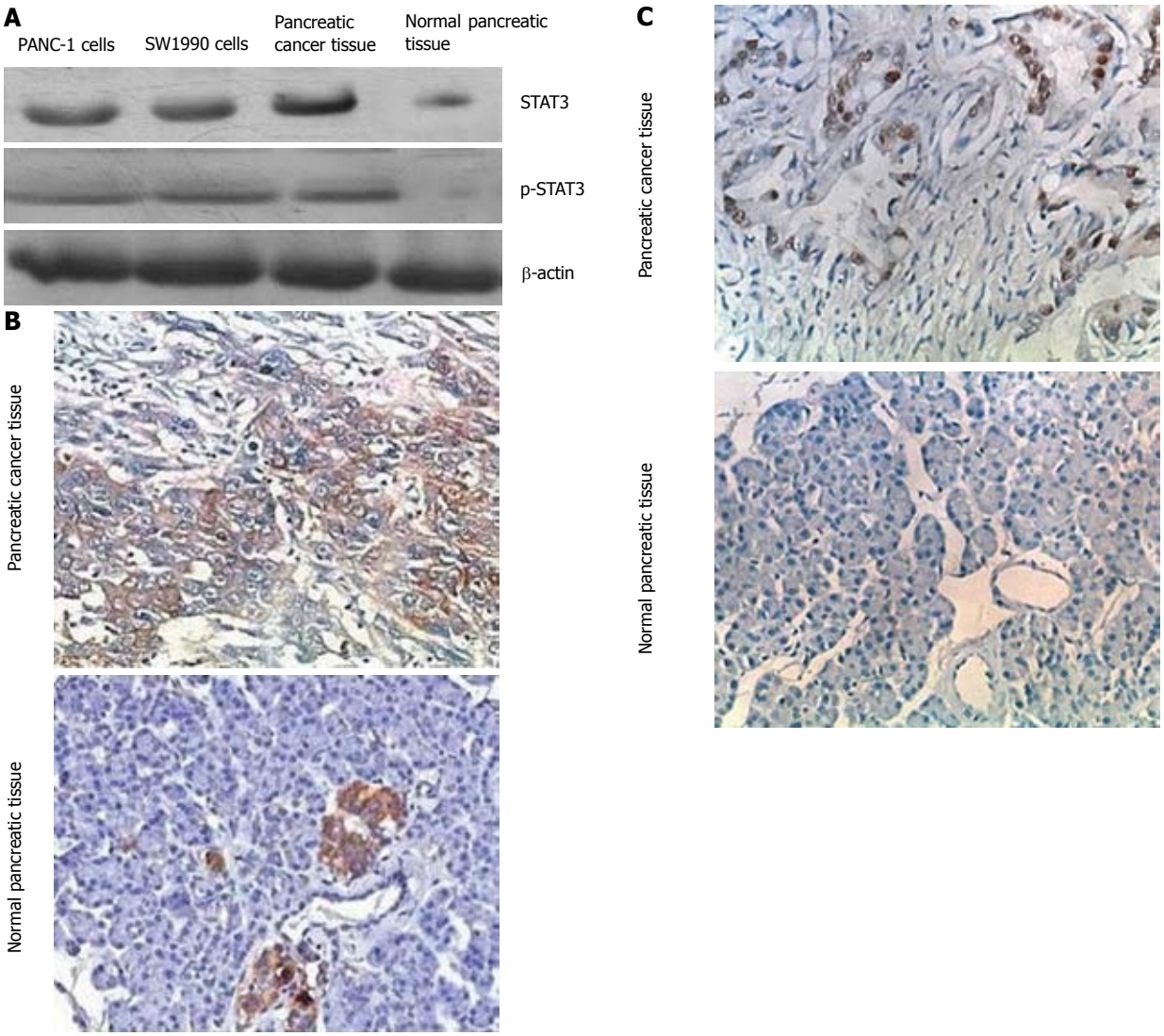

Figure 1 Signal transduction and activation of transcription 3 expression in human pancreatic cancer cells. A: Western blotting analysis of STAT3 and p-STAT3 expression in PANC-1, SW1990, pancreatic cancer, and normal pancreatic tissue with 100 μg total protein for each sample; B: Immunohistochemical 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

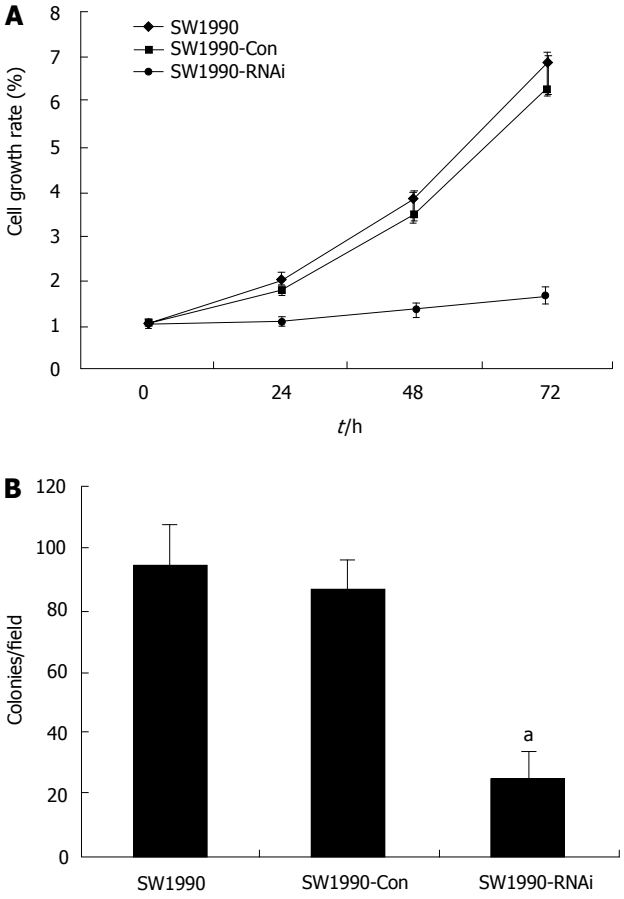

Figure 2 Effects of silence of signal transduction and activation of transcription 3 gene on cell proliferation and anchorage-independent growth of pancreatic cancer cells. A: Cell growth curve of pancreatic cancer cells. SW1990 cells and stably-transfected cells (SW1990-Con and SW1990-RNAi) were subjected 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.

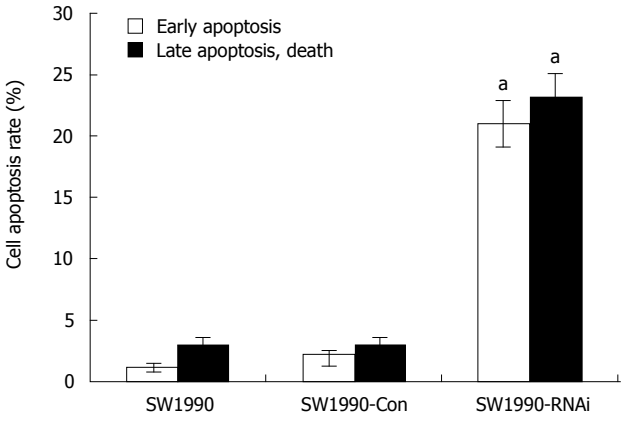

Figure 3 Effects of silence of signal transduction and activation of transcription 3 gene on apoptosis of pancreatic cancer cells. SW1990 cells and stably-transfected cells (SW1990-Con and SW1990-RNAi) were collected to analyze the cell apoptosis with flow cytometry. More apoptotic cells were detected 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

