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Novel image-enhanced endoscopy with i-scan technology

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Abstract

i-scan technology is the newly developed image-enhanced endoscopy technology from PENTAX, Japan. This consists of three types of algorithms: surface enhancement (SE), contrast enhancement (CE), and tone enhancement (TE). SE enhances light-dark contrast by obtaining luminance intensity data for each pixel and applying an algorithm that allows detailed observation of a mucosal surface structure. CE digitally adds blue color in relatively dark areas, by obtaining luminance intensity data for each pixel and applying an algorithm that allows detailed observation of subtle irregularities around the surface. Both enhancement functions work in real time without impairing the original color of the organ, therefore, SE and CE are suitable for screening endoscopy to detect gastrointestinal tumors at an early stage. TE dissects and analyzes the individual RGB components of a normal image. The algorithm then alters the color frequencies of each component and recombines the components to a single, new color image. This is designed to enhance minute mucosal structures and subtle changes in color. TE works in real time and consists of three modes such as TE-g for gastric tumors, TE-c for colonic tumors, and TE-e for esophageal tumors. TE is suitable mainly for detailed examination of the lesions that are detected in a screening endoscopy. i-scan technology leads us to easier detection, diagnosis and treatment of gastrointestinal diseases.

INTRODUCTION

Following recent advances in endoscopic treatment of gastrointestinal tumors, early detection and accurate diagnosis of tumors have been increasing in importance. Under such circumstances, a new endoscopic image enhancement technology i-scan has been developed by PENTAX (HOYA Corporation), Japan. This paper describes the features of this i-scan technology, and shows actual images.

i-SCAN

i-scan technology is classified as a digital contrast method among endoscopic imaging techniques^[1]. i-scan has three modes of image enhancement, i.e. surface enhancement (SE; enhancement of the structure through recognition of the edges); contrast enhancement (CE; enhancement of depressed areas and differences in structure through colored presentation of low density areas); and tone enhancement (TE; enhancement tailored to individual organs through modification of the combination of RGB components for each pixel). For SE and CE, switching

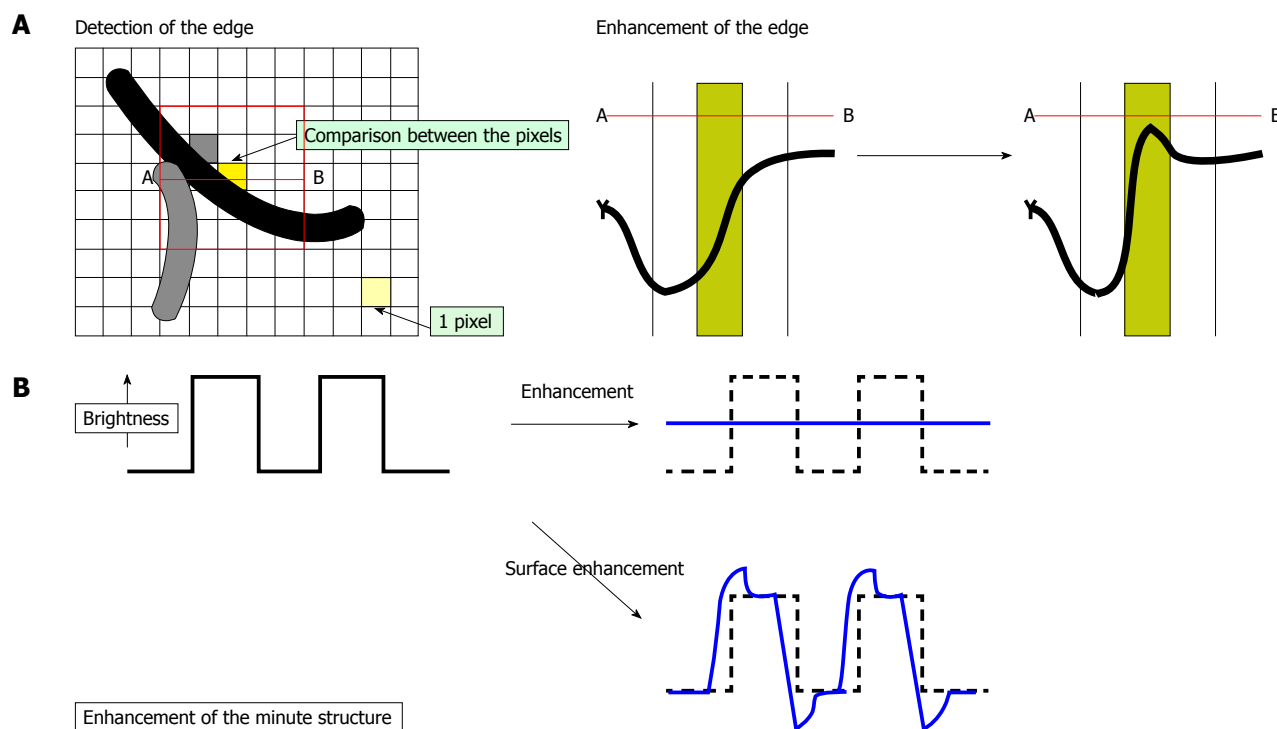


Figure 1 Principle of surface enhancement (SE). A: Enhancement of the edge; B: Enhancement of the minute structure.

among three enhancement levels (low, medium and high) is possible. For TE, switching among three objects (esophagus, stomach and colon) is possible. The three modes (SE, CE and TE) are arranged in series, therefore, it is possible to apply two or more of these three modes at one time. Switching the levels or modes of enhancements can be done on a real-time basis, without any time lag by pushing a relevant button, thus enabling efficient endoscopic observation.

SE

With SE, the difference in luminance intensity between the pixels concerned and the surrounding pixels is analyzed and the edge components are enhanced (Figure 1A). With ordinary enhancement, minor changes in structure are perceived as noise, and the area that shows such changes is smoothed out. With SE, on the other hand, adjustment of the noise erasure function allows more evident enhancement of the edges, which corresponds to minor changes in structure (Figure 1B). As compared to normal images, SE images do not differ in brightness and differ little in color. Furthermore, SE allows more extensive observation of minute glandular structures, which makes it easier to check changes on the basis of structural differences (Figure 2A and B).

CE

With CE, areas lower in luminance intensity compared to surrounding pixels are identified on the basis of pixel-wise luminance intensity data, followed by relative enhancement of the B component through the slight suppression of R and G components in this low luminance area (Figure 3A and B). As a result of CE, the low

luminance area is stained slightly bluish white and minute irregularities on the mucosal surface are enhanced. Even in the case of flat mucosa, the minute glandular structure can be enhanced because the color is changed, which reflects very minute depressions at the gland duct opening. Processing of images with CE does not cause a change in image brightness or a marked change in the color of the images. It causes only slight bluish-white staining of depressed areas (Figure 2C).

TE

With conventional endoscopy, the white reflective rays from the mucosa are caught by the CCD at the tip of the endoscope and are displayed on the monitor. With TE, on the other hand, the RGB components of an ordinary endoscope image are disintegrated into each component (R, G and B), and each component thus isolated is converted independently along the tone curve, followed by a re-synthesis of the three components to yield a reconstructed image (Figure 4A). The tone curve is depicted by plotting input (on the x axis) against output (on the y axis) (Figure 4B). The tone curve can be changed into various forms by modification of the parameters. The tone-curve forms can be divided roughly into S and J types. Figure 4B is an image yielded from modification of the R component alone; this example clearly shows how the image changes as the tone curve is switched from S to J type or vice versa. If the tone curve assumes an S type, the high R-component area is shifted to a further higher range of R to enhance the color tone R, or the low R-component area is shifted to a further lower range of R to elevate the sensitivity to GB components, thus allowing clear enhancement of the differences in

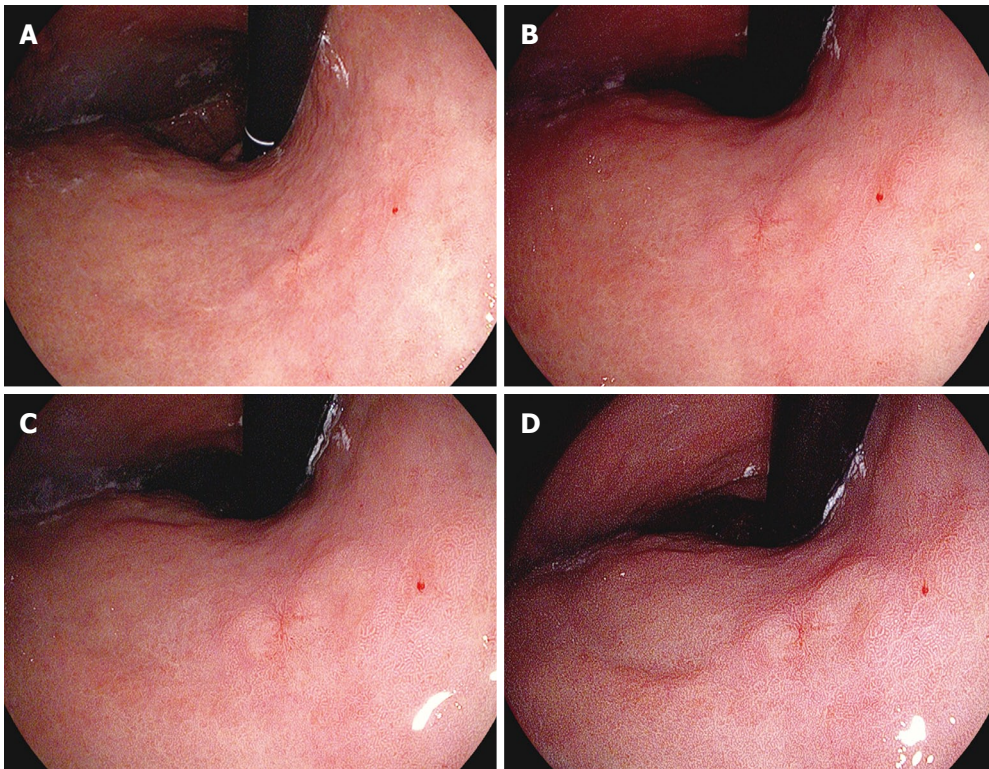


Figure 2 Images using SE and contrast enhancement (CE). A: Conventional image; B: SE image; C: CE image; D: SE + CE image.

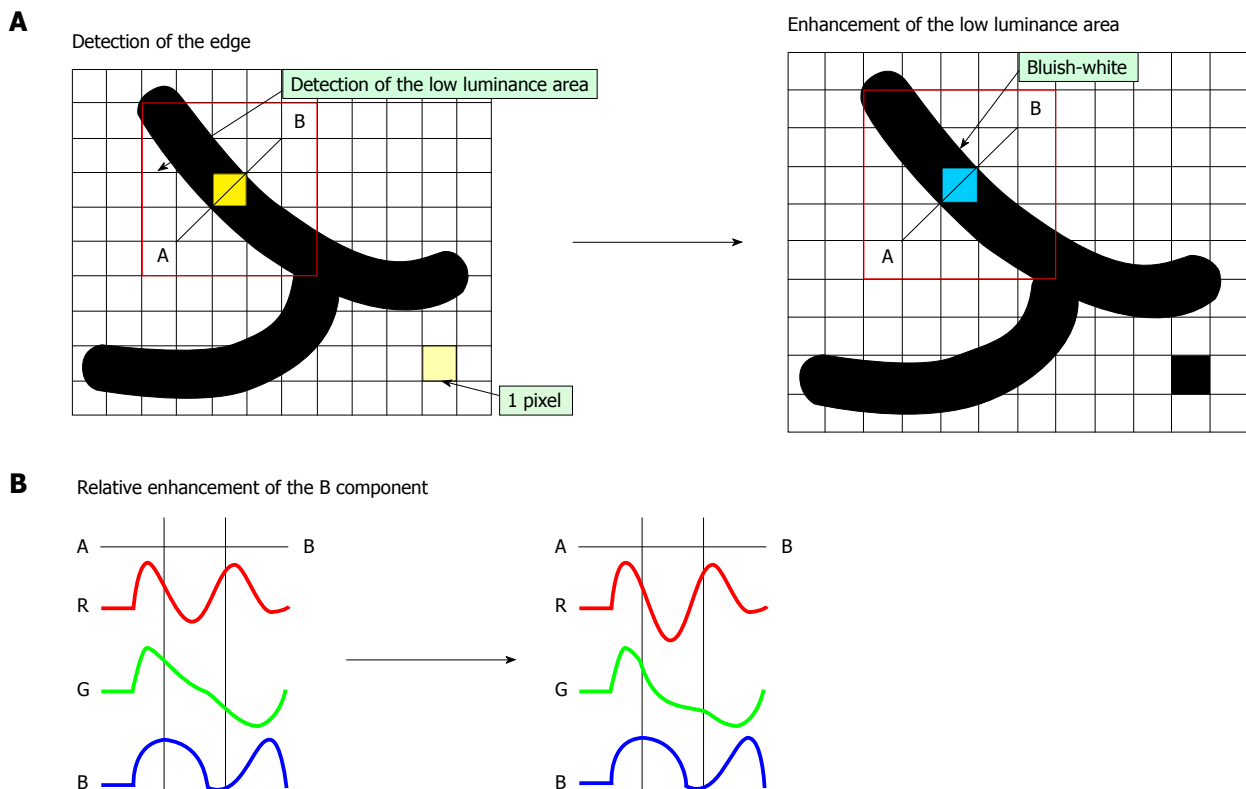


Figure 3 Principle of CE. A: Enhancement of the low luminance area; B: Relative enhancement of the B component.

color tone. If the tone curve assumes a J-type form, the R component is shifted completely to a low R range, to elevate the overall sensitivity to GB components and the brightness/darkness contrast. At present, three types of TE are available, including TE-e (for esophagus), TE-g

(for stomach) and TE-c (for intestines). With TE-e, the J-type tone curve, which suppresses the maximum output, is adopted for the R component, to elevate G/B contrast and make structural changes clearer (Figure 5A). With TE-g, the J-type tone curve, which suppresses the

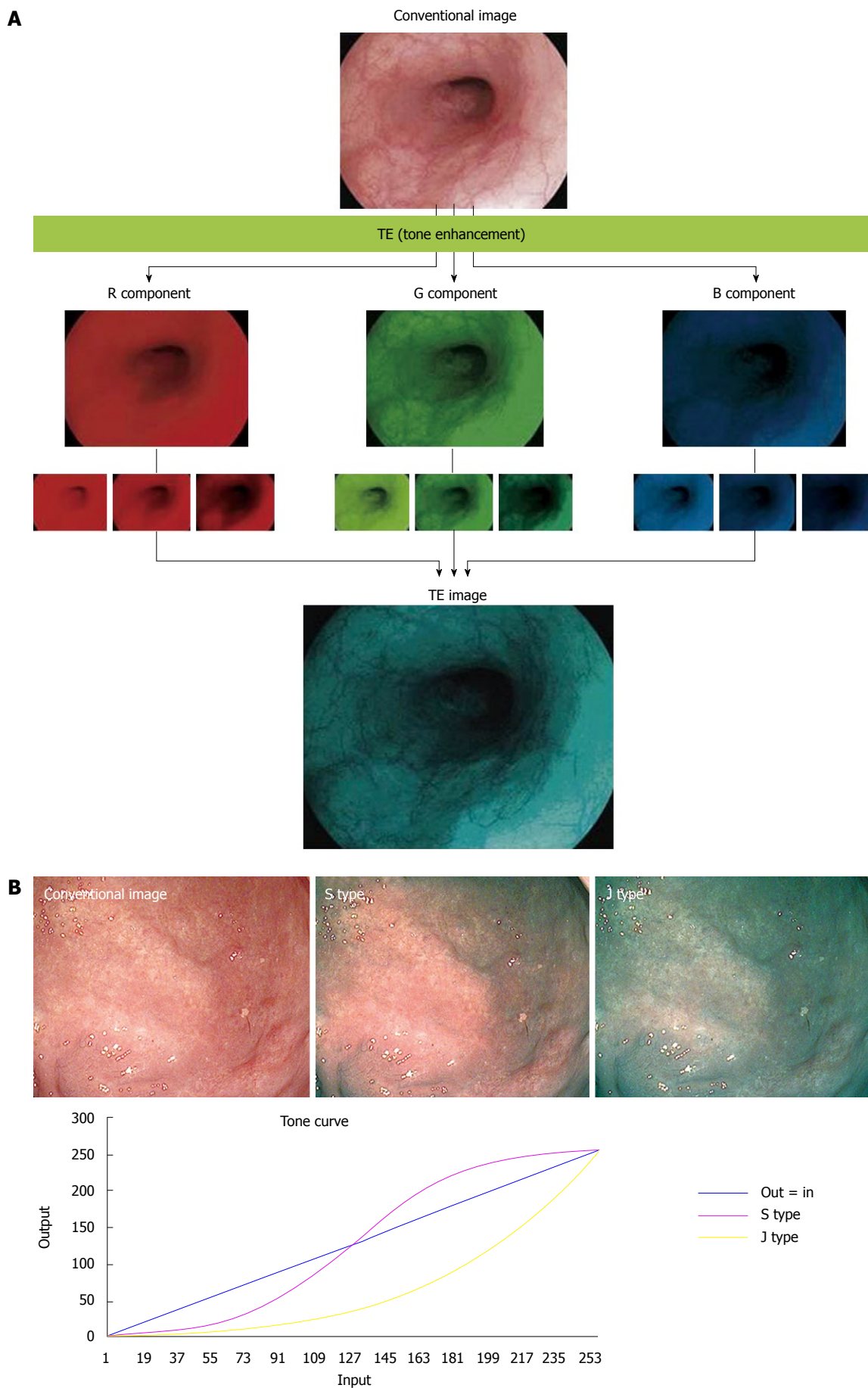


Figure 4 Principle of tone enhancement (A) and tone curve (B). TE: Tone enhancement.

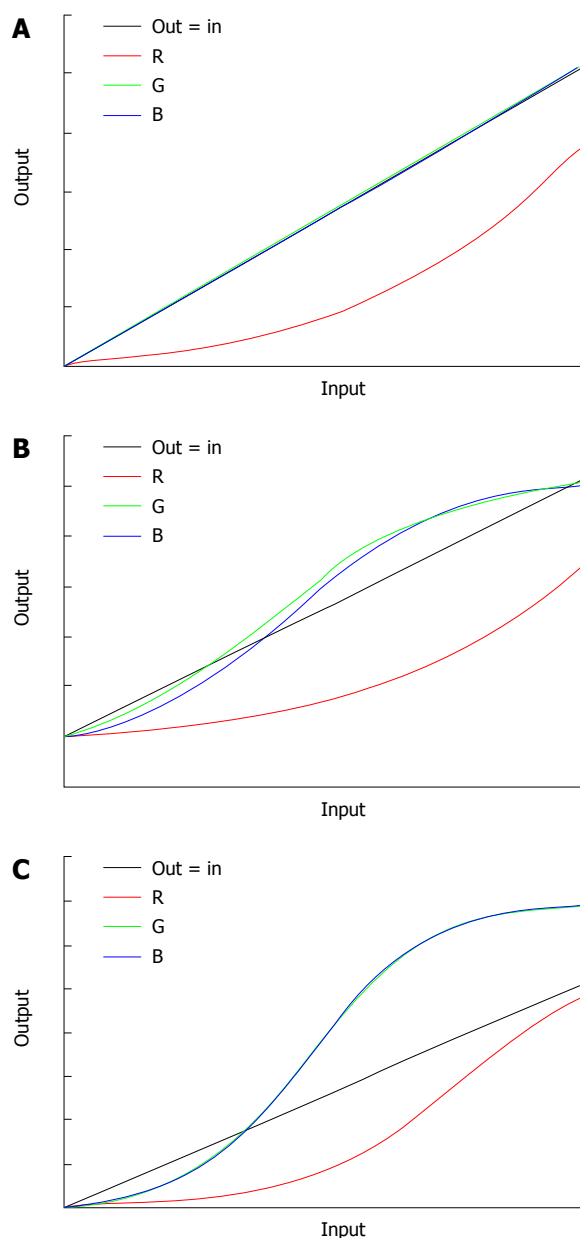


Figure 5 Tone curve. A: TE-e; B: TE-g; C: TE-c.

maximum output, is adopted for the R component to elevate the G/B contrast, and the S-type tone curve is adopted for G and B components to elevate color contrast. In this way, TE-g yields images that allow easy identification of a lesion because it can increase the contrast for even minor differences in color tone (Figure 5B). With TE-c, the J-type tone curve is adopted for the R component, but the output is higher than that with TE-g, which results in a slightly reddish image. The S-type tone curve is adopted for the G and B components with TE-g, and the output is set higher to allow a more evident depiction of the structure of the gland opening in the medium to high range (Figure 5C).

i-SCAN METHOD OF USE

SE and CE are expected to provide a useful means of

screening because they can improve discernment of lesions without altering the color tone markedly and reducing the brightness of images. At our hospital, SE and CE are applied at the same time. Noise increases as enhancement becomes more intense, therefore, we usually apply the lowest levels of SE (low) + CE (low) (Figure 2D). After a lesion is found, TE is applied as a simple means of making changes in the color tone and structure more evident. If the structure or irregularities need to be further enhanced during TE, the level of SE and CE is elevated. TE can be effected quite simply and instantaneously by pushing the button. It may be used to assess the necessity of more detailed testing by means of dye spraying, magnified observation, or biopsy.

USEFULNESS OF i-SCAN

Narrow band imaging (NBI) is an endoscopic imaging technique that emphasizes the mucosal microvasculature and identifies vascular alterations, by placing narrow bandpass filters in front of a conventional white-light source to obtain tissue illumination at selected, narrow wavelength bands^[2,3]. Although NBI is useful for detecting and diagnosing early esophageal cancers with^[4] or without magnifying endoscopy^[5], and identifying the demarcation line and predicting the histological characteristics of gastric cancers with magnifying endoscopy^[6-8], narrow band images are much darker than conventional white-light images, particularly in large luminal diameter regions of the gastrointestinal tract. i-scan images are as bright as conventional white-light images, therefore, i-scan is able to observe much larger areas in a distant view compared with NBI. Moreover, i-scan does not need magnifying endoscopy to observe the demarcation of the lesion. Consequently, i-scan might be more useful for performing screening endoscopy and diagnosing the extent of a lesion, particularly that of a large superficial lesion in the stomach.

On the other hand, chromoendoscopy came into clinical use 40 years ago as a method of identifying lesions and observing them in detail^[9]. In particular, indigo carmine is the dye most frequently used for chromoendoscopy in Japan. Although this method is useful for recognizing a lesion and diagnosing the margin of a lesion more easily than conventional endoscopic observation^[10,11], it may not be time consuming. i-scan can be effected quite simply and instantaneously by pushing a button, therefore, it is an easy method for screening or detailed inspection, and may reduce both time and costs.

i-SCAN CASES

Case 1

On the ordinary image without i-scanning, a slightly irregular mucosal structure was visible on the right wall of the middle thoracic esophagus (Figure 6A). If SE (low) + CE (low) were applied to this image, the irregular mucosal structure could be discerned as an area structurally different from the surrounding normal area, with almost the entire circumference of the boundary

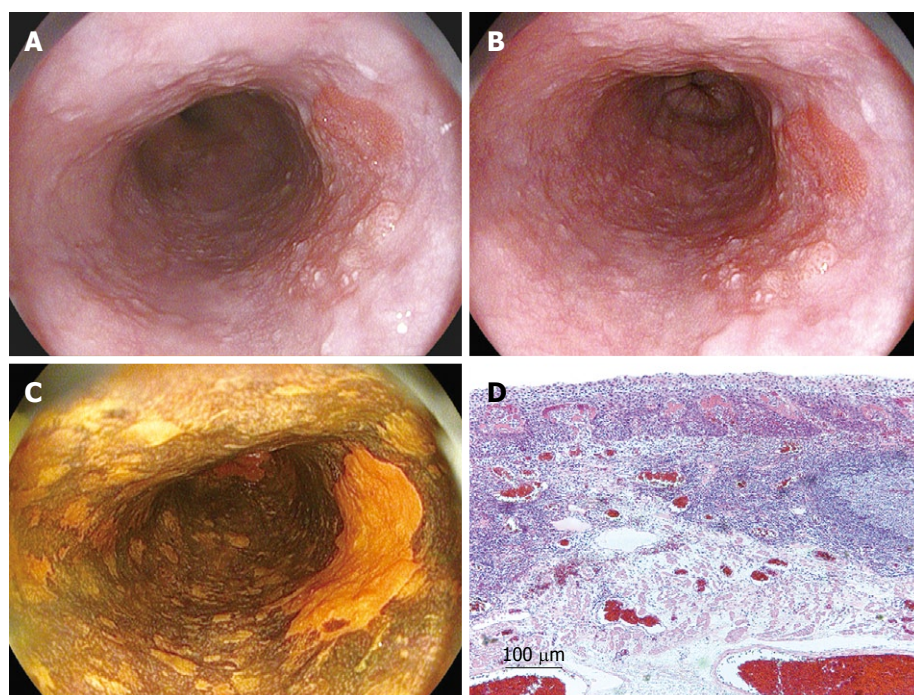


Figure 6 Case 1 (esophageal cancer).

A: Conventional image: A slightly reddish irregular area was recognized in the right wall of the thoracic middle esophagus; B: SE + CE image: As compared with conventional image, the lesion could be recognized as an irregular area with clear boundaries; C and D: TE-e image: As compared with conventional image and SE + CE image, the demarcation line of the lesion could be recognized more clearly as a reddish irregular area. This was pathologically diagnosed as carcinoma *in situ* that corresponded to this region.

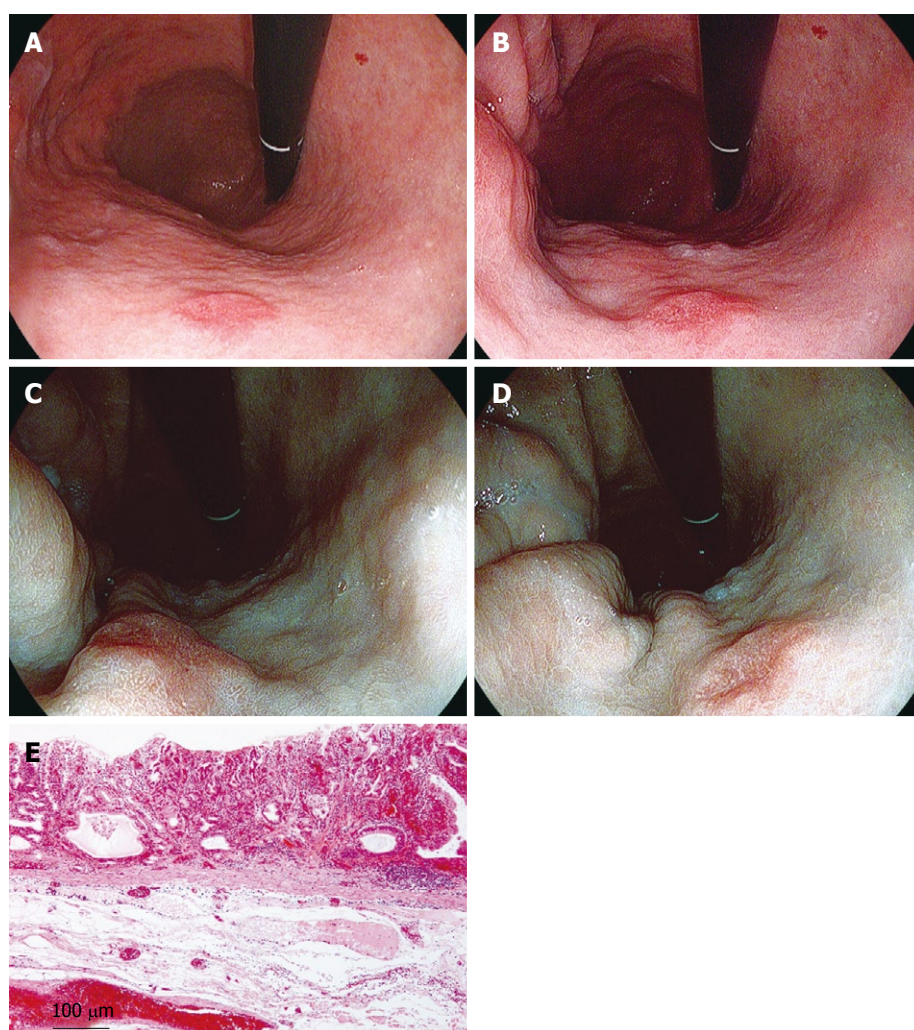


Figure 7 Case 2 (gastric cancer).

A: Conventional image: A red depressed lesions was visible on the anterior wall of the lower gastric body, but it was difficult to identify the demarcation of it; B: SE + CE image: The differences of the mucosal structure and color of the lesion could be observed; C, D: TE image: The demarcation of the lesion could be identified more clearly, observing changes in mucosal structure and color tone; E: Pathological diagnosis indicated a well to moderately differentiated adenocarcinoma, localized in the mucosal layer.

visible (Figure 6B). If TE-e was applied to this area (Figure 6C), the irregular mucosal structure was visible clearly as a

structure differing in color tone from the well-demarcated surrounding area. Endoscopic resection was eventually

performed on this lesion, which was rated as carcinoma *in situ* that corresponded to this region (Figure 6D).

Case 2

On an ordinary image without i-scanning, a red depressed lesion with marginal elevation was visible on the anterior wall of the lower gastric body, although the demarcation of the lesion could not be recognized clearly (Figure 7A). If the same area was observed with SE (low) + CE (low), the differences of the mucosal structure and color of the lesion could be observed, although the boundary was not clear (Figure 7B). If TE-g was applied to this image (Figure 7C and D), the normal mucosa changed color into brown or green, the reddish area remained reddish, and the minute glandular structure was clearly visible. Therefore, the difference in color tone from the normal area became clearer and it was possible to make a full-circumferential assessment of the boundary of a lesion based on a check of changes in mucosal structure and color tone. The lesion was finally resected endoscopically and was rated histopathologically as a well to moderately differentiated adenocarcinoma localized in the mucosal layer (Figure 7E).

CONCLUSION

SE and CE can elevate the capability of discerning lesions during screening. TE allows detailed evaluation of the lesion found during screening. All of these three enhancement modes can be switched on/off instantaneously, which reduces the mental stress of the examiners. In addition, because these techniques can reduce the frequency of tests involving dye spraying and biopsy, they are advantageous also for patients. We believe that i-scan can elevate the quality of endoscopic diagnosis and treatment.

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Inflammatory bowel disease in the dog: Differences and similarities with humans

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Abstract

Inflammatory bowel diseases (IBD) represent important chronic conditions affecting the gastrointestinal tract in man. However, similar disorders are found in several animal species and the IBD affecting dogs are particularly important. These are encompassed by an umbrella of probably several different entities with common symptoms, some of which seem to share striking similarities with human conditions. This review will focus on the actual knowledge of IBD in dogs, and attempt to identify differences and similarities with human IBD conditions.

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Key words: Colitis; Dog; Inflammatory bowel diseases

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INTRODUCTION

Inflammatory bowel diseases (IBD) are well known gastrointestinal disorders in humans; however, these disorders are also present and often investigated in animals^[1]. IBD in man [Crohn's disease (CD) and ulcerative colitis (UC)] can be defined as chronic idiopathic multifactorial diseases^[2,3]. CD is typically a disease of ileum and colon, but can also affect other areas of the digestive tract^[4], forms granulomas and involves the whole intestinal wall, while UC is an ulcerative and inflammatory disease usually limited to superficial layers (mucosa, superficial part of the submucosa) of the colon^[5].

Dogs may also develop IBD^[6], being part of the chronic enteropathies if lasting more than three weeks^[7], and are properly defined when there is histological demonstration of mucosal inflammation and all other possible causes of enteritis/infiltrates have been investigated and excluded^[7-10]. Enteritis is classified depending on which predominant cells infiltrate the intestinal wall and where this infiltration takes place^[11].

Thus, chronic diseases of the small intestine that can be included among IBD are lymphocytic-plasmacytic enteritis (LPE), eosinophilic enteritis and eosinophilic gastro-enteritis (EGE)^[10]. With regard to the large intestine, four main conditions are recognized as IBD in dogs: lymphocytic-plasmacytic colitis, eosinophilic colitis,

histiocytic ulcerative colitis (HUC) (mainly PAS-positive macrophages), and regional granulomatous colitis (mainly PAS-negative macrophages)^[10,12-16]. However, it is worth noting that such diseases may frequently involve both the small and the large intestine, or even include the stomach: lymphocytic-plasmacytic enterocolitis, eosinophilic enterocolitis (EEC) or gastroenterocolitis (EGEC) and granulomatous gastroenteritis^[8,10,13,15].

In dogs, another important chronic enteritis, whose causes have not yet been clearly defined, but have been widely investigated and for which underlying allergic factors have been hypothesized, is the so-called food-responsive diarrhoea (FRD)^[6]. This disease is especially important because it is often included in the differential diagnosis of IBD.

IBD is considered to have the same incidence in males and females, and middle-aged dogs seem to be more affected^[10]. With regard to breed predisposition, this has been suggested for some specific forms of IBD such as immunoproliferative enteropathy in Basenjis, protein-losing enteropathy and associated protein-losing nephropathy in Soft-Coated Wheaten Terriers, and HUC in boxers^[10,11]. However, although HUC displays a higher predisposition in boxers^[17], it has also been described in other breeds of dogs such as French Bulldog, Doberman Pinscher, Mastiff and Alaskan Malamute^[16,18].

PATHOGENESIS

Information in dogs

In dogs, the development of IBD is thought to originate as a consequence of a deregulation of mucosal immunity in predisposed animals^[14]. The loss of tolerance to antigens (food, intestinal bacteria, *etc.*) is one of the most studied mechanisms that could justify the development of chronic intestinal inflammation^[8,11,19]. The immune-mediated basis of the disease can be inferred by the response to the administration of immunomodulant drugs; the presence of increased IgE positive cells in diseased dogs compared to healthy dogs is a further aspect that also suggests the involvement of hypersensitivity reactions in the pathogenesis of canine IBD^[20], as well as the increased concentration of eosinophils and mast cells in many dogs with EGE^[8]. Interruption of the mucosal barrier, independently of the primary cause (bacterial, chemical, *etc.*), can also lead to further antigen exposure, allowing the process to become chronic^[21], and is enforced by decreased apoptosis of lymphocytes, as demonstrated in dogs with IBD compared to control dogs^[22].

Homeostasis inside the digestive tract is maintained by the equilibrium between the reactions to pathogens and to commensal bacteria or other inoffensive luminal antigens (tolerance) that are mediated by different molecules^[23]. The presence of mucosal tolerance to harmless antigens is very important, because depending on its absence the subsequent inflammatory response can be exaggerated and even detrimental. Such tolerance is probably based on the fact that the antigen is presented or not, contextually to other danger signals^[10,24]. The difference between toler-

ance and reaction is also based on pattern recognition receptors (PRRs)^[25], which are able to recognize microflora according to their pathogen-associated molecular patterns or microbe-associated molecular patterns^[26].

Similar to humans, the study of IBD in affected dogs has led to the hypothesis that genetic factors and enteric bacteria can play a pivotal role in the pathogenesis of these disorders, owing to the abnormal intestinal response to commensal microflora^[26]. Once stimulated, PRRs such as Toll-like receptors (TLRs)^[27] start their pro-inflammatory activity, and a recent study showed that three TLRs (2, 4, and 9) stimulated by bacteria are up-regulated in dogs with IBD^[26]. These results are similar to those following the activation of TLR4 which has been demonstrated in humans suffering from IBD^[28]. In these patients, both genetic predisposition and environmental factors are considered important elements in the development of the disease. Moreover, as already well known in humans^[29,30], a recent study showed that in IBD dogs, small intestinal bacteria are different from those found in healthy dogs^[31], strengthening the idea of a correlation between microflora and IBD.

Thus, even in small animals, and similar to that in man, intestinal lymphocyte subset distribution and major histocompatibility complex class II antigens, as well as cytokine gene expression and other markers, have yielded interesting and sometimes overlapping results^[9,32-36]. For instance, one study showed that dogs with IBD display a larger number of IgE positive cells than healthy dogs, in a manner similar to that for interleukin 4 (IL-4) expression in man with IBD^[20]. In addition, the modulation of the expression of intestinal *lamina propria* lymphocytes P glycoprotein (P-gp) seems to play a similar role in both human and dog IBD. In fact, in IBD patients scarcely responsive to steroid treatment, P-gp is highly expressed, and in dogs showing a good response to treatment this protein is modestly represented^[37].

As previously documented in humans, in IBD dogs the investigation of specific subsets of cell populations led to the demonstration of decreased numbers of mast cells (MCs) and of an increase in both CD3+ cells and IgG+ plasma cells^[38].