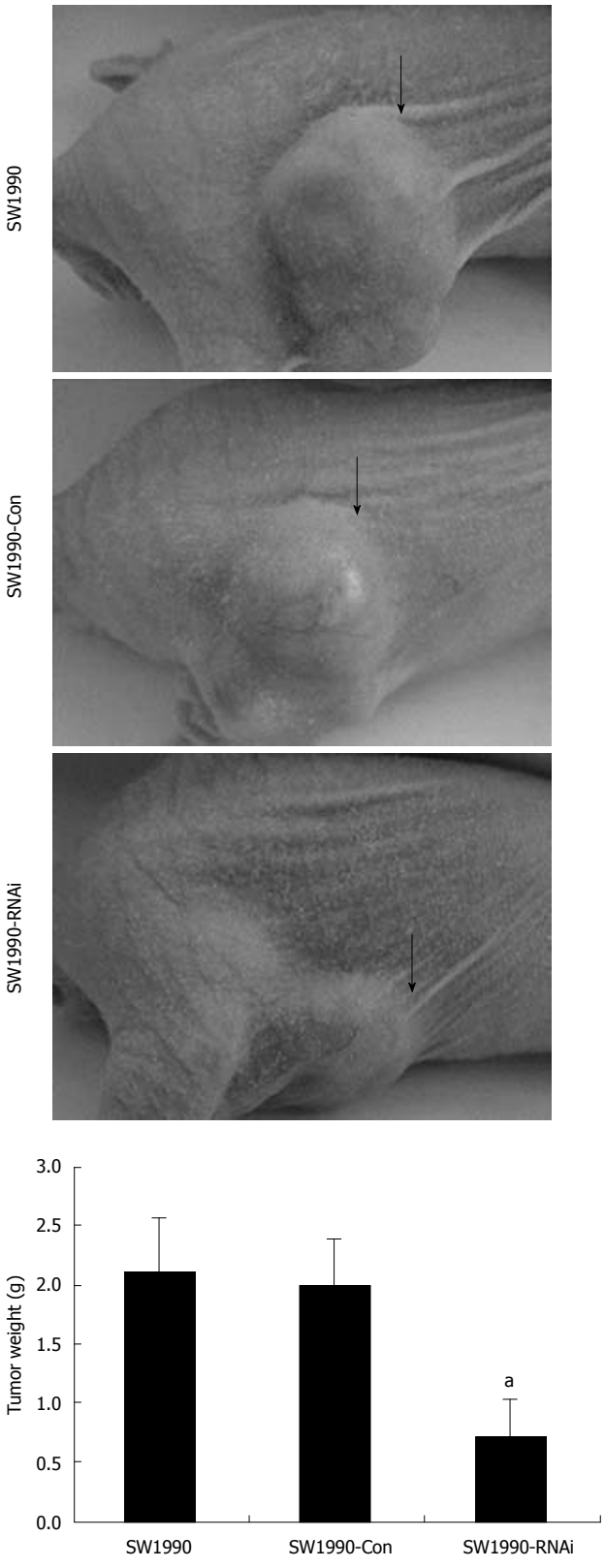

Figure 4 Effects of silence of signal transduction and activation of transcription 3 gene on tumorigenicity *in vivo*. *In vivo* assay was done as described in materials and methods. Six weeks after injection, tumors were harvested and analyzed. Tumors from pRNAT-STAT3-siRNA-Ⅱ–infected SW1990 cells were 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.

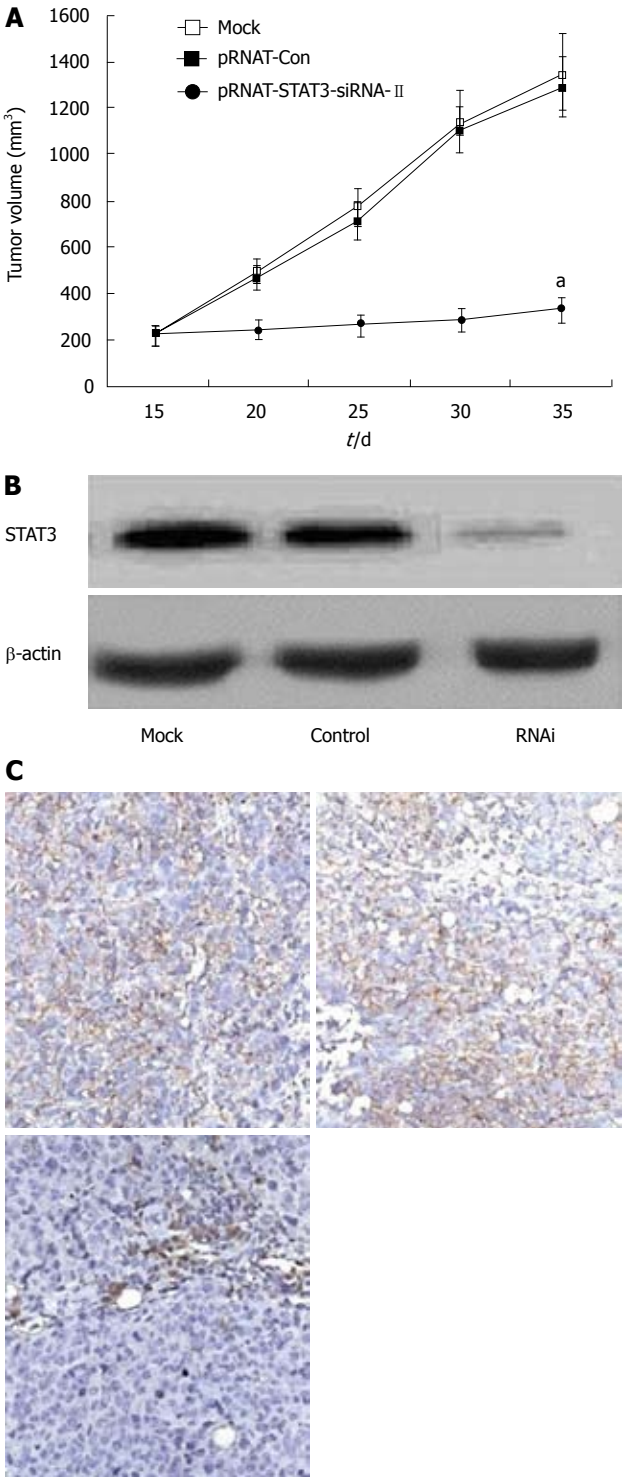

Figure 5 Effects of silence of signal transduction and activation of transcription 3 gene on tumor growth *in vivo*. A: Tumor growth curve after injection with mock, pRNAT-Con, and pRNAT-STAT3-siRNA-Ⅱ cells. Intratumoral electroinjection of pRNAT-STAT3-siRNA-Ⅱ resulted in significant inhibition of tumor 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.

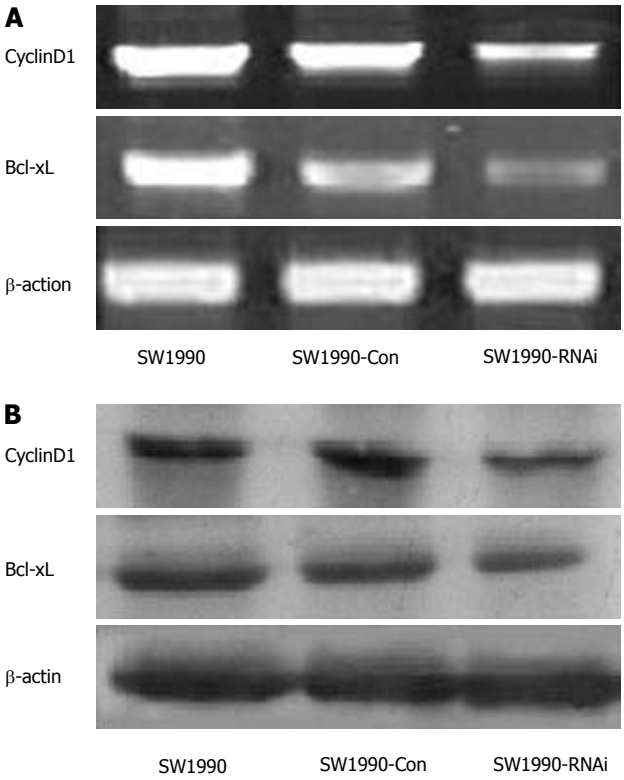

Figure 6 Effects of silence of signal transduction and activation of transcription 3 gene on the expression of CyclinD1 and Bcl-xL. A: Reverse transcription polymerase chain reaction analysis. The RNA samples (2 μg in each) extracted from SW1990 cells, SW1990 cells transfected with a control vector (SW1990-Con), 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.

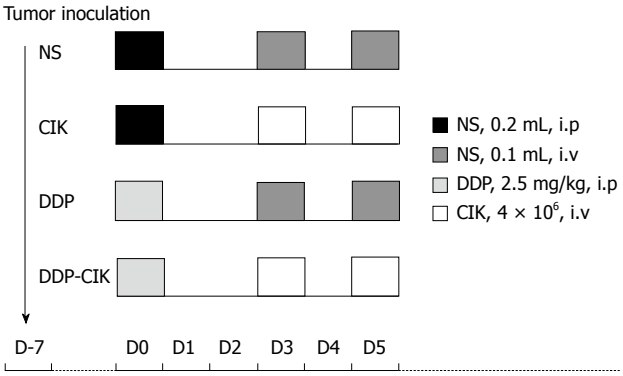

Figure 1 Treatment scheme. BALB/c WT or BALB/c nu/nu mice were injected s.c with 1 × 10^6 CT-26 cells on Day 7 and were treated with the respective regimens according to the treatment scheme. Group cytokine-induced killer cells (CIK) received two i.v infusions of 4 × 10^6 CIK cells at a 1-d interval; 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-

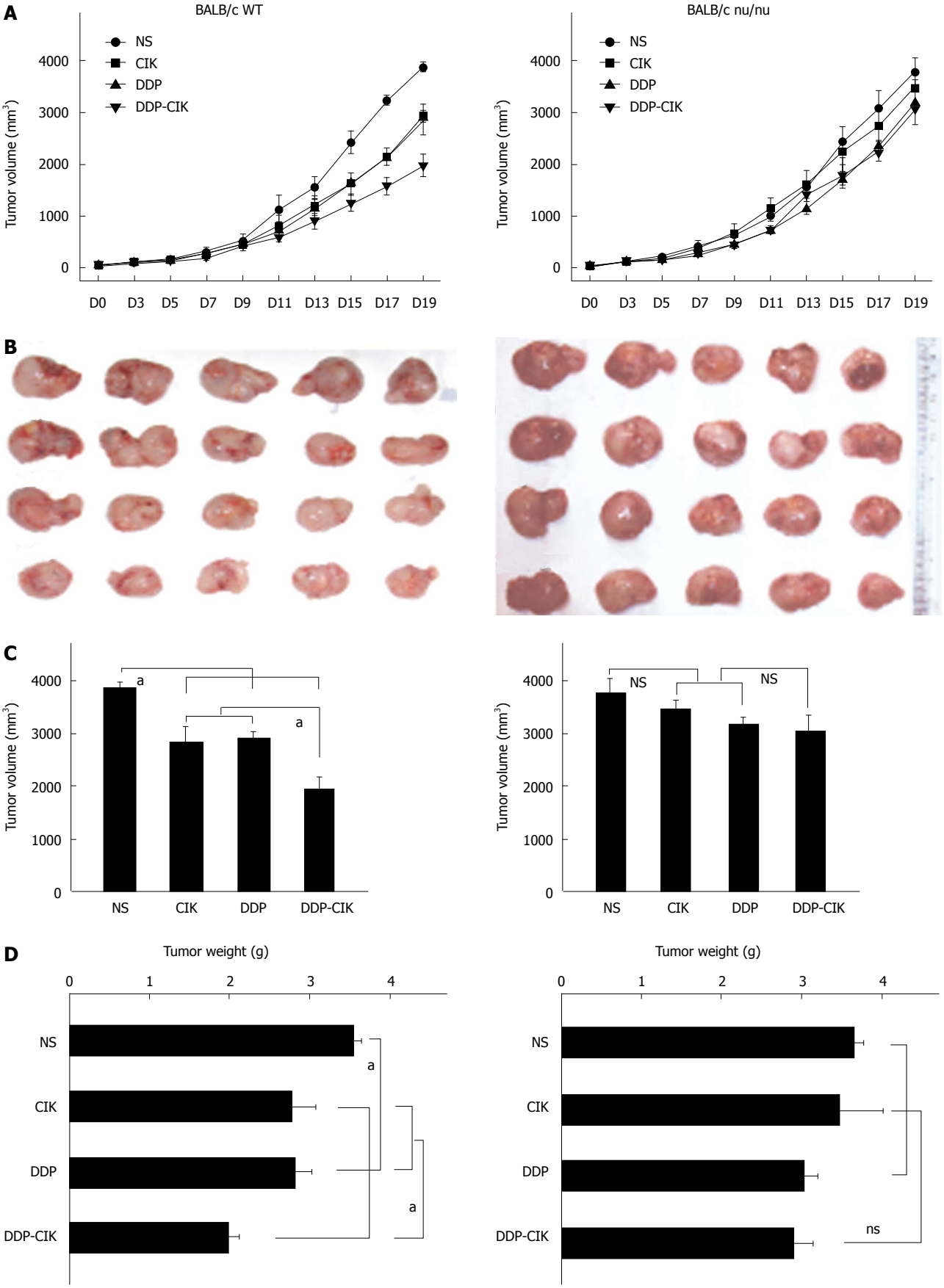

Figure 2 Anti-tumor effect of cisplatin and cytokine-induced killer cells therapy in BALB/c WT and nude mice. BALB/c WT and BALB/C nu/nu mice were inoculated s.c. with 1 × 10^6 CT-26 cells on Day 7 and treated according to the treatment scheme. Tumor size was monitored every other day (A). On Day 19, 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. $^aP < 0.05$.

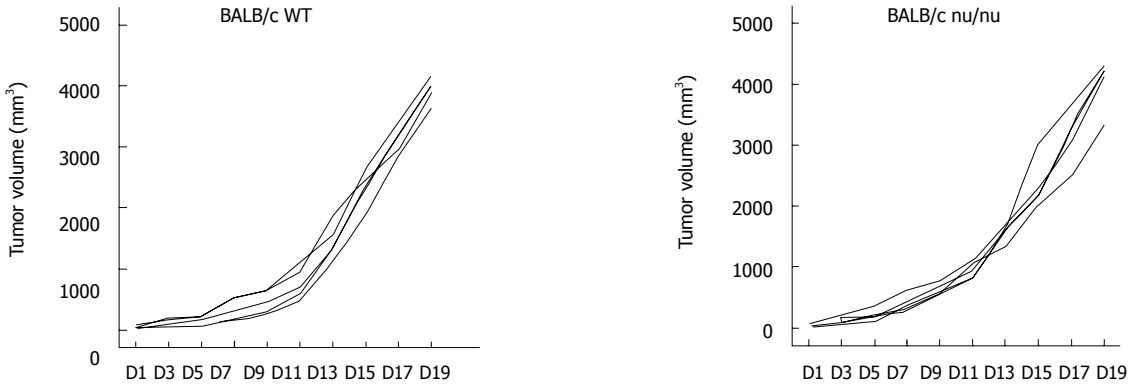

Figure 3 Intrinsic tumor growth pattern of CT-26 carcinomas in untreated BALB/c WT and nude mice. BALB/c WT (wild type) and BALB/c nu/nu mice were inoculated s.c with 1 × 10^6 CT-26 cells on Day 7 and tumor size was monitored every other day. The inherent growth patterns in both strains of mice are shown.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