In veterinary medicine, encouraging results also originate from the study of nuclear receptors (NRs, presumably also involved in the genesis of IBD in man), such as peroxisome proliferator-associated receptor α (PPAR α) and especially of NR target genes [such as multi-drug-resistance gene-1 (*MDR1*)], multiple drug resistance-associated proteins (MRP2), cytochrome P450 (CYP3A12), and phenol-sulfating phenolsulfotransferase (SULT1A1)^[39,40]. In a recent study it was highlighted that MRP2, CYP3A12, SULT1A1, and PPAR α are more expressed in FRD and/or IBD dogs and that *MDR1* can also differentiate between them^[21]. Moreover, in a study on German shepherd dogs with IBD, the mRNA expression of many cytokines such as IL-2, IL-5, IL-12p40, interferon- γ (IFN- γ), tumour necrosis factor- α (TNF- α), and transforming growth factor- β 1 (TGF- β 1) has been shown to be higher in diseased animals compared to controls^[34]; however, it must also be kept in

mind that other studies reconsidered the increased expression of some cytokines in dogs with IBD^[35,36].

Information in humans

IBD in man, particularly CD, probably recognise a genetic predisposition and many genes such as *NOD2/CARD15*, *HLA* and *IL-1-Ra* genes have been investigated and are thought to be implicated in CD development^[41-43]. However, environmental factors^[44,45] and gut microbiota can also be involved in IBD^[46-49], as shown by the fact that antibacterial drugs may help in controlling symptoms^[50-52]. An important point for the perpetuation of damage in human IBD is that once stimulated, lymphocytes tend to accumulate in the *lamina propria* because they seem to be more resistant to apoptosis^[53]. Moreover, an imbalance between proinflammatory and anti-inflammatory cytokines may also play a role^[54].

In man, nuclear factor- κ B (NF- κ B) has also been associated with IBD^[55], and it has been shown that IL-23 can lead to the differentiation of Th17 lymphocytes producing IL-17 with subsequent activation of NF- κ B pro-inflammatory signals^[56]. In a recent study, the link between IBD and NF- κ B has also been hypothesized in dogs, where biopsy samples from dogs with IBD showed that the presence of NF- κ B activation in *lamina propria* macrophages was higher than in the control group^[23].

Differences between species

An important development in human medicine has shown the different involvement of lymphocyte subtypes (Th1 and Th2) in IBD, with Th1 being mainly implicated in CD while Th2 predominate in UC^[57], with subsequent different cytokine activation^[23]. In dogs, different to man, there is mixed activation of Th1 and Th2 in IBD^[6,23,38], leading to different expression of some cytokines, even though it has recently been hypothesized that different Th cells can be involved in different IBD types^[8]. Some authors have shown that MC can be increased or decreased, depending on the predominant cellular infiltrate, and that these are predominantly reduced in LPE, and increased in EGE, thus hypothesizing a possible different genesis for the two types of IBD (Th1 for LPE and Th2 for EGE)^[8].

Even though the above studies signify an active interest in IBD pathogenesis, it is also evident that the mechanisms behind the disease are far from understood, both in man and dogs. HUC, a peculiar condition, in which a central role for bacteria has been proposed due to the presence of intralesional microbes and PAS + macrophages, similar to Whipple's disease in man^[7].

CLINICAL AND DIAGNOSTIC ASPECTS

Clinical manifestations of IBD in dogs are numerous and nonspecific; the most common clinical signs are weight loss, persistent or recurrent vomiting and/or diarrhoea^[8], frequently associated with symptoms that are an expression of eventual complications, such as ascites (if hypoalbuminemia is present) or pallor of mucous membranes (in the case of chronic gastrointestinal bleeding)^[11,58].

Before reaching a diagnosis of IBD it is important to exclude all other possible causes of chronic enteritis^[6,59] by complete clinical examination, laboratory tests and instrumental investigations, including biopsy samples for histological assessment. Diet correction is also an important tool to exclude or eventually confirm FRD^[23].

Since in dogs the diagnosis of IBD is by exclusion, it is obvious that many tests performed during the diagnostic *iter* (for example blood, urine and faeces examinations) are necessary to exclude other causes of inflammation, and are rarely specific for IBD, thus, not overestimating the incidence of such a diagnosis^[10]. This aspect is very important, because if the cause of the chronic enteritis is misdiagnosed and it is treated as an IBD, it is unlikely to resolve^[58].

In diagnosing IBD, instrumental diagnostics are important and often of paramount importance, even though of the three most used techniques, i.e. radiology (X-R), ultrasonography (US) and endoscopy, only the latter yields more specific information for the diagnosis of IBD (Figure 1), especially as it allows biopsy samples, which are indispensable to distinguish the various subtypes of mucosal infiltration^[60]. X-R and US (which give important information on gut layering and wall thickness) seem to be more helpful for the exclusion of other possible causes^[61], and because the importance of intestinal wall thickening in dogs with IBD has recently been reevaluated^[10,62,63].

After having excluded the most common causes of chronic enteropathies, intestinal biopsies, obtained surgically or endoscopically depending on circumstances (endoscopy is less invasive, but sampling is limited in terms of site of execution and/or of dimension), can allow the diagnosis of IBD^[10] (Figure 2). However, it is important to stress that biopsy samples are not unequivocally interpretable^[6,10,64], even though recent work helped in clarifying such interpretation by providing a histopathological score for mucosal changes in dogs^[65].

In humans, to diagnose and/or to monitor IBD, several serological markers such as anti-neutrophil cytoplasmic antibody (p-ANCA), anti-*Saccharomyces cerevisiae* (ASCA), outer membrane porin C antibody (anti-OmpC), anti-laminaribioside antibody, anti-mannobioside antibody, and anti-chitobioside antibody, anti-pancreas Ab and anti-caliciform intestinal cells Ab, faecal calprotectin, lactoferrin and polymorphonuclear neutrophil elastase are available and have been tested, although not all of these are employed in clinical practice^[66-68].

Together with the clinicopathological diagnosis/monitoring of IBD, in veterinary medicine several markers such as p-ANCA and ASCA, have also been investigated for this purpose. However, even if these were indicative of IBD^[69], no correlation with symptoms or pathological aspects was documented for p-ANCA^[19]. Conversely, in dogs with IBD mucosal permeability measured through the administration of lactulose and rhamnose has been correlated to the histological gravity of the lesions^[70]. The serum concentrations of folate and cobalamin have also been investigated, and although

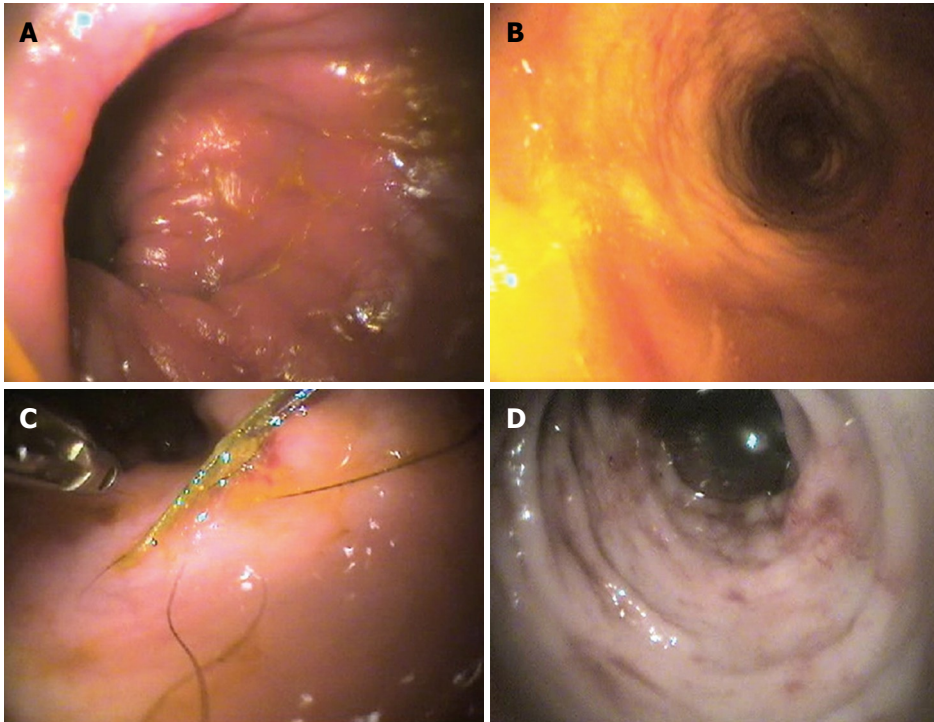


Figure 1 Representative endoscopic images in the dog. A: Lymphocytic-plasmacytic colitis; B: Macrofollicular and diffuse interstitial lymphocytic colitis; C: Histiocytic colitis; D: Neutrophilic-eosinophilic colitis.

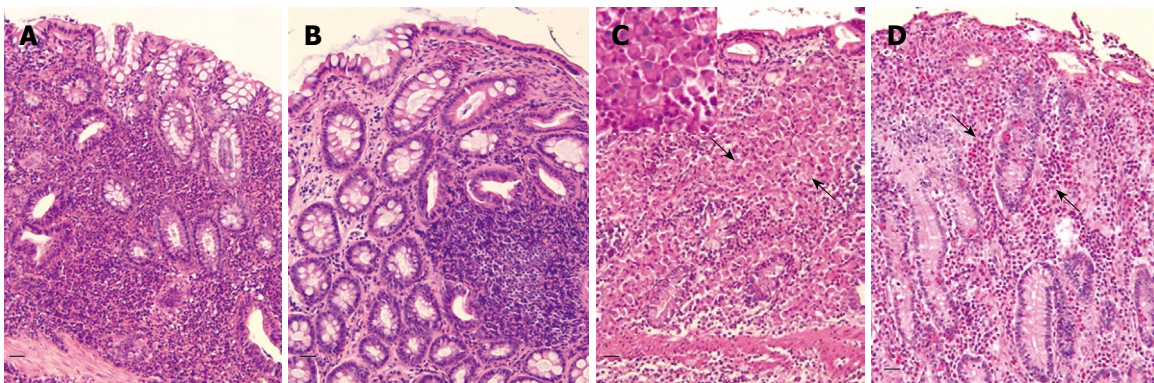


Figure 2 Representative histologic images in the dog (HE, bar = 50 μ m). A: Lymphocytic-plasmacytic colitis. Note the interstitial diffuse pattern of infiltrate represented by a large amount of lymphocytes mixed with plasma cells and some macrophages; B: Lymphocytic-plasmacytic colitis (follicular variant); C: Histiocytic colitis. Severe mucosal abnormalities with loss of crypts and diffuse infiltration by large macrophages (arrows) that in the insert (PAS stain) are shown as the main cells infiltrating the lamina propria; D: Eosinophilic colitis. Note the presence of a large number of eosinophils (arrows).

they represent nonspecific findings^[71] these are important for supplementation during treatment^[10]. Moreover, IgG and nitrite concentrations in colonic lavage fluid can be higher in dogs with IBD than in controls, with important implications in the diagnosis and monitoring of the disease^[72]. Other markers, such as microalbuminuria and C-reactive protein, yielded less encouraging results, since they showed no correlation with symptoms or pathological assessment^[73]. As stated above, IBD in dogs can be accompanied by protein loss, which is easily verified by measuring serum albumins; the presence of a new test to dose α 1-proteinase inhibitor (α 1-PI) in faeces may allow the earlier identification of protein loss, because this protein has a molecular mass similar to that of albumin,

but has the advantage of persisting unaltered in stool^[71].

If diagnosis of the disease necessitates a long and complete diagnostic plan, important tools for the clinical evaluation of IBD are the canine chronic enteropathy activity index and the canine IBD activity index (CIBDAI) (similar to the Crohn's disease activity index in man^[74]) that also allows follow-up of the patient during treatment^[75-77], even though the CIBDAI does not seem to correlate with histopathologic grade^[73]. One important aspect in managing IBD is that it is very difficult to foresee the outcome of the patient; fortunately, many important markers have recently been studied as prognostic indicators, such as canine pancreatic lipase immunoreactivity (cPLI), cobalamin and albumin concentration^[78-80].

TREATMENT

As in human medicine, the main difficulties in treating dogs with IBD originate from an incomplete understanding of the pathophysiological basis of these diseases; thus, it is not surprising that in many cases therapeutic protocols are partially borrowed from those adopted in man^[23,78]. Usually, the therapeutic protocols of choice for IBD in dogs depend on the seriousness of the disease and, eventually, on the resistance to some drugs. Some patients, such as those with FRD, may improve or even resolve their condition following the simple administration of a specific diet, such as those based on hydrolysed proteins^[58].

In general, the first approach in dogs with IBD usually involves some dietary modification, the use of prebiotics-probiotics^[10,11], antimicrobials and, eventually, corticosteroids (prednisolone or, more recently, budesonide)^[58]. Metronidazole represents an important therapeutic agent, because of its simultaneous antimicrobial and immunomodulatory action^[10,58]. Other immunomodulatory or anti-inflammatory drugs, such as azathioprine, cyclosporin, chlorambucil, cyclophosphamide and 5-aminosalicylates can be administered as an alternative or in association with the steroids, depending on the molecule^[10,12,58,78]. When treating a dog with IBD it is also important to add symptomatic therapeutic measures such as gastroprotectors, antiemetics, motility modulators, *etc.*, and to correct any possible imbalance (for example, by means of cobalamin or electrolyte supplementation)^[10,58].

Considering that the knowledge of the cytokine patterns involved in IBD human patients represents an important tool in the development of new therapeutic strategies^[61], this could also supply new therapeutic tools in veterinary medicine^[10,58]; the results of a recent study on intestinal biopsies from dogs with IBD, assessing growth hormone receptor and insulin-like growth factor (IGF-1/-2) mRNA expression, gave further perspectives for IBD treatment^[82].

CONCLUSION

To date, the relationship between IBD in humans and dogs has not been completely defined, especially with regard to pathophysiology and therapeutic protocols; thus, of the aspects investigated in man only a few could probably be successfully translated to dogs. In fact, IBD in the latter displays several and some strikingly different forms with respect to humans, in whom these diseases display more standardized clinical, endoscopic, and pathologic aspects.

However, as suggested by some authors^[83,84], the parallel study of IBD in animals could also lead to important information in man^[85], and hopefully develop better therapeutic measures to alleviate these troubles in our canine friends and companions.

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Even low-grade inflammation impacts on small intestinal function

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Abstract

Independent of the cause and location, inflammation - even when minimal - has clear effects on gastrointestinal morphology and function. These result in altered digestion, absorption and barrier function. There is evidence of reduced villus height and crypt depth, increased permeability, as well as altered sugar and peptide absorption in the small intestine after induction of inflammation in experimental models, which is supported by some clinical data. Identification of inflammatory factors which may promote the process of gastrointestinal dysfunction as well as clinical research to verify experimental observations of inflammatory modulation of gastrointestinal function are required. Moreover, nutritional strategies to support functional restitution are needed.

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Key words: Small intestine; Digestion; Absorption; Motility; Permeability; Low-grade inflammation; Cytokines

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INTRODUCTION

The gut mucosa is a highly organized structure that is adapted to the absorption of dietary components from the gastrointestinal lumen. It is maintained by a critical balance between epithelial cell proliferation, differentiation, migration and apoptosis^[1]. A complex interplay between multiple signaling events within epithelial cells controlling extracellular signals either from neighboring cells (cell-cell or cell-matrix contacts) or from a distant origin (e.g. hormones) maintains the specific organization and function of these cells.

Inflammation, regardless of its location or extent, sends many local and systemic signals, which in turn may cause changes in the intestine. Inflammation alters the metabolic status and elevates energy expenditure, fat metabolism and protein catabolism^[2,3]. The intestine, however, has an inherent ability to adapt morphologically and functionally in response to internal and external environmental stimuli^[4]. The adaptations include modifications of the brush border membrane fluidity and permeability as well as up- and down-regulation of carrier-mediated transporter proteins.

Inflammatory processes initiate a cascade of intestinal events which not only influence the mucosa itself but may also affect the function and integrity of remote organs and tissues. Interleukin-1 β (IL-1 β), for example, has both direct central and indirect peripheral depressant effects on appetite mechanisms, whereas plasma concentrations

of tumor necrosis factor α (TNF- α) are associated with increases in energy expenditure^[5,6]. Lymphocyte activation leads to increased glucose consumption which stimulates major glucose-transporter proteins (GLUT) and insulin receptors^[7,8]. Increased expression of insulin receptors is essential for immune cell division, size and survival, and IL-7 seems to be essential in this process^[9].

This review gives a brief overview of some observations of the morphological and functional changes in the non-inflamed part of the small intestine that are caused by inflammatory events in other parts of the body.

MORPHOLOGICAL ALTERATIONS IN THE INTESTINAL MUCOSA

Gastrointestinal dysfunction is a common complication in patients with traumatic brain injury (TBI)^[10]. It is proposed that TBI sets off a sequence of local and systemic inflammatory events. Hang *et al.*^[11,12] found increased TNF- α and IL-6 concentrations in the jejunal tissue of mice after parietal brain contusion induced by a weight-dropping method. In this TBI model, Hang *et al.*^[11] demonstrated rapidly developing histopathological damage of the gut mucosa, including apoptosis of epithelial cells, loss of tight junctions and mitochondrial damage. They found reduced jejunal villus height, crypt depth and surface area as early as 3 h following induction of brain injury.

Morphological alterations have been confirmed in rats. Chen *et al.*^[13] induced TBI by a weight-dropping method in male Wistar rats. Ileal tissue was analyzed 5 d after brain injury. The villus height and crypt depth of ileal samples were significantly decreased compared to healthy controls. The authors also noticed increased inflammatory markers, such as IL-1 β , TNF- α , IL-6 and intracellular adhesion molecule-1 expression, in the ileal tissue.

Sundaram *et al.*^[14] found similar alterations in the mucosal structure in the non-inflamed part of rabbit ileum after colitis induced by intragastrically inoculated microbes. Despite a clear increase in proinflammatory IL-10 in duodenal, jejunal and ileal tissue samples, however, neither Barada *et al.*^[15] nor Mourad *et al.*^[16] observed any histological alterations in the non-inflamed small intestine of colitis-induced rats. Colitis was induced by intracolonic administration of iodoacetamide or 2,4,6-trinitrobenzene sulfonic acid.

Morphological alterations in the small intestine in inflammatory bowel disease (IBD) patients are conflicting. Whereas Salem & Truelove^[17] and Arvanitakis^[18] found histological changes in the non-inflamed part of the jejunum in almost 70% of IBD patients, Soulé *et al.*^[19] only observed morphological abnormalities in less than 5% of patients.

In chronic heart failure patients, the presence of endothelial dysfunction is associated with increased levels of inflammatory markers^[20]. This may also induce mor-

phological changes in the intestine. Sandek *et al.*^[21] reported thickening of non-inflamed ileal walls and of all parts of the large intestine in chronic heart failure patients compared with control subjects of similar age. The increase in wall thickening was about 30% in the terminal ileum and almost 50% in the transverse colon. Increased bowel wall thickness in the small intestine is a frequent finding in various inflammatory-related conditions, such as acute ischemic colitis, IBD and food hypersensitivity^[22,23].

To summarize, many of the above-mentioned studies in animal models assume the contributory effects of inflammatory mediators to be, at least partly, behind the observed morphological changes in the small intestine. However, there is no clear opinion as to the degree or location of inflammation or the quantity or quality of morphological changes in the small intestine. In addition, the possible route of effects, whether e.g. *via* circulating mediators or the autonomic nervous system, remains to be clarified.

ALTERATIONS OF PERMEABILITY

Tight junctions are dynamic structures that limit the passive absorption of hydrophilic molecules from the intestinal lumen, thereby being crucial in intestinal barrier mechanisms.

Proinflammatory cytokines IL-1 β and TNF- α increased epithelial tight junction permeability *in vitro* in Caco-2 cells dose- and time-dependently^[24,25]. This was mediated by an increase in myosin L chain kinase (MLCK) expression and activity, which in turn was mediated by a nuclear factor- κ B-dependent increase in MLCK gene transcription^[26,27]. In a rabbit jejunal *ex vivo* model, however, added IL-8 had no effect on passive tissue permeability measured with Cr-EDTA in Ussing-type chambers^[28]. Nevertheless, cytokines affected neurotransmitter release and content, suggesting the leading role of the autonomic nervous system^[29].

Intestinal mucosal function can be assessed *in vivo* by measuring the permeability of the mucosal barrier to small or large solutes as in a lactulose-mannitol test. Mannitol (M), a small sugar, passes through the cell membranes. Lactulose (L), a large molecule, is absorbed paracellularly through the tight junctions. Increased absorption of lactulose can reflect mucosal leakiness, and decreased absorption of mannitol can indicate a decreased functional absorptive area. In a rat model of TBI, the L/M ratio increased significantly at 3 h following brain contusion and reached a peak level at 72 h, indicating rapidly increased permeability and disruption of tight junctions between epithelial cells^[11].

Feighery *et al.*^[30] measured ileal permeability *ex vivo* in Sprague Dawley rats after inducing brain injury. After cortical contusion, ileal segments were removed and mounted in Ussing chambers within 30 min. Significantly reduced ileal barrier function was observed by monitoring fluxes of C-mannitol crossing epithelial tight

junctions. The authors assumed this to be mediated by opening of the tight junctions caused by disturbances in the autonomic nervous system or possibly also by hemorrhagic shock. In addition, cerebral stroke leads to immunodepression promoting gut barrier dysfunction, which has led to increased permeability in a mouse model^[31].

In a colitis rat model, Fries *et al.*^[32] found leaky tight junctions in the non-inflamed duodenum and ileum where permeability was increased over 70% compared with healthy controls. This increase correlated positively with the macroscopic colon damage score. In this study, colitis was induced by intrarectal trinitrobenzene sulfonic acid. Changes in the permeability of the non-inflamed part of the small intestine have been previously noticed in Crohn's disease (CD) patients^[33]. Söderholm *et al.*^[34], however, found no changes in baseline permeability in surgical segments of non-inflamed distal ileum of CD patients. In spite of this finding, intestinal tight junctions of CD patients were noticed to be more reactive to luminal stimuli of sodium caprate (a constituent of milk fat affecting tight junctions) compared with ileal segments from colon cancer patients, who served as non-inflamed controls. The authors assumed that the altered function of the small intestinal tight junctions was mediated *via* disturbed cytoskeletal contractility in inflamed patients.

Chronic heart failure patients showed a 35% increase in small intestinal permeability measured by the L/M test and a 29% decrease of D-xylose absorption, which reflects a reduced activity of passive carrier-mediated transport^[21]. This may be due to diminished gut circulation contributing to local edema and leading to reduced barrier function and dysfunction of transport proteins, but proinflammatory cytokines can also influence gut epithelial hyperpermeability. Furthermore, the possible effect of long-term medication cannot be excluded with these patients.

To sum up, increased permeability has been noticed in many experimental animal models of inflammation, which is supported by some clinical observations. Altered permeability is most likely mediated by open tight junctions, but the role of inflammatory agents and the autonomic nervous system in this phenomenon remains to be studied. Changes in the barrier function may have implications for nutrient absorption as well as for multiple organ failure caused by increased systemic delivery of luminal endotoxins.

ALTERATIONS OF INTESTINAL MOTILITY

Gastrointestinal motility is regulated by circulating and local hormones as well as neurotransmitters, mainly of the enteric nervous system. Release of these agents, however, is regulated by the central nervous system (CNS). Brain-gut peptides, such as cholecystokinin (CCK), vasoactive intestinal peptide (VIP) and calcitonin gene-related peptide (CGRP), are important mediators in the regulation of gastrointestinal motility. Several kinds

of stress, such as trauma, cold, and pain, can induce the release of these peptides from the CNS and enteric nerves^[35]. Even psychological stress inhibits small intestinal motility by regulating the expression of CCK and VIP^[36].

Evidence of inflammatory impact on gut motility and gastrointestinal transit is scarce. However, Hang *et al.*^[37] measured gastric distension, delayed gastric emptying and intestinal dilatation in rats after brain contusion induced by a weight-dropping method. They also noticed significant changes of brain-gut peptides CCK, VIP and CGRP both in plasma and jejunum tissue, which were assumed to be involved in the pathogenesis of gastrointestinal dysmotility due to systemic stress.

ALTERATIONS OF NUTRIENT ABSORPTION

There is experimental and clinical evidence that intestinal inflammation is often associated with altered malabsorption of fluids, electrolytes and nutrients - in the inflamed as well as the non-inflamed parts of the intestine^[16-18,33,38-40].

Lactose and sucrose are the major disaccharides in the human diet. Before absorption, they are hydrolyzed to monosaccharides by the microvillus membrane enzymes, lactase and sucrase. Luminal factors, hormones and growth factors regulate the expression of these enzymes along the longitudinal and vertical axes of the small intestine. The iodoacetamide-induced colitis model of Jurjus *et al.*^[41] showed a transient reduction in jejunal lactase and sucrase expression on days two and four after colitis induction in rats. The same observation was made for the jejunal aminopeptidase and GLUT-5 expressions. Aminopeptidases are involved in protein digestion in the jejunum and ileum, and GLUT-5 is a fructose transporter of the small intestine. The expression of lactase, aminopeptidases and brush border GLUT-5 returned to normal in the jejunum following the resolution of colonic inflammation. However, sucrase and crypt GLUT-5 expression remained lower for four or more days. Jurjus *et al.*^[41] suggest that the reduced enzyme expressions are probably due to decreased translation or increased degradation, or both, since transcriptional changes were not noticed. Previously, a significant reduction in disaccharidase activity in the jejunum and ileum of IBD patients has been noticed^[18,33].

Chronic heart failure patients have reduced intestinal sugar absorption, determined by the follow-up of urinary recovery of orally administered D-xylose^[21]. This indicates diminished absorption due to alterations in passive carrier-mediated transport. However, the effects of circulation and heart function cannot be excluded. There is also an increased risk of anemia among heart failure patients, but the mechanisms for this phenomenon are not entirely clear^[42]. In addition to renal dysfunction and decreased renal blood flow, circulating neurohormonal

and proinflammatory cytokines appear to contribute to anemia in chronic heart disease.

Interleukins may modulate intestinal transport function. Some proinflammatory cytokines have been reported to exert significant effects on central and peripheral regulation of glucose metabolism, which may be related to the increased metabolic demands of inflamed tissue. IL-6, IL-1 α and IL-8 seem to improve the absorption of glucose *ex vivo* in rabbit jejunal tissue^[28]. Intracerebroventricular injection of IL-1 α enhances whole-body glucose metabolism and increases insulin levels^[43]. No alterations were seen after intravenous administration of the same dose. Anti-inflammatory IL-10 appears to have no role in the regulation of jejunal nutrient transport^[28].

TNF- α is an important inflammatory mediator that plays a central role in triggering inflammatory reactions. Absorption of monosaccharide galactose in incubated rat intestinal rings is impaired by proinflammatory IL-1 β and TNF- α by 30%^[44]. In an experimental model of sepsis, intravenous administration of TNF- α inhibited intestinal D-fructose absorption in the rabbit jejunum by decreasing the expression of the GLUT-5 transporter at the brush border membrane^[45]. It also inhibited D-galactose transport across the apical membrane of the enterocyte in rabbit intestinal tissue preparations and brush border vesicles by reducing the amounts of Na⁺/glucose cotransporter protein (SGLT1) in the plasma membrane through a mechanism in which several protein-like kinases are involved^[46].

Proinflammatory cytokines IL-1 α and IL-8 significantly increased L-proline absorption *in vitro* in the rabbit jejunal tissue^[28]. However, in rats with intracolonic iodoacetamide-induced colitis, jejunal alanine absorption was reduced 30%-40% at two days following iodoacetamide administration^[47].

Brain injury in a rat model increased the transport and uptake of dipeptides, even though the villus height and surface area were demonstrated to be significantly decreased^[48]. This process was carried out by increases in the intestinal brush border transporter PepT1, which transfers dipeptides from the intestinal lumen to enterocyte cytoplasm. Rat intestinal PepT1 can be up-regulated under the influence of many factors including the dietary protein load^[49]. Up-regulation of this oligopeptide transporter in the intestine suggests that the gut can increase the luminal transport of dietary proteins in the form of di- and tripeptides during intestinal failure, independent of changes in the mucosal surface area. This has pharmacological implications as well, since PepT1 mediates the intestinal absorption of some peptide-like drugs^[50]. There is some evidence that PepT1 may even have a role in inflammatory processes, since it is up-regulated in the colonic epithelial cells of patients with chronic ulcerative colitis and CD^[51].

To summarize, the observations of changed absorptive capacity of the small intestine are mainly from animal models. Maldigestion of carbohydrates and changes in transporters of sugars and peptides have been noticed.

Table 1 Summary of *in vivo* evidence of changes in small intestinal function induced by inflammation markers

Alterations in intestinal	Inflammation model		
	TBI	IBD, colitis	Chronic heart failure with low-grade inflammation
Morphology	A, H	A?, H?	H
Permeability	A	A, H?	H
Motility	A		
Absorption	A	A, H	H

Most observations in this summary are, however, from a single study. TBI: Traumatic brain injury; IBD: Inflammatory bowel disease; A: Evidence from animal studies; H: Evidence from clinical studies; ?: Conflicting results.

The clinical relevance of these observations remains to be elucidated.

CONCLUSION

Systemic or local inflammation may influence the intestinal absorptive area, epithelial cells, and barrier function *via* released inflammatory mediators and products from activated immune cells. Knowledge regarding the interplay of inflammation and intestinal function is scant. The molecular mechanisms of these changes may differ in acute and chronic inflammation.

Even if the number of observations of morphology, permeability, motility and digestion is limited, the results are interesting and indicate possible clinical relevance. Table 1 summarizes the existing *in vivo* evidence. Most of the observations, however, have been made after TBI which is an extreme model for studying inflammatory responses in the intestine. Interesting early clinical results of altered intestinal morphology and function have been obtained in chronic heart failure patients. Chronic heart failure is associated with increased levels of inflammatory markers related to so-called low-grade or minimal inflammation.

More research is needed to verify these mainly experimental observations and to establish their possible clinical relevance.

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Factors influencing intercellular spaces in the rat esophageal epithelium

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Abstract

AIM: To evaluate the effect of acute stress, hydrochloric acid, ethanol, aspirin, and prednisolone on the intercellular spaces of the esophageal epithelium.

METHODS: Part I, male Sprague-Dawley rats were randomly divided into eight groups and treated with the damaging or control factors. The esophagus of each rat was macroscopically inspected. Histological changes in mucosal biopsies were examined by light microscopy, and the widths of intercellular spaces were determined by transmission electron microscopy (TEM). Part II, in part I, we found that acute stress and aspirin induced dilated intercellular spaces (DIS) of the esophageal epithelium. Therefore, the effect of acid suppression pretreatment with esomeprazole on esophageal epithelial DIS induced by water immersion and restraint stress (WRS) and aspirin was further investigated to determine the association of DIS with acid reflux. After administration of 0.9% sodium chloride solution or esomeprazole solution orally for five days, rats underwent WRS or intragastric administration of aspirin solution. Esophageal epithelial intercellular spaces were investigated by TEM.

RESULTS: (1) The five damaging factors produced no lesions or inflammation in esophageal mucosa of rats under either gross or routine histological inspections. Esophageal epithelial intercellular space diameters in stress and aspirin groups were significantly greater, nearly three or two-fold respectively, than those in their corresponding control groups (stress model: $0.38 \pm 0.05 \mu\text{m}$ vs $0.13 \pm 0.02 \mu\text{m}$, $P < 0.01$; aspirin model: $0.32 \pm 0.12 \mu\text{m}$ vs $0.19 \pm 0.05 \mu\text{m}$, $P < 0.01$). Neither intragastric administration of hydrochloric acid or ethanol, nor hypodermic injection of prednisolone produced DIS compared with their corresponding control groups (hydrochloric acid model: $0.24 \pm 0.03 \mu\text{m}$ vs $0.19 \pm 0.05 \mu\text{m}$, $P > 0.05$; ethanol model: $0.25 \pm 0.10 \mu\text{m}$ vs $0.19 \pm 0.05 \mu\text{m}$, $P > 0.05$; prednisolone model: $0.20 \pm 0.03 \mu\text{m}$ vs $0.14 \pm 0.03 \mu\text{m}$, $P > 0.05$); and (2) No significant difference in the intercellular space diameters was observed between the group pretreated with esomeprazole and the control group, in both the stress and aspirin models (stress model: $0.35 \pm 0.05 \mu\text{m}$ vs $0.37 \pm 0.05 \mu\text{m}$, $P > 0.05$; aspirin model: $0.24 \pm 0.02 \mu\text{m}$ vs $0.27 \pm 0.03 \mu\text{m}$, $P > 0.05$).

CONCLUSION: Acute stress and aspirin can induce DIS of the esophageal epithelium in rats, and it is not correlated with acid reflux.

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Key words: Esophagus; Dilated intercellular spaces; Stress, acute; Aspirin; Reflux, acid

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INTRODUCTION

Gastroesophageal reflux disease (GERD) covers a broad range of symptoms and signs arising from the reflux of gastric contents into the esophagus. Gastric acid is the most important injury factor in the refluxate. In patients with GERD, only one third of those with heartburn have endoscopic evidence of erosive esophagitis (EE), and the remainder have non-erosive reflux disease (NERD). Mucosal damages are not visible under endoscopy in patients with NERD^[1]. Several diagnostic tests have been established to diagnose GERD, but there is not a gold standard for diagnosing NERD. Many existing methods, such as ambulatory 24-h esophageal pH or bile monitoring, are not satisfactory due to their poor sensitivity, specificity, and reproducibility^[2]. For example, about 50% of patients with NERD were found to have physiological values of acid exposure time with ambulatory 24-h esophageal pH monitoring^[3-5]. Therefore, more effective methods need to be developed for the diagnosis of NERD.

Dilated intercellular spaces (DIS) have been observed in both the early stage of acid-perfused rabbit esophageal epithelium^[6] and the esophageal biopsies of patients with GERD, including NERD and EE^[7,8]. Studies reported that three or six months of acid suppression therapy with omeprazole led to a recovery of DIS in more than 90% patients with GERD, accompanied by regression of heartburn^[9]. Thus, DIS has been considered as a feature of esophageal epithelial damage induced by gastric acid reflux, and serves as a marker for new methods to diagnose GERD, especially NERD^[10-14].

However, the specificity of DIS is questionable. In patients with other esophageal disorders, either with or without acid reflux, DIS was also observed, suggesting that refluxed acid is not the only factor that results in DIS^[15,16]. A number of common damaging factors, such as ethanol, hydrochloric acid, nonsteroidal anti-inflammatory drugs (NSAIDs), corticosteroids, and stress, have been known to induce stomach lesions. The aim of this study was to evaluate the effect of acute stress, hydrochloric acid, ethanol, aspirin, and prednisolone on the intercellular spaces of the esophageal epithelium in rats.

MATERIALS AND METHODS

Animals

Adult male Sprague-Dawley (S-D) rats, weighing 190-210 g, were used in the experiments. The animals were kept in individual cages, and provided with food and water *ad libitum*. They were housed in an air-conditioned room with a temperature of $23 \pm 1^\circ\text{C}$, humidity of 55%, and a 12 h light/dark cycle. All rats were acclimated under the same conditions for three days before study.

Models

In part I we evaluated the effect of different damaging factors on the intercellular spaces of the esophageal epithelium in rats. Subsequently, the effect of esomeprazole pretreatment on esophageal epithelial DIS induced by

water immersion and restraint stress (WRS) and aspirin was further investigated (part II).

Part I : After a three-day acclimation, 45 rats were randomly divided into eight groups (five or six rats per group) and treated as follow: (1) Normal control group (NC): rats remained untreated in their home cage for seven hours, and subsequently were anesthetized using diethyl ether; (2) Normal saline (NS) intragastric administration group (NSIG): rats were anesthetized one hour after intragastric administration of 1 mL of 0.9% sodium chloride solution (NS); (3) NS hypodermic injection group (NSHD): rats were given hypodermic injection of 2 mL of 0.9% sodium chloride solution at cervical part once a day for four days, and anesthetized eight hours after the last injection; (4) WRS group: the four limbs of each rat were bounded on a board, and the rats were immersed in water in a head-up vertical position up to the level of the xiphoid process at a temperature of $20 \pm 1^\circ\text{C}$ for seven hours before they were anesthetized^[17,18]; (5) Hydrochloric acid intragastric administration group (HCLIG): rats were anesthetized one hour after intragastric administration of 1 mL of 0.7 mol/L hydrochloric acid; (6) Ethanol intragastric administration group (EIG): rats were anesthetized one hour after intragastric administration of 1 mL of 100% ethanol; (7) Aspirin intragastric administration group (AIG): rats were anesthetized one hour after intragastric administration of 1 mL of aspirin solution (300 mg/kg, dissolved in 2% sodium bicarbonate solution); and (8) Prednisolone hypodermic injection group (PHD): rats were given hypodermic injection of 2 mL of prednisolone acetate injection (250 mg/kg per day) at cervical part once a day for four days, and anesthetized eight hours after the last injection. All rats were fasted for 24 h but allowed free access to water before anesthesia. The esophagi and stomachs of rats were isolated, opened, and macroscopically inspected. Esophageal mucosal biopsies 0.5-1 cm above Z-line were then obtained to prepare paraffin sections for light microscopy and ultra-thin sections for transmission electron microscopy (TEM).

Part II : (1) WRS model: 12 rats were randomly divided into two groups averagely: control group (C) and esomeprazole group (E), which were given 0.9% sodium chloride solution (2 mL, once a day) or esomeprazole solution (5 mg/kg per day, dissolved in 2 mL of distilled water, once a day) orally for four days, respectively. Rats were then fasted for 24 h but allowed free access to water. On the 5th day, two hours after 0.9% sodium chloride solution or esomeprazole solution were administered, all rats underwent WRS for seven hours as described in part I, and were then sacrificed under ethyl ether inhalation. Esophageal mucosal biopsies 0.5-1 cm above Z-line were taken to measure the width of intercellular spaces with TEM; and (2) Aspirin model: 12 rats were divided into two groups, and treated as described in WRS model for the first four days. On the 5th day, rats received intragastric administration of 1 mL of aspirin solution (300 mg/kg, dissolved in 2% sodium bicarbonate solution) two hours after 0.9% sodium chloride solution or esome-

prazole solution were administered. One hour later, the rats were anesthetized using ethyl ether. Esophageal mucosal biopsies 0.5-1 cm above Z-line were taken to measure the width of intercellular spaces with TEM.

Histology

Biopsies of esophageal mucosal tissues were fixed in formalin immediately after being removed from the rats, and were then embedded in paraffin wax. Serial 5 μm sections were cut and stained with hematoxylin and eosin (HE) for routine histological evaluation by light microscopy.

Transmission electron microscopy

Specimens of esophageal mucosae were fixed in glutaraldehyde immediately after being removed from the rats, rinsed, and processed for TEM. The specimens were post-fixed in 1% buffered osmium tetroxide. They were then dehydrated through a graded acetone series and embedded in Araldite. Blocks were trimmed, and ultra-thin sections on copper grids were post-stained with uranyl acetate and lead citrate. Each specimen was analyzed with a transmission electron microscope (JEOL JEM-1230) and then photographed at an accelerating voltage of 80 kV. Photographs of at least 10 fields were magnified at $\times 5000$.

Ten transmission electron photomicrographs of biopsy specimens from each rat were obtained. In particular, the suprabasal layer of the esophageal mucosa was examined in each image. Photographs with an internal scale marker were digitized and then each field was evaluated using Adobe Photoshop 7.0 software. According to Tobey *et al*^[7], at least 10 randomly selected perpendiculars transects to adjacent membranes were drawn and measured in each image for a total of 100 measurements in each case. Each transect was drawn at a distance not closer than 1 μm .

Statistical analysis

The measurements obtained were used to calculate the mean intercellular space scores and the mean scores of the minimal and maximal intercellular spaces for each subject and for all cases as a whole. Scores are reported as mean \pm SD. Statistical significances were determined using one-way analysis of variance (ANOVA) and LSD-*t* test for comparisons among multiple groups, and using Student's *t*-test for unpaired samples between two groups. A *P*-value of less than 0.05 was considered statistically significant.

RESULTS

Effect of different damaging factors on intercellular spaces of the esophageal epithelium

Multiple congestion, erosions, ulcers, and hemorrhage in the gastric mucosa were observed grossly in the WRS, HCLIG, EIG, AIG, and PHD groups, while in the other three control groups the gastric mucosa remained intact with no apparent lesions. The esophageal mucosae of all the groups were also examined by gross inspection, and no evident inflammation or lesions were found in any rat. In addition, under light microscopy with magnifica-

Table 1 Effect of damaging factors on intercellular space diameters of esophageal epithelium (μm , mean \pm SD)

Groups	Cases	Mean IS	Mean of minimal IS	Mean of maximal IS
NC	5	0.13 \pm 0.02	0.09 \pm 0.02	0.18 \pm 0.03
NSIG	5	0.19 \pm 0.05	0.10 \pm 0.01	0.29 \pm 0.12
NSHD	5	0.14 \pm 0.03	0.09 \pm 0.02	0.20 \pm 0.02
WRS	6	0.38 \pm 0.05 ^b	0.20 \pm 0.06 ^b	0.64 \pm 0.08 ^b
HCLIG	6	0.24 \pm 0.03	0.14 \pm 0.02	0.40 \pm 0.10
EIG	6	0.25 \pm 0.10	0.16 \pm 0.07	0.37 \pm 0.13
AIG	6	0.32 \pm 0.12 ^d	0.19 \pm 0.07 ^d	0.47 \pm 0.17 ^d
PHD	6	0.20 \pm 0.03	0.13 \pm 0.02	0.30 \pm 0.07

^b*P* < 0.01 *vs* NC; ^d*P* < 0.01 *vs* NSIG. IS: Intercellular spaces; NC: Normal control group; NSIG: Normal saline (NS) intragastric administration group; NSHD: NS hypodermic injection group; WRS: Water immersion and restraint stress group; HCLIG: Hydrochloric acid intragastric administration group; EIG: Ethanol intragastric administration group; AIG: Aspirin intragastric administration group; PHD: Prednisolone hypodermic injection group.

tion of $\times 100$ and $\times 400$, no histological evidences of inflammation (intraepithelial inflammatory infiltration, epithelial cellular swelling, submucous dropsy, telangiectasia, papillae elongation, or hyperplasia of the basal layer) were observed in the esophageal mucosa from all eight groups.

The mean and mean scores of the minimal and maximal intercellular space diameters of esophageal epithelium were determined using TEM (Table 1, Figure 1A-H). The mean intercellular space diameter in the WRS group was nearly three times greater than that in the NC group ($0.38 \pm 0.05 \mu\text{m}$ *vs* $0.13 \pm 0.02 \mu\text{m}$, *P* < 0.01). The mean intercellular space diameter in the AIG group was greater, by nearly two-fold, than that in the NSIG group ($0.32 \pm 0.12 \mu\text{m}$ *vs* $0.19 \pm 0.05 \mu\text{m}$, *P* < 0.01). The comparison of mean scores of the minimal and maximal intercellular space diameters showed similar results to the comparison of mean between WRS and NC groups, and AIG and NSIG groups. No difference in mean and mean scores of the minimal and maximal intercellular space diameters was observed between HCLIG and NSIG groups, EIG and NSIG groups, and PHD and NSHD groups (*P* > 0.05).

Effect of esomeprazole pretreatment on esophageal epithelial DIS induced by WRS and aspirin

Esomeprazole inhibits gastric acid secretion with suppression of esophageal acid exposure, therefore the effect of esomeprazole pretreatment on esophageal epithelial DIS induced by WRS and aspirin was further investigated to determine the association of DIS with acid reflux.

In both the WRS model and the aspirin model, no significant difference in mean and mean scores of the minimal and maximal intercellular space diameters was observed between the group pretreated with esomeprazole and the control group (*P* > 0.05) (Table 2, Figure 1I-L).

DISCUSSION

DIS of esophageal epithelium in patients with GERD was first reported 30 years ago^[19]. It was later detected at

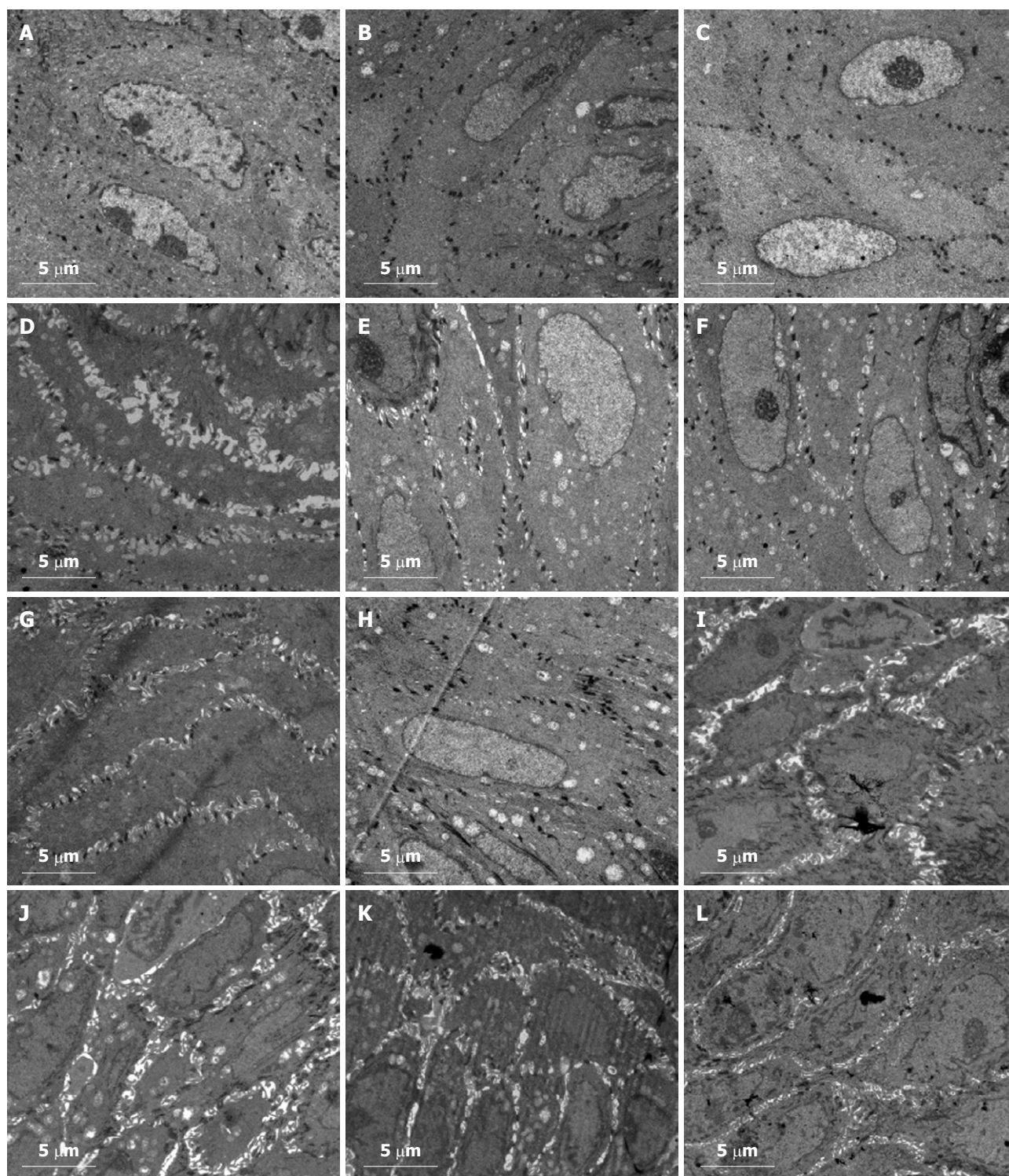


Figure 1 Transmission electron photomicrographs of the esophageal epithelium, showing intercellular spaces in rats of different groups in Part I (A-H) and Part II (I-L) (magnified at $\times 5000$ by transmission electron microscopy). A: Normal control group; B: Normal saline (NS) intragastric administration group; C: NS hypodermic injection group; D: Water immersion and restraint stress (WRS) group; E: Hydrochloric acid intragastric administration group; F: Ethanol intragastric administration group; G: Aspirin intragastric administration group; H: Prednisolone hypodermic injection group; I: NS + WRS group; J: Esomeprazole + WRS group; K: NS + aspirin group; L: Esomeprazole + aspirin group.

the early stage of acid-induced epithelial injury of rabbit esophagus^[6,20]. Using TEM, Tobey *et al.*^[7] demonstrated that DIS was a feature of reflux damage to the human esophageal epithelium, irrespective of whether the patient had erosive or non-erosive disease. However, TEM is expensive and thereby difficult to apply to routine biopsies.

Scientists have been trying to establish an equivalent of TEM with light microscopy to detect DIS^[21-23]. Although DIS can be seen under light microscopy, TEM has higher resolution and better quality of visualization. In 2003, DIS was shown to be an extremely sensitive marker of damage in GERD, duodenal gastro-esophageal reflux (DGER)

Table 2 Effect of esomeprazole pretreatment on esophageal epithelial DIS induced by WRS and aspirin (μm , mean \pm SD)

Damaging factors	Mean IS		Mean of minimal IS		Mean of maximal IS	
	Control group	Esomeprazole group	Control group	Esomeprazole group	Control group	Esomeprazole group
WRS	0.37 \pm 0.05	0.35 \pm 0.05	0.27 \pm 0.05	0.25 \pm 0.04	0.49 \pm 0.05	0.45 \pm 0.07
AIG	0.27 \pm 0.03	0.24 \pm 0.02	0.19 \pm 0.02	0.18 \pm 0.02	0.35 \pm 0.05	0.32 \pm 0.03

and NERD, and hence was thought to be the most appropriate marker to evaluate damage in NERD^[24].

In patients with GERD, contact with, and damage to, the esophageal epithelium by gastric acidic refluxate causes microscopic defects, such as DIS. DIS allows luminal acid to diffuse between the intercellular spaces, access the esophageal nociceptors in the intraepithelial sensory nerve endings, and trigger a signal by virtue of changing the pH in the intercellular environment, thereby eliciting symptoms of heartburn and pain^[25]. Among the antisecretory drugs, proton pump inhibitors (PPIs) offer rapid symptomatic relief in the highest percentage of patients with GERD, due to their inhibition of gastric acid secretion with suppression of esophageal acid exposure. Studies demonstrated that three and six months of acid suppression therapy with omeprazole, respectively, led to a complete recovery of DIS in 92.1% and 97.4% of the patients with GERD, accompanied by regression of heartburn^[9]. Similar results were also shown in other two studies^[26,27]. In addition, recently studies have also shown that dilation of intercellular space diameter occurred along the distal and proximal esophageal epithelium in NERD patients and could be responsible for the enhanced perception of proximal acid reflux^[2]. In an experimental model of non-erosive acid-damage esophageal epithelium in rabbits, DIS developed in association with, and as a marker of, reduced trans-epithelial resistance and increased shunt permeability^[28]. All these findings indicate that DIS could be a sensitive histopathological marker of NERD and be related to acid reflux.

However, the specificity of DIS to diagnose NERD is questionable, as it is also present in patients without GERD but with other esophageal disorders. Takubo *et al.*^[16] evaluated nine histological changes that have been previously described in the esophageal mucosa of patients with GERD, including DIS, and found that there was no significant difference in the incidences of DIS between the GERD and esophageal cancer patients. DIS was also found in children with esophagitis due to causes other than acid reflux (Candida infection, food allergy, and eosinophilic esophagitis)^[15]. We designed this study to evaluate whether the common damaging factors, including acute stress, hydrochloric acid, ethanol, aspirin, and prednisolone, can induce DIS of the esophageal epithelium and investigated the association of DIS provoked by any of these factors with acid reflux.

Stress, ethanol, NSAIDs, and corticosteroids are common exogenous injury factors, while acid is an important endogenous injury factor. They have been known to induce damage to the gastric mucosa at certain densities and doses. In this study, lesions were grossly observed

in the gastric mucosa of rats under these conditions, but the esophageal mucosa remained intact, with no evident lesions or inflammation, under both gross and histological inspections. Possibly the contact time of gastric and esophageal mucosa with stimulants is different, or the sensitivities of different epithelial types to stimulants are different.

Stress has been known to induce all kinds of mental and physical disorders. Up to 60% of patients with GERD report an increase in symptoms related to stressful life events^[4,29,30]. WRS has been widely used to induce gastric lesions in animals, providing a model system for studying stress-induced ulcers in humans^[31,32]. This model involves elements of physical stress in addition to psychological stress. Factors known to play a role in the induction of gastric lesions by WRS include gastric acid, oxygen-derived free radicals, cytokines, and bioactive amines^[33]. We found that the intercellular space diameter of the esophageal epithelium in the WRS group was nearly three times greater than that in the control group ($P < 0.01$), suggesting that acute stress is able to induce DIS of the esophageal epithelium. This result also correlates with recent studies by Farré *et al.*^[34]. Twenty percent to thirty percent of the patients treated with NSAIDs have symptoms in the digestive tract. NSAIDs are able to block cyclo-oxygenase-1 (COX-1) and inhibit the synthesis of physiological prostaglandins (PGs); or damage the mucosa of digestive tract directly as a poor organic acid. In our study, the intercellular space diameter of esophageal epithelium in the aspirin intragastric administration group was significantly greater than that in the NS intragastric administration group ($P < 0.01$), indicating that aspirin could provoke esophageal epithelial DIS. Combined with the results showing there were no gross lesions or histological evidence of cell necrosis and inflammation in the esophageal mucosa, both in the stress group and the aspirin group, we speculate that DIS is an early and sensitive marker of esophageal damage, and appears before the changes observable in gross or detectable with routine histological inspections. In a recent study, Shebani *et al.*^[35] showed increased intercellular space diameters in the epithelium in bronchial biopsies of asthmatics versus healthy controls, also suggesting that DIS might simply represent the early epithelial response to diverse insults, regardless of its nature and the type of epithelium.

Acid is the most important endogenous injury factor, and the major component is hydrochloric acid. Gastric mucosal injury induced by ethanol is considered "non-acid related" injury, because acid does not make the main contribution in the injury^[36-38]. We could not detect significant differences in the esophageal epithelial inter-

cellular space diameters between the hydrochloric acid intragastric administration group and the NS intragastric administration group ($P > 0.05$), or between the ethanol intragastric administration group and the NS intragastric administration group ($P > 0.05$) in our study. This discrepancy is likely due to the dose and action time we used in our experiment. Possibly hydrochloric acid and ethanol were not refluxed or the refluxed time was too short under our conditions. Though gastric mucosal lesions were produced in rats, there were no evident lesions or inflammation either in gross or in routine histological inspections. In addition, the intercellular space diameters of the esophageal epithelium did not change. Further studies regarding continuous perfusion with hydrochloric acid or ethanol into the animal's esophagus are required to investigate the effect of hydrochloric acid and ethanol on intercellular spaces of the esophageal epithelium. Our study also showed that there was no difference in the intercellular space diameter between the prednisolone hypodermic injection group and the NS hypodermic injection group ($P > 0.05$). However, other studies showed that incidence rates of peptic ulcers usually increase after treatment with corticosteroids^[39]. We currently do not know the exact reason for this difference. It is possible that prednisolone has no effect on intercellular spaces of the esophageal epithelium. Further studies need to be conducted to determine the reason.

PPIs provide more effective acid suppression and are more effective at reaching target pH values > 4 for ≥ 18 h per day^[40]. Our study showed that there were no significant differences in intercellular space diameters of the esophageal epithelium of rats between the group pretreated with esomeprazole and the control group both in the WRS model ($P > 0.05$) and the aspirin model ($P > 0.05$). This suggests that pretreatment with esomeprazole had no effect on DIS of esophageal epithelium induced by acute stress or aspirin. DIS was still present after acid suppression, indicating that DIS induced by acute stress and aspirin is not correlated with acid reflux. Similar results were also observed by Bradley *et al.*^[29], who showed that acute stress did not increase esophageal acid exposure. Besides acid reflux, other noxious contents of the refluxate, such as bile, were reported to be able to impair mucosal integrity and provoke dilated intercellular spaces after short exposure of the esophageal mucosa to bile acid in both acidic and weakly acidic conditions^[41]. Moreover, more than 50% of patients with NERD have normal esophageal acid exposure^[4]. No difference in intercellular space diameters was observed between NERD pH-positive and pH-negative patients^[8]. Therefore DIS is not related solely to acid reflux.

In conclusion, we find that acute stress and aspirin can induce DIS of the esophageal epithelium in rats, which appears before macroscopically observable changes and before changes detectable with routine histological inspection. This ultrastructural change provoked by acute stress and aspirin is not related to acid reflux, suggesting that other damaging factors can also induce DIS of esophageal epithelium. Therefore DIS is an early, sensitive, but non-specific, ultrastructural feature of NERD. These results

are beneficial for the diagnosis and differential diagnosis of NERD, and provide initial insight to the mechanism of this disease.

COMMENTS

Background

The incidence of gastroesophageal reflux disease (GERD) is rising rapidly. Symptoms of GERD arise from the exposure of increased acid gastric contents into the lower part of the esophageal mucosa. In patients with GERD, two-thirds of them have non-erosive reflux disease (NERD) with invisible mucosal damages under endoscopy. However, there is not a gold standard to diagnose NERD due to the poor sensitivity, specificity, and reproducibility of many existing methods. A prime candidate for this role is dilated intercellular spaces (DIS) in esophageal epithelium, which has been a recent research hotspot.

Research frontiers

DIS has been considered as a feature of esophageal epithelial damage induced by gastric acid reflux, and serves as a marker for new methods to diagnose NERD. However, the specificity of DIS is questionable. In this study, the esophageal mucosal damage in response to various factors in rats, including acute stress, hydrochloric acid, ethanol, aspirin, and prednisolone, were evaluated.

Innovations and breakthroughs

Recent studies have highlighted the importance of refluxed gastric acid in esophageal epithelial DIS. This study demonstrated that acute stress and aspirin can induce DIS of the esophageal epithelium in rats, and it appeared before changes that can be seen in gross and routine histological inspections. Furthermore, DIS induced by acute stress and aspirin was not correlated with acid reflux. Thus, our study suggests that DIS is an early, sensitive, but nonspecific, ultrastructural feature of NERD.

Applications

By showing that DIS is not related solely to acid reflux, this study is beneficial for the diagnosis and differential diagnosis of NERD, and provides initial insight to the mechanism of this disease.

Peer review

A well done investigation in rats that examines esophageal mucosal damage in response to various factors.

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Mindin is upregulated during colitis and may activate NF- κ B in a TLR-9 mediated manner

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Abstract

AIM: To investigate the regulation of mindin expression and the signaling pathway involved during inflammation.

METHODS: C57BL/6 mice were treated with 3% dextran sulfate sodium (DSS) in drinking water for 6 d to induce acute colitis, and then the colon was harvested for histological analysis or for RNA isolation. mRNA expression of mindin and nuclear factor (NF)- κ B p65 was analyzed by quantitative real time polymerase chain reaction (RT-PCR) and mindin expression construct was confirmed by Western blotting. Mouse macrophage and intestinal epithelial lineage cells were stimulated with different cytokines and toll-like receptor (TLR) ligands, before pNF- κ B-luciferase activity was assessed using the Dual-Luciferase reporter assay system.

RESULTS: mRNA expression of mindin was upregulated 4.7 ± 1.1 fold compared with the baseline dur-

ing DSS-induced intestinal inflammation in the mice. Stimulation with CpG-ODN (a known TLR-9 ligand) induced 4.2 ± 0.3 fold upregulation of mindin expression in RAW 264.7 cells. Full-length of mindin was cloned from cDNA of mouse mesenteric lymph node, then the pCMV-Mindin-Flag expression vector was established and the protein expression level was confirmed. Transfection of the mindin construct and stimulation with CpG-ODN significantly increased the NF- κ B-luciferase activity by 2.5 ± 0.3 and 4.5 ± 0.5 fold in RAW264.7 and CMT93 cells, respectively ($P < 0.01$).

CONCLUSION: Mindin expression is upregulated during intestinal inflammation and may induce NF- κ B promoter activation in a TLR-9 mediated manner.

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Key words: Mindin; Nuclear factor- κ B promoter activity; Toll-like receptor-9

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INTRODUCTION

Mindin (also called spondin 2) is a member of the mindin-F-spondin family of secreted extracellular matrix proteins^[1]. Mindin was initially identified in zebrafish and was found to selectively accumulate in the basal lamina. Subsequently, the genes encoding rat and human mindin were cloned^[2,3]. Mouse Spon2 cDNA encodes an open reading frame of 330 amino acids with a calculated molecular mass of 36 kDa. Spon2 mRNA is abundantly

expressed in the spleen and lymph nodes^[4].

To investigate the involvement of mindin in the immune system, mindin-deficient mice have been generated^[4]. Mice lacking mindin are resistant to lipopolysaccharide (LPS)-induced septic shock and exhibit an impaired ability to clear bacterial infections^[4]. Macrophages and mast cells show defective responses to a broad spectrum of microbial stimuli. Mindin also functions as an opsonin for macrophage phagocytosis of bacteria^[4]. Mice lacking mindin exhibit defective clearance of influenza virus, whereas mindin-deficient macrophages show impaired activation following influenza infection^[5]. Thus, mindin is a pattern recognition molecule that is critical for initiating innate immune responses to both bacterial and viral pathogens^[5,6].

Mindin-deficient mice display severely impaired recruitment of neutrophils and macrophages to inflammation sites^[7]. These effects are mediated through the interaction of mindin with $\alpha_M\beta_2$ and $\alpha_4\beta_1$ integrins on neutrophils. Mindin-integrin interactions also have a key function in T-cell priming by dendritic cells (DCs). DCs from mindin-deficient mice show an impaired capacity to prime CD4⁺ T cells due to inefficient engagement of T lymphocytes. Additionally, these DCs have reduced levels of the Rho guanosine triphosphatases Rac1 and Rac2, which control DC priming capability^[8]. Mindin regulates Rac1 and Rac2 expression by signaling through $\alpha_4\beta_1$ and $\alpha_5\beta_1$ integrins on DCs^[9].

Despite these previous studies indicating that mindin is important in both innate and adaptive immunity, information regarding its regulation is poorly understood and activation of signaling pathways are not well investigated.

The innate immune system uses pattern-recognition receptors (PRRs) to recognize pathogen-associated molecular patterns (PAMPs)^[10,11]. Several cell surface PRRs such as toll-like receptors (TLRs) are essential in innate immune recognition and the microbial components recognized by TLRs have been identified in recent decades^[12-16].

Our data demonstrate that mRNA expression of mindin is upregulated during dextran sulfate sodium (DSS)-induced acute intestinal inflammation and *in vitro* co-stimulating data suggest that mindin may induce nuclear factor (NF)- κ B promoter activation in a TLR-9 mediated manner.

MATERIALS AND METHODS

Mice and induction of acute colitis

C57BL/6 mice were purchased from the laboratory animal center, Shanghai, China. All procedures involving experimental animals were performed in accordance with protocols approved by the Committee for Animal Research of the University of Xiamen and complied with the Guide for the Care and Use of Laboratory Animals (NIH publication No. 86-23, revised 1985). Mice were housed in a specific pathogen-free animal facility and at 8 to 12 wk of age were treated with 3% (wt/vol) DSS (MP Biomedicals, Irvine, CA) for 6 d, then the colon was harvested, two tissue pieces 0.5 cm in length were dissected out, 2 cm away from the anus of each study mouse. One

piece of colon tissue was fixed in 4% neutral buffered formalin, cut into 6 μ m thick sections and then stained with hematoxylin and eosin (HE) for light microscopic examination and another piece was prepared for RNA isolation. All experiments were repeated at least 3 times.

Cell culture and stimulation

RAW 264.7 (purchased from Sigma-Aldrich), CMT93 and HEK293 cells (both purchased from ATCC, USA) were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and a 0.5% penicillin G/streptomycin mixture at 37°C in a 5% CO₂ atmosphere. About 1×10^5 of RAW 264.7 and CMT93 cells were cultured in 12-well plates and stimulated with the cytokines; 10 ng/mL of mouse recombinant tumor necrosis factor- α (TNF- α) and interleukin-1 β (IL-1 β) (both from R&D Systems); and different TLR ligands consisting of 0.05 μ g/mL of Pam3-CSK4, 5 μ g/mL of peptidoglycans (PGN), 0.1 μ g/mL of LPS, 0.5 μ g/mL of flagellin (*S. typhimurium*) and 5 μ g/mL of CpG-ODN 1585 (all of the ligands were purchased from Invivogen), for 8 h, then cells were harvested for RNA isolation.

Quantitative real time polymerase chain reaction

The total RNA from mice colon tissue or RAW 264.7 cells stimulated with different cytokines and TLR ligands was extracted using Trizol reagent (InvitroGen). The cDNA templates were synthesized using the iScript™ cDNA synthesis kit and quantitative polymerase chain reaction (PCR) reactions were performed with iQ™ SYBR green PCR supermix (both from Bio-Rad). Quantitative real time (RT)-PCR analysis was performed using the Bio-Rad iQ5 system and gene expression levels for each individual sample were normalized to GAPDH. Mean relative gene expression was determined and differences were calculated using the 2^{- $\Delta\Delta C_t$} method as described previously^[17]. The following oligonucleotides were used as primers: mindin forward, 5'-CAATGGGCTGAGGGACITTTG and reverse, 5'-TCTCTCCTGCAGCTTCGATCTC, NF- κ B p65 forward, 5'-AGGCTTCTGGGCCTTATGTG and reverse, 5'-TGCTTCTCTCGCCAGGAATAC and GAPDH forward, 5'-TGGCAAAGTGGAGATTGTTGCC and reverse, 5'-AAGATGGTGATGGGCTTCCCG.

For the RT-PCR analysis of mindin expression in different organs, the following primers were employed: mindin forward, 5'-CAGCCCTGACTGGTITTTGTGGGC and reverse, 5'-CCCTGGGACTCTGCTGTAGCCGCA CG and hypoxanthine phosphoribosyltransferase (HPRT) forward, 5'-GTTGGATACAGGCCAGACTTTGTGTG and reverse, 5'-GATTCAACTTGCCTCATCTTAGGC.

Establishment of mindin expression vector

Full-length of mindin was amplified by PCR from a cDNA template from mouse mesenteric lymph node (MLN) using upstream (containing *Bam*HI site) of 5'-CGGGATCCCGTGATGGAAAACGTGAGTCTTG and downstream (containing *Eco*RI site) of 5'-CGGAA TTCCGACGCAGTTATCTGGGGCACACT primers. Then the fragment was inserted into the *Bam*HI and

*Eco*RI site of the pCMV-Flag-tag 4C (Stratagen) vector, which resulted in the construction of a pCMV-Mindin-Flag expression vector, confirmed by sequencing.

Western blotting analysis

HEK293 cells were transfected with pCMV-Flag, pCMV-Mindin-Flag clone 1 and clone 2 for 24 h, then Western blotting analysis was performed as described previously^[18]. Briefly, 2×10^5 of transfected HEK293 cells were lysed in RIPA buffer (50 mmol/L Tris pH 8, 0.1% SDS, 0.5% deoxycholate, 1% NP-40, 150 mmol/L NaCl, 1 tablet complete mini protease inhibitor/10 mL, Roche Diagnostics GmbH, Germany). Next, equal amounts of total cell lysates were electrophoresed and transferred to Pall Fluoro Trans W membrane (WAKO, Japan). The membranes were incubated with anti-Flag antibody. After washing, the membrane was incubated with horseradish peroxidase-conjugated secondary antibody and visualized using an enhanced chemiluminescence detection system (ECL Advance, Amersham Bioscience, UK).

Dual-luciferase assay

About 5×10^4 of the RAW 264.7 and CMT93 cells were cultured in 24-well plates and transfected with 0.01 μ g of pNF- κ B-Luc (firefly luciferase) (Clontech), 0.001 μ g of pRL-0 vector (Promega) and 0.25 μ g of pCMV-mindin-Flag or pCMV-Flag vectors with Lipofectamine 2000 (Invitrogen, Carlsbad, CA). After 12 h of transfection, the cells were additionally stimulated with 0.05 μ g/mL of Pam3-CSK4, 5 μ g/mL of PGN, 0.1 μ g/mL of LPS, 0.5 μ g/mL of flagellin and 5 μ g/mL of CpG-ODN 1585 for 12 h. Then, luciferase assays were performed using the Dual-Luciferase reporter assay system (Promega, Madison, WI) according to the manufacturer's instructions. *Renilla* luciferase activity was used as an internal control. All experiments were carried out at least 3 times.

Statistical analysis

The data were expressed as the mean \pm SD. Groups were compared using Student's *t*-test. $P < 0.05$ was considered statistically significant.

RESULTS

Mindin mRNA expression is upregulated during acute intestinal inflammation

To investigate the regulation of the mindin gene during acute colitis, we used a colitis model in which 3% of DSS is added to drinking water for six days, which results in epithelial damage and acute inflammation. HE staining of the colon tissue (Figure 1A) revealed that DSS treatment induced severe colitis compared with normal tissue, characterized by epithelial and muscle hypertrophy with patchy lymphocytic infiltrates extending into the muscle layers. Quantitative mRNA expression of NF- κ B p65 (Figure 1C) showed over 15 ± 2.5 fold upregulation compared with control group, supporting the inflammation at the mRNA level. Then we used the same cDNA samples to analyze the mindin expression,

and, surprisingly, mRNA expression of mindin was upregulated in all of the colitis mice (Figure 1B, left graph, $n = 3$, each group). When quantitatively analyzed from triplicate samples of the study mice (Figure 1B, right graph), mindin expression was upregulated 4.7 ± 1.1 fold compared with the baseline during intestinal inflammation.

Mindin mRNA expression is upregulated by CpG-ODN stimulation

To further define the specific regulators of mindin expression *in vitro*, we employed the mouse leukemic monocyte macrophage cell line, RAW 264.7, and stimulated the cells with different cytokines and TLR-ligands. The results show that cytokines of TNF- α and IL-1 β , and the TLR-2, -6, -5 ligands of Pam3-CSK4, PGN and flagellin do not induce upregulation of mindin expression (Figure 2A and B). Of interest, regarding the TLR-9 ligand of CpG-ODN, this ligand induced 4.2 ± 0.3 fold upregulation of mindin mRNA expression. This result suggests that a TLR-9 mediated signaling pathway is involved in this regulation.

Mindin induces NF- κ B promoter activation in a TLR-9 mediated manner

We analyzed mindin expression in multiple mice organs and it was abundantly expressed in intestine, Peyer's patches, spleen, MLN, lung and heart tissue (Figure 3A), in agreement with previous documentation^[4].

To precisely investigate whether the TLRs are involved in a mindin-mediated signaling pathway, we cloned the full-length of mindin from cDNA of mouse MLN and established the pCMV-Mindin-Flag expression vector. HEK293 cells were transfected with pCMV-Flag, pCMV-Mindin-Flag clone 1 and clone 2 for 24 h, followed by Western blotting analysis. As shown in Figure 3B, around 37 kDa of mindin-Flag fusion protein was detected in both clone 1 and 2 lanes and clone 1 was employed in a further experiment, with no detection of protein on control lane at left side.

To define whether NF- κ B pathway activation is involved in this process, RAW 264.7 and CMT93 cells were transfected with pNF- κ B-Luc (firefly luciferase), pRL-0 vector and pCMV-mindin-Flag or pCMV-Flag control vectors. Twelve hours after transfection, stimulation took place with different TLR ligands of Pam3-CSK4, PGN, LPS, flagellin and CpG-ODN 1585 for an additional 12 h, then luciferase assays were performed.

As shown in Figure 3C and D, luciferase activity of NF- κ B promoter was significantly increased by 2.5 ± 0.3 fold and 4.5 ± 0.5 fold, in cells transfected with mindin and stimulated with CpG-ODN, for RAW264.7 and CMT93 cells respectively, compared with the control. LPS induced 2.6 ± 0.2 fold activity in CMT93 cells, but there was no significant difference between mindin and control group. No significant induction of NF- κ B luciferase activation was noted in mindin-transfected cells which underwent Pam3-CSK4, PGN, or flagellin stimulation. This result suggests that mindin induces NF- κ B promoter activation in a TLR-9 mediated manner.