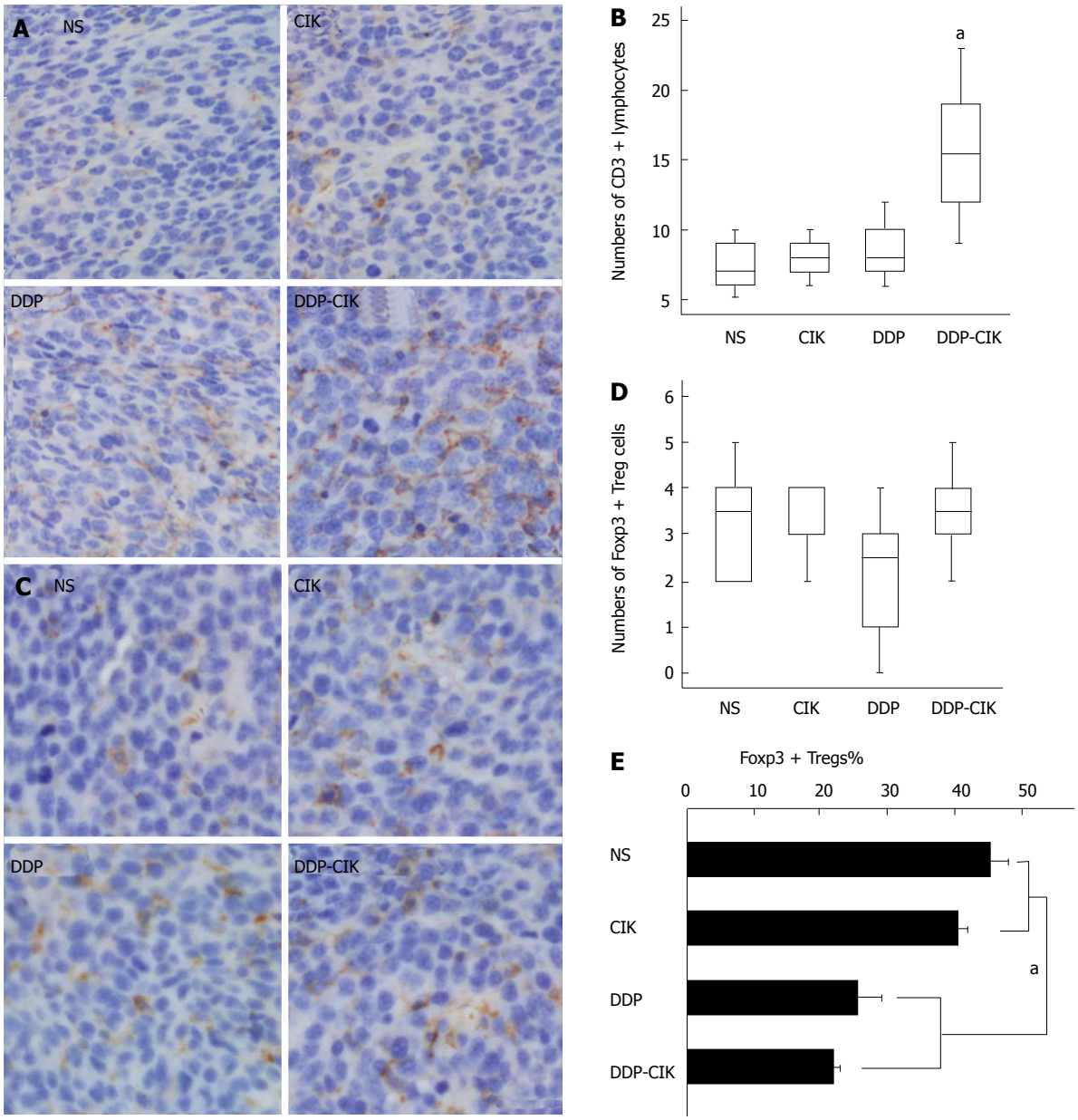

Figure 4 Intratumoral infiltration of lymphocytes after combined therapy. BALB/c WT mice were injected s.c. with 1 × 10^6 CT-26 cells and the treatment protocols were initiated 7 d later. On Day 19, consecutive tumor sections were prepared and analyzed by CD3 (A) and FoxP3 (C) staining. Ten individual 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

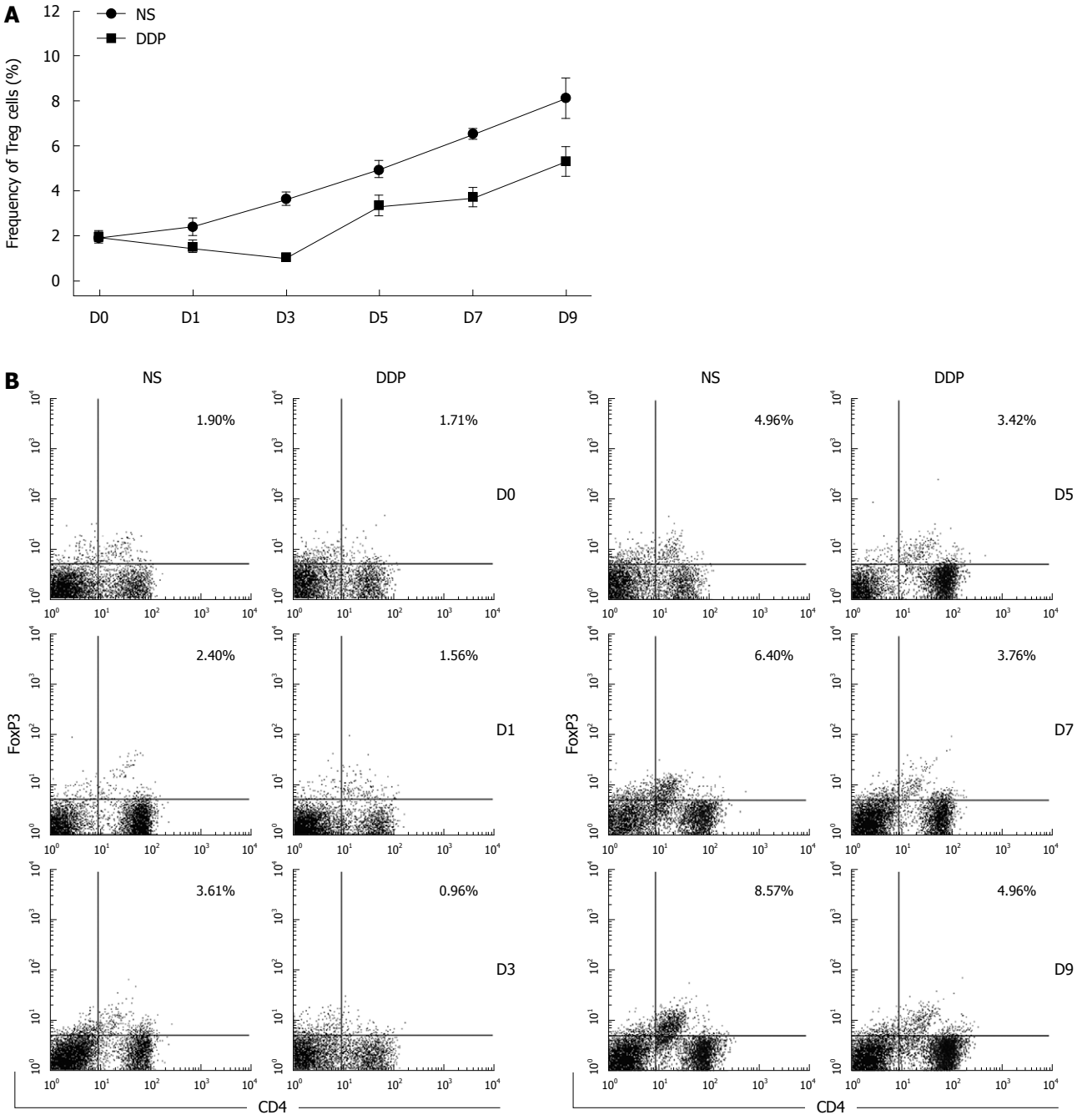

Figure 5 Dynamic changes in the percentage of splenic Tregs after cisplatin (DDP) administration. BALB/c WT mice were injected s.c. with 1 × 10^6 CT-26 cells 7 d before treatment. The tumor-bearing mice were treated with DDP (2.5 mg/kg, i.p., D0) and the spleen single cell suspensions prepared to analyze 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