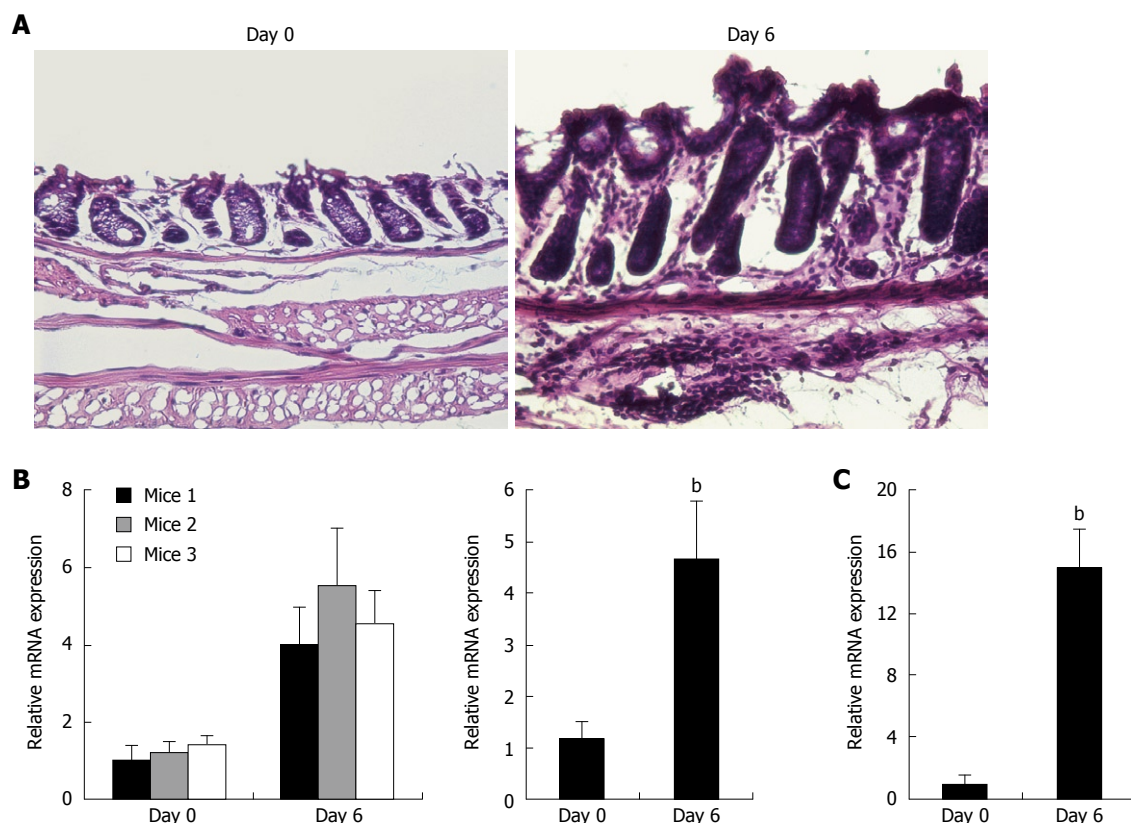


Figure 1 Mindin mRNA expression is upregulated during acute intestinal inflammation. A: Histological analysis of normal tissue and of acute inflammation specimen after 6 d of DSS. HE staining of colonic sections (magnification 40 ×); B: Quantitative mRNA expression of mindin in study mice (left graph, $n = 3$ of each group, triplicate samples from each mouse, right graph combines the data from each group); C: Relative mRNA expression of NF-κB p65 in study groups. Bars represent mean \pm SD; $^bP < 0.01$ vs day 0.

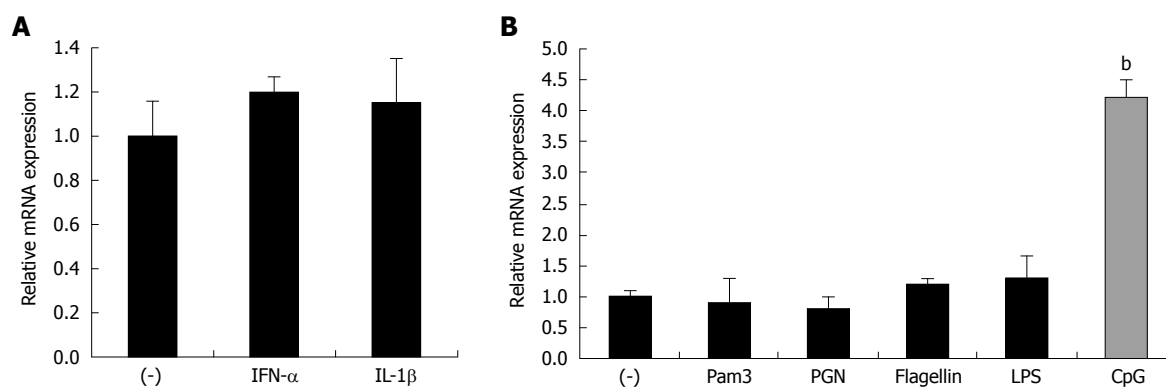


Figure 2 Mindin mRNA expression is upregulated by CpG-ODN stimulation. About 1×10^5 RAW 264.7 cells were cultured in 12-well plates and stimulated with cytokines of mouse recombinant tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β), toll-like receptor (TLR) ligands of Pam3-CSK4, peptidoglycans (PGN), lipopolysaccharide (LPS), flagellin and CpG-ODN 1585 (B) for 8 h, then cells were harvested for RNA isolation and relative mRNA expression of mindin was analyzed using quantitative real time polymerase chain reaction (RT-PCR). In each group, bars represent mean \pm SD; $^bP < 0.01$ vs unstimulated cells.

DISCUSSION

Our data demonstrated that mRNA expression of mindin is upregulated during DSS-induced acute intestinal inflammation and *in vitro* co-stimulating data suggested that mindin may induce NF-κB promoter activation in a TLR-9 mediated manner.

The immune system uses many sensors to detect and report microbial invaders. Most of these sensors are associated with immune cells, but the extracellular matrix

also seems to be essential for this sentinel duty^[19]. Investigators generated mice deficient in mindin; these mice have severe defects in many of their immune responses. The mindin-deficient cells are hyporesponsive to many PAMPs, including lipotechoic acid, peptidoglycan, zymosan, mannan and CpG DNA^[7]. Other results have indicated that the F-spondin domain mediates integrin binding and identified the binding site by mutagenesis, suggesting that mindin-integrin interactions are critical for inflammatory cell recruitment *in vivo*^[20,21].

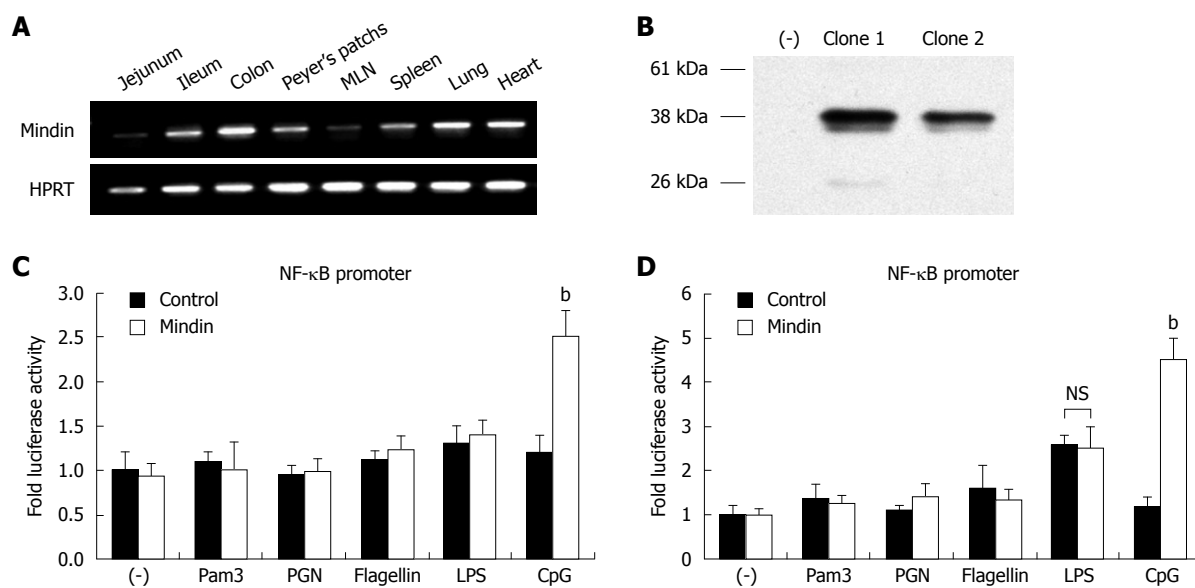


Figure 3 Mindin induces nuclear factor (NF)-κB promoter activation in a TLR-9 mediated manner. A: Mindin mRNA expression in mouse multiple organs, HPRT expression as a total RNA control; B: HEK293 cells were transfected with pCMV-Flag (lane at left side), pCMV-Mindin-Flag clone 1 and clone 2 for 24 h and Western blotting analysis was performed. RAW 264.7 (C) and CMT93 (D) cells were transfected with pNF-κB-Luc (firefly luciferase), pRL-0 vector and pCMV-mindin-Flag or pCMV-Flag control vectors. Twelve hours after transfection, cells were stimulated with the different TLR ligands of Pam3-CSK4, PGN, LPS, flagellin and CpG-ODN 1585 for an additional 12 h, then luciferase assays were performed. NS: Not significant. ^b*P* < 0.01 vs control.

These studies indicate the essential role of mindin in both innate and adaptive immunity, but information regarding its regulation is poorly understood. Also, the role of other cytokines and the interaction of DCs with T cells need to be further investigated, and different organ-specific inflammation models need to be established. DSS is thought to induce colitis by inducing mucosal erosion which is observed 5 d after DSS administration, allowing commensal bacteria to infiltrate into the lamina propria. Our data showed that DSS-induced colitis is associated with an increased activation of NF-κB. Hall *et al.*^[22] demonstrate that commensal bacterial DNA can suppress Treg cell conversion *via* TLR9-mediated activation of lamina propria dendritic cells and thus potentially disrupt intestinal homeostasis, and also show a relationship with mindin expression^[22,23]. Our data demonstrated that mRNA expression of mindin is upregulated during DSS-induced acute intestinal inflammation and that CpG-ODN stimulation induced 4.2 ± 0.3 fold upregulation of mindin expression in RAW 264.7 cells, suggesting that mindin has indispensable diverse roles in mucosal immunity.

The activation of known signaling pathways in response to LPS is largely unaffected in mindin-deficient cells, implicating that mindin may bind to a distinct receptor and activate other TLRs or different signal transduction pathways. Production of TNF and IL-6 induced by PAMPs is reduced in mindin-deficient macrophages indicating that mindin-induced signaling is as important as those well-defined signaling pathways that are largely transduced through TLRs^[7,10]. However, the exact mechanism of mindin-mediated TLR recognition of microbial components remains unclear. Recent findings suggest that NF-κB has not only proinflammatory but also tissue-protective functions in inflammatory diseases. NF-κB has multiple, often opposing functions in the intestine^[24].

Antiapoptotic actions of NF-κB in intestinal epithelial cells dominate tissue responses to many acute inflammatory and injurious challenges, whereas proinflammatory and cell survival functions of NF-κB in macrophages and T cells govern chronic intestinal inflammation. Our data showed that luciferase activity of the NF-κB promoter is significantly increased in mice macrophage and intestinal epithelial cell lineages stimulated with exogenous mindin and CpG-ODN, but not in the cells stimulated with other TLR ligands, suggesting that mindin induces NF-κB promoter activation through a TLR-9 mediated pathway. While basolateral TLR stimulation mobilizes an inflammatory cascade, apical TLR-9 stimulation delivers negative signals that curtail inflammatory responses to basolateral stimulation *via* different TLRs^[25]. Mindin was initially identified in zebrafish and was found to selectively accumulate in the basal lamina^[1]. Taken together, our data suggest that while basolateral TLR-9 signaling is fully capable of inducing a NF-κB-mediated pro-inflammatory response, apical TLR-9 signaling does not induce an inflammatory response due to a defect in NF-κB activation.

However, while mindin binds to some kinds of bacteria and integrins, it is still unclear whether mindin functions as a coreceptor for several PPRs or other novel receptors and we urge further broad studies to address these questions. Our results raise the importance of the TLR-9 mediated pathway and provide a clue to help define more precisely the function and signaling pathways of the mindin protein.

COMMENTS

Background

Mindin is a member of the mindin-F-spondin family of secreted extracellular matrix proteins. Mindin mRNA is abundantly expressed in the spleen and lymph nodes. Mindin-deficient mice are resistant to lipopolysaccharide-induced

septic shock and exhibit an impaired ability to clear bacterial infections. Mindin-deficient macrophages and mast cells show defective responses to a broad spectrum of microbial stimuli. Mindin also functions as an opsonin for macrophage phagocytosis of bacteria. Despite previous studies indicating that mindin is important in both innate and adaptive immunity, information regarding its regulation is poorly understood and activation of signaling pathways are not well investigated.

Research frontiers

Mindin has an indispensable role in both innate and adaptive immunity. This study investigated regulation of mindin expression and the signaling pathway involved.

Innovations and breakthroughs

Despite previous studies indicating that mindin is important in both innate and adaptive immunity, information regarding its regulation is poorly understood and activation of signaling pathways are not well investigated. The data of this study demonstrated that mRNA expression of mindin is upregulated during dextran sulfate sodium-induced acute intestinal inflammation and *in vitro* co-stimulating data suggested that mindin may induce nuclear factor (NF)- κ B promoter activation in a toll-like receptor (TLR)-9 mediated manner.

Applications

While mindin binds to some kinds of bacteria and integrins, it is still unclear whether mindin functions as a coreceptor for several PPRs or other novel receptors and we urge further broad studies to address these questions. The results of this study raise the importance of the TLR-9 mediated pathway and provide a clue to help define more precisely the function and signaling pathways of the mindin protein.

Terminology

Mindin is an extracellular matrix protein. NF- κ B has not only proinflammatory but also tissue-protective functions in inflammatory diseases. Basolateral TLR-9 signaling is fully capable of inducing a NF- κ B-mediated pro-inflammatory response.

Peer review

In this work the authors propose that mindin expression is upregulated during intestinal inflammation and may induce NF- κ B promoter activation through a TLR-9 mediated manner. The initial observation is interesting, but the authors should include the biological and clinical significance of the mindin upregulation in colitis in the discussion section.

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Mechanisms mediating CCK-8S-induced contraction of proximal colon in guinea pigs

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Abstract

AIM: To investigate the effects of sulfated cholecystokinin octapeptide (CCK-8S) on the contractile activity of guinea-pig proximal colon.

METHODS: Proximal colonic smooth muscle (PCSM) strips were obtained from adult female guinea pigs and contractile response of PCSM strips was recorded using a polyphysiograph. PCSM cells were isolated by enzymatic digestion. Resting potential (RP), action potential (AP), large conductance potassium channel currents (I_{BKCa}) and L-type calcium currents (I_{Ca-L}) were recorded by patch-clamp techniques.

RESULTS: (1) CCK-8S (10^{-7} mol/L) enhanced the mean contractile amplitude of colonic circular muscle and longitudinal muscle (LM) strips by $56.53\% \pm 11.92\%$

($P = 0.038$) and $65.93\% \pm 12.98\%$ ($P = 0.019$), respectively, as well as the mean frequency of LM by $31.69\% \pm 13.58\%$ ($P = 0.023$), which were significantly attenuated by pretreating strips with devazepide, nifedipine, iberiotoxin, thapsigargin (TG) and BAPTA-AM (BA) respectively; (2) CCK-8S (10^{-7} mol/L) increased the AP amplitude by $38.6\% \pm 3.2\%$ ($P = 0.015$), decreased AP duration by $36.9\% \pm 8.7\%$ ($P = 0.026$), and depolarized the RP from -61.3 ± 2.7 mV to -29.8 ± 5.9 mV ($P = 0.032$); and (3) Compared with the normal control group, CCK-8S (10^{-7} mol/L) enhanced the peak current of I_{BKCa} by $18.7\% \pm 2.1\%$ (from 916 ± 183 pA to 1088 ± 226 pA; at $+60$ mV; $P = 0.029$), which was inhibited by respective pretreatment with iberiotoxin and devazepide. Additionally, CCK-8S (10^{-7} mol/L) intensified the peak current of I_{Ca-L} by 40% (from 60 ± 8 pA to 84 ± 11 pA; at $+10$ mV; $P = 0.012$), compared to the normal control group, which was apparently suppressed by respective pretreatment with nifedipine, devazepide, TG and BA. In the respective presence of heparin and staurosporine, CCK-8S did not significantly enhance I_{BKCa} and I_{Ca-L} .

CONCLUSION: The results suggest that CCK-8S promotes guinea-pig proximal colon contraction by CCK1 receptors, following activation of the inositol triphosphate-protein kinase C signal transduction pathway.

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Key words: Muscle contraction; Potassium channels; Calcium channels; Colon; Cholecystokinin octapeptide

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INTRODUCTION

Cholecystokinin (CCK), a gut-brain peptide, plays an important role in regulating a variety of physiological functions such as the stimulation of exocrine and endocrine secretion, mediation of emotion and behavior, and the regulation of gut motor functions^[1-3]. Previous studies investigating the targets for CCK in the gastrointestinal tract have focused mainly on the gallbladder and pancreas. However, recent research has shown that CCK and its related peptides are implicated in the pathophysiology of functional digestive diseases, such as irritable bowel syndrome (IBS). Several early reports also have indicated that either an altered release of CCK or abnormal responses to this peptide could be responsible for the symptoms of IBS^[3-5].

It has been reported that sulfated CCK octapeptide (CCK-8S) is the predominant and major molecular form of CCK peptides^[6,7] and it actions on the gastrointestinal tract through two receptor subtypes, designated as CCK1 and CCK2^[3]. Previous studies have shown that some mechanisms could be involved in the effect of CCK on smooth muscle motility in the gastrointestinal tract. First, it has been reported that CCK1 receptors appear to be involved mainly in the control of gut motility and visceral sensation^[2,3,8-10]. CCK2 receptors mainly contribute to the release of histamine from enterochromaffin-like cells in the stomach^[11], and the stimulation of acid secretion from parietal cells^[12,13]. Second, Morton *et al.*^[14] have demonstrated that CCK-induced contraction of the human colon and gallbladder smooth muscle is mediated solely through the CCK1 receptor, whereas Fornai *et al.*^[3,15] have reported that CCK2 receptors mediate the inhibitory action of the peptide on the contractile activity of human distal colon, *via* the nitric oxide pathway. Furthermore, activation of protein kinase C (PKC) can enhance L-type calcium current (I_{Ca-L}) in a variety of smooth muscles^[16]. It has been shown that CCK can activate phospholipase C through binding to its distinct receptors^[7,17]. CCK-8S-evoked $[Ca^{2+}]_i$ concentration increase in gastric antral smooth muscle cells (SMCs) depends on the release of $[Ca^{2+}]_i$ stores that are regulated positively by CCK1 receptors *via* PKC-mediated phosphorylation of inositol trisphosphate (IP_3) type 3 receptor^[7]. Released Ca^{2+} in turn activates I_{Ca-L} , which ultimately results in the contraction of the gastric smooth muscle^[7]. However, the direct electrophysiological effects of CCK on the contractile activity of the colon and the crosstalk between the CCK-8S-triggered PKC and Ca^{2+} signaling pathways in colon SMCs remain unclear.

Therefore, in the present study, we investigated the effects of CCK-8S on the contraction of proximal colon smooth muscle strips, resting potential (RP), action potential (AP), large conductance potassium channel current (I_{BKCa}) and I_{Ca-L} in smooth muscle cells (SMCs) of proximal colon. In addition, the role of the IP_3 -mediated protein kinase C (PKC) pathway in the physiological responses of SMCs to CCK-8S was also studied.

MATERIALS AND METHODS

Experimental animals

Adult female guinea pigs (250-300 g) were obtained by mail from the Experimental Animal Center of Wuhan University and housed under controlled conditions and temperature ($25 \pm 2^\circ\text{C}$). The animal-testing protocol used in the present investigation was approved by the Institutional Animal Care and Use Committee of Wuhan University.

Reagents and solutions

The physiological saline solution (CaPSS) contained (in mmol/L): NaCl 135.0, KCl 5.0, $CaCl_2$ 2.0, $MgCl_2$ 1.2, glucose 10.0, and HEPES 10.0 (adjusted to pH 7.4 with NaOH). The Ca^{2+} -free PSS contained similar ingredients, without $CaCl_2$. Tyrode buffer contained (in mmol/L): NaCl 147.0, KCl 4.0, $CaCl_2$ 2.0, NaH_2PO_4 0.42, Na_2HPO_4 2.0, $MgCl_2$ 1.05, and glucose 5.5 (adjusted to pH 7.4 with NaOH). KB solution contained (in mmol/L): EGTA 0.5, HEPES 10.0, $MgCl_2$ 3.0, KCl 50.0, glucose 10.0, L-glutamate 50.0, taurine 20.0, and KH_2PO_4 20.0 (adjusted to pH 7.4 with NaOH). Digestive solution contained: 0.1% collagenase II, 0.1% trypsin inhibitor and 0.2% BSA dissolved in Ca^{2+} free-PSS. Internal pipette solution used in experiments investigating APs and RPs contained (in mmol/L): K-aspartate 120.0, HEPES 10.0, EGTA 10.0, Na_2ATP 5.0, $MgCl_2$ 4.0, and $CaCl_2$ 3.0 (adjusted to pH 7.4 with KOH). Internal pipette solution of large conductance potassium channel contained (in mmol/L): KCl 20.0, K-aspartate 110.0, $MgCl_2 \cdot 6H_2O$ 1.0, $Mg-ATP$ 5.0, Na_2 creatine phosphate 2.5, EGTA 2.5, and 2.0 $\mu\text{g/mL}$ nystatin (adjusted to pH 7.4 with KOH). Internal pipette solution of L-type calcium channel contained (in mmol/L): CsCl 135.0, $MgCl_2$ 4.0, HEPES 10.0, Na_2-ATP 2.0, EGTA 10.0, tetraethylammonium (TEA) 20.0, and 2.0 $\mu\text{g/mL}$ nystatin (adjusted to pH 7.35 with Tris-HCl). All these chemicals together with CCK-8S, nifedipine, iberiotoxin, thapsigargin (TG) and BAPTA-AM (BA) were purchased from Sigma (St Louis, MO, USA). Devazepide and CI 988 were purchased from Tocris Bioscience (UK).

Preparation of isolated colonic smooth muscle strips

Guinea pigs were sacrificed by ether, followed by cervical exsanguination. The proximal colon was removed, cleaned and opened along the mesenteric border and then placed in Ca^{2+} -free PSS bubbled with carbogen (95% O_2 /5% CO_2). The smooth muscle strips (3 mm \times 10 mm) were obtained after the mucosa and submucosa were excised.

Preparation of isolated colonic SMCs

Single SMCs were isolated by enzymatic digestion as described previously^[18]. The strips of proximal colon were pinned to the base of the sylgard surface of a Petri dish and the mucosa was carefully dissected away under an anatomical microscope. The tissue was cut into small strips (about 2 mm wide and 5-6 mm long) and placed in Ca^{2+} -free PSS solution that contained 0.12% (w/v) collagenase

II supplemented with 0.2% soybean trypsin inhibitor and 0.2% bovine serum albumin (BSA), and incubated for 25 min at 37°C. After completion of digestion, the segments were washed five times in a Ca^{2+} -free PSS solution and then triturated gently with a fire-polished Pasteur pipette to create a cell suspension. Cells were stored at 0–4°C and used within 8 h.

Contraction recording of proximal colonic smooth muscle (PCSM) strips

Each fresh smooth muscle strip was mounted in an organ bath and connected to an isometric force transducer (JZJOIH, Chengdu, China). The organ baths contained 10 mL Tyrode's buffer at 37°C and were constantly warmed by a circulating water jacketed at 37°C, and bubbled with carbogen (95% O_2 /5% CO_2). One end of the strip was fixed to a hook on the bottom of the chamber, while the other end was connected by a thread to an external isometric force transducer at the top. Each muscle strip was placed under a resting preload of 1.0 g and allowed to equilibrate for 60 min, with solution change every 20 min. The frequencies of contraction were calculated by counting the contraction waves per minute. The mean contractile amplitude and frequency of spontaneous contractions were recorded (control values) and compared with the mean contractile amplitude and frequency when exposed to treatment (response values). The results were presented as the changed percentage (changed percentage = $100\% \times (\text{response value} - \text{control value})/\text{control value}$)^[19].

Measurement of RPs and APs in isolated colonic SMCs

Several drops of cell suspensions were placed in a recording chamber that was mounted with an inverted microscope. Cells were superfused with CaPSS (3 mL/min) after adhering to the coverslip. Membrane potentials were recorded by EPC-10 amplifier (HEKA, Lambrecht, Germany). Cells were patch-clamped in current clamp mode after a 10-min equilibration. The AP stimulus pattern was composed of a 600-pA current step for 8 ms; the holding current of which was 0 pA. After several minutes equilibration, the current-clamp mode was transformed into the whole-cell voltage-clamp mode and the RP was shown in the V-membrane screen. Drugs were added subsequently to the cell suspension to observe relative effects on AP and RP when their values were stable.

Nystatin-perforated whole-cell patch-clamp recordings

Several drops of cell suspensions were placed in a recording chamber that was mounted with an inverted microscope (Olympus, Japan). After adhering to the coverslip, the cells were infused with Tyrode's buffer (3 mL/min). Pipettes were made using a micropipette puller (PC-10; Narishige, Japan). Typical pipette resistances were 3–5 M Ω . A gigaseal was formed with negative suction. Capacitance was compensated for and the residual capacitance current was removed digitally. Whole-cell currents were recorded by using a nystatin-perforated whole-cell patch-clamp configuration with an EPC-10 amplifier (HEKA, Germany). The

effects of CCK-8S at different concentrations were investigated on I_{BKCa} and $\text{I}_{\text{Ca-L}}$. Data were filtered at 200 Hz, digitized at 10 kHz (filter 1) and 2.9 kHz (filter 2), and stored in the computer for subsequent analyses. All the experiments were conducted at $25 \pm 2^\circ\text{C}$.

Statistical analysis

Statistical analyses were performed using SPSS for Windows, version 15.0. Results are expressed as the mean \pm SD of n strips or cells. Data were compared using Student's t tests. $P < 0.05$ was considered statistically significant.

RESULTS

Effects of CCK-8S on the contraction of PCSM strips

CCK-8S enhanced the resting tension of PCSM strips when applied for 3–5 min (Figure 1). CCK-8S (10^{-7} mol/L) increased the mean contractile amplitude of circular muscle (CM) and longitudinal muscle (LM) strips by $56.53\% \pm 11.92\%$ and $65.93\% \pm 12.98\%$, respectively, as well as the mean frequency of LM by $31.69\% \pm 13.58\%$ ($n = 15$ for each group, $P = 0.038, 0.019, 0.023$), but CCK-8S had little influence on the frequency of CM ($n = 15$, $P = 0.087$) (Figure 1, Table 1). CCK-8S-intensified effects on proximal colonic strips were significantly attenuated when these strips were pretreated with CCK1 receptor antagonist devazepide (10^{-7} mol/L), L-type calcium channel inhibitor nifedipine (10^{-5} mol/L), Ca^{2+} -ATPase inhibitor TG (10^{-5} mol/L), or intracellular calcium chelator BA (10^{-5} mol/L) ($n = 15$ for each group, $P < 0.05$). Pretreating CM and LM strips with iberitoxin (10^{-6} mol/L), a selective BKCa channel blocker, did not inhibit the CCK-8S-induced increase in the contractile amplitude of CM and LM strips ($n = 15$ for each group, $P = 0.096, 0.078$ vs CCK-8S group), but decreased their frequency ($n = 15$ for each group, $P = 0.036, 0.041$ vs CCK-8S group) (Figure 1, Table 1), whereas superfusion with the CCK2 receptor antagonist CI 988 (10^{-7} mol/L) did not block the CCK-8S-intensified effect on CM and LM strips ($n = 15$ for each group, $P > 0.05$) (Figure 1, Table 1).

Effects of CCK-8S on RPs and APS of PCSMCs

CCK-8S (10^{-7} mol/L) depolarized RP of SMCs from -61.3 ± 2.7 mV to -29.8 ± 5.9 mV ($n = 15$, $P = 0.032$) (Figure 2). In the presence of devazepide (10^{-7} mol/L), CCK-8S (10^{-7} mol/L) did not depolarize RP (-59.4 ± 3.8 mV, $n = 15$, $P = 0.065$ vs control group) (Figure 2). When SMCs were preincubated with CI 988, CCK-8S (10^{-7} mol/L) depolarized RP to -32.8 ± 4.2 mV ($n = 15$, $P = 0.029$ vs control group) (Figure 2). The AP amplitude was expressed as peak value, and the mean amplitude in the control group (CaPSS) was 169.9 ± 12.3 mV ($n = 15$) (Figure 3A and D). After the addition of CCK-8S (10^{-7} mol/L), the AP amplitude was increased by $38.6\% \pm 3.2\%$ [from 169.9 ± 12.3 mV (in control CaPSS) to 235.5 ± 11.6 mV, $n = 15$, $P = 0.015$] (Figure 3A–C and E), and fast repolarization time (repolarizing to 90% of the peak value of AP, T_{90}) was shortened by $36.9\% \pm 8.7\%$ [from 48.42 ± 3.38 ms (in control CaPSS) to 30.53 ± 4.15 ms, $n = 15$, $P = 0.026$]

Table 1 Effects of CCK-8S on the contractile amplitude and frequency of CM and LM strips of proximal colon (mean \pm SD)

	CM strips		LM strips	
	Amplitude	Frequency	Amplitude	Frequency
CCK-8S	56.53 \pm 11.92 ^a	0.87 \pm 1.52	65.93 \pm 12.98 ^a	31.69 \pm 13.58 ^a
Devazepide + CCK-8S	3.68 \pm 1.17	1.92 \pm 0.83	2.09 \pm 0.78	1.57 \pm 1.07
Nifedipine + CCK-8S	-79.26 \pm 5.93 ^c	-19.82 \pm 3.92 ^c	-78.69 \pm 6.42 ^c	-21.58 \pm 2.87 ^c
Iberiotoxin + CCK-8S	49.93 \pm 11.81	-36.57 \pm 17.35 ^c	57.47 \pm 10.92	-23.82 \pm 5.97 ^c
TG & BA + CCK-8S	-98.12 \pm 0.72 ^d	-97.42 \pm 2.73 ^d	-97.57 \pm 1.25 ^d	-96.11 \pm 3.26 ^d
CI 988 + CCK-8S	53.29 \pm 0.52	1.48 \pm 0.17	57.48 \pm 11.27	28.96 \pm 9.53

^a P < 0.05 *vs* control group; ^c P < 0.05, ^d P < 0.01 *vs* CCK-8S group. CCK: Cholecystokinin; CM: Circular muscle; LM: Longitudinal muscle; TG: Thapsigargin; BA: BAPTA-AM.

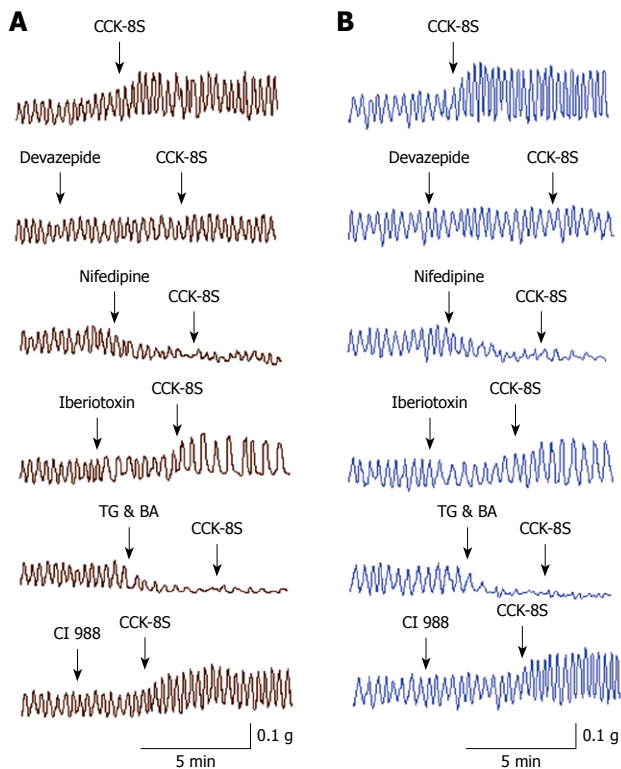


Figure 1 Effects of CCK-8S on the resting tension of guinea pig proximal colonic smooth muscle (PCSM) strips. A: Representative traces of the effect of CCK-8S (10^{-7} mol/L) on the resting tension of circular muscle (CM) strips. CCK-8S increased the mean contractile amplitude of CM strips, which was inhibited by pretreatment with CCK1 receptor antagonist devazepide (10^{-7} mol/L), L-type calcium channel inhibitor nifedipine (10^{-5} mol/L), Ca^{2+} -ATPase inhibitor thapsigargin (TG) (10^{-5} mol/L) and intracellular calcium chelator BAPTA-AM (BA) (10^{-5} mol/L). Pretreating CM strips with iberiotoxin (10^{-6} mol/L), a selective potassium channel inhibitor, did not block the CCK-8S-induced increase in the contractile amplitude of CM strips but decreased the frequency; B: Representative traces of the effect of CCK-8S (10^{-7} mol/L) on the resting tension of longitudinal muscle (LM) strips. The CCK-8S-induced increase in the amplitude and frequency of LM strips was inhibited by pretreatment with devazepide (10^{-7} mol/L), nifedipine (10^{-5} mol/L) or TG (10^{-5} mol/L) and BA (10^{-5} mol/L). Pretreating LM strips with iberiotoxin (10^{-6} mol/L) did not attenuate the CCK-8S-induced increase in the contractile amplitude of LM strips but decreased the frequency. CCK2 receptor antagonist CI 988 had no effect on the CCK-8S-induced contraction of CM and LM strips.

(Figure 3A, B, D and F), which were blocked when SMCs were pretreated with 10^{-7} mol/L devazepide (171.2 ± 13.4 mV, 47.18 ± 3.45 ms, $n = 15$, $P = 0.006$ *vs* CCK-8S group, $P = 0.074$ *vs* control group) (Figure 3E and F). In

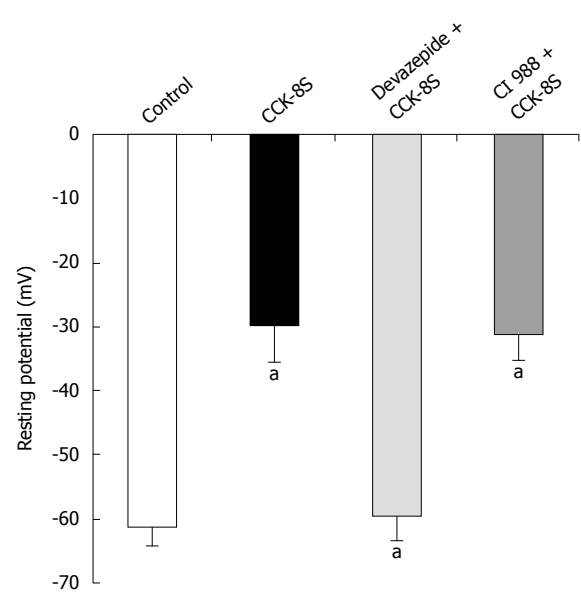


Figure 2 Effects of CCK-8S on resting potential (RP) of PCSMCs. RP rose from -61.3 ± 2.7 mV to -29.8 ± 5.9 mV after superfusion with CCK-8S (10^{-7} mol/L). In the presence of devazepide (10^{-7} mol/L), CCK-8S (10^{-7} mol/L) did not depolarize RP (-59.4 ± 3.8 mV). When SMCs were pretreated with CI 988, CCK-8S (10^{-7} mol/L) depolarized RP to -29.8 ± 5.9 mV. ^a P < 0.01 *vs* control group (CaPSS).

contrast, CI 988 did not inhibit the CCK-8S-evoked effect on AP amplitude and T_{90} (228.8 ± 12.9 mV, 31.26 ± 3.97 ms, $n = 15$, $P = 0.083$ *vs* CCK-8S group) (Figure 3E and F). After SMCs were incubated with nifedipine (10^{-5} mol/L), the enhancement of AP amplitude by CCK-8S was inhibited (75.6 ± 8.3 mV, $n = 15$, $P = 0.039$ *vs* CCK-8S group) (Figure 3A and E). In addition, superfusion with iberiotoxin (10^{-6} mol/L) significantly attenuated the effect of CCK-8S on T_{90} (46.54 ± 4.88 ms, $n = 15$, $P = 0.026$ *vs* CCK-8S group; $P = 0.079$ *vs* control group) (Figure 3B and F).