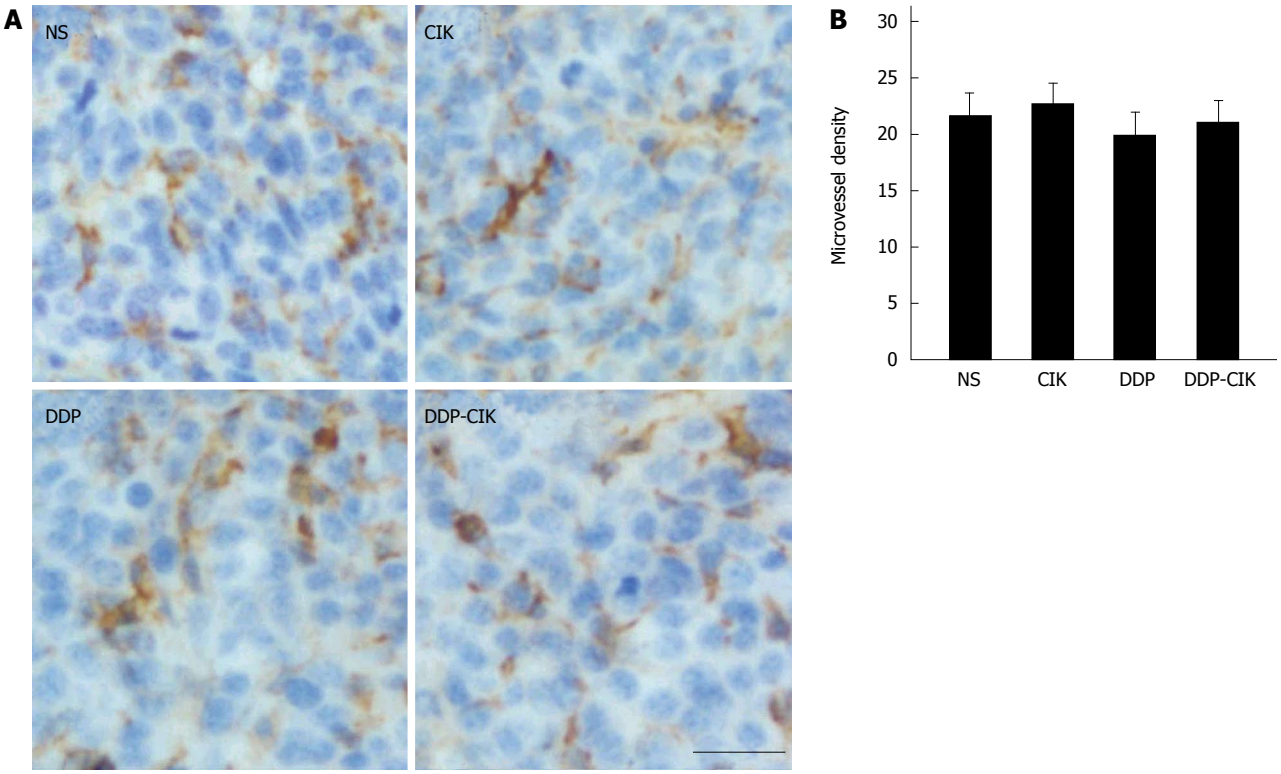

Figure 6 Tumor microvessel density after combination therapy. BALB/c WT mice were injected s.c. with 1 × 10^6 CT-26 cells and the treatment protocols were initiated 7 d later. On day 19, tumor sections were prepared and analyzed by CD31 staining (A). Ten individual fields (0.16 mm^2) at × 400 magnification 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

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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

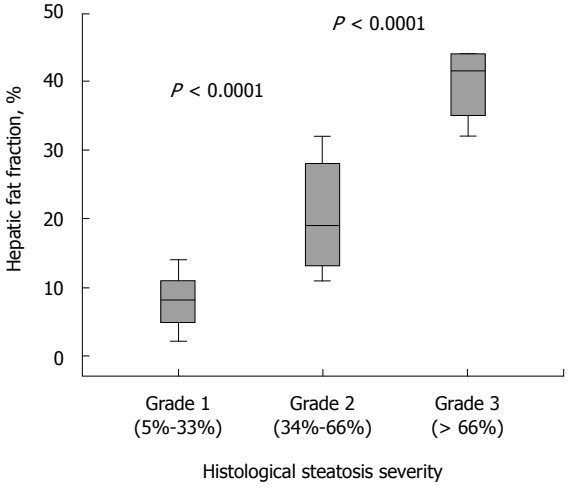

Figure 1 Magnetic resonance imaging hepatic fat fraction according to the histopathological results (grade of steatosis). Boxplots give the median value (black), 25th and 75th percentiles (lower and upper limits of the box), and lower and upper adjacent values (whiskers).

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

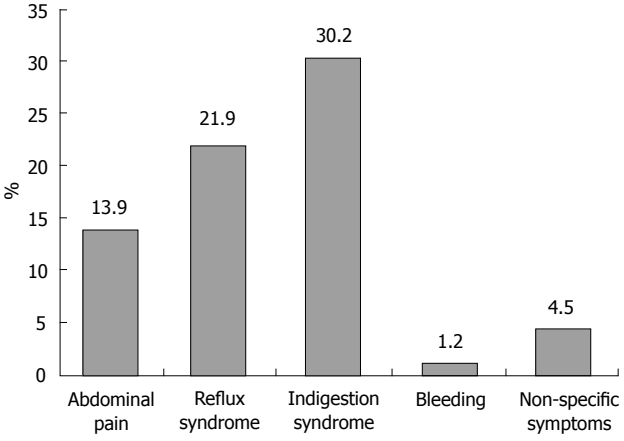

Figure 1 Prevalence of upper gastrointestinal symptoms according to the upper gastro-intestinal symptom questionnaire for the elderly score clusters.

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.

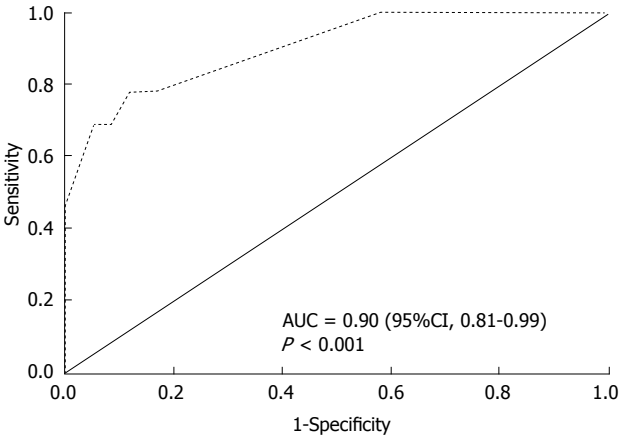

Figure 1 Multivariate model receiver operating characteristics curve. AUC: Area under the curve; CI: Confidence interval.