Effects of CCK-8S on I_{BKCa} in PCSMCs

With nystatin-perforated whole-cell voltage-clamp recordings, I_{BKCa} was evoked by using a depolarizing step pulse from a holding potential of -60 mV to $+100$ mV for 400 ms, with an interpulse interval of 10 s (Figure 4A). To reduce the amount of non- Ca^{2+} -dependent delayed rectifier K^{+} currents through inactivation, cells were held at

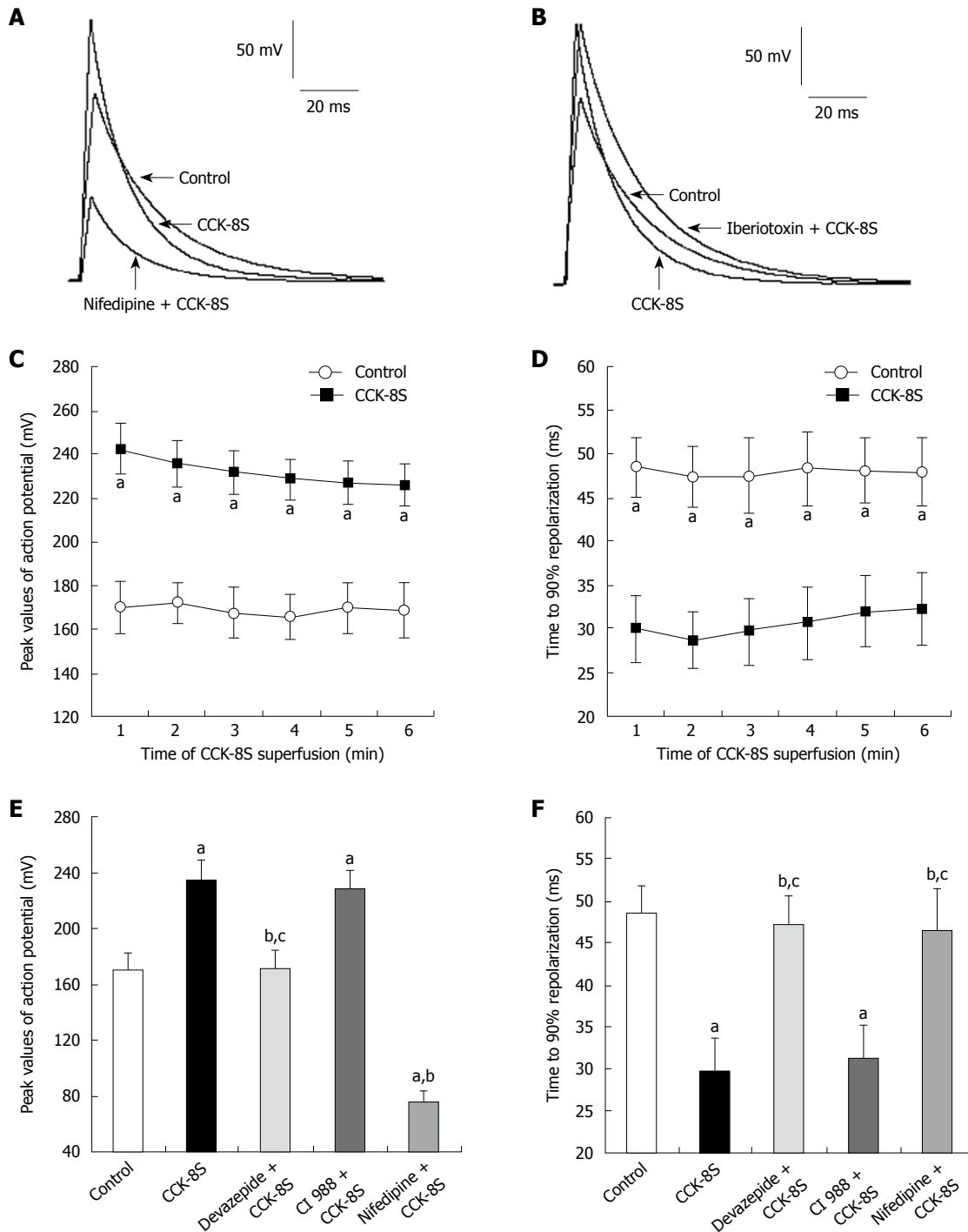


Figure 3 Effects of CCK-8S on AP of PCSMCs. A-C and E: CCK-8S (10^{-7} mol/L) suppressed the amplitude of AP to $38.6\% \pm 3.2\%$ ($n = 15$, $P < 0.01$); A, B, D and F: CCK-8S (10^{-7} mol/L) decreased fast repolarization times (T_{90}) by $36.9\% \pm 8.7\%$ ($n = 15$, $P < 0.01$); A and E: Nifedipine (10^{-5} mol/L) blocked the effects of CCK-8S on the amplitude of AP but failed to have any effect on T_{90} ; B and F: Iberiotoxin (10^{-6} mol/L) blocked the effects of CCK-8S on T_{90} but failed to have any effect on the amplitude of AP; E and F: Pretreating SMCs with devazepide (10^{-7} mol/L) blocked the CCK-8S-evoked effect on AP amplitude and T_{90} , whereas CI 988 had no such effect. ^a $P < 0.01$ vs control group (CaPSS); ^b $P < 0.01$ vs CCK-8S group; ^c $P > 0.05$ vs control group.

0 mV for at least 2 min before being subjected to step depolarization^[18,19], and superfused with 3 mmol/L 4-aminopyridine in the extracellular solutions^[18,20]. The application of iberiotoxin (10^{-5} mol/L) markedly blocked the inward current by 79% (at +60 mV, 192 ± 37 pA) (Figure 4A), which demonstrated that this current was I_{BKCa} . CCK-8S increased I_{BKCa} in a concentration-dependent manner ($n = 8$, $P < 0.05$, at 10^{-8} , 10^{-7} and 10^{-6} mol/L; $EC_{50} = 3.5 \times$

10^{-8} mol/L; Figure 4B). Compared with the CaPSS control group, CCK-8S (10^{-7} mol/L) enhanced peak I_{BKCa} depolarized to +60 mV by about $18.7\% \pm 2.1\%$ (from 916 ± 183 pA to 1088 ± 226 pA, $n = 8$, $P = 0.029$) (Figure 4A). Although this enhancement effect was blocked by pretreatment with 10^{-7} mol/L devazepide (908 ± 109 pA, $n = 8$, $P = 0.012$ vs CCK-8S group, $P = 0.083$ vs control group) (Figure 4A), the CCK2 receptor antagonist CI 988

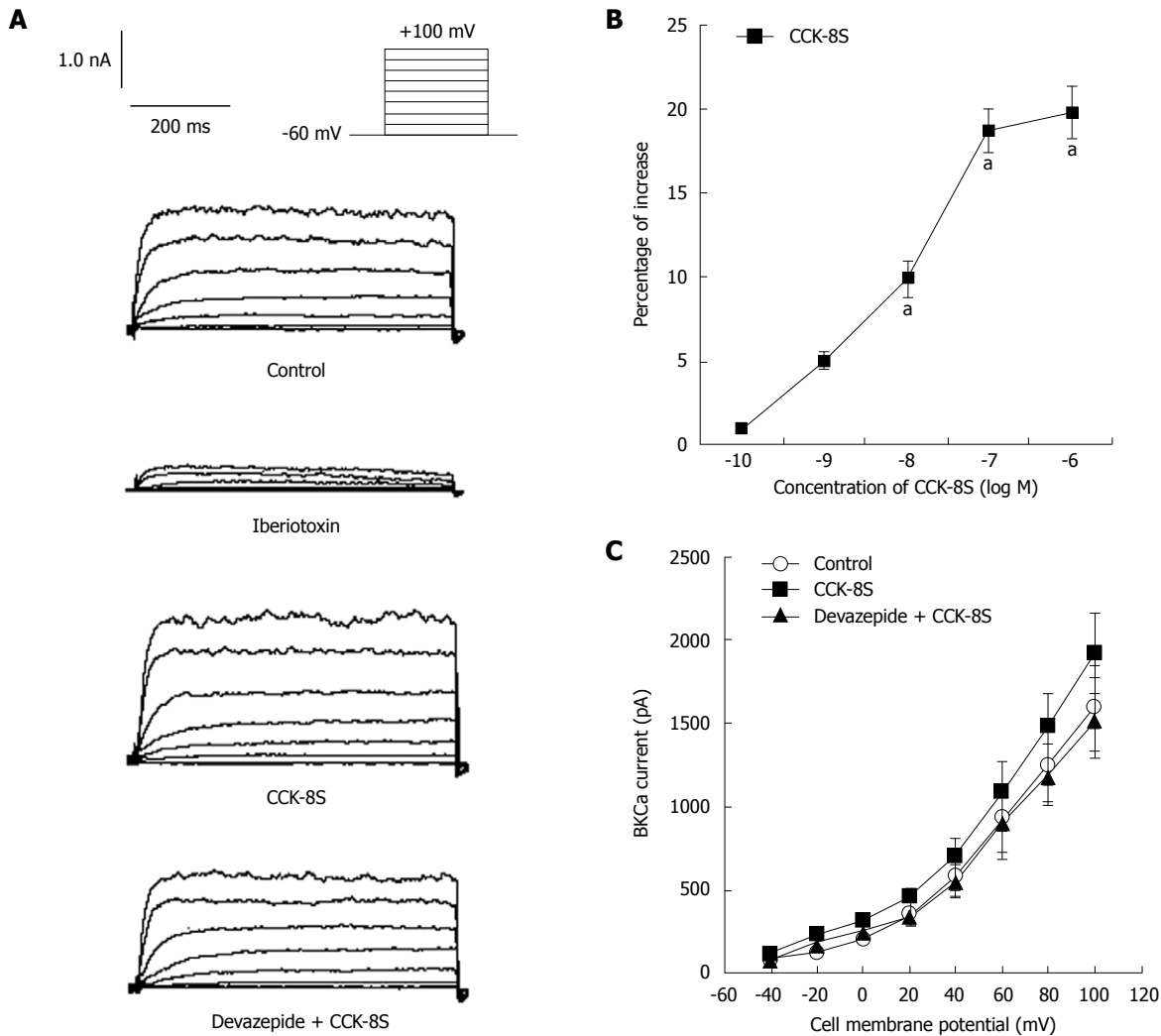


Figure 4 Effects of CCK-8S on I_{BKCa} of PCSMCs. A: Current traces of I_{BKCa} were recorded from -60 mV to +100 mV during 400 ms depolarization; B: CCK-8S increased I_{BKCa} in a concentration-dependent manner ($EC_{50} = 3.5 \times 10^{-8}$ mol/L); CCK-8S (10^{-7} mol/L) enhanced I_{BKCa} depolarized from -60 mV to +60 mV (from 916 ± 183 pA to 1088 ± 226 pA; $n = 8$, $P < 0.01$); C: Current-voltage relationship of average peak I_{BKCa} under each condition. CCK-8S-evoked enhancement of I_{BKCa} was suppressed by devazepide (10^{-7} mol/L). $^a P < 0.05$ vs control group (CaPSS).

(10^{-7} mol/L) had no effect (1052 ± 196 pA, $n = 8$, $P = 0.098$ vs CCK-8S group).

When heparin (10^{-6} mol/L), an inhibitor of IP_3 receptors, was added to the pipette solution, I_{BKCa} in cells depolarized to +60 mV was 813 ± 126 pA. CCK-8S (10^{-7} mol/L) did not enhance I_{BKCa} (879 ± 117 pA, $n = 8$, $P = 0.074$ vs control group, $P = 0.016$ vs CCK-8S group) (Figure 5A and B). To investigate whether the CCK-8S-intensified effect on I_{BKCa} was mediated by the PKC pathway, SMCs were pretreated with the PKC inhibitor staurosporine (10^{-6} mol/L), and I_{BKCa} in cells depolarized to +60 mV was 835 ± 112 pA. CCK-8S (10^{-7} mol/L) had no effect on I_{BKCa} (887 ± 120 pA; $n = 8$; $P = 0.069$ vs control group; $P = 0.041$ vs CCK-8S group; Figure 5C and D).

Effects of CCK-8S on I_{Ca-L} in PCSMCs

With nystatin-perforated whole-cell voltage-clamp recordings, I_{Ca-L} was evoked by using a depolarizing step pulse from a holding potential of -40 mV to +30 mV for 400 ms, with an interpulse interval of 10 ms (Figure 6A). I_{Ca-L} reached a maximum value at around +10 mV and the in-

ward current was markedly blocked by 80% by nifedipine (10^{-5} mol/L), which indicated that this current was I_{Ca-L} (Figure 6C). CCK-8S increased the amplitude of I_{Ca-L} in a concentration-dependent manner ($n = 8$, $P < 0.05$, at 10^{-8} , 10^{-7} and 10^{-6} mol/L; $EC_{50} = 3.2 \times 10^{-8}$ mol/L; Figure 6B). When CCK-8S (10^{-7} mol/L) was applied for 3-6 min, the amplitude of I_{Ca-L} was augmented by about 40% (from 60 ± 8 pA to 84 ± 11 pA), compared to the normal controls (at +10 mV, $n = 8$, $P = 0.012$) (Figure 6A). Although this enhancement effect was completely inhibited by 10^{-7} mol/L CCK1 receptor antagonist devazepide (61 ± 9 pA; at +10 mV, $n = 8$, $P = 0.023$ vs CCK-8S group, $P = 0.079$ vs control group) (Figure 6C), the CCK2 receptor antagonist CI 988 (10^{-7} mol/L) had no effect (84 ± 11 pA, $n = 10$, $P = 0.079$ vs CCK-8S group). CCK-8S-intensified I_{Ca-L} was reduced by $91.7 \pm 5.6\%$ by 10^{-5} mol/L TG and BA (7 ± 5 pA, at +10 mV, $n = 8$, $P = 0.006$ vs CCK-8S group) (Figure 6C). The current-voltage relationships are shown in Figure 6D. The peak current amplitudes of I_{Ca-L} under each condition were normalized relative to the maximum control current amplitude.

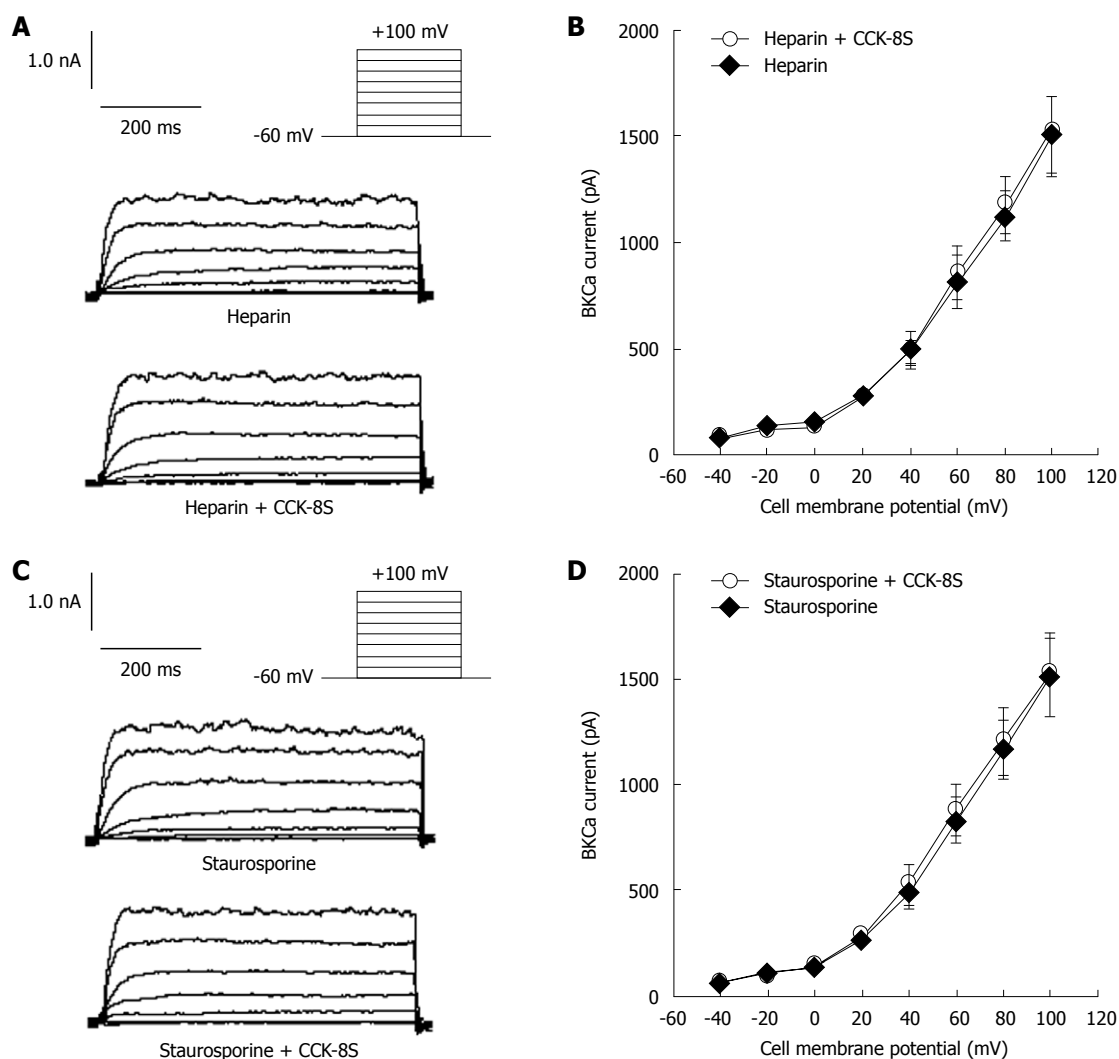


Figure 5 Heparin and staurosporine prevented CCK-8S-intensified I_{BKCa} in PCSMCs. **A** and **C**: Current traces of I_{BKCa} were evoked by a step from -60 mV to +60 mV; **B**: When heparin (10^{-6} mol/L) was present in the pipette solution, CCK-8S (10^{-7} mol/L) did not increase I_{BKCa} ; **D**: CCK-8S (10^{-7} mol/L) failed to increase I_{BKCa} in the presence of staurosporine (10^{-6} mol/L).

When 10^{-6} mol/L heparin was added to the pipette solution, CCK-8S (10^{-7} mol/L) had no effect on I_{Ca-L} (63 ± 12 pA, at +10 mV, $n = 8$, $P = 0.183$ *vs* control group, $P = 0.042$ *vs* CCK-8S group) (Figure 7). When SMCs were pretreated with 10^{-6} mol/L PKC inhibitor staurosporine for 10 min, CCK-8S (10^{-7} mol/L) did not increase I_{Ca-L} (65 ± 10 pA, at +10 mV, $n = 8$, $P = 0.215$ *vs* control group; $P = 0.032$ *vs* CCK-8S group) (Figure 7).

DISCUSSION

In the present study, we demonstrated that: (1) CCK-8S prompted the contraction of guinea pig PCSM strips and intensified I_{BKCa} and I_{Ca-L} by CCK1 receptors *via* activation of the IP_3 -PKC signal transduction pathway; and (2) CCK-8S increased AP amplitude through enhancing I_{Ca-L} , and accelerated fast repolarization of AP by increasing I_{BKCa} .

CCK has been implicated in the pathophysiology of functional digestive diseases, such as IBS^[1-3] and exerts its effects by two specific receptors, designated as CCK1 and CCK2, which are expressed in the colon and have

a high affinity for CCK-8S^[3,8-15,21,22]. However, it remains unknown which receptor plays a more important role in mediating colon contractility. Our results showed that CCK-8S not only promoted the mean contractility and frequency of longitudinal PCSM strips, but also the mean contractility of circular PCSM strips. Devazepide and CI 988 are CCK antagonists that are selective for the CCK1 and CCK2 receptor, respectively, and are used widely in scientific research^[23,24]. Pretreatment with CCK1 receptor antagonist devazepide markedly abolished CCK-8S-intensified contraction of PCSM strips, whereas CCK2 receptor antagonist CI 988 had little effect, thus indicating that CCK1 instead of CCK2 receptor plays a major role in mediating the motility of the proximal colon. It is consistent with the effect of CCK-8S on the gastric antral smooth muscle^[7] and human ascending colonic smooth muscle^[14].

Elevation in $[Ca^{2+}]_i$ concentration is an important function in the regulation of cell contraction and can be accomplished by release from internal calcium stores, extracellular Ca^{2+} influx across the plasma membrane, or both^[25,26]. Ca^{2+} -ATPase inhibitor TG and $[Ca^{2+}]_i$ chela-

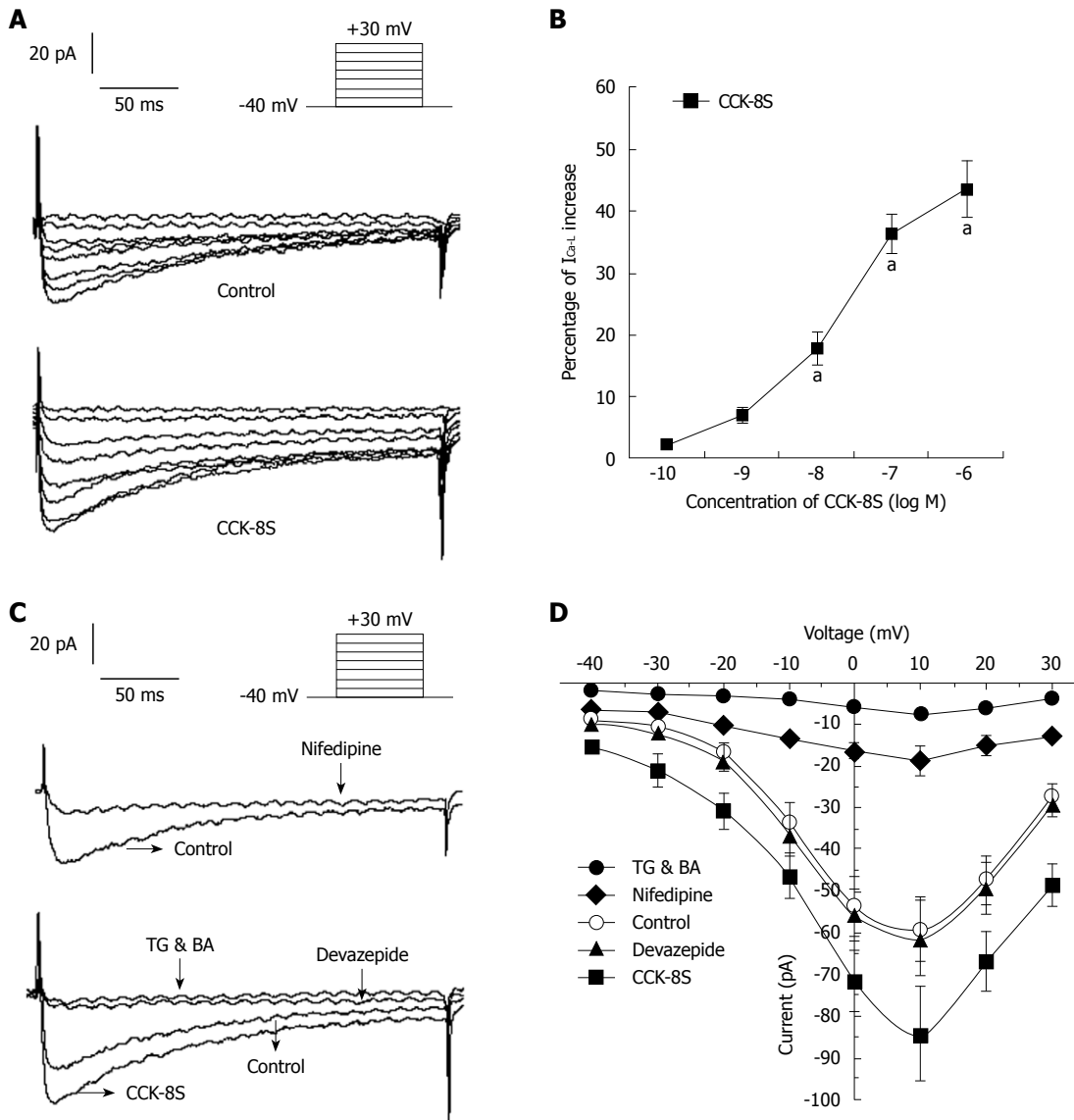


Figure 6 Effects of CCK-8S, nifedipine and devazepide on I_{Ca-L} in PCSMCs. A: Current traces of I_{Ca-L} were recorded from -40 mV to +30 mV during 400 ms depolarization; B: CCK-8S enhanced I_{Ca-L} in a concentration-dependent manner ($EC_{50} = 3.2 \times 10^{-8}$ mol/L); CCK-8S (10^{-7} mol/L) increased I_{Ca-L} depolarized from -40 mV to +10 mV (from 60 ± 8 pA to 84 ± 11 pA; $n = 8$); C: CCK-8S-intensified I_{Ca-L} was markedly suppressed by devazepide (10^{-7} mol/L), nifedipine (10^{-5} mol/L), TG and BA (10^{-5} mol/L); D: Current-voltage curves of whole-cell patch clamp under each condition. Compared with control, CCK-8S (10^{-7} mol/L) significantly enhanced I_{Ca-L} , whereas devazepide, nifedipine, TG and BA inhibited I_{Ca-L} . ^a $P < 0.05$ vs control group (CaPSS).

tor BA are used to deplete $[Ca^{2+}]_i$ and are often used in experiments to explore the role of intracellular calcium stores and signaling pathways^[7,27,28], whereas nifedipine is often used to examine the role of extracellular Ca^{2+} influx^[7]. We found that CCK-8S-evoked contraction of PCSM was significantly antagonized by pretreatment not only with CCK1 receptor antagonist devazepide, but also with nifedipine, TG and BA, but CCK2 receptor antagonist CI 988 had little effect, thus indicating that CCK-8S acts on the CCK1 receptor and enhances extracellular Ca^{2+} influx by L-type calcium channels and promotes Ca^{2+} release from intracellular calcium stores by intracellular signaling pathways.

APs are responsible for the contractile activity of colon SMCs. The upstroke of the AP is mainly the result of calcium entry through L-type calcium channels, and the number of APs within a certain period of time can be

regarded as an indicator of contractility of gastrointestinal SMCs^[18,29]. In this study, we demonstrated that CCK-8S enhanced AP amplitude and shortened the period of fast repolarization, by increasing AP generation as well as depolarizing RP. All these effects could be inhibited by pretreatment with CCK1 receptor antagonist devazepide, but CCK2 receptor antagonist CI 988 had little influence. It is also well known that contraction of gastrointestinal SMCs results from the close interaction between the two mechanisms: an initial peak caused by calcium release from stores and a plateau phase caused by calcium influx^[30-33]. Based on these studies, we found that CCK-8S intensified I_{Ca-L} in a concentration-dependent manner, which was significantly attenuated by pretreatment with devazepide and nifedipine, but CI 988 exerted little effect, indicating that extracellular calcium entry through L-type calcium channels is essential for contraction of PCSM cells, and

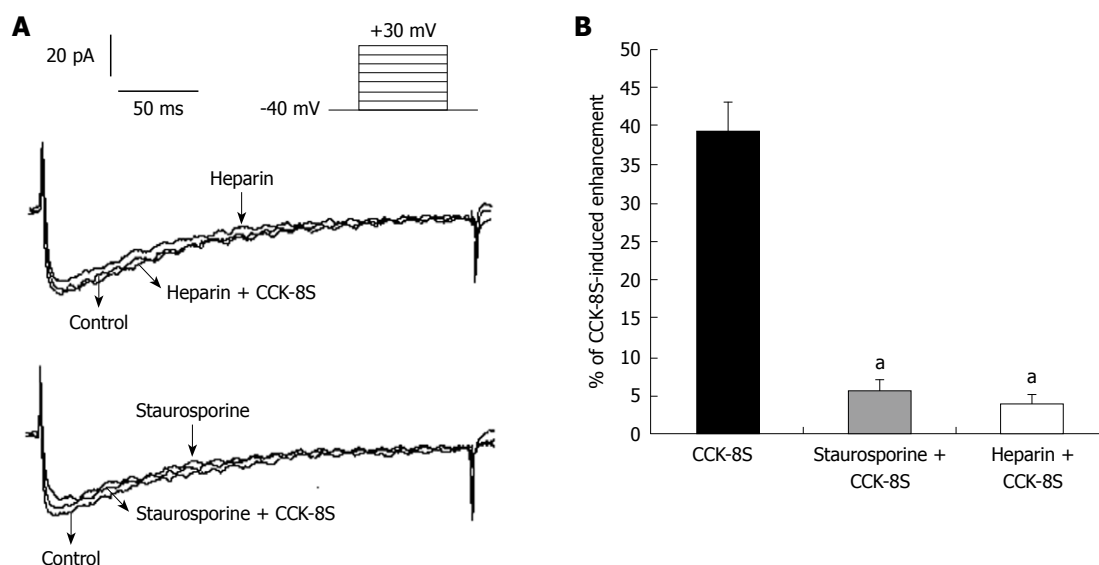


Figure 7 Heparin and staurosporine prevented CCK-8S-intensified I_{Ca-L} in PCSMCs. A: Current traces of I_{Ca-L} were evoked by a step from -40 mV to $+10$ mV. When heparin (10^{-6} mol/L) was present in the pipette solution, CCK-8S (10^{-7} mol/L) did not increase I_{BKCa} ; CCK-8S (10^{-7} mol/L) failed to increase I_{BKCa} in the presence of staurosporine (10^{-6} mol/L); B: CCK-8S-induced enhancement of I_{Ca-L} in the absence and presence of staurosporine and heparin, respectively. ^a $P < 0.05$ vs CCK-8S group.

the increase in I_{Ca-L} induced by CCK-8S through CCK1 receptors can depolarize membrane potentials, which may contribute to the decrease in RP. We also found that the depletion of $[Ca^{2+}]_i$ by the Ca^{2+} -ATPase inhibitor TG and $[Ca^{2+}]_i$ chelator BA completely blocked CCK-8S-intensified I_{Ca-L} and contraction of PCSM. Moreover, the characteristic of the current-voltage curve was not significantly altered. Thus, we can suppose that $[Ca^{2+}]_i$ stores are primarily responsible for the CCK-8S-induced contraction, and this can lead to depolarization of the membrane, which in turn leads to calcium influx through L-type calcium channels, to cause a further increase in $[Ca^{2+}]_i$ and, ultimately, contraction of PCSMCs.

Large-conductance calcium activated potassium channels have been identified in guinea pig colonic SMCs, and are associated with fast repolarization (repolarizing to 90% of the peak value of AP, T_{90})^[18,30]. We thus studied whether CCK-8S could also affect I_{BKCa} . We showed that CCK-8S shortened the time to T_{90} and augmented I_{BKCa} in a concentration-dependent manner, whereas this effect was inhibited by potassium channel blocker iberiotoxin, and the CCK1 receptor antagonist devazepide, but the CCK2 receptor antagonist CI 988 exerted little effect. Furthermore, the characteristics of the current-voltage curve were not apparently altered. Our results demonstrated that CCK-8S shortened the refractory period and accelerated the repolarization of the AP by increasing I_{BKCa} , mediated by activation of CCK1 receptors, which may enhance the contractile frequency and ultimately result in a synergistic effect on the contraction.

It is well established that IP_3 and PKC play an important role in the regulation of smooth muscle movement. Activation of phosphatidylinositol (4,5) biphosphate can generate IP_3 , which binds to IP_3 receptors in the sarcoplasmic reticulum (SR) and then induces the release of Ca^{2+} from the intracellular calcium stores, which immediately activates PKC to exert its biological effects^[7,33].

Likewise, serotonin promotes contraction of colonic myocytes, mostly as a result of Ca^{2+} release from SR following activation of the IP_3 pathway^[18]. In addition, CCK-induced gallbladder muscle contraction can be blocked by the PKC inhibitor staurosporine^[33]. In agreement with these results, we demonstrated that heparin, an inhibitor of IP_3 receptors, blocked CCK-8S-intensified I_{Ca-L} and I_{BKCa} in isolated PCSMCs. When cells were pretreated with the PKC inhibitor staurosporine, CCK-8S had no effect on I_{Ca-L} and I_{BKCa} . These results may further indicate that CCK-8S regulates I_{Ca-L} and I_{BKCa} by activating the IP_3 -PKC signal transduction pathway. However, the exact mechanism by which CCK-8S enhances the activity of IP_3 -mediated PKC has not been elucidated.

In conclusion, CCK-8S evokes contraction of the proximal colon in guinea pigs, mainly by promoting Ca^{2+} release from the SR and increasing Ca^{2+} influx through L-type calcium channels, *via* the IP_3 -PKC signal transduction pathway by activation of CCK1 receptors. In addition, the decrease in AP duration is caused by the acceleration of fast repolarization of AP by increased I_{BKCa} , and the increase in AP amplitude results from enhancement of I_{Ca-L} , which both ultimately contribute to the contraction induced by CCK-8S.

COMMENTS

Background

Cholecystokinin (CCK) acts as a hormone and neurotransmitter in the gastrointestinal tract, and regulates gut motility *via* two receptor subtypes, which have been characterized as CCK1 and CCK2, although the direct electrophysiological effect of CCK on the contractile activity of colon remains undetermined. The authors investigated how CCK regulates colon contraction through CCK receptors.

Research frontiers

CCK has been implicated in the pathophysiology of functional digestive diseases. In this study, the authors demonstrated the electrophysiological mechanisms that mediate CCK octapeptide (CCK-8S)-induced contraction and the relationship between the CCK-8S-triggered protein kinase C (PKC) and Ca^{2+} signaling pathways in colon smooth muscle cells.

Innovations and breakthroughs

The present study has proved that: (1) CCK-8S elicited stimulant effects on the motor activity of guinea pig proximal colon through CCK1 receptors, following activation of the inositol trisphosphate (IP₃)-PKC signal transduction pathway; and (2) the decrease in action potential (AP) duration was caused by acceleration of fast repolarization of the AP by increased large conductance potassium channel currents, and the increase in AP amplitude was caused by enhancement of L-type calcium current, which both ultimately contribute to the contraction induced by CCK-8S. This is believed to be the first report on the electrophysiological mechanisms of proximal colon contraction evoked by CCK-8S in guinea pigs.

Applications

The results of this study indicate that CCK stimulates proximal colon contraction through CCK1 receptors following activation of the IP₃-PKC signal transduction pathway, which could be useful in further study of functional digestive diseases, such as irritable bowel syndrome.

Peer review

This appears to be a well executed piece of work. The authors have established clearly that CCK-8S stimulates the contraction of colonic circular and longitudinal muscle through the CCK1 receptor, and that enhanced Ca effects are involved.

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Blocking effects of siRNA on VEGF expression in human colorectal cancer cells

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normal tissues ($P = 0.008$, $P = 0.000$, $P = 0.000$). The expression of VEGF was positively correlated with both lymph node metastasis and clinical stage ($P = 0.009$ and $P = 0.025$, respectively). Immunocytochemistry showed that the expression of VEGF was weakly positive and Western blotting indicated a significant reduction in VEGF-siRNA cell protein levels. VEGF-siRNA cell growth inhibition was assessed by the MTT assay, and the tumor cell proliferation rate was significantly different at 24, 48, and 72 h after transfection. FCM results showed that the VEGF-siRNA group had an apparent aneuploid peak.

CONCLUSION: VEGF, FLT-1 and FLK-1 are associated with colorectal carcinogenesis. siRNA silencing of the VEGF gene suppresses proliferation, and induces apoptosis in HCT116 cells. The results suggest that VEGF may be a new gene therapy target for colorectal cancer.

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Abstract

AIM: To investigate the expression of vascular endothelial cell growth factor (VEGF) and its receptors Fms-like tyrosine kinase 1 (FLT-1) and fetal liver kinase 1 (FLK-1) in colorectal carcinoma (CRC), and the blocking effects of small interfering RNAs (siRNAs) on VEGF expression in human colorectal cancer HCT116 cells.

METHODS: Immunohistochemical staining for VEGF, FLT-1 and FLK-1 proteins was performed in 82 cases of CRC and 14 normal colorectal mucosae. A siRNA targeting VEGF was synthesized and transfected into HCT116 cells using lipofectamine 2000. Immunocytochemical staining and Western blotting analyses were performed to detect the expression of VEGF protein. The suppressive effect of the siRNA on cell proliferation was detected using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltertrazolium bromide (MTT) assay. Cellular apoptosis was detected using flow cytometry (FCM).

RESULTS: The expression of VEGF, FLT-1 and FLK-1 in tumor tissues was significantly higher than that in

Key words: Colorectal carcinoma; Vascular endothelial cell growth factor; Fms-like tyrosine kinase 1; Fetal liver kinase 1; Small interfering RNA

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INTRODUCTION

Colorectal carcinoma (CRC) is one of the most common malignant diseases in China and has a poor survival

rate. The carcinogenesis and development of CRC is a complex process attributable to numerous factors. Angiogenesis plays an important role in tumor growth. Neovascularization is regulated by multiple factors, but the most important one is vascular endothelial cell growth factor (VEGF), which exerts its effects of increasing microvascular growth and permeability *via* specific receptors^[1]. Fms-like tyrosine kinase 1 (FLT-1) and fetal liver kinase 1 (FLK-1) are the most important among these receptors. At present, the main treatment for CRC is excision of the primary tumor, followed by radiotherapy and chemotherapy. However, in many cases, this still leaves problems such as metastatic lesions and subsequent cancer recurrence. Recently, following the development of modern molecular biology, in-depth researches have been conducted in developing new strategy of CRC treatment at a genetic level. In particular, RNA interference (RNAi) technology has the potential to become an effective treatment for tumors^[2], and has established a new area of clinical therapy for CRC^[3]. In this study, immunohistochemical staining using a streptavidin-peroxidase method for the VEGF, FLT-1, and FLK-1 proteins was performed on 82 CRC and 14 normal colorectal mucosal samples in an attempt to explore their roles in CRC. Moreover, a VEGF-targeting small interfering RNA (siRNA) was transferred into human colorectal cancer HCT116 cells to explore its effects on proliferation and apoptosis.

MATERIALS AND METHODS

Patients and samples

Eighty-two CRC specimens and matched adjacent normal mucosae were obtained from the Department of Pathology, First Affiliated Hospital, Anhui Medical University between 2007 and 2008. None of the patients had been treated with radiotherapy or chemotherapy before surgery. Samples were taken from areas of the tumor tissue with no evidence of hemorrhage or putrescence. Samples of matched normal mucosa were collected from the surgical cutting edge, which was approximately 3-5 cm away from the cancerous lesion. The clinical diagnosis of all 82 patients was confirmed by histological examination after surgery. The colon cancer cell line, HCT116, was obtained from the Chinese Academy of Sciences.

Immunohistochemistry

The mouse anti-human VEGF monoclonal antibody (clone VG1), and rabbit anti-human FLT-1 and FLK-1 polyclonal antibodies (clone RAB-0525, clone RAB-0522) were purchased from Maixin Co. (China). Immunohistochemistry was performed as follows: Sections were deparaffinized and rehydrated for 3 × 3 min in xylene; 3 × 2 min in 100% ethanol; 2 min in 95% ethanol, 2 min in 75% ethanol and, finally, 2 × 1 min in distilled water. Antigens were recovered by heating in a microwave oven for 15 min, and the sections were washed for 3 × 5 min with phosphate buffered solution (PBS). Endogenous peroxidase activity was blocked by soaking the slides in

a solution of 3% hydrogen peroxide for 15 min at room temperature (RT), followed by washing for 3 × 5 min with PBS. Non-immune animal blood serum (50 μL) was added to each section for 15 min at RT, followed by primary antibody (50 μL). Slides were incubated overnight at 4°C in a humidified chamber, then washed for 3 × 10 min in PBS. Biotin-labeled secondary antibody (50 μL) was added to each section and kept for 15 min at 37°C, followed by 3 × 5 min washes with PBS. Samples were incubated for 15 min at RT and washed for 3 × 5 min in PBS, before addition of 100 L freshly prepared 3,3-diaminobenzidine (DAB) for approximately 5-20 min. The reaction was stopped by washing in cold water. Slides were counterstained with hematoxylin followed by a sealing procedure using neural gum. A known-positive section served as the positive control, and sections in which the primary antibody was omitted served as negative controls. VEGF, FLT-1 and FLK-1 protein expressions were observed in the cytoplasm and graded according to Volm's standard^[4]: (1) Counting the percentage of positive cells within the total number in each high power field (no positive cells = grade 0, less than 25% positive = grade 1, 25%-50% = grade 2, > 50% = grade 3); and (2) The intensity of staining: no positively stained cells = grade 0, weak staining = grade 1, moderate staining = grade 2, strong staining = grade 3. Aggregate analysis of the results from (1) and (2): grade 0 = negative (-), grade 1-2 = weakly positive (+), grade 3-4 = moderately positive (++), grade 3-4 = strongly positive (+++). The intensity of the staining was estimated independently by two pathologists.

siRNA design

The sequence of the VEGF-targeting siRNA was as follows: Sense 5'-GGAGUACCCUGAUGAGAUCd TdT-3', Antisense 5'-GAUCUCAUCAGGGUACUC CdTdT-3'. All siRNAs were purchased from Shanghai ShineGene Molecular Biotechnology Co., Ltd. Each siRNA was resuspended in water and the stock solutions (20 μmol/L) were stored at 4°C until use.

Cell culture

The colon cancer cell line, HCT116, was grown as a monolayer in RPMI-1640 (Hyclone) containing 10% fetal bovine serum. Cells were maintained at 37°C in a humidified 5% CO₂ incubator.

Transfection of siRNA

siRNA transfection was done according to the protocol supplied by Invitrogen. Briefly, 1 × 10⁵ cells were seeded into six-well plates containing antibiotic-free medium and incubated overnight. For each well, 5 μL siRNA was mixed with 125 μL OPTI-MEM I. The mixture was then combined with a solution of 5 μL lipofectamine in 125 μL OPTI-MEM I and, after a 20-minute incubation at RT, the mixture was applied to the cells in a final volume of Opti-MEM I, making the final concentration of 100 nmol/L for each siRNA. After incubation for 6 h at 37°C, RPMI-1640 supplemented with serum was added.

Cells were then cultured for an additional 24 h at 37°C before analysis.

Immunocytochemistry

After 48 h transfection, HCT116 cells were fixed in cold methanol/acetone (1:1) at -20°C for 20 min and washed twice in PBS. The fixed cells were treated with 3% hydrogen peroxide for 5 min to eliminate endogenous peroxidase activity. After blocking with 1.5% horse serum for 20 min, the cells were incubated with primary anti-Survivin antibody (Santa Cruz) for 30 min at RT, or overnight at 4°C. The cells were then incubated with biotinylated goat anti-mouse IgG (Dako), followed by peroxidase-labeled streptavidin solution (Dako) for 20 min at RT. Antibodies were visualized using DAB (Sigma). All cells were counterstained with hematoxylin and the experiments were repeated three times. Negative control slides omitting the primary antibody were included for each staining.

Western blotting

Anti-VEGF and anti- β -actin antibodies were obtained from Zhongshan Goldenbridge Biotechnology Co., Ltd. (Santa Cruz). For Western blotting analysis, cells were harvested 48 h after transfection, washed with cold PBS, and lysed with phenylmethanesulfonyl fluoride lysis buffer. After centrifugation at 12000 r/min for 30 min, the supernatant was analyzed for protein content using the bicinchoninic acid reagent. Total protein (50 μ g) was electrophoresed on a 10% SDS-PAGE gel, and transferred onto a polyvinylidene fluoride membrane. The membranes were blocked with PBS buffer containing 5% fat-free milk and 0.1% Tween20 for 30 min at RT, and then incubated with primary anti-VEGF antibody for at least 1 h at RT, or overnight at 4°C. Finally, the membranes were washed three times with PBS containing 0.1% Tween20, incubated with peroxidase-conjugated secondary antibodies, and developed using the ECL reagent (Pierce).

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltertrazolium bromide (MTT) assay

Cells were grown in 96-well plates (1×10^3 cells/well) and then transfected with VEGF-targeting siRNA. Control cells were treated with DMEM containing 0.1% dimethyl sulfoxide (DMSO). At 24, 48, and 72 h following siRNA transfection, 20 μ L of MTT was added to each well to a final concentration of 0.5%. After a 4-h incubation at 37°C in the dark, 150 μ L DMSO was added to each well for 10 min to dissolve the formazan crystals. The absorbance was measured using an ELISA reader (EXL800, USA) at 490 nm. All experiments were repeated three times. The viability of the siRNA-transfected cells was expressed as a percentage of population growth plus the standard error of the mean relative to that of untransfected control cells. Cell death caused by siRNA-transfection was calculated as a percentage of inhibition as follows: % inhibition = $(1 - \text{mean experimental absorbance} / \text{mean control absorbance}) \times 100$.

Flow cytometric analysis

At 48 h after transfection, samples (1×10^6 cells) were fixed in 70% (v/v) ethanol, washed, and RNase (0.25 mg/mL) and Tween20 [0.1% (v/v)] were added for 30 min at RT. The cells were then stained with propidium iodide and analyzed using a flow cytometer (EPICS XL; Beckman). A sub-G0-G1 peak was detected on the DNA plots using the Cell Quest software. The peak indicates the apoptosis rate of the cells.

Statistical analysis

The statistical software package SPSS 13.0 was used. Data were analyzed using either the χ^2 test or Fisher's exact test. Non-parametric Spearman rank correlation analysis was used for analysis of ranked data. The rates were compared using Z tests. A *P* value < 0.05 was regarded as significant.

RESULTS

Expression of VEGF, FLT-1 and FLK-1 proteins in CRC and normal mucosa

Of the 82 CRC cases examined, 57 (69.5%) expressed VEGF protein. Among them, three cases (5.2%) were weakly positive (+), 30 cases (52.6%) were moderately positive (++), and 24 cases (42.1%) were strongly positive (+++). Of the 14 normal mucosa cases, only 4 (28.6%) were weakly positive for VEGF protein expression. These results confirmed that VEGF protein was present at significantly higher levels in CRC cases than in normal mucosa (*P* < 0.01). Also, FLT-1 (82.9%) and FLK-1 (81.7%) protein expression was significantly higher in CRC cases than in normal mucosa (*P* < 0.01) (Figure 1 and Table 1). We also found a positive correlation between FLT-1 and FLK-1 expression (*r* = 0.457, *P* < 0.05) in the CRC samples.

Relationship between VEGF, FLT-1 and FLK-1 protein expression and clinical pathological features in CRC

The positive rate for VEGF protein in the lymph node-positive group (86.7%) was significantly higher than that in the lymph node-negative group (59.6%, *P* < 0.01), and there were significant differences between the stage A, B, and C + D groups (57.9%, 60.6%, and 86.7%, respectively, *P* < 0.05). The expression of VEGF protein was not correlated with age, gender, depth of tumor invasion, or degree of differentiation (*P* > 0.05). Moreover, the expression of the FLT-1 and FLK-1 proteins was not correlated with any clinical pathological features of CRC (*P* > 0.05, Table 2).

Expression of VEGF protein in HCT116 cells after transfection

The cells positively stained by the anti-VEGF antibody accounted for 92%-100%. The normal control group, Lipofectamine 2000 control group, and mismatched control group all showed strongly positive cytoplasmic

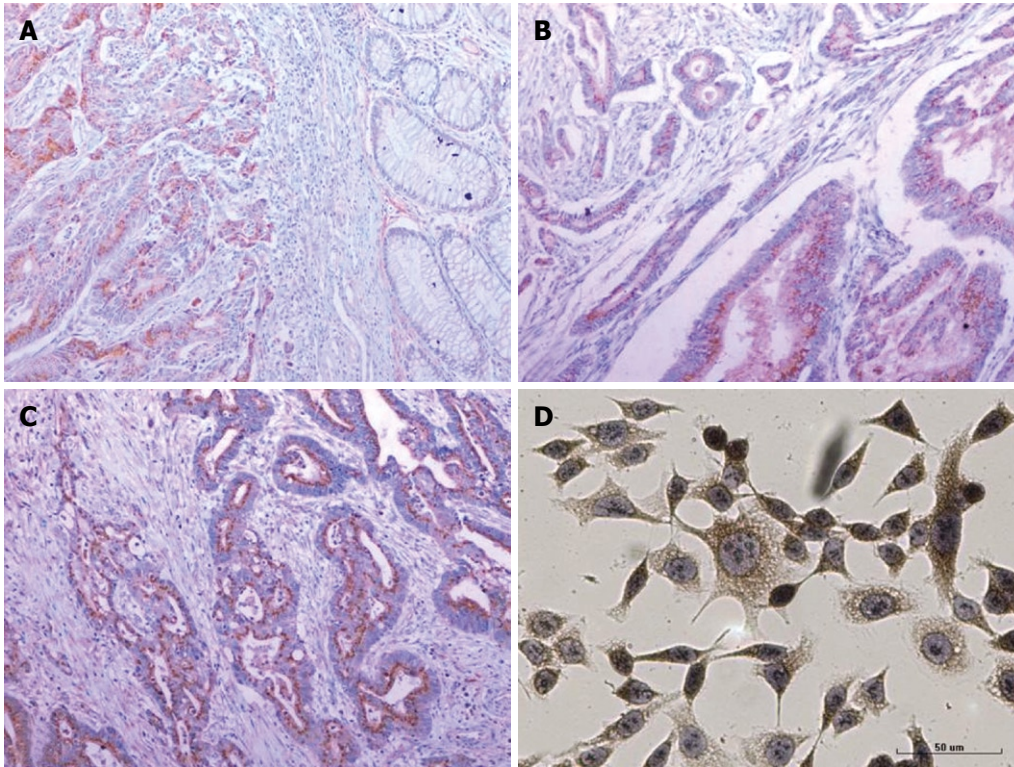


Figure 1 Immunohistochemical staining of colorectal carcinoma (CRC) tissue sections and immunocytochemical staining of HCT116 cells. The expression of vascular endothelial cell growth factor (VEGF) (SP \times 100, A), Fms-like tyrosine kinase 1 (FLT-1) (SP \times 100, B) and fetal liver kinase 1 (FLK-1) (SP \times 100, C) is positive in the CRC sections and strongly positive in HCT116 cells (SP \times 400, D).

Table 1 Expression of VEGF and its receptors FLT-1 and FLK-1 in CRC and normal mucosa <i>n</i> (%)							
	<i>n</i>	VEGF		FLT-1		FLK-1	
		Negative	Positive	Negative	Positive	Negative	Positive
Normal mucosa	14	10	4 (28.6)	9	5 (35.7)	10	4 (28.6)
CRC	82	25	57 (69.5) ^b	14	68 (82.9) ^b	15	67 (81.7) ^b

^b*P* < 0.01 vs normal mucosa. VEGF: Vascular endothelial cell growth factor; FLT-1: Fms-like tyrosine kinase 1; FLK-1: Fetal liver kinase 1; CRC: Colorectal carcinoma.

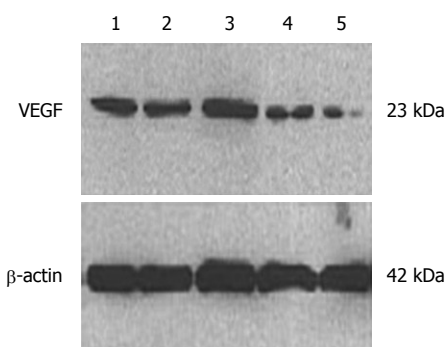


Figure 2 Expression of VEGF in HCT116 cells after VEGF-small interfering RNA (siRNA) transfection. 1: Normal control group; 2: Lipofectamine 2000 control group; 3: Mismatched control group; 4: VEGF-siRNA group; 5: 5-FU group. β -actin: Internal control.

expression of VEGF (dark brown yellow), while the VEGF-siRNA group showed only weakly positive cytoplasmic expression (light brown yellow). Western

blotting showed that VEGF-siRNA transfected cells expressed significantly less VEGF than the normal control, Lipofectamine 2000 control, or the mismatched control groups (Figures 1 and 2).

Effect of siRNA on cell proliferation

The proliferation rate of the HCT116 cell line 24, 48, and 72 h after transfection with VEGF-siRNA ($22.43\% \pm 3.08\%$, $24.53\% \pm 1.94\%$, and $28.53\% \pm 2.00\%$, respectively) was significantly higher than those of the normal control group (0.00, 0.00, 0.00) and the mismatched control group ($5.92\% \pm 0.49\%$, $6.10\% \pm 0.90\%$, $6.46\% \pm 0.60\%$, respectively) as assessed by the MTT assay. These effects were time-dependent. These results clearly showed that cell proliferation was markedly inhibited in the VEGF-siRNA-transfected group (Figure 3).

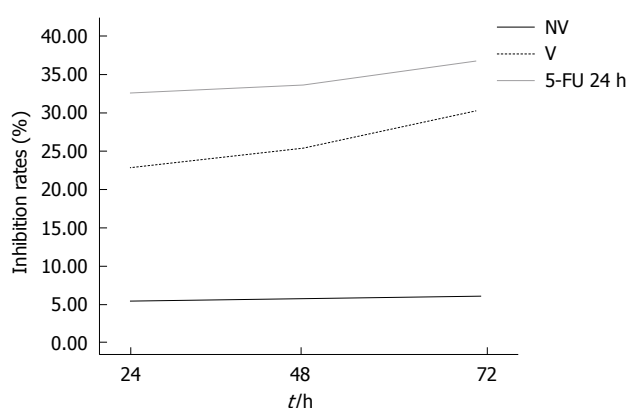
Cell apoptosis detected by flow cytometry (FCM)

To test for apoptotic cells, we used FCM to look for an apoptotic sub-G0-G1 (M1 value) peak at 48 h after

Table 2 Relationship between VEGF expression and its receptors FLT-1 and FLK-1 and clinical pathological features of CRC *n* (%)

	<i>n</i>	VEGF		FLT-1		FLK-1	
		Negative	Positive	Negative	Positive	Negative	Positive
Age (yr)							
< 60	41	14	27 (65.9)	10	31 (75.6)	9	32 (78.0)
≥ 60	41	11	30 (73.2)	4	37 (90.2)	6	35 (85.4)
Gender							
Male	49	13	36 (73.5)	11	38 (77.6)	10	39 (79.6)
Female	33	12	21 (63.6)	3	30 (90.9)	5	28 (84.8)
Depth of tumor invasion							
Not exceed muscular layer	25	10	15 (60.0)	5	20 (80.0)	5	20 (80.0)
Exceed muscular layer	57	15	42 (73.7)	9	48 (84.2)	10	47 (82.5)
Lymph node metastasis							
Negative	52	21	31 (59.6)	8	44 (84.6)	8	44 (84.6)
Positive	30	4	26 (86.7) ^b	6	24 (80.0)	7	23 (76.7)
Degree of differentiation							
Well	20	10	10 (50.0)	4	16 (80.0)	5	15 (75.0)
Moderate	41	10	31 (75.6)	5	36 (87.8)	5	36 (87.8)
Poor	21	5	16 (76.2)	5	16 (76.2)	5	16 (76.2)
Duke's stage							
A	19	8	11 (57.9) ^a	4	15 (78.9)	4	15 (78.9)
B	33	13	20 (60.6)	4	29 (87.9)	4	29 (87.9)
C + D	30	4	26 (86.7)	6	24 (80.0)	7	23 (86.7)

^a*P* < 0.05 *vs* stage B and C + D groups; ^b*P* < 0.01 *vs* the lymph node-negative group.

**Figure 3** Inhibition of HCT116 cell proliferation at different time points after VEGF-siRNA transfection (%).

siRNA transfection. The results showed that, compared with the normal control group ($M1 = 0.59$), and the mismatched group ($M1 = 0.63$), the VEGF-siRNA group displayed an apparent aneuploid peak ($M1 = 13.88$) (Figure 4), suggesting the apoptosis of the cells transfected with VEGF-siRNA.

DISCUSSION

Angiogenesis is necessary for tumors to grow beyond a certain size, and is a prerequisite for tumor invasion and metastasis. VEGF plays a critical role in the process of angiogenesis. VEGF is a glycoprotein of 34-50 kDa, first purified from cattle hypophysis folliculo-stellate cells in 1989^[5,6]. VEGF stimulates the proliferation and migration of vascular endothelial cells, and modeling of the tumor neovasculature. VEGF acts *via* specific recep-

tors, including FLT-1 and FLK-1. FLT-1 is mainly found in vascular endothelial cells^[7]. FLT-1 has a stronger affinity for VEGF than FLK-1, but is unable to activate the proliferation signal in vascular endothelial cells, so probably acts as a down-regulator of angiogenesis triggered by VEGF^[8]. By binding and activating FLK-1, VEGF stimulates the proliferation of endothelial cells, increases vascular permeability and induces neovascularization^[2,6]. Therefore, FLK-1 plays a more important role in tumor angiogenesis. Our study shows that the expression of VEGF, FLT-1 and FLK-1 were all significantly higher in CRC than in normal tissues ($P < 0.01$). The expression of VEGF was positively correlated with lymph node metastasis and with clinical stages of disease ($P < 0.05$). This indicates that VEGF, FLT-1 and FLK-1 are all associated with colorectal carcinogenesis.

One technique that has been used recently for the rapid and efficient identification of gene function is RNAi^[9]. This refers to a group of related post-transcriptional gene silencing mechanisms in which the terminal effector is a short antisense RNA^[10]. The use of RNAi in mammalian cells involves the transfection of an annealed 21-mer comprising sense and antisense RNA oligonucleotides (siRNAs) that correspond to a portion of the genes of interest. These RNAs then bind specifically to the cellular RNA and activate a process that leads to the degradation of the mRNA, and a subsequent 80%-90% decrease in the levels of the corresponding protein. Thus, RNAi is an important technique that specifically down-regulates the expression of cellular genes and has opened the door to the therapeutic use of siRNAs. In our study, we used RNAi technology to transfect a siRNA targeted against VEGF into HCT116 cells using Lipofectamine 2000. Immunocytochemistry

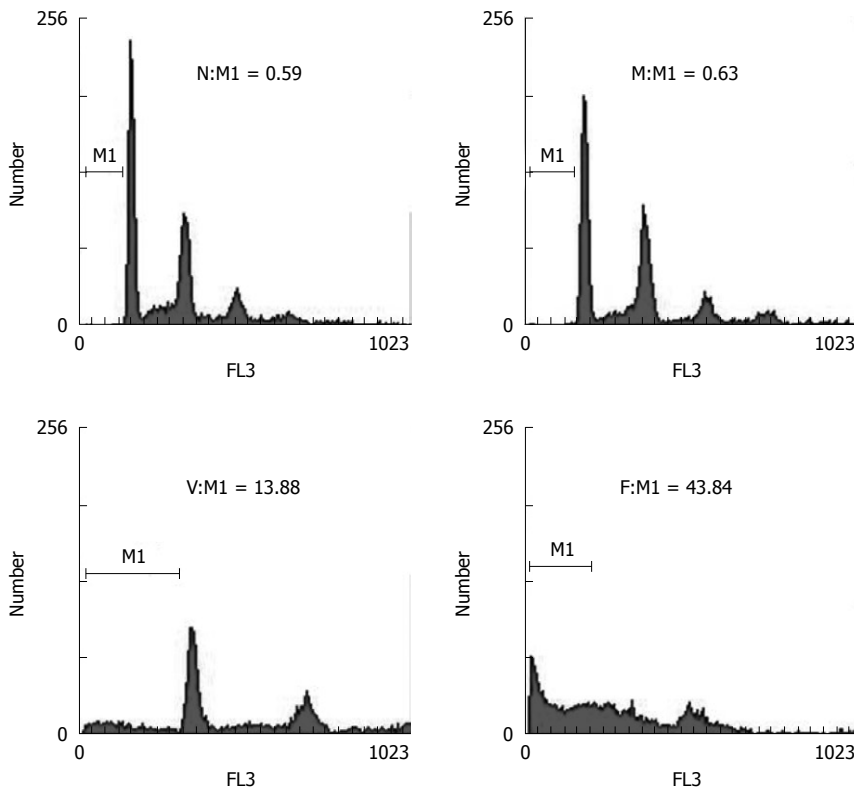


Figure 4 Apoptosis of HCT116 cells measured by flow cytometry (FCM). The percentage of the pre-G0-G1 population (peak) is shown for each histogram. The peak indicates the apoptosis rate of the cells. N: Normal control group; M: Mismatched group; V: VEGF-siRNA group; F: 5-FU group.

and Western blotting both showed that the expression of the VEGF protein was significantly reduced. Our results indicate that VEGF-siRNA can actively suppress VEGF protein expression and inhibit cell proliferation, all consistent with current literature^[11,12].

The appearance of the tumor cell proliferation is an important indicator of its biological function. In our study, blocking the VEGF expression using RNAi resulted in significant inhibition of cell growth in a time-dependent manner. A recent study showed that VEGF is closely associated with apoptosis. Castro-Rivera *et al*^[13] found that VEGF₁₆₅ could suppress apoptosis in lung cancer and breast cancer cells. Pidgeon *et al*^[14] found that VEGF could up-regulate bcl-2 levels in breast cancer cells, thus inhibiting apoptosis in both tumor cells and vascular endothelial cells. Our results show that the rate of apoptosis in the VEGF-siRNA group was significantly higher than that in the normal control group. This suggests that siRNA can effectively silence the VEGF gene and promote apoptosis in CRC cells.

In summary, our study suggests that VEGF is associated with colorectal carcinogenesis. Blocking VEGF expression using RNAi technology significantly reduces VEGF protein levels, suppresses the proliferation of HCT116 cells, and induces apoptosis. Our data indicate that siRNA may have therapeutic potential for inhibiting gene expression, and that VEGF is an attractive therapeutic target for the treatment of colon cancer.

COMMENTS

Background

Colorectal carcinoma (CRC) is one of the most common malignant diseases in

China and has a poor survival rate. Angiogenesis plays an important role in the growth of tumors. Neovascularization is regulated by multiple factors, the most important one is vascular endothelial cell growth factor (VEGF). VEGF acts to increase microvascular growth and permeability through binding to specific receptors, including Fms-like tyrosine kinase 1 (FLT-1) and fetal liver kinase 1 (FLK-1). Recently, RNA interference (RNAi) technology has been regarded as an effective tool that can be used against tumors, and has established a new domain for CRC clinical therapy.

Research frontiers

VEGF plays an important role in tumor angiogenesis through binding to vascular endothelial growth factor receptors, including FLT-1 and FLK-1. RNAi refers to a group of related post-transcriptional gene silencing mechanisms in which the terminal effector is a short antisense RNA. RNAi is an important method for specifically down-regulating the expression of cellular genes, and has opened the door to the therapeutic use of small interfering RNAs (siRNAs).

Innovations and breakthroughs

In this study, immunohistochemical staining for the VEGF, FLT-1, and FLK-1 proteins was performed in CRC samples to explore their roles in carcinogenesis. Also, a VEGF-targeting siRNA was transfected into human colorectal cancer HCT116 cells using Lipofectamine 2000 to investigate the effect of VEGF-targeted siRNA on the proliferation and apoptosis of HCT116 cells.

Applications

As indicated in this study, siRNA may have therapeutic potential for inhibiting the expression of specific genes, and VEGF is an attractive therapeutic target.

Peer review

In this manuscript, the authors demonstrate the relationship between the expression of VEGF and its receptors, FLT-1 and FLK-1, in CRC. The series of experiments was well planned and well performed, and the manuscript well written.

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Predictors of loss of hepatitis B surface antigen in HIV-infected patients

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Abstract

AIM: To study factors associated with loss of hepatitis B surface antigen (HBsAg) in patients co-infected with human immunodeficiency virus (HIV) and hepatitis B virus (HBV).

METHODS: We retrospectively reviewed the medical records of 5681 patients followed up at two New York City HIV clinics from January 1999 to May 2007. Clinical and laboratory parameters including baseline and follow-up HIV viral loads, CD4 cell counts, alanine transaminase levels, demographics, presence of hepatitis C infection, and treatment with highly active antiretroviral therapy dually active against both HIV and HBV infection, were analyzed to determine factors associated with loss of HBsAg.

RESULTS: Three hundred and fifty five patients (355/5681, 6.84%) were co-infected with HIV and HBV and were evaluated. Of these, 226 patients with more than 12 mo follow-up were included in further analysis to determine

factors associated with loss of HBsAg in the long-term follow-up. In the univariate analysis, baseline CD4 cell count was associated with loss of HBsAg ($P = 0.052$). Cox regression analysis revealed that loss of HBsAg was associated with baseline CD4 cell count > 500 cells/mm³ ($P = 0.016$, odds ratio: 76.174, 95% confidence interval: 2.233-2598.481).

CONCLUSION: Our study showed an interesting association of loss of HBsAg in HIV-HBV co-infected patients with higher CD4 cell count, suggesting that T-cell cytolytic activity against HBV may still be effective in clearing HBV infection.

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Key words: Human immunodeficiency virus; Hepatitis B; Viral antigens; Surface antigens

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Psevdos G Jr, Kim JH, Suh JS, Sharp VL. Predictors of loss of hepatitis B surface antigen in HIV-infected patients. *World J Gastroenterol* 2010; 16(9): 1093-1096 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v16/i9/1093.htm> DOI: <http://dx.doi.org/10.3748/wjg.v16.i9.1093>

INTRODUCTION

Co-infection with human immunodeficiency virus (HIV) and hepatitis B virus (HBV) poses a treatment challenge. In Western Europe and the United States, chronic HBV infection has been found in 6%-14% of HIV-positive patients^[1]. Increased HBV carriage rates, greater levels of HBV viremia, more rapid decline in hepatitis B surface antibody (HBsAb), increased reactivation episodes, faster progression to liver cirrhosis, and development of hepatocellular carcinoma at a younger age leading to increased

liver-related morbidity and mortality, are all characteristic of HIV and HBV co-infected patients. Also, there are lower incidences of spontaneous loss of HBV e antigen (HBeAg) or HBV surface antigen (HBsAg) because of impaired host innate and adaptive immunity^[2-4]. However, factors associated with HBsAg loss in HIV and HBV co-infected patients remain unclear. In this study, our aim was to analyze the characteristics of patients dually infected with HIV and HBV, and to determine factors associated with loss of HBsAg on follow-up.

MATERIALS AND METHODS

We conducted a retrospective medical chart review of 5681 patients followed in 2 HIV/AIDS clinics that comprise the Center for Comprehensive Care at St. Luke's-Roosevelt Hospital Center in New York City, from January 1999 to May 2007. All HIV-infected patients had positive enzyme-linked immunosorbent assay (ELISA) [HIVAB™ HIV-1/HIV-2 (rDNA) EIA, Abbott Laboratories, Abbott Park, IL, USA] and confirmatory Western blotting test (HIV 1/HIV 2 Western blot/Immunoblot, Quest Diagnostics, USA). HIV and HBV co-infection was defined as having both positive HIV infection and HBsAg serology as determined by an ELISA kit (VITROS® Immunodiagnostics, Ortho-Clinical Diagnostics, USA) for at least 6 mo of follow-up. Hepatitis C virus (HCV) serology was determined using ELISA (VITROS® Immunodiagnostics, Ortho-Clinical Diagnostics, USA). We compared patients that lost HBsAg to the rest of the cohort, at baseline and at time of loss of HBsAg. Clinical and laboratory parameters including baseline and follow-up HIV viral loads, CD4 cell counts, alanine aminotransferase (ALT) levels, HCV co-infection, demographics, and duration of anti-HBV therapy were analyzed to determine factors associated with loss of HBsAg. In the earlier study years, HBV DNA viral load, HBV genotype, and HBeAg determinations were not available for every patient, therefore these variables were excluded from the final analysis.

Statistical analysis

Statistical analysis was performed using SPSS, version 15.0 for Windows (SPSS). Dichotomous variables were compared using Fisher's Exact test or the Pearson χ^2 test. For continuous variables, the independent *t* test was used. Univariate analysis and then multivariate Cox regression analysis, with data censored at the time of loss of HBsAg, were conducted to determine factors associated with loss of HBsAg. Odds ratio (OR) and 95% confidence interval (CI) were calculated. A *P* value < 0.05 was considered to be statistically significant.

RESULTS

Of the 5681 HIV infected patients in our cohort, 355 patients (355/5681, 6.84%) who were co-infected with both HIV and HBV were evaluated. Of these, 226 patients with more than 12 mo follow-up were included in the further analysis to determine factors associated with

Table 1 Demographic, virological, immunologic characteristics, and outcomes of HIV and HBV co-infected patients in 2 groups according to HBsAg status

	Persistent HBsAg	Loss of HBsAg	<i>P</i>
Number of patients (%)	205 (90.7)	21 (9.3)	
Median age (IQR)	46 (42-51)	41 (33-55)	0.305
Gender (%)			0.353
Female	32 (15.6)	5 (23.8)	
Male	173 (84.4)	16 (76.2)	
Ethnicity (%)			
White	25 (12.2)	3 (14.3)	0.731
Hispanic	42 (20.5)	6 (28.6)	9.404
Black	134 (65.4)	12 (57.1)	0.453
Other	4 (2.0)	0 (0.0)	
HIV risk factor (%)			
Hetero	59 (28.8)	5 (23.8)	0.630
MSM	81 (39.5)	12 (57.1)	0.118
IDU	27 (13.2)	3 (14.3)	0.747
IDU and hetero	10 (4.9)	0 (0.0)	
IDU and MSM	6 (2.9)	0 (0.0)	
Other	5 (2.4)	0 (0.0)	
Unknown	17 (8.3)	1 (4.8)	1.000
HCV co-infection (%)			0.789
HCV positive	49 (23.9)	4 (19.0)	
HAART (%)			0.746
No HAART	30 (14.6)	2 (9.5)	
HAART	175 (85.4)	19 (90.5)	
ALT baseline			0.469
Median ALT baseline (IQR)	44.50 (26.00-79.00)	24.50 (17.50-100.50)	
CD4 baseline			
Median CD4 baseline (IQR)	270 (115-439)	271 (63-594)	0.449
CD4 baseline > 500 (%)	25 (12.2)	5 (23.8)	0.078
HIV viral load baseline			
Median HIV VL (log) (IQR)	4.47 (3.01-5.45)	4.03 (2.70-5.39)	0.841
HIV VL ≤ 400 (%)	40 (19.5)	3 (14.3)	0.763

Hetero: Heterosexual; MSM: Male sex with male; IDU: Intravenous drug use; HIV: Human immunodeficiency virus; HBV: Hepatitis B virus; HCV: Hepatitis C virus; HBsAg: Hepatitis B surface antigen; HAART: Highly active antiretroviral therapy with dually active agents against both HIV and HBV including lamivudine, tenofovir or both for more than 6 mo; ALT: Alanine aminotransferase; CD4 baseline: CD4 cell count baseline cells/mm³; HIV VL: HIV viral load copies/mL; IQR: Interquartile range.

loss of HBsAg in the long-term follow-up. The patients were observed for a mean duration of 45.6 mo (range, 20.8-61.1 mo). During the follow-up period, 21 (9.3%) of the 226 co-infected patients lost HBsAg. Of these 21 patients, 8 patients (38.1%) were noted to have developed HBsAb.

Table 1 shows demographic, virological and immunologic characteristics, and outcomes of the HIV and HBV co-infected patients in 2 groups according to HBsAg status. There were no differences in terms of age, gender, ethnicity, risk factor, HCV co-infection, CD4 cell count, HIV viral load, and ALT level at baseline between these 2 groups. Additionally, patients were categorized by CD4 cell count and HIV viral load. With regard to the patients with a CD4 cell count baseline > 500 cells/mm³, 25 patients (25/205, 12.2%) had persistent HBsAg, and 5 patients (5/21, 23.8%) had seroclearance of HBsAg. Although a higher CD4 cell count, > 500 cells/mm³, was observed in the HBsAg seroclearance group, this difference was

Table 2 Univariate analysis of factors associated with loss of HBsAg

	<i>P</i>
ALT baseline	0.737
HCV co-infection	0.660
HIV VL baseline	0.981
HIV VL end of follow-up	0.790
CD4 cell counts baseline	0.052
CD4 cell count end of follow-up	0.064
CD4 cell count gain	0.118
HAART	0.764

not statistically significant ($P = 0.078$). With regard to the patients with a baseline HIV viral load ≤ 400 copies, 40 patients (40/205, 19.5%) had persistent HBsAg, and 3 patients (3/21, 14.3%) had seroclearance of HBsAg, but there was no statistical significance, $P = 0.763$.

One hundred and ninety four HIV and HBV co-infected patients (194/226, 85.8%) received dually active antiretroviral therapy against HIV and HBV, including either lamivudine or tenofovir or both, for more than 6 mo as part of their highly active antiretroviral therapy (HAART) for HIV. Treatment with these dually active antiretroviral drugs did not differ between the 2 groups.

During the study period, more deaths in the persistent HBsAg group were noted (21/205, 10.2%) than in the HBsAg seroclearance group (1/21, 4.8%) although this was not statistically significant ($P = 0.702$).

In the univariate analysis, baseline CD4 cell count was associated with loss of HBsAg ($P = 0.052$). Other factors, including baseline ALT, presence of HCV co-infection, baseline HIV viral load, HIV viral load at end of follow-up, CD4 cell count at end of follow-up, CD4 cell count gain, and treatment with dually active antiretrovirals were not correlated with loss of HBsAg (Table 2).

Cox regression analysis revealed that baseline CD4 cell count > 500 cells/mm³ ($P = 0.016$, OR: 76.174, 95% CI: 2.233-2598.481) was associated with loss of HBsAg (Table 3).

DISCUSSION

Our clinics serve a large number of ethnically diverse HIV-infected patients in New York City. In our study, the prevalence of HIV and HBV co-infection was 6.84%. These results are consistent with previous data^[1], suggesting that our adult study cohort was representative of the current HIV and HBV co-infection epidemic in the United States.