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

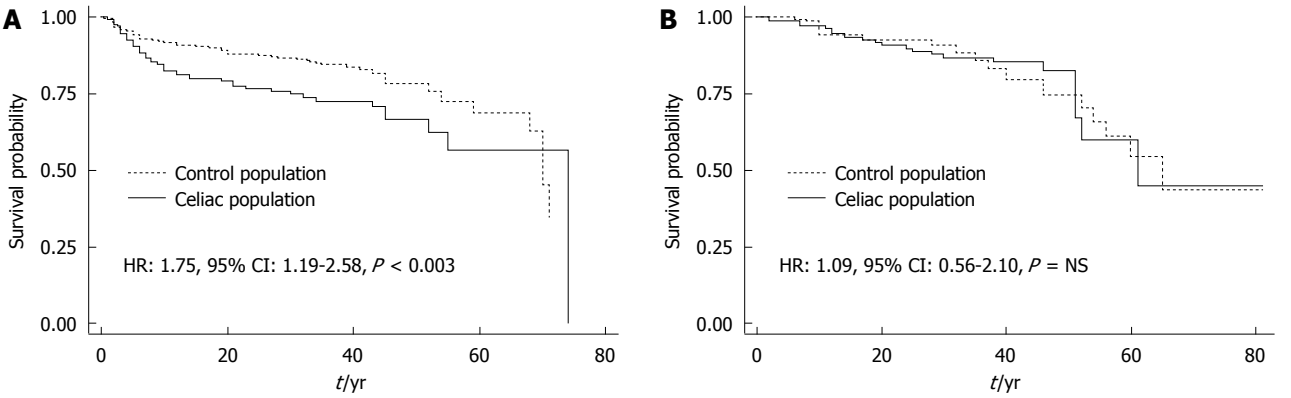

Figure 1 Kaplan-Meier curves of time to first fracture for patients according to clinical presentation in celiac disease patients and matched control population before the time of celiac disease diagnosis. A : Classic presentation celiac disease (CD) patients; B: Atypical/silent presentation CD patients. 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-

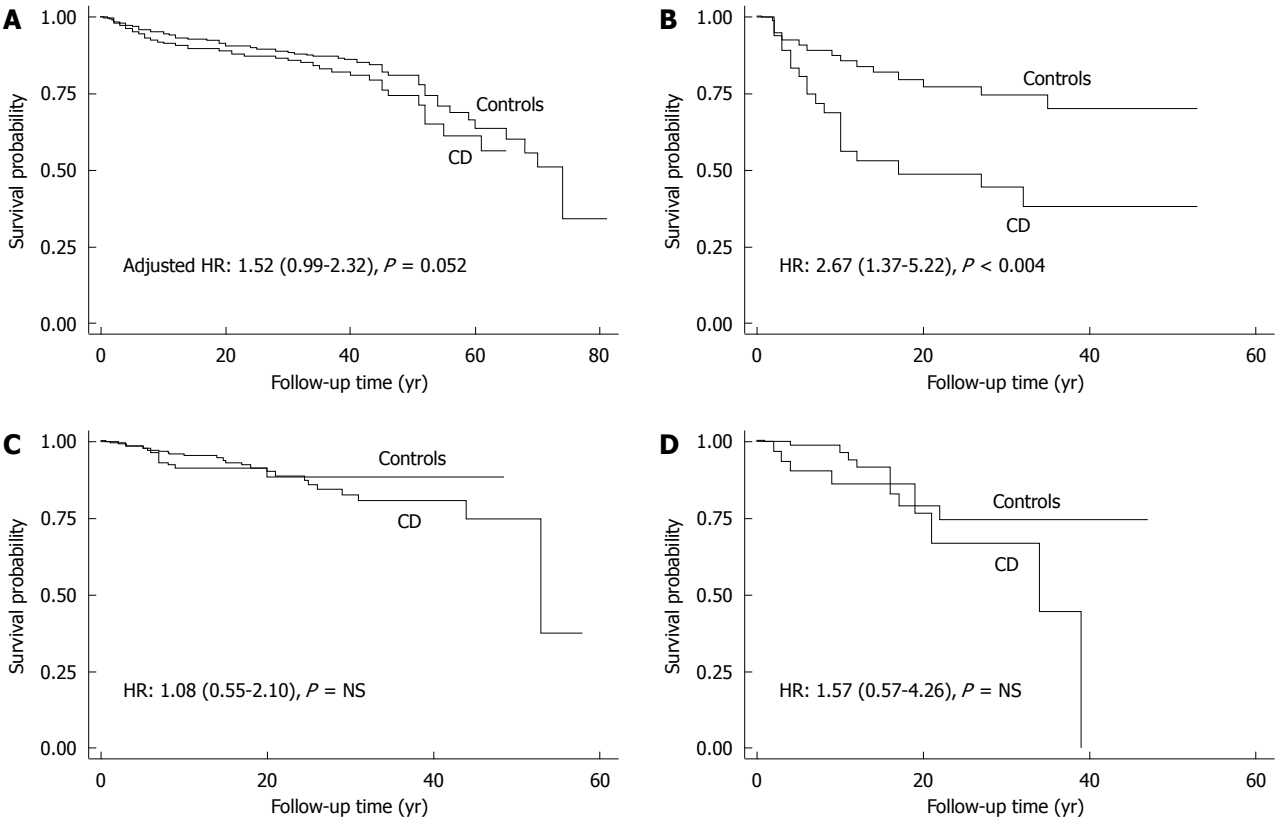

Figure 2 Kaplan-Meier curves showing time of first fracture according to gender in celiac disease population *versus* control group. A: Females before diagnosis; B: Males before diagnosis; C: Females after diagnosis; D: Males after diagnosis. CD: Celiac disease; HR: Hazard ratio; NS: Not significant.

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

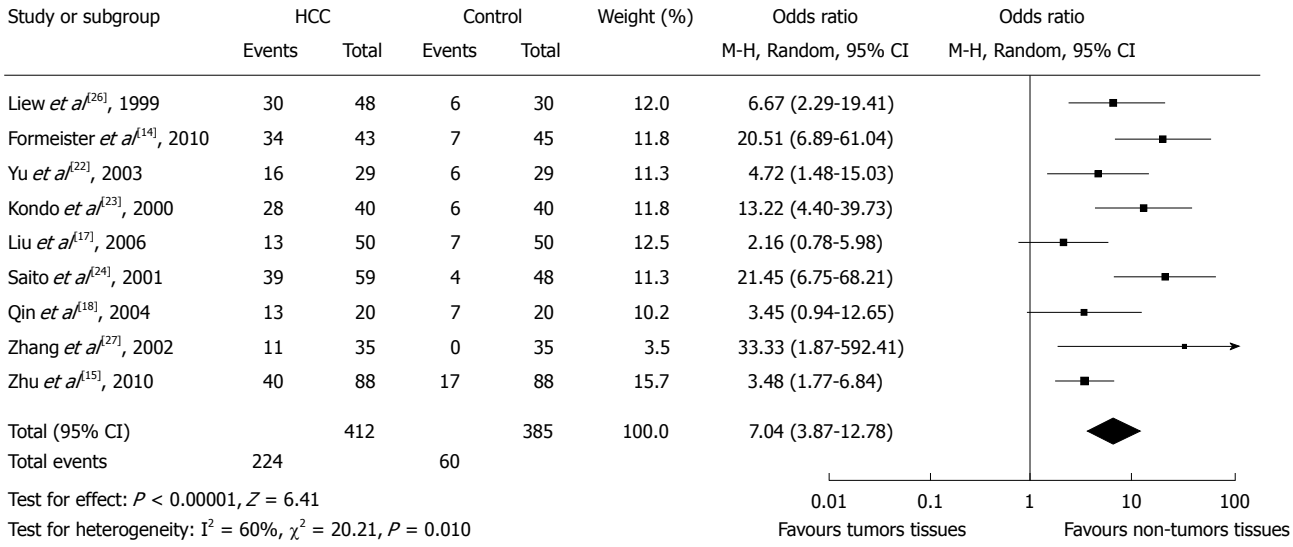

Figure 1 Pooled analysis of p16 hypermethylation in tumorous liver tissues and non-tumorous liver tissues of hepatocellular carcinoma patients. HCC: Hepatocellular carcinoma.