The results of our study demonstrates that higher baseline CD4 cell count was significantly associated with loss of HBsAg in HIV and HBV co-infected patients. These results correspond with earlier studies which reported that higher initial CD4 cell count was associated with HBsAg seroconversion in HIV and HBV co-infected patients receiving lamivudine therapy^[5,6]. CD4 T-cell responses are believed to play a critical role in maintaining actively functioning cytotoxic T-lymphocytes (CTLs), which aid in release of antiviral cytokines^[7]. Inter-

Table 3 Multivariate Cox regression analysis of factors associated with loss of HBsAg

	Odds ratio (95% CI)	<i>P</i>
ALT baseline		
Normal ALT (ref)		
Elevated ALT	0.157 (0.013-1.829)	0.139
HCV co-infection		
HCV negative (ref)		
HCV positive	0.073 (0.002-2.802)	0.160
HIV VL baseline (copies/mL)		
HIV VL ≤ 75	3.018 (0.028-325.768)	0.644
HIV VL 76-500	9.195 (0.037-2289.798)	0.431
HIV VL 501-100000	1.026 (0.009-118.554)	0.991
HIV VL > 100000 (ref)		
HIV VL end of follow up (copies/mL)		
HIV VL ≤ 75	1134.708 (0.000-1.43E+151)	0.968
HIV VL 76-500	4403.835 (0.000-5.55E+151)	0.962
HIV VL 501-100000	3338.767 (0.000-4.19E+151)	0.963
HIV VL > 100000 (ref)		
CD4 baseline (cells/mm ³)		
CD4 < 200 (ref)		
CD4 200-350	0.983 (0.042-22.809)	0.992
CD4 351-500	0.000 (0.000-1.35E+034)	0.834
CD4 > 500	76.174 (2.233-2598.481)	0.016
CD4 end of follow-up (cells/mm ³)		
CD4 < 200 (ref)		
CD4 200-350	9.113 (0.141-587.310)	0.299
CD4 351-500	0.521 (0.018-15.301)	0.705
CD4 > 500	0.003 (0.000-0.631)	0.034
CD4 gain (cells/mm ³)		
CD4 gain ≤ 150 (ref)		
CD4 gain > 150	2.800 (0.122-64.365)	0.520
HAART		
No HAART (ref)		
HAART	0.117 (0.004-3.105)	0.199

Data for 226 patients. Data censored at the time of HBsAg loss.

estingly, HBV-specific CD4 T-cell responses are thought to be reduced in HIV and HBV co-infected patients^[8]. However, a recent study showed that the frequency and quality of HBV-specific T-cell responses increased with a higher CD4 cell count and there was no relationship between circulating HBV-specific T cells and liver damage as measured by fibrosis scores^[9]. Therefore a functioning immune system in HIV and HBV co-infected patients with very high CD4 cell count may enable a robust T-cell cytolytic response with production of anti-HBV cytokines such as interferon-gamma to help clear HBV infection in HIV co-infected patients.

Our study showed no significant association between elevated baseline ALT levels and loss of HBsAg. These results differ from those of Lau *et al*^[10] and Bonino *et al*^[11], which have shown a greater treatment response to either interferon or lamivudine in the setting of elevated ALT levels in chronic HBV patients without hepatitis C or HIV co-infection. However, none of our HIV and HBV co-infected patients were treated with interferon. It is also important to note that 53 patients out of our 226 studied patients (23.5%) were also co-infected with HCV. Therefore, findings from previous studies are not directly applicable to our study. A recent review article suggested that baseline ALT levels were not associated with treatment response in patients treated with nucleos(t)ide ana-

logues^[12]. There is conflicting and limited data regarding the utility of baseline ALT levels as a predictor of treatment response especially in the HIV and HBV co-infected patients. Further study is necessary to clarify the role of baseline ALT levels as a predictor of treatment response in this population.

Current guidelines^[13-15] recommend that HIV and HBV co-infected patients should be started on antiretroviral therapy with dually active agents such as lamivudine, tenofovir as part of HAART for HIV treatment regardless of CD4 cell count when treatment of HBV is indicated, based on HBV DNA levels, histological lesions, and serum ALT levels. Also, a significant number (50%) of expert panel members in the guidelines favor starting HAART in patients with CD4 cell count > 500 cells/mm³. Therefore, our study results would support initiating HAART earlier in the HIV and HBV co-infected patients with dually active agents against both HIV and HBV, even in patients with CD4 cell count > 500 cells/mm³ to maximize the T-cell response to both HIV and HBV.

There were some limitations to our study. It was a retrospective chart review. As previously mentioned, many patients did not have determination of HBV genotype, HBV viral load, HBeAg, or HBeAb. Therefore, we were not able to analyze these factors in our univariate and multivariate Cox regression analysis.

In summary, our study showed an interesting association of HBsAg loss in HIV-HBV co-infected patients with higher CD4 cell count, suggesting that T-cell cytolytic activity against HBV may still be effective in clearing HBV infection.

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COMMENTS

Background

Co-infection with human immunodeficiency virus (HIV) and hepatitis B virus (HBV) is associated with increased morbidity and mortality.

Research frontiers

The aim of this study was to determine factors that influence loss of hepatitis B surface antigen (HBsAg) in an HIV and HBV co-infected population.

Innovations and breakthroughs

In this large retrospective study, a strong association between high baseline CD4 cell count (CD4 cell count > 500 cells/mm³) and seroclearance of HBsAg in HIV and HBV co-infected patients was found, suggesting that very high CD4 cell count may enable a robust T-cell cytolytic response with production of anti-HBV cytokines such as interferon-gamma to help clear HBV infection in HIV co-infected patients.

Applications

The authors' study results would support initiating highly active antiretroviral therapy earlier in HIV and HBV co-infected patients with dually active agents against both HIV and HBV, even in patients with CD4 cell count > 500 cells/mm³, to maximize the T-cell response to both HIV and HBV.

Terminology

HBsAg seroclearance, loss of HBsAg; one of the criteria used for determination of resolution of hepatitis B virus infection.

Peer review

This is a large retrospective cohort study demonstrating that HBsAg seroclearance was associated with higher baseline CD4 cell counts in patients with HIV-HBV co-infection.

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Omeprazole induces gastric transmucosal permeability to the peptide bradykinin

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Abstract

AIM: To investigate omeprazole-induced transepithelial gastric leak and its effects on the permeability of the peptides bradykinin and oxytocin.

METHODS: Rat gastric corpus tissue was isolated and mounted in an Ussing chamber apparatus to evaluate the permeability of ^3H -bradykinin, ^3H -oxytocin, and ^{14}C -EDTA in the presence or absence of omeprazole. Thin-layer chromatography was performed to identify any metabolic breakdown products of the peptides resulting from permeation through the gastric tissue, and thereby calculate the true flux of the peptide.

RESULTS: The flux rate of intact ^3H -bradykinin increased substantially after omeprazole addition (109.5%) compared to the DMSO vehicle control (14%). No corresponding change in flux of intact ^3H -oxytocin was observed under the same conditions (11.9% and 6.4% in the DMSO- and omeprazole-treated conditions, respectively). After exposure to omeprazole, the flux rate of ^{14}C -EDTA also increased dramatically (122.3%) compared to the DMSO condition (36.3%).

CONCLUSION: The omeprazole-induced gastric leak allows for transmucosal permeability to charged molecules as well as non-electrolytes. This induced leak will allow certain peptides to permeate.

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Key words: Proton pump inhibitor; Paracellular; Drug delivery; Tight junction; Transepithelial

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INTRODUCTION

Our group has previously reported in patient-based (*in vivo*) and animal (*ex vivo*) studies that proton pump inhibitors (PPIs) induce a paracellular transepithelial leak in the gastric corpus^[1,2]. The leak allows unchanged, non-electrolyte probes (mannitol, sucrose, polyethyleneglycol) as large as 4000 Da to cross, and is bidirectional in nature. We have also investigated the medical significance of the

PPI-induced leak and have shown that the small molecule drug, digoxin, used to treat congestive heart failure and supraventricular arrhythmias, can cross the gastric mucosa in the presence of omeprazole^[3]. Hopkins *et al.*^[4] earlier speculated that PPIs may allow small molecule drugs to permeate across the gastric mucosa. This finding suggests that omeprazole has the potential to alter blood kinetics of certain orally-delivered small molecule drugs whose blood levels must be carefully titrated, and co-administration may therefore pose a significant clinical risk. Kiley *et al.*^[5] have very recently shown the clinical ramifications of such PPI-induced leak coinciding with PPI-mediated inhibition of liver cytochrome degradation of certain small molecule drugs such as digoxin. It is currently unknown, however, whether the possession of charge would prevent molecules from permeating through this induced leak. The aforementioned 4000 Da molecular weight “ceiling” on the omeprazole-induced leak was based on study of uncharged molecules only. This led us to investigate a small, charged (non-metabolizable) probe, EDTA (MW 380). The exact charges on the EDTA molecule vary depending upon pH^[6].

The leak induced by PPIs may carry drug delivery potential, due largely to the fairly safe profile of PPIs^[7]. Although the specific mechanism of the gastric leak is unknown, the leak seems to be benign to the tissue, carrying with it no morphological damage to gastric mucosa, a major benefit for a drug delivery vehicle. This current study also examines the gastric permeability of two characteristic small peptides, bradykinin (MW 1060) and oxytocin (MW 1007) (Figure 1) in the presence and absence of omeprazole. If omeprazole were to allow these peptides to leak across the gastric mucosa into the bloodstream, they would thereby avoid proteolytic digestion by trypsin and other digestive enzymes present in the duodenum.

MATERIALS AND METHODS

Animals, tissue dissection, Ussing chambers, incubation salines

Sprague Dawley male rats weighing approximately 400 g were sacrificed by decapitation and the stomach was quickly removed, cut along the greater curvature, and flushed of luminal contents. The serosal membrane was then stripped from the corpus region and two equal sized pieces of corpus mucosa were mounted in two separate Ussing chambers with exposed tissue areas of 1.13 cm²^[2]. Chambers were connected to 15 mL gas-lift reservoirs filled with un-buffered saline aerated with 100% O₂ and bicarbonate-buffered saline (pH 7.3) aerated with 95% O₂/5% CO₂ on the mucosal and serosal sides, respectively. Un-buffered saline was used on the mucosal side to allow for luminal acidification. Saline temperature was held constant at 37°C by a jacketed water bath surrounding the reservoirs. Thirty minutes at 37°C was allowed for tissue equilibration. In paired experiments, one tissue served as (vehicle) “control”, and the other as omeprazole-treated “experimental”.

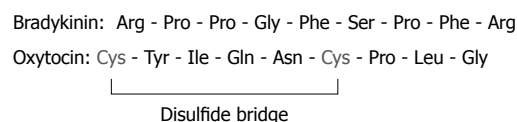


Figure 1 Amino acid structures of bradykinin (top) and oxytocin (bottom). Note the presence of the disulfide bridge in oxytocin.

Electrophysiology and mucosal pH

Electrical parameters were monitored throughout the duration of the experiment. Tissues were maintained under open circuit conditions. Ag/AgCl electrodes were bridged to saline in chambers with 4% agarose bridges stored in 1 mol/L NaCl. Tissue voltage (potential difference), short circuit current, and transepithelial resistance were recorded approximately every five minutes for the duration of the experiment, by passing a one-second current pulse equal to biological current to bring the potential difference to zero using a current/voltage clamp (McGrath Research & Technology). Transepithelial resistance was calculated by dividing open circuit potential difference by short circuit current (Ohm's law).

Mucosal and serosal saline pH measurements were taken at approximately 5 min intervals using a manual pH electrode (Denver Instruments) to ensure that the tissue was secreting acid. Mucosal pH readings were also taken before and after the addition of omeprazole to document the action of omeprazole on inhibiting acid secretion. Tissue viability was confirmed at the end of each experiment by tissue exposure to 10 mmol/L amiloride in the mucosal fluid compartment in order to inhibit short circuit current.

Transepithelial drug flux studies

After 30 min of tissue equilibration time in Ussing chambers, dibutyl cyclic AMP (dBcAMP) (Sigma) was added at a final concentration of 1 mmol/L to the serosal fluid chambers to stimulate acid secretion. Forty minutes following dBcAMP addition, protease inhibitors, pepstatin (1.8 µmol/L) and leupeptin (5 µmol/L), were added to the mucosal and serosal sides to prevent extracellular degradation of the peptide molecules of interest. Radiolabeled peptides under study [³H-bradykinin, ³H-oxytocin (PerkinElmer)] along with their unlabeled forms were added five minutes after the addition of protease inhibitors to the mucosal fluid of both chambers to achieve final concentrations of 0.3 mmol/L bradykinin or 5 µmol/L oxytocin in the mucosal fluid. The addition of the isotope marked the beginning of the flux period. Five hundred microliters of samples were taken from the serosal fluid compartment (opposite from which the isotope was added) for liquid scintillation counting (LSC). Samples were only taken from the mucosal fluid compartment at the end of the experiment to determine the specific activity of radioactivity initially added. The amount of radioactivity present in the mucosal compartment does not significantly change throughout the duration of the experiment. Serosal samples were initially taken at 45 min intervals for 90 min for the ³H-bradykinin

and ^3H -oxytocin fluxes to establish the basal permeability rate ($\text{pmoles}/\text{min per cm}^2$) across the tissue. About $200\text{ }\mu\text{mol}/\text{L}$ omeprazole (Sigma) (in DMSO) was then added to the mucosal and serosal sides of one chamber and an equivalent amount of DMSO (Sigma) was also added to both sides of the control chamber. Another series of serosal fluid samples were taken for LSC at 45 min intervals for the remaining 90 min of ^3H -bradykinin and ^3H -oxytocin flux experiments to determine flux rate of the radiolabeled drugs after omeprazole addition. In all cases of fluid sampling for liquid scintillation counting, an equal amount of fresh buffered saline was added back to the serosal side to prevent hydrostatic gradients from developing across the tissue. The flux of each isotope ($\text{pmole}/\text{min per cm}^2$) was determined by calculating the linear regression slope of the graph of cpm *vs* time, and converting cpm to picomoles after determining the specific activity of the drug in the mucosal fluid.

A similar flux protocol was followed for ^{14}C -EDTA experiments, with the following differences: ^{14}C -EDTA (ARC, Inc.) was added to the serosal fluid compartment 45 min after the addition of dBcAMP (no protease inhibitors were added) along with its unlabeled form to achieve a final concentration of $10\text{ }\mu\text{mol}/\text{L}$. Five hundred and fifty microliters of samples were taken from the mucosal fluid compartment (opposite from which the isotope was added) for LSC every 15 min for 75 min. About $200\text{ }\mu\text{mol}/\text{L}$ omeprazole (or DMSO) was then added and another series of mucosal fluid samples were taken every 15 min for the remaining 45 min of the flux period. Fresh unbuffered saline was added back to the mucosal fluid after each sample was taken.

Thin-layer chromatography

Additional saline samples were taken from the mucosal and serosal fluid compartments one minute before the addition of omeprazole/DMSO and at the end of the entire flux period to allow for thin-layer chromatography (TLC) analysis of radioactivity that crossed the tissue. TLC was performed to account for the potential contribution of radiolabeled metabolites to total radioactivity crossing the epithelial barrier, in the event of drugs entering gastric epithelial cells and metabolites being effluxed. Saline samples were taken from both surfaces of the tissue to determine possible degradation of the molecule upon exposure to the mucosal surface of the tissue as well as to analyze the chemical nature of the radioactivity that crossed the tissue. Saline samples from bradykinin and oxytocin flux experiments were concentrated by evaporation (Savant Speed Vac) to dryness and then resuspended in small volumes of double deionized water. TLC was performed on silica gel 60 plates with a 254 fluorescent indicator (Kodak) by spotting very small amounts of the concentrated sample. Plates were then placed in a sandwich tank and a variety of mobile phases were employed, including butanol/acetic acid/water (120:30:50), isopropanol/water/ NH_4OH (120:30:1), isopropanol/water (120:30), and ethyl acetate/methanol/water (81:11:8). After completion of the mobile phase,

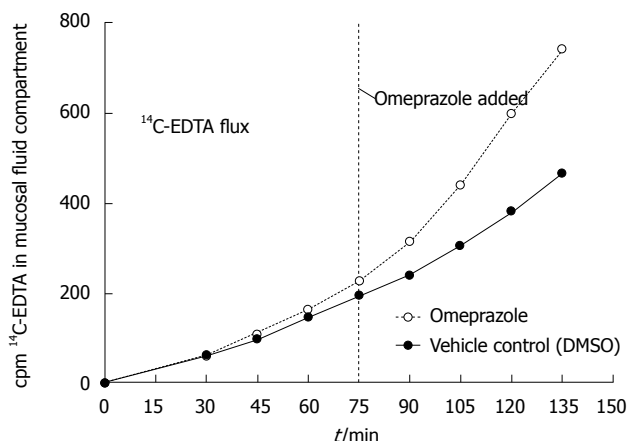


Figure 2 Effect of $200\text{ }\mu\text{mol}/\text{L}$ omeprazole on $10\text{ }\mu\text{mol}/\text{L}$ ^{14}C -EDTA flux rate across rat corpus mucosa. The flux rate of ^{14}C -EDTA dramatically increases over time after the addition of omeprazole compared to the DMSO vehicle control. Representative of three experiments and three separate animals. Samples were taken at 15 min intervals beginning at 30 min after the addition of the isotope. Omeprazole and DMSO were added to their respective chambers 75 min after the addition of ^{14}C -EDTA. The total flux time was 135 min. ^{14}C -EDTA was added to the serosal fluid compartment and samples were taken from the mucosal fluid compartment.

plates were sprayed with ninhydrin reagent to visually detect the location of proline or tyrosine, likely amino acid degradation products of bradykinin or oxytocin, respectively, and examined under a 254 UV lamp for detection of bradykinin or oxytocin, indicated by blocked fluorescence.

RESULTS

Ex vivo analyses indicate that omeprazole increases the rate at which the non-metabolizable, paracellular probe, ^{14}C -EDTA, diffuses across rat gastric mucosa compared to the vehicle control (DMSO). The amount of radio-labeled EDTA in the mucosal fluid compartment increased linearly over time in both conditions, but upon the addition of omeprazole to one chamber, there was a dramatic increase in the rate of diffusion of radioactive EDTA seen entering the mucosal fluid compartment (Figure 2). The observed increase in flux after treatment with omeprazole is continuous and nearly immediate in each of three experiments. When comparing the post-omeprazole flux values to the post-DMSO flux values, the flux rate of EDTA after the addition of omeprazole increased by $122.3\% \pm 8.5\%$ *vs* $36.3\% \pm 11.5\%$ for DMSO (Table 1). This evidences that the paracellular leak induced by omeprazole does allow a charged particle, ^{14}C -EDTA, to diffuse across the gastric mucosa. In Table 1 the values listed show the picomoles/min per cm^2 of ^{14}C -EDTA crossing the gastric mucosa in the DMSO and omeprazole conditions, with corresponding percent increases after the addition of each compound. Data from three experiments (and three animals) are shown. There is a consistent dramatic percent increase from the pre- to post-omeprazole addition compared to the pre- to post-DMSO (control) condition. The mean \pm SE of the mean

Table 1 Percent increase in flux of ^{14}C -EDTA (10 $\mu\text{mol/L}$) after the addition of 200 $\mu\text{mol/L}$ omeprazole

	Flux value ($\rho\text{mole/min per cm}^2$)				% increase in flux	
	Pre-DMSO	Post-DMSO	Pre-omeprazole	Post-omeprazole	After DMSO	After omeprazole
Experiment 1	1.78	2.03	1.52	3.18	14.0	109.0
Experiment 2	1.65	2.36	1.58	3.48	43.0	120.0
Experiment 3	1.50	2.28	1.69	4.03	52.0	138.0
mean \pm SE	1.64 \pm 0.08	2.22 \pm 0.10	1.60 \pm 0.05	3.56 \pm 0.25	36.3 \pm 11.5	122.3 \pm 8.5

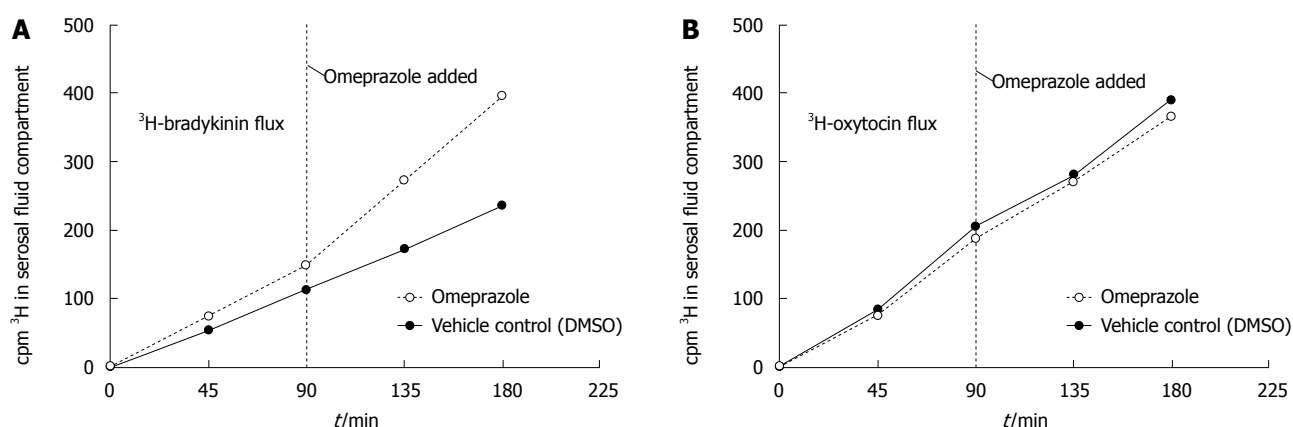


Figure 3 ^3H -bradykinin (panel A) or ^3H -oxytocin (panel B) was added to the mucosal fluid compartment at $t = 0$ to begin the flux period of 180 min. Omeprazole or DMSO was added to the tissue 90 min after the addition of ^3H -peptide. Samples were taken every 45 min for the total 180 min flux period (pre- and post-omeprazole/DMSO) from the serosal fluid compartment for LSC and TLC. Omeprazole increases the rate at which total radioactivity crosses the gastric mucosa compared to the paired vehicle control (DMSO) in a ^3H -bradykinin flux experiment (panel A) whereas total radioactivity crossing the tissue in a ^3H -oxytocin flux experiment (panel B) remains unchanged after the addition of omeprazole or DMSO. Both panel A and panel B represent two separate experiments and animals.

is also listed from the three experiments.

The total amount of tritium diffusing across the gastric mucosa (in a mucosal to serosal direction) also increased in the presence of omeprazole compared to DMSO in a ^3H -bradykinin flux experiment (Figure 3A), but did not increase under the same conditions in a ^3H -oxytocin flux (Figure 3B). Since, unlike EDTA, peptides are subject to metabolism by the gastric tissue, TLC was performed to analyze the total radioactivity from each flux experiment and differentiate intact ^3H -bradykinin or ^3H -oxytocin (data not shown) from ^3H -metabolites. TLC results revealed the presence of ^3H -metabolites in the serosal fluid after a 180 min ^3H -bradykinin flux experiment; however no substantial difference in relative proportions of ^3H -bradykinin and ^3H -metabolites between the omeprazole and DMSO conditions was seen (Figure 4A and B). Intact ^3H -bradykinin migrated with a known pure ^3H -bradykinin and unlabeled bradykinin standard (Figure 4C). After TLC analysis, flux values were adjusted to reflect the total amount of radiolabeled intact bradykinin or oxytocin crossing the gastric mucosa over the 180 min flux period. After the addition of omeprazole, there was a $109.5\% \pm 38.5\%$ (range) increase in the rate of ^3H -bradykinin flux compared to the DMSO vehicle control increase of only $14.0\% \pm 12.0\%$ (range) (Table 2). Both bradykinin experiments performed yielded similar results, with omeprazole increasing ^3H -bradykinin flux dramatically more than DMSO did. There was, however, no dramatic

difference in flux rate of ^3H -oxytocin after the addition of omeprazole or DMSO, with percent increases in flux being $11.9\% \pm 42.6\%$ (range) and $6.4\% \pm 9.9\%$ (range) in the DMSO- and omeprazole-treated conditions, respectively (Table 3). These figures reflect correction of the data for oxytocin metabolites in the 3H-cpm coming into the serosal fluids compartment (Figure 4D-F), as was done for the bradykinin flux measurements. It can thus be concluded that permeability to bradykinin is increased by omeprazole, but permeability to oxytocin is not.

Tables 2 and 3 show the actual flux values in mole/min per cm^2 and fmole/min per cm^2 pre- and post-omeprazole (or DMSO) addition. All values are corrected after TLC analysis of radioactivity. Two experiments were performed for each peptide (^3H -bradykinin and ^3H -oxytocin).

DISCUSSION

In previous patient-based^[1] and animal model studies^[2,3] our group has shown that proton pump inhibitors induce a transmucosal, paracellular “leak” in the gastric corpus. Using nonelectrolyte probes, it was shown that molecules as large as 4 kDa can permeate through this induced leak. Although there are many biomedical considerations to such leak, one possibility is that the leak could be used for oral administration of molecules (e.g. peptides, proteins) that normally could not cross the GI barrier into the

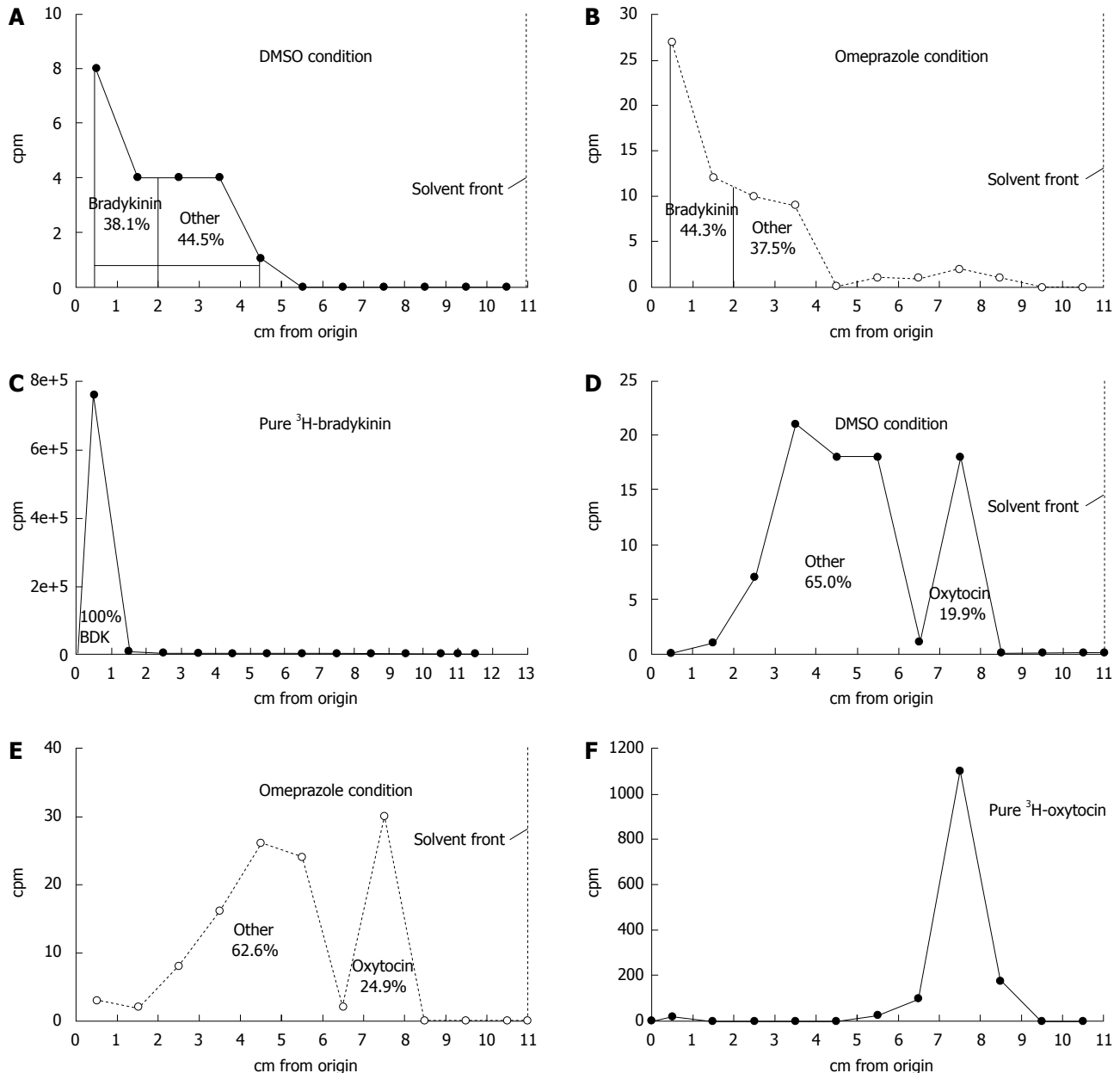


Figure 4 Thin-layer chromatography analysis of ^3H crossing the tissue from selected ^3H -bradykinin and ^3H -oxytocin flux experiments. The radiochromatograms shown are representative of the total radioactivity present in the serosal fluid compartment at the end of a 180 min flux period from the DMSO (A, D) and omeprazole (B, E) conditions. There are no obvious differences in the proportion of ^3H -peptide and ^3H -metabolites in either condition. The chromatographic profiles of pure ^3H -bradykinin (C) and pure ^3H -oxytocin (F) are also shown. 1 cm strips of the chromatogram were cut from the origin to the solvent front. Each strip was counted in a scintillation counter to determine the cpm. The location of ^3H -bradykinin or ^3H -oxytocin on the chromatogram was detected by its blocking of fluorescence at the origin under a short-wave UV lamp. All radiochromatograms were run in an isopropanol/water/ NH_4OH (120:30:1) solvent system on silica gel 60 plates with a 254 fluorescent indicator.

bloodstream and/or are metabolized in the lumen (e.g. duodenum) before uptake is possible. The field of drug delivery has long pursued agents capable of inducing transmucosal permeation without damaging the mucosal tissue.

In this study we have shown that a relatively small, charged molecule (EDTA) can pass through the omeprazole-induced gastric leak, suggesting that the possession of charge by a molecule will not prevent it from crossing through the leak. It made it reasonable to assume that other charged molecules would likely also diffuse through

the “pore”. We can only speculate, however, that the same would be true for a larger, charged molecule. If a charged molecule with a larger radius than EDTA (one closer to the size of the opening) were to come in contact with the omeprazole-induced pore, it may be more likely repelled because of its close proximity to the lining of the pore. In other words, if a charged molecule is small enough compared to the pore, it does not come close enough to be repelled by fixed charges lining the pore.

In the EDTA studies reported here the radioactivity coming across the gastric mucosa after each flux

Table 2 Percent increase in flux of ^3H -bradykinin (0.3 mmol/L) after the addition of 200 $\mu\text{mol/L}$ omeprazole

	Flux value ($\rho\text{mole/min per cm}^2$)				% increase in flux	
	Pre-DMSO	Post-DMSO	Pre-omeprazole	Post-omeprazole	After DMSO	After omeprazole
Experiment 1	10.2	10.4	12.4	21.2	2.0	71.0
Experiment 2	5.0	6.3	5.0	12.4	26.0	148.0
mean \pm range	7.6 \pm 2.6	8.35 \pm 2.1	8.7 \pm 3.7	16.8 \pm 4.4	14.0 \pm 12.0	109.5 \pm 38.5

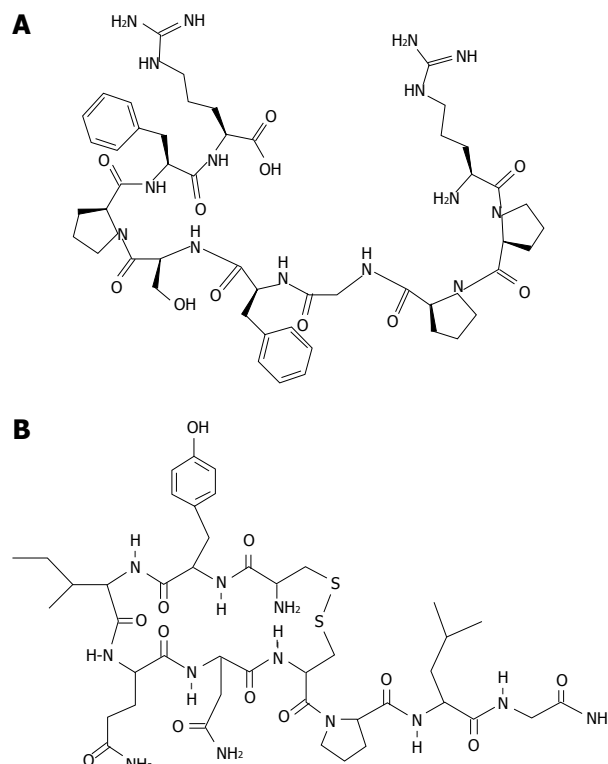
Table 3 Percent change in flux of ^3H -oxytocin (5 $\mu\text{mol/L}$) after the addition of 200 $\mu\text{mol/L}$ omeprazole

	Flux value (fmole/min per cm^2)				% increase in flux	
	Pre-DMSO	Post-DMSO	Pre-omeprazole	Post-omeprazole	After DMSO	After omeprazole
Experiment 1	20.9	32.3	38.7	45.0	54.5	16.3
Experiment 2	51.5	35.7	31.3	30.2	-30.7	-3.5
mean \pm range	36.2 \pm 15.3	34.0 \pm 1.7	35.0 \pm 3.7	37.6 \pm 7.4	11.9 \pm 42.6	6.4 \pm 9.9

experiment would be all true EDTA because EDTA does not permeate cell membranes, is metabolized only negligibly and is known to only travel paracellularly^[8,9]. We saw only one peak of radioactivity (data not shown), but because of the many forms molecular EDTA takes on at different pHs, consistent co-migration results were difficult to attain. Given the fact that EDTA will alter its charge as a function of pH it is worth highlighting that EDTA fluxes in this study were conducted in the serosal to mucosal direction. Serosal pH in these studies was maintained at pH 7.3. However in this direction, EDTA must traverse the intercellular space before presenting to the tight junction barrier. We do not know the exact pH of the intercellular fluid, but note that it is in communication with the pH 7.3 serosal fluid compartment.

Because charge was thus found to not be exclusionary for passage through the leak induced by omeprazole, the permeability of two small, similar size peptides (bradykinin and oxytocin) in the presence and absence of omeprazole was also investigated. Similar to EDTA, based on their amino acid moieties, peptides can have dynamically changing charges at different pHs. We observed that bradykinin (MW 1060) was found to diffuse through the omeprazole-induced leak while oxytocin (MW 1007) did not show an increase in flux in the presence of omeprazole. There are obvious differences in the molecular structures of bradykinin and oxytocin (Figure 5) albeit their sizes are very similar. Bradykinin is an open chain of amino acids, while oxytocin is more globular and rigid, containing a disulfide bridge. Also noteworthy is that bradykinin, but not oxytocin, has basic amino acids at its C and N termini. These structural points may be pivotal to permeation through a PPI-induced “pore”.

Because the gastric leak induced by omeprazole appears to be benign to the tissue, with no histological pathology or increased occurrence of apoptosis, it is possible it may have value as a drug delivery vehicle for certain biological drugs. Melittin has been used in epithelial cell culture as a barrier manipulator, but was

**Figure 5** Molecular structure of bradykinin (A) and oxytocin (B).

found to cause hemolysis and cytotoxicity in the process of enhancing permeability^[10]. The local toxicity caused by sodium caprate, another drug delivery agent, is a major downfall in its use in absorption enhancement^[11]. Yamamoto *et al.*^[12], in fact, reported a linear relationship between efficacy of various absorption enhancers and their toxicity. Contrary to these other barrier manipulators that are shown to enhance the barrier with negative local and systemic repercussions, omeprazole is a well-tolerated, widely-used, FDA-approved medication that opens the gastric paracellular barrier (tight junction) upstream from the duodenum with seemingly no physiological downside.

The leak exhibits a size limit, is highly specific to what types of molecules permeate, and is reversible upon discontinuation of PPI therapy. We postulate that these specificity characteristics are precisely why this leak occurs with little or no tissue morbidity.

We suspect that this PPI-induced transepithelial gastric leak to bradykinin is paracellular and not transcellular because we already demonstrated that the PPI-induced leak is bidirectional^[2]. In addition, the gastric epithelium is not specialized to transport molecules transcellularly like the duodenum. The gastric mucosa is not absorptive, as the majority of gastrointestinal absorption occurs downstream in the small intestine. Finally, however, note that there is evidence that as a result of PPIs raising gastric luminal pH, the protonation of certain molecules may be changed causing an alteration of their diffusion through the gastric epithelium^[13]. This remains another possible mechanism of how bradykinin may have crossed the gastric mucosa in the presence of omeprazole elevating mucosal fluid pH.

The ability of bradykinin to permeate suggests that this leak may be useful as a drug delivery vehicle for synthetic peptides. The inability of oxytocin to pass suggests that the structure, charge, and hydrophobicity of a peptide will matter considerably to its paracellular permeation, and the exact criteria for permeation of future candidate peptides need to be determined. Future studies will look into establishing both the inclusionary and exclusionary structural criteria of peptide passage through the leak, as well as examine if the leak admits oligonucleotide permeation.

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COMMENTS

Background

The very recently discovered transmucosal gastric leak caused by proton pump inhibitor (PPI) drugs may have use in allowing oral delivery of certain peptides and oligonucleotides that might otherwise have to be injected. PPIs have little if any morbid effects on the gastric mucosa, and thus the induced leak appears to not be harming the tissue.

Research frontiers

PPIs are very widely utilized drugs for treatment of reflux disease and gastritis. The ability of PPIs to induce a gastric leak is just beginning to be explored. In this manuscript a positive aspect of such leak, namely a delivery route for other oral medications that normally have no way into the bloodstream, is explored.