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

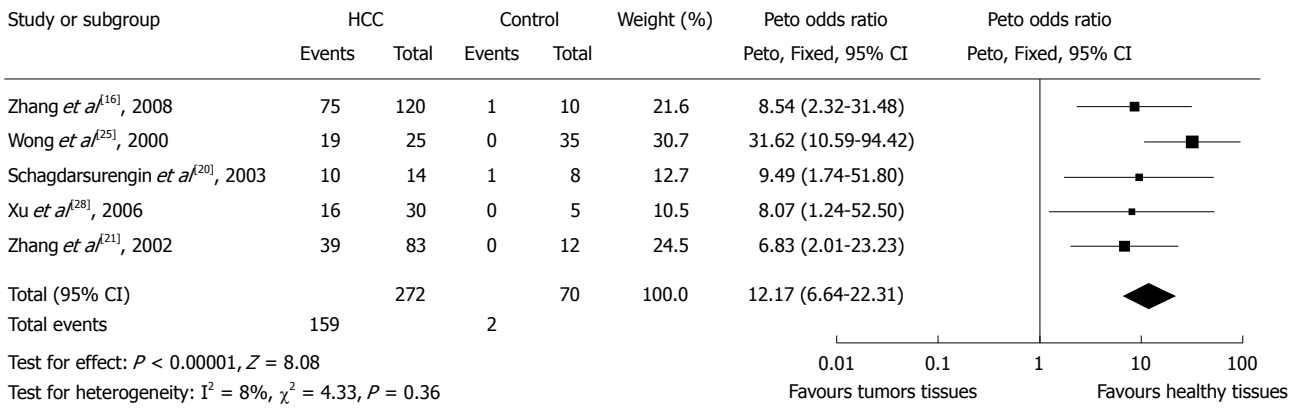

Figure 2 Pooled analysis of p16 hypermethylation in tumorous liver tissues of hepatocellular carcinoma patients and healthy liver tissues of patients with other diseases. HCC: Hepatocellular carcinoma.

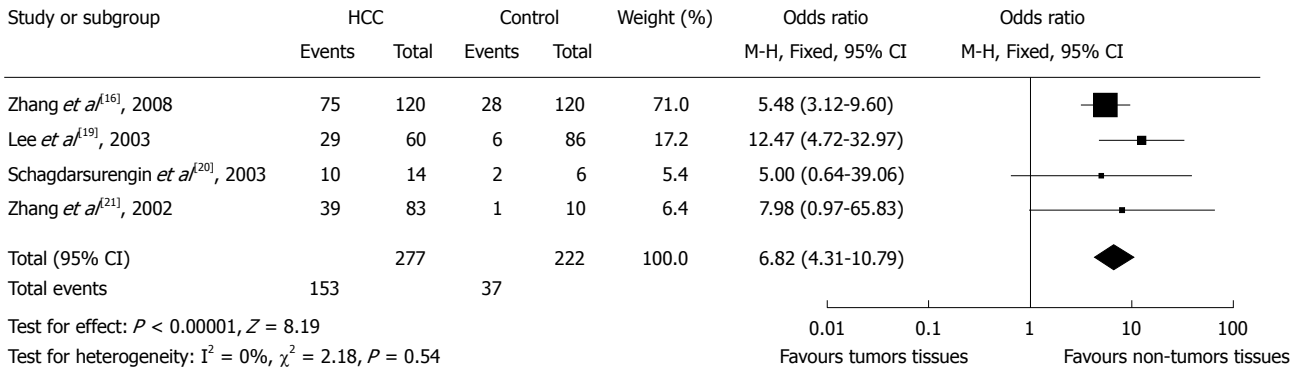

Figure 3 Pooled analysis of p16 hypermethylation in tumorous liver tissues of hepatocellular carcinoma patients and liver tissues of patients with non-tumorous liver diseases. HCC: Hepatocellular carcinoma.

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.

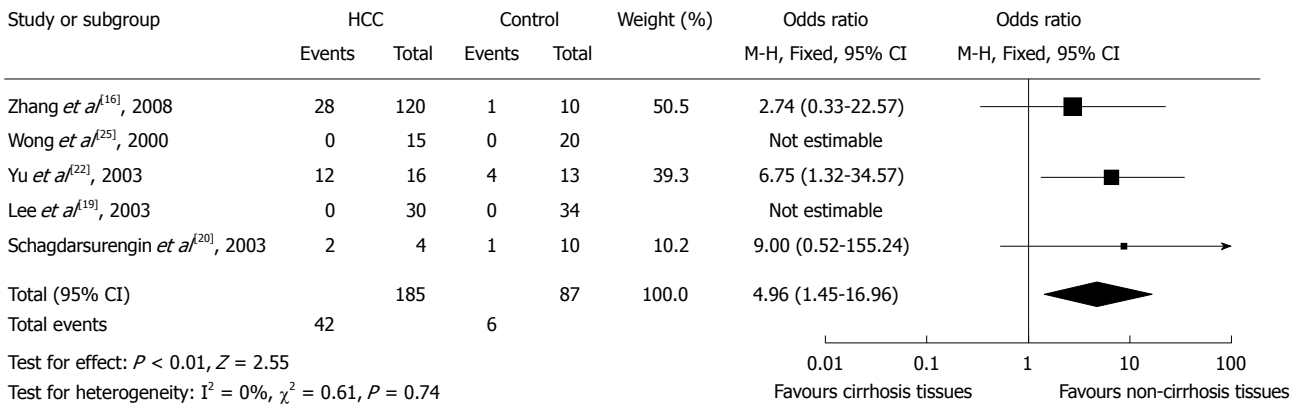

Figure 4 Pooled analysis of p16 hypermethylation in cirrhotic liver tissues and non-cirrhotic liver tissues. HCC: Hepatocellular carcinoma.

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.

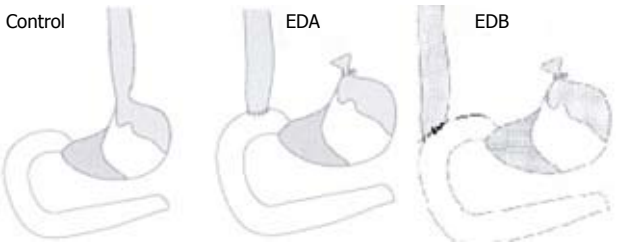

Figure 1 Animal model establishment. Esphagoduodenostomy group A (EDA) for duodeno-gastro-esophageal reflux of low pH value and esphagoduodenostomy group B (EDB) for high pH value respectively, and control for the sham operation group.

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%.

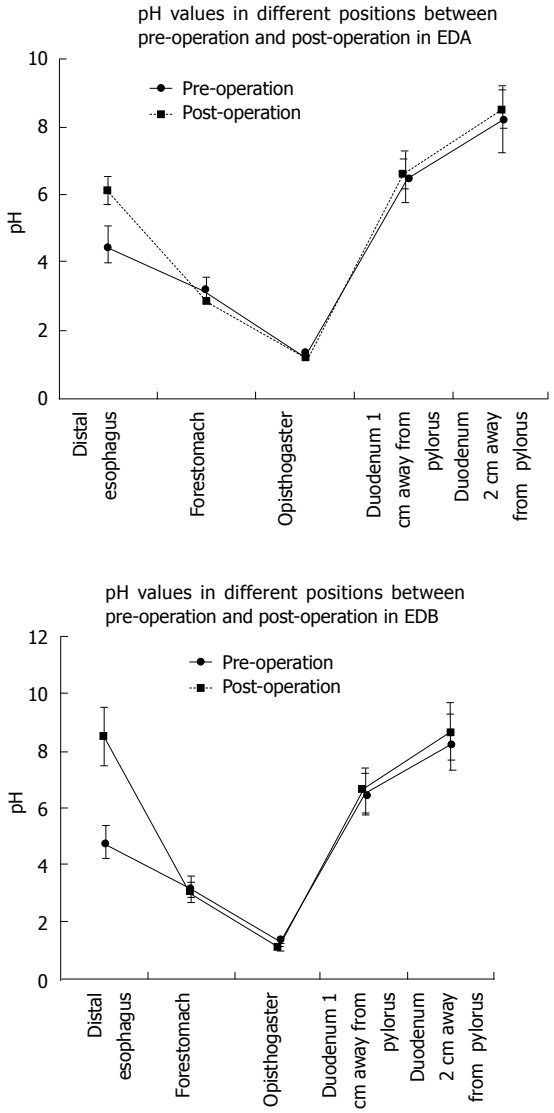

Figure 2 pH in different positions of esophagus, stomach and duodenum.

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

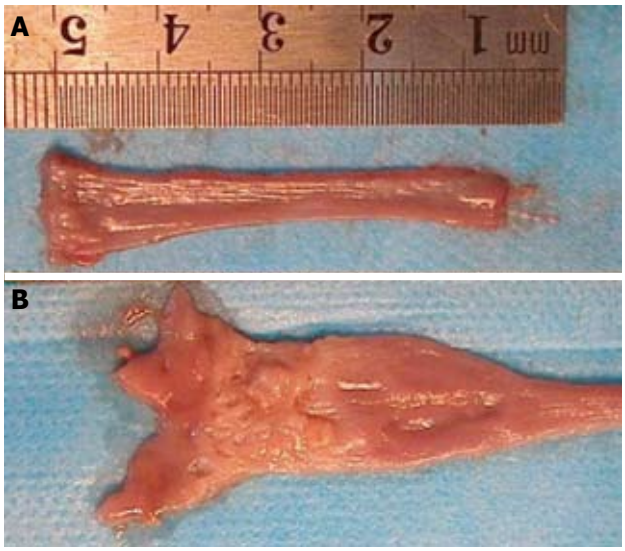

Figure 3 Gross specimens of the sham operated-group(shown in picture A) and animal model group(shown in picture B).

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

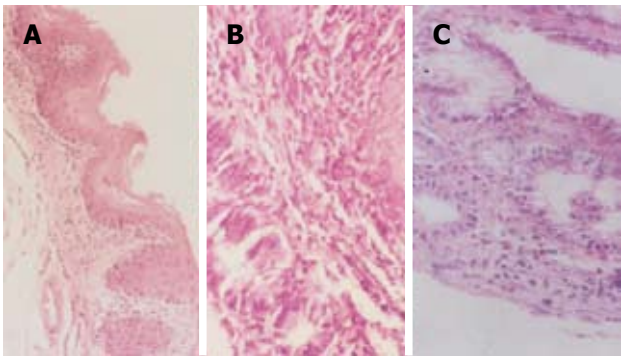

Figure 4 The changes of the esophagus mucosa in the sham operated group and the animal model groups under the light microscope (200 ×). A: Normal esophagus in the sham operated group; B: Esophagitis and barrett esophagus in the model group; C: Intestinal metaplasia with dysplasia and esophageal adenocarcinoma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

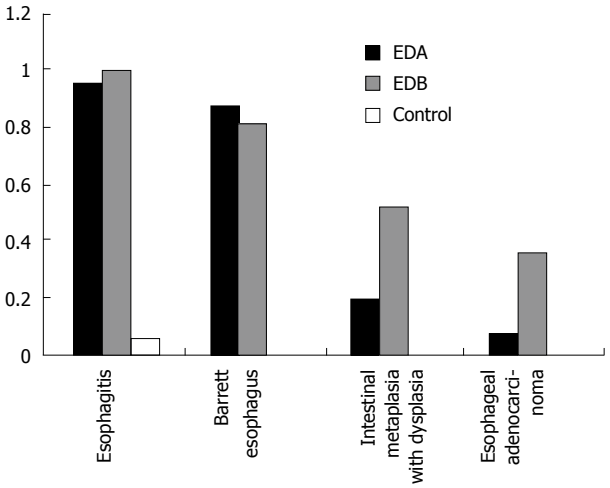

Figure 5 The incidence rates of diseases of esophagitis, Barrett esophagus, intestinal metaplasia with dysplasia and esophageal adenocarcinoma 40 wk after operation. The incidence of esophagitis, barrett esophagus, intestinal metaplasia with dysplasia and esophageal adenocarcinoma 40 wk after operation 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

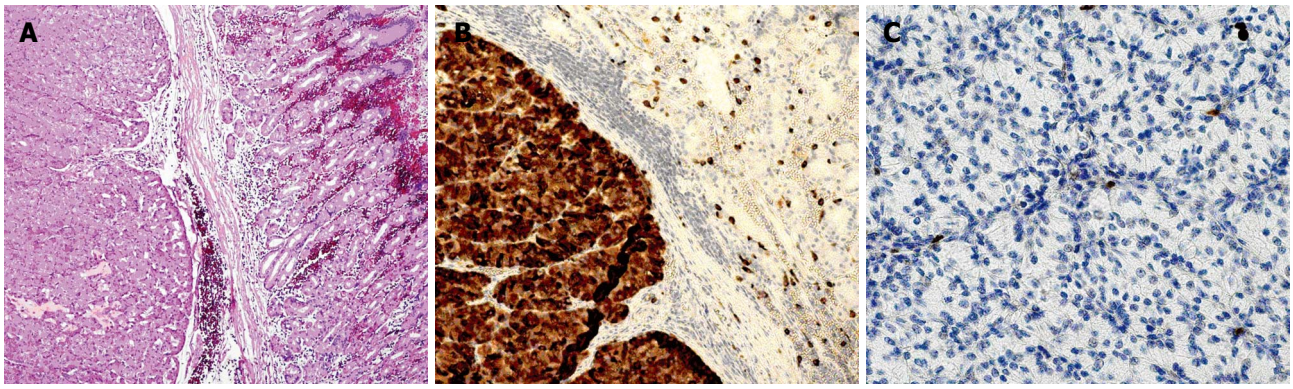

Figure 1 Gastric carcinoid. A: Infiltration of the muscularis mucosae; Hematoxylin-eosin stain. Magnification, × 50; B: Tumor and normal mucosa adjacent to tumor immunostained for VMAT-2. Virtually all tumor cells positive. Magnification, × 100; C: Tumor immunostained for Ki-67. < 1% tumor cells positive.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

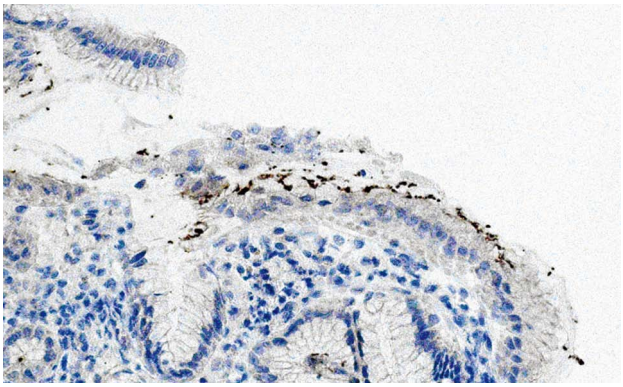

Figure 2 Signs of *Helicobacter pylori* infection in biopsy from antral mucosa. Magnification, × 200.

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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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

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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

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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
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

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22nd European Society of Gastrointestinal and Abdominal Radiology Annual Meeting and Postgraduate Course, Venise, Italy

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4th Congress of the Gastroenterology Association of Bosnia and Herzegovina with international participation, Hotel Holiday Inn, Sarajevo, Bosnia and Herzegovina

June 11-12, 2011
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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
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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

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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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