Innovations and breakthroughs

In previous work, this leak was shown to be bidirectionally symmetric and allow only molecules less than 10 kDa to pass. In this current study the leak is shown to allow charged molecules in general to pass, and specifically allow the peptide, bradykinin, to pass. The inability of the similarly-sized peptide, oxytocin, to pass through the leak suggests that the leak possesses considerable specificity in the types of molecules it will admit.

Applications

The field of drug delivery has long sought agents that can "open up" epithelial barriers but not cause morbidity for the tissues in the process. It is a significant problem given the physiological importance of these barriers and the noxious nature of many constituents of luminal fluid compartments.

Terminology

These studies utilize the Ussing chamber technique wherein gastric mucosal epithelial tissue is placed between two fluid-filled chambers. The passage across the tissue of radiolabeled probes, in this case, peptides, is then observed and measured, as a function of PPI exposure. These molecules are in all probability moving paracellularly across the tissue, and likely diffusing through altered epithelial tight junctions.

Peer review

This is a very interesting paper in which the investigators examined the gastric permeability of two characteristic small peptides, bradykinin and oxytocin in the presence or absence of omeprazole. This is a carefully conducted study by a well qualified group of investigators.

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New means to monitor the effect of glucocorticoid therapy in children

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Abstract

AIM: To study the individual effects of glucocorticoid (GC) therapy on the state of immune activation in patient serum.

METHODS: We developed a novel assay in which the effect of corticosteroid-treated patient serum on healthy donor peripheral blood mononuclear cells (target cells) was studied, with a panel of markers for effector [interferon (IFN) γ and interleukin (IL)-5] and regulatory T cells (FOXP3 and glucocorticoid-induced tumor necrosis factor receptor, GITR). The study group comprised 19 children with inflammatory bowel disease. The individual effect of patient serum on target cells was analyzed prior to GC therapy and 2 wk later.

RESULTS: The effect of GC therapy mediated by patient serum was seen as a decrease in the target

cells expression of regulatory T-cell-related markers GITR (median suppression 24%, range of suppression 1%-63%, in 2 cases increase of 6% and 77%, $P < 0.01$ for mitogen-activated target cells) and FOXP3 (median suppression 33%, range of suppression 0%-79%, in one case an increase of 173%, $P < 0.05$ for resting cells), and secretion of IFN γ [from a mean of 87 700 pg/mL (SD 33 900 pg/mL) to 60 900 pg/mL (SD 44 200 pg/mL) in mitogen-activated target cells, 13 of the cases showed a decrease, $P < 0.01$]. The total or weight-related prednisolone dose did not correlate with the patient-serum-induced changes in the target cell markers.

CONCLUSION: GC response could be monitored at an individual level by studying the effect of patient serum on signaling pathways of target immune cells.

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Key words: Glucocorticoid-induced tumor necrosis factor receptor; FOXP3; Inflammatory bowel disease; Children

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INTRODUCTION

Since the description of the life-saving effect of glucocorticoids (GCs) in the treatment of inflammatory bowel disease (IBD), GCs have been the mainstay in the treatment of moderate and severe forms of the disease^[1]. Although widely used, several patients show an

inadequate response to GCs, a phenomenon that could not have been foreseen. In patients with GC resistance, therapeutic effect is lacking. Those considered to be steroid-dependent benefit from the treatment but their disease flares up immediately after lowering the GC dose or discontinuation of the therapy. Among IBD patients, up to 31%-45% seem to become steroid-dependent^[2-4]. Other treatment options have not overcome the need for GCs in IBD, a disease that is constantly on the increase in many western countries^[5,6].

The pathogenesis of IBD is multifactorial. An inappropriate response of a mucosal immune system to the indigenous flora and luminal antigens plays a key role that leads to activation of innate immune cells that direct the effector and regulatory T-cell responses of adaptive immunity^[7].

GC action is mediated by a GC-receptor complex that is translocated into the nucleus. The genomic actions are classified as transrepression, that is, inhibition of the synthesis of the regulatory proteins, which results from the interaction between the activated GC-receptor complex and transcription factors, such as nuclear factor- κ B or activator protein-1, and transactivation, that is, induction of the synthesis of regulatory proteins that leads to downregulation of inflammatory cytokines, such as tumor necrosis factor- α , interleukin (IL)-12 and interferon (IFN) γ , and upregulation of anti-inflammatory cytokines, such as IL-10, and transforming growth factor β ^[8-11]. GCs have also non-genomic interactions with cellular membranes^[12].

Therapeutic response to GCs at an individual level is unpredictable. Here, we searched for new means to monitor the stage of immune activation during GC therapy. We developed a novel assay, in which the effect of patient serum on donor peripheral blood mononuclear cells (PBMCs) was studied using a panel of markers for effector (IFN γ and IL-5) and regulatory T cells (FOXP3 and glucocorticoid-induced tumor necrosis factor receptor, GITR). We found that attenuation of inflammation during GC therapy was reflected in these markers.

MATERIALS AND METHODS

Subjects

Nineteen consecutive pediatric patients with active IBD (Table 1) were prospectively introduced to oral prednisolone (Leiras, Finland), with a constant once daily dose of 1 mg/kg (range 15-60 mg) for 2 wk, as described in detail elsewhere^[13,14]. The need for GCs was based on the physician's global assessment of severe disease. After the 2-wk study protocol^[13,14], the clinicians tapered the GC dose and adjusted the therapy. At the start of GC therapy, the maintenance therapies (Table 1) were unaltered. All patients had undergone gastrointestinal endoscopy to establish the diagnosis of IBD^[15].

Blood samples

The first blood sample was taken prior to the first GC dose on the day that the clinician instructed the start of GC therapy. The second sample was taken after 2 wk

and the two samples were compared individually. Routine samples for blood count, hemoglobin, erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP) were obtained at the start of GC therapy and after 2 wk.

Laboratory assay

PBMCs and the plasma from the blood of healthy donors and patients were separated by Ficoll-Paque (Amersham Biosciences, Freiburg, Germany) centrifugation according to the protocol of the manufacturer. PBMCs were suspended in 1 mL of medium (RPMI with HEPES buffer (Gibco, Carlsbad, CA, USA) with 0.002 mmol/L glutamine (Gibco), 25 μ L/mL gentamicin (Gibco) and 5% serum), for counting. PBMCs were frozen at -136°C with DMSO (Sigma-Aldrich Corp, St. Louis, MO, USA). These cells from healthy donors were used as target cells. Target cells were diluted in cell culture medium (described above) without serum. Target cells, unactivated or activated with phytohemagglutinin (PHA, 5 μ g/mL) were cultured as duplicates in the presence of the IBD patients' inactivated (56°C for 35 min) serum, at a concentration of 8%, for 72 h at 37°C in a humidified atmosphere with 50 mL/L CO₂. After culture, the supernatants and target cells were collected by centrifugation at 400 g for 7 min. RT lysis buffer (Sigma-Aldrich) was added to the target cells, and the supernatants were stored at -70°C. The target cell assay was repeated using PBMCs from two different healthy donors (26-year-old woman and 31-year-old man) as target cells. Results represent one series of experiments.

ELISA for IFN γ and IL-5

IL-5 and IFN γ were measured with ELISA in duplicate from the supernatants collected from the target cell cultures incubated in the presence of patient serum. IFN γ and IL-5 were detected as described previously^[16,17]. We subtracted the non-stimulated value from the stimulated value to obtain the Δ value for statistical analysis.

Quantitative reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA was isolated from cell samples with GenElute Mammalian total RNA Miniprep kit (Sigma-Aldrich), and RNA concentration was measured by a spectrophotometer (ND-1000; NanoDrop Technologies Inc, Wilmington, DE, USA). Reverse transcription was performed by using TaqMan Reverse Transcription reagents (Applied Biosystems, Foster City, CA, USA), with additional treatment of total RNA at 10 ng/ μ L with DNase I (0.01 U/ μ L) (Roche Diagnostics, Mannheim, Germany) to eliminate genomic DNA. Quantitative RT-PCR was performed using pre-designed FAM-labeled TaqMan Gene Expression Assay reagents (Applied Biosystems) and the ABI Prism 7700 Sequence Detection System (Applied Biosystems) in triplicate wells. Assay reagents for FOXP3 (Hs00203958_m1), GATA3 (Hs00231122_m1), T-BET (Hs00203436_m1), GITR (Hs00188346_m1), IFN γ (Hs00174143_m1), IL-5 (Hs00542562_m1), IL-10 (Hs00174086_m1), STAT-5b (Hs00273500_m1), and 18s RNA (Hs99999901_s1) were

Table 1 Clinical characteristics of 19 children with IBD treated with oral prednisolone

Patient No.	Age (yr)	Sex	Diagnosis	Disease extension at diagnosis	Disease duration at inclusion (yr)	Maintenance medication at inclusion
1	14	Male	UC	Left-sided	2.9	5-ASA
2	14	Female	UC	Pancolitis	0.2	5-ASA
3	5.6	Female	UC	Distal	0.8	No
4	18	Male	UC	Pancolitis	5.6	5-ASA
5	17	Male	UC	Pancolitis	2.2	5-ASA
6	15	Female	UC	Pancolitis	3	5-ASA
7	15	Male	UC	Pancolitis	0.8	5-ASA
8	14	Male	UC	Pancolitis	0.1	5-ASA
9	14	Male	UC	Pancolitis	0	5-ASA
10	13	Female	UC	Pancolitis	3.7	5-ASA, AZA
11	12	Male	UC	Pancolitis	0	5-ASA
12	12	Male	UC	Pancolitis	0	5-ASA
13	12	Female	UC	Pancolitis	1.3	5-ASA
14	9.4	Male	UC	Pancolitis	4.2	5-ASA
15	7.4	Male	UC	Pancolitis	1.8	5-ASA
16	7.2	Male	UC	Pancolitis	1.3	5-ASA
17	15	Male	CD	Pancolitis	0	5-ASA
18	12	Female	CD	Pancolitis	10	5-ASA
19	16	Male	IC	Pancolitis	1.2	5-ASA

IBD: Inflammatory bowel disease; 5-ASA: 5-aminosalicylic acid; AZA: Azathioprine; IC: Indeterminate colitis; UC: Ulcerative colitis.

used. The quantitative value obtained from the TaqMan run was the threshold cycle Ct, which indicated the number of PCR cycles at which the amount of amplified target molecule exceeded a predefined fluorescence threshold value. The difference value (ΔC_t) was the normalized quantitative value of the expression level of the target gene, which was achieved by subtracting the Ct value of the housekeeping gene (18S) from that of the target gene. An exogenous cDNA pool calibrator was collected from PHA-stimulated PBMCs, and considered as an interassay standard to which normalized samples were compared. $\Delta\Delta C_t$ was the difference between the ΔC_t of the analyzed sample and that of the calibrator. Calculation of $2^{-\Delta\Delta C_t}$ gave a relative amount of the target gene in analyzed sample compared with the calibrator, both normalized to an endogenous control (18S). For presentations, the relative amount of target genes was multiplied by 1000 and expressed as relative units. When fold change of gene expression following GC therapy was expressed, it was calculated by dividing the relative expression after therapy by that before therapy. For test reliability, those samples with mRNA expression under 10 ng/ μ L ($n = 14$ for resting and $n = 1$ for activated target cells) between triplicate wells were excluded from the statistical analysis.

Statistical analysis

We used the Wilcoxon rank test, two-tailed Mann-Whitney U test, two-tailed Spearman's rho correlation test, and χ^2 test when appropriate (SPSS version 13.0). In the few cases with IFN γ greater than the detection limit of 120 000 pg/mL, this high value was used in statistical analysis. $P < 0.05$ was set for statistical significance.

Ethics

The study was approved by the Ethics Committee of Helsinki University Central Hospital and by the Institu-

tional Review Board. The families attending the study signed an informed consent form.

RESULTS

Serum-induced changes in gene expression of target cells

The median expression of FOXP3-specific mRNA in the resting target cells was suppressed by 33% (range of suppression 0%-79%, in one case, an increase of 173%, $P < 0.05$) and the median expression of GITR-specific mRNA by 46% (range 2%-78%, in three cases, an increase of 6%-124%, $P = 0.050$), when the effect of serum after GC was compared to that before therapy (Figure 1). In PHA-activated target cells, the median expression of GITR-specific mRNA was suppressed by 25% compared to the pre-treatment serum (range of suppression 1%-63%, in two cases, an increase of 6%-77%, $P < 0.01$).

The total or weight-related prednisolone dose did not correlate with the serum-induced GITR or FOXP3 gene expression of the target cells.

Serum-induced changes in secretion of IFN γ and IL-5 from target cells

IFN γ secretion from target cells was lower when cultured with patient serum taken 2 wk after the start of GC therapy, compared to the culture with the serum sample taken before GC therapy. This decrease was seen in resting target cells [from a mean of 68 pg/mL (SD 110 pg/mL) to 1.5 pg/mL (SD 6.7 pg/mL)]; however, because of the low secretion of IFN γ in resting target cells, detectable levels were only seen in seven cases, six of whom showed a decrease, $P < 0.05$. In activated target cells, 13 cases showed a decrease from a mean of 87 700 pg/mL (SD 33 900 pg/mL) to 60 900 pg/mL

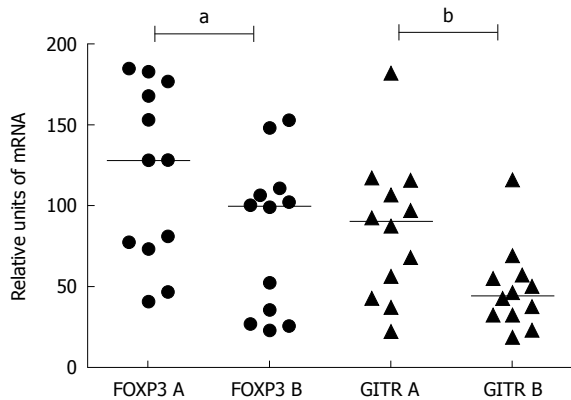


Figure 1 GITR and FOXP3 gene expression in target cells when cultured with patient serum taken before (A) and 2 wk after (B) GC therapy. For test reliability, the patients with low mRNA expression < 10 ng/ μ L ($n = 7$) were excluded from the Figure and the statistical analyses. ^a $P < 0.05$, ^b $P = 0.050$.

Table 2 Concentration of IFN γ and IL-5 (pg/mL)

Patient No.	IFN γ A PHA	IFN γ B PHA	IL-5 A PHA	IL-5 B PHA
1	7200	19000	11	28
2	99000	8500	0.0	0.0
3	41000	12000	77	19
4	82000	17000	150	9.3
5	94000	36000	150	11
6	> 120000	> 120000	54	21
7	> 120000	100000	140	38
8	63000	28000	1.2	1.2
9	59000	34000	99	34
10	110000	120000	75	89
11	> 120000	84000	8.1	1.2
12	> 120000	85000	12	3.5
13	91000	19000	84	17
14	40000	62000	73	130
15	91000	6600	10	7.2
16	> 120000	> 120000	15	2.3
17	90000	63000	69	33
18	> 120000	97000	33	0.0
19	68000	120000	89	160

Measured with ELISA in the supernatants of healthy donor PBMCs (target cells, stimulated with PHA) cultured with patient serum taken before (A) and 2 wk after oral GC therapy (B).

(SD 44200 pg/mL) ($P < 0.01$, Table 2). The decrease in serum-induced IL-5 secretion from activated target cells decreased after GC therapy was close to the level of statistical significance [from a mean of 60 pg/mL (SD 50 pg/mL) to 32 pg/mL (SD 46 pg/mL); 12 cases showed a decrease, $P = 0.055$, Table 2]. Serum-induced IL-5 secretion from resting target cells was undetectable.

The total or weight-related prednisolone dose did not correlate with IFN γ or IL-5 secretion from target cells.

Changes of cytokines and transcription-factor-specific mRNA in patient PBMCs

There were no statistically significant changes in the mRNA levels of the cytokines IFN γ , IL-5 and IL-10, or the transcription factors FOXP3, GITR, GATA3, STAT-5b and T-bet, in PBMCs derived from patients with IBD before and after 2 wk of GC therapy.

ESR, CRP and leukocyte count

ESR decreased from a median of 29 (range 0-64) mm/h to 12 (range 4-36) mm/h ($P < 0.01$), and CRP decreased from a median of 10 (range 5-75) mg/L to 5 (range 5-10) mg/L ($P < 0.01$) during 2 wk GC therapy. ESR or CRP did not correlate with the expression of GITR or FOXP3 from target cells, or with IFN γ or IL-5 secretion from target cells.

DISCUSSION

We found that serum from a GC-treated patient modulated *in vitro* the response of healthy donor-derived PBMCs and simulated the effects of GC treatment on the patient immune system. The attenuation of systemic inflammation during GC therapy was demonstrated with this assay at an individual level. The effect of GC treatment was mediated by patient serum, which caused a decrease in the target cell expression of regulatory T-cell-associated markers GITR^[18] and FOXP3^[19], and in the secretion of Th1 cytokine IFN γ .

The serum-induced decrease in cytokine and signaling molecule expression in the target cells was not attributed to the GC dose. This suggests that the observed changes in the levels of cell signaling markers were not solely due to GCs, but rather reflected the net systemic effect in patient serum. It has been reported previously that neither the level of GC in plasma or serum or serum GC bioactivity correlate with therapeutic responses^[14,20,21]. To study whether serum-induced effects on target cells were mediated by GC receptors, we co-cultured the target cells with patient serum and GC receptor antagonist mifepristone^[13,22]. Disappointingly, the results were uninformative as the GC receptor antagonist suppressed the target cell cytokine production and the treatment-mediated effects could not be assessed (data not shown).

It is highlighted that we did not see any direct effects of GC therapy on PBMCs derived from the patients. The PBMC population is heterogeneous and comprises naïve and memory T cells and effector and regulatory T cells. Thus, the net effect of GCs might be covered when studied in patient-derived PBMCs. Instead, we found that the effect of GC treatment was demonstrated as a patient-serum-induced decrease of FOXP3 and GITR in the target cells.

Earlier studies of the effect of GC therapy have focused on the direct changes induced in patient PBMC or lymphocyte subtypes such as regulatory T cells. The observation of GC-induced changes in regulatory T-cell-related FOXP3 is controversial, and there are no data from pediatric IBD. In an *in vitro* study on regulatory T cells of healthy donors, the administration of dexamethasone on purified CD4⁺ T cells induced a transient increase in the expression of FOXP3^[23]. Accordingly, in asthmatic patients, an increase in FOXP3 mRNA expression has been observed in freshly isolated and purified CD4⁺ T cells after oral GC administration^[23]. However, in another study, the counts of FOXP3-expressing CD4⁺CD25^{high} T cells and mRNA expression of GITR in PBMCs were

unaltered after 4 wk treatment with inhaled steroids^[24]. Furthermore, in a study of patients with allergic rhinitis, FOXP3-positive cells from nasal mucosa biopsies decreased significantly after several weeks treatment with intranasal GCs^[25]. These controversial findings of regulatory T-cell activation in different tissues and during different courses of GC therapy underline the importance of further studies to increase our understanding of the anti-inflammatory effects of GCs.

In line with our finding of a decrease in target cell IFN γ secretion, downregulation of pro-inflammatory cytokines, including IFN γ , has been reported in an *in vitro* study using PBMCs from healthy donors stimulated with dexamethasone and studied with DNA microarray chip analysis, online PCR and flow cytometry^[26].

Recently, it has been shown that, in severe pediatric colitis, the response to intravenous GC could be predicted already on day 3, with the aid of clinical activity scores^[27]. However, for oral GC therapy, there are no such data. We assessed the immunological responses of our patients at 2 wk, a time interval that was initially chosen for practical reasons. Therefore, it warrants further study to investigate if our findings could be detected during the first week of GC therapy.

To conclude, we developed a new cell culture assay to study the effect of GC therapy at an individual level, using serum samples taken from patients during GC therapy. We consider that the patient-serum-induced changes in the target cells reflect the net effect of the metabolism of GC and its immunological effects, and this mirrors the systemic inflammatory activity of the disease in the patient's circulation during GC therapy.

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COMMENTS

Background

Corticosteroids are widely used in the treatment of several diseases, for example, asthma, rheumatic disease and inflammatory bowel disease (IBD). Corticosteroids are effective in suppressing active inflammation but not all patients respond to treatment, and various side effects are common. To date, the therapeutic effect cannot be foreseen and there are no specific means to monitor the therapeutic response at an early phase of the treatment.

Research frontiers

IBD is a chronic disorder with an increasing incidence in children. In pediatric patients, the disease course is often aggressive and the majority of patients need corticosteroids to suppress their symptoms. There is an urgent need for new means to monitor therapeutic responses to improve patient care.

Innovations and breakthroughs

The authors describe a novel assay that gives the possibility to monitor therapeutic responses to corticosteroids, using a serum sample from the patient. Disease activity is reflected in the serum that is used in an *in vitro* cell assay to stimulate human white blood cells. In cell culture, it is possible to measure how the effect of patient serum on specific white blood cell markers (T

cells) varies according to the given therapy. With this novel assay, it is possible to study the individual effect of corticosteroid therapy.

Applications

The results suggest that the effect of corticosteroid therapy on disease activity can be measured from patient serum at an early phase of the therapy. The authors studied pediatric patients with IBD, and further study is warranted to establish whether this kind of method is suitable for other patients with corticosteroid treatment.

Peer review

This is a well written and interesting manuscript.

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Prediction of flare-ups of ulcerative colitis using quantitative immunochemical fecal occult blood test

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symptoms (19 patients), and the flare-up predictive type (15 patients). In patients with the flare-up predictive type, the values of I-FOBT were generally low during the study period with stable symptoms. Two to four weeks before the flare-up of symptoms, the I-FOBT values were high. Thus, in these patients, I-FOBT could predict the flare-up before symptoms emerged.

CONCLUSION: Flare-up could be predicted by I-FOBT in approximately 20% of UC patients. These results warrant periodical I-FOBT in UC patients.

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Key words: Flare-ups; Immunochemical fecal occult blood test; Inflammatory bowel disease; Prediction; Ulcerative colitis

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Abstract

AIM: To examine the feasibility of predicting the flare-up of ulcerative colitis (UC) before symptoms emerge using the immunochemical fecal occult blood test (I-FOBT).

METHODS: We prospectively measured fecal hemoglobin concentrations in 78 UC patients using the I-FOBT every 1 or 2 mo.

RESULTS: During a 20 mo-period, 823 fecal samples from 78 patients were submitted. The median concentration of fecal hemoglobin was 41 ng/mL (range: 0-392500 ng/mL). There were three types of patients with regard to the correlation between I-FOBT and patient symptoms; the synchronous transition type with symptoms (44 patients), the unrelated type with

INTRODUCTION

Ulcerative colitis (UC) is a chronic intestinal disorder of unknown etiology with a typically relapsing course. Although many UC patients often relapse with flare-ups of symptoms such as diarrhea and bloody stools despite appropriate maintenance therapies, the prediction of flare-ups a few weeks or months before symptoms emerge is impossible. If we could recognize relapses in the early stage, i.e. during the asymptomatic phase,

treatment of the relapse could be started in the early stage and might allow the patient to enter remission again more easily.

The fecal occult blood test (FOBT) is generally used as a screening method for colorectal cancer (CRC)^[1,2]. In many countries, the guaiac-based manual test is mainly used and validated to decrease mortality due to CRC^[3-5]. Recently, however, mainly in Japan, the test has developed into an immunochemical method with instruments for automatic measurement. Immunochemical FOBT (I-FOBT) has been reported to be more sensitive and specific for CRC detection than the guaiac-based test, and its effectiveness has also been accepted and widely used in Western countries^[6-21].

In this study, therefore, we measured fecal hemoglobin concentrations in UC patients in remission using an I-FOBT instrument each time they visited our hospital for regular UC-related check-ups. The aim of our study was to examine the feasibility of the prediction and prophylaxis of UC relapse by comparing fecal hemoglobin concentrations and symptoms in UC patients.

MATERIALS AND METHODS

Patients

Between April 2006 and November 2007, all UC patients who visited Okayama University Hospital were requested to prepare and bring fecal samples each visit. A total of 110 patients routinely came to our hospital every month or every 2 mo for determination of their disease status. Of these, a total of 78 patients with submitted fecal samples were analyzed in this study. The study protocol was approved by the institutional review board of Okayama University Graduate School of Medicine, Dentistry, and Pharmaceutical Sciences.

Assessment of disease

The diagnosis of UC was based on conventional criteria^[22]. Patients without a definite diagnosis of UC (e.g. indeterminate colitis) were excluded. The duration of disease was defined as the period between the time of onset and the first hospital visit during the study period.

Clinical relapse was defined as follows: active phase with worsening symptoms or emerging new symptoms that required treatment with newly prescribed or increasing doses of 5-aminosalicylic acid, salazosulfapyridine, corticosteroids, azathioprine/6-mercaptopurine, cyclosporine, tacrolimus or infliximab, including enemas or suppositories with such ingredients. The start of apheresis therapy (leukocytapheresis) was also defined as a clinical relapse.

Fecal sampling

Details of the method used for the fecal samplings were described previously^[17-20]. Fecal samples were prepared on the morning of or the day before the visit days. Patients were asked to prepare a fecal sample from a stool specimen by using the collection kit provided by

the manufacturer (Eiken Chemical, Tokyo, Japan). The I-FOBT sampling probe is inserted into an 8 cm × 2 cm test tube-shaped container. The patient inserts the probe into several different areas of the stool and then reinserts it firmly into the tube to seal it. The probe tip with the fecal sample is suspended in a standard volume of hemoglobin-stabilizing buffer.

Instrument for I-FOBT analysis

Submitted stool samples were immediately processed and examined using the OC-SENSOR neo (Eiken Chemical). The OC-SENSOR neo is the further-developed and large-scale version of the OC-SENSOR μ , which was used in previous reports^[17-20].

We assessed patient symptoms and disease activity using the partial Mayo score. Schroeder *et al.*^[23] developed the Mayo score in 1987, which comprises 4 items: stool frequency, rectal bleeding, findings of flexible proctosigmoidoscopy, and physician's global assessment. Each response is graded on a four-point scale ranging from "0" to "3". A score of zero means stool frequency is normal, no rectal bleeding is seen, findings of flexible proctosigmoidoscopy are normal if inactive disease is present, and physician's global assessment is normal. The total worst score is 12 points. This Mayo score is in common use in many clinical trials, and has noninvasive versions: the partial Mayo score (which excludes the mucosal appearance item from the 4-item index). Total worst score of the partial Mayo score is 9 points.

Classification of patients according to I-FOBT data

The patients were classified into three types according to I-FOBT data: synchronous transition with symptoms, the unrelated type with symptoms, and the flare-up predictive type. We first determined the patients who had the flare-up predictive type (the third type). Those patients with an I-FOBT value which was high an average of 1 mo before the flare-up of symptoms were assigned to this type.

After determining the third type, the remaining patients were classified into the first or second type by univariate regression analysis using I-FOBT values and the patient's partial Mayo scores. The first type (synchronous transition with symptoms): Univariate regression analyses appeared significant or produced a *P* value of < 0.20. The second type (unrelated type with symptoms): Univariate regression analyses produced a *P* value of \geq 0.20.

Statistical analysis

Statistical analysis was conducted using the SAS[®] System for Windows, version 9.2 (SAS Institute, Cary, NC, USA). Trends were assessed with the exact trend test to compare three discrete variables. Continuous variables were compared among the three patient groups using the Kruskal-Wallis test. Comparisons of continuous variables were performed using Mann-Whitney's *U* test. The results were considered to be statistically significant when

P values were less than 0.05. To determine the correlation between the value of I-FOBT and the partial Mayo score, a univariate regression analysis was performed.

RESULTS

Patients

A total of 78 patients were analyzed. These patients comprised 33 men and 45 women, with a median age of 40 years. The median disease duration was 9 years (range: 1-37 years). Of the 78 patients, 40 (51%) had pancolitis, 22 (28%) had left-sided colitis, and 16 (21%) had proctitis. During the study period, a total of 823 fecal samples were submitted by 78 patients, with a median of 10 samples submitted per patient. The frequency of submission of fecal samples was 0.74 times per month for each patient.

Range of I-FOBT values

The quantified value of the I-FOBT for each fecal sample was immediately determined by the OC-SENSOR neo. The median concentration was 41 ng/mL (0-392 500 ng/mL), and the distribution of the 823 fecal samples is shown in Figure 1. In general, stools with hemoglobin concentrations of more than 1000-10 000 ng/mL were recognized as bloody stools.

Correlation between the value of I-FOBT and patient symptoms

We examined the correlation between the value of I-FOBT and the partial Mayo score in each patient by univariate regression analysis. We found three types of correlations between the two parameters: synchronous transition with symptoms, the unrelated type with symptoms, and the flare-up predictive type.

First type (synchronous transition with symptoms):

In this type of patient, the values of I-FOBT were stable and did not fluctuate widely when patient symptoms were stable. However, the values increased or decreased along with deterioration or improvement of patient symptoms, respectively. These patients exhibited high I-FOBT values when their symptoms became worse. In turn, once their symptoms were relieved, the value of I-FOBT decreased. Forty-four patients (56%) were categorized into this type.

Second type (unrelated type with symptoms): The values of I-FOBT in this type of patient were not parallel with the symptoms. Of 78 patients, 19 (24%) were classified into this type. The values of I-FOBT in these patients were likely to be extremely high, although their symptoms were generally stable.

Third type (flare-up predictive type): In these patients, the values of I-FOBT were generally low during the period with stable symptoms. Before the flare-up of symptoms, the I-FOBT value was high, although the symptoms

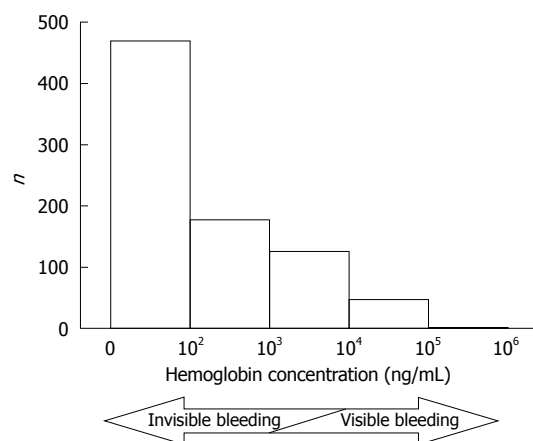


Figure 1 A histogram of hemoglobin concentrations in 823 fecal samples. The distribution of hemoglobin concentration in 823 fecal samples from 78 UC patients is shown. The median concentration was 41 ng/mL (0-392 500 ng/mL). More than 50% of fecal samples exhibited values between 0 and 100 ng/mL. Approximately 20% were between 100-1000 ng/mL, 15% were between 1000-10 000 ng/mL, and the remaining 7% of samples exhibited values more than 10 000 ng/mL. In general, stools with a hemoglobin concentration more than 1000-10 000 ng/mL were recognized as bloody stools.

Table 1 Characteristics of the three types of patients according to the correlation between I-FOBT value and patient symptoms

	First type	Second type	Third type	<i>P</i>
<i>n</i>	44	19	15	
Male/female	23/21	3/16	7/8	0.034 ^a
Age (yr)	39 (18-79)	40 (16-66)	46 (27-70)	0.54 ^b
Disease duration (yr)	9 (1-37)	9 (1-28)	8 (1-30)	0.88 ^b
Disease extent				
Pancolitis	23 (52)	8 (42)	9 (60)	0.22 ^a
Left-side colitis	10 (23)	6 (32)	6 (40)	
Proctitis	11 (25)	5 (26)	0 (0)	
Total fecal samples	427	204	192	
Submitted fecal samples/person	9 (4-20)	10 (4-19)	12 (6-26)	0.13 ^b
I-FOBT result (ng/mL) ^c	9 (0-57 616)	486 (0-142 639)	189 (0-392 500)	< 0.0001 ^b

Data are medians with ranges in parentheses or numbers with percentages in parentheses. ^aExact trend test; ^bKruskal-Wallis test; ^cMann-Whitney's *U* test, 1st type *vs* 2nd type, *P* < 0.0001; 2nd type *vs* 3rd type, *P* = 0.021; 1st type *vs* 3rd type, *P* < 0.0001.

at that point had not worsened. An average of 1 mo after the rise in I-FOBT value (i.e. the day of the next consultation), the patient's symptoms became worse, and additional UC therapies or admission was required. Therefore, the I-FOBT values in these patients could predict the worsening of symptoms nearly 1 mo in advance. Fifteen patients (19%) were classified in this type.

Characteristics of the three types of patients

The demographic and clinical variables of the three types of patients are shown in Table 1. There was a higher proportion of females in the second-type group compared with the first- and third-type groups (*P* = 0.034). Age, disease duration, disease extent, and clinical

course were not significantly different among the three groups. I-FOBT results were significantly higher in the second-type group compared with the first- and third-type groups ($P < 0.0001$).

DISCUSSION

We were able to predict relapse before clinical symptoms emerged in approximately 20% of UC patients by quantifying fecal hemoglobin concentration on consultation days. The feasibility of predicting relapse is attractive, because it would permit earlier and therefore possibly more effective treatment. If colonoscopy is performed at the time when fecal hemoglobin concentration increases, but no symptoms of flare-up are observed, clinical relapse can be detected at the presymptomatic stage. If we start treatment for relapse at this stage, the severity of flare-ups could be reduced. Moreover, prophylaxis of flare-ups may be possible. Thus, follow up of the third-type of patients with I-FOBT would be beneficial.

Although many laboratory parameters (erythrocyte sedimentation rate, CRP, platelet count, white blood cell count, α_1 -antitrypsin) have been proposed as predictors of clinical relapse in UC^[24-31], it is difficult for these markers to predict relapse before symptoms emerge as they are not sufficiently specific or sensitive to inflammation in the colon. In contrast, increases in I-FOBT values are parallel with or predictive of the deterioration of symptoms in more than 80% of UC patients.

One promising predictive marker of relapse in UC is fecal calprotectin concentration. Tibble *et al.*^[32] and Costa *et al.*^[33] reported that in clinically quiescent UC patients followed for 12 mo, median fecal calprotectin levels at the beginning of the study in patients who relapsed were significantly higher than those in patients who did not relapse. In these reports, however, fecal calprotectin was measured once, and the concentration could be a marker of relapse that would occur several months after the measurement. Calprotectin is a granulocyte cytosolic protein, and a high fecal concentration of calprotectin indicates patients who are at risk of relapse. On the other hand, I-FOBT directly measures the fecal hemoglobin concentration, and a sequential change in fecal hemoglobin concentration could be a marker of relapse. Moreover, I-FOBT measurement was automated within a short time during outpatient consultation, and the instrument can be used as a screening method for CRC. Thus, sequential I-FOBT measurement is easier and more useful than the measurement of fecal calprotectin concentration in predicting early relapse.

There are limitations to this study. First, we adopted the 1-d method of I-FOBT. In CRC screening, the 2- or 3-d method is usually recommended because these methods are superior to the 1-d method in terms of sensitivity for colorectal neoplasia^[34-36]. Therefore, in the present UC study the examination of two or three samples of feces in one consultation day may be more reliable. Second, I-FOBT can not be used in women during menstruation

as the value of fecal hemoglobin concentration may be inaccurate.

In conclusion, we prospectively followed-up UC patients with I-FOBT. In some patients, an increase in fecal hemoglobin concentration was a useful marker of early relapse. The potential for sequential measurement is also an advantage of follow-up with I-FOBT. To confirm the usefulness of I-FOBT in the follow-up of UC patients, comparative studies are needed between patients with and without I-FOBT submission.

COMMENTS

Background

Ulcerative colitis (UC) is a chronic intestinal disorder of unknown etiology with a typically relapsing course. Although many UC patients often relapse despite appropriate maintenance therapies, the prediction of flare-ups a few weeks or months before symptoms emerge is impossible.

Research frontiers

Many laboratory parameters (erythrocyte sedimentation rate, CRP, platelet count, white blood cell count, α_1 -antitrypsin) have been proposed as predictors of clinical relapse of UC, but it is difficult for these markers to predict relapse before symptoms emerge as they are not sufficiently specific or sensitive to inflammation in the colon.

Innovations and breakthroughs

This is the first study to report the feasibility of prediction of UC relapse using the immunochemical fecal occult blood test (I-FOBT).

Applications

Starting treatment for relapse in the early stage may allow the patient to enter remission again more easily than if treatment is started after the symptoms of flare-up emerge. In addition, the prophylaxis of flare-ups by modulating the maintenance therapy to anticipate flare-ups by a few days or weeks may become possible.

Terminology

FOBT is generally used as a screening method for colorectal cancer. Recently, mainly in Japan, the test has developed into an immunochemical method with instruments for automatic measurement.

Peer review

This study aimed to predict flare up of UC by estimating hemoglobin at regular intervals using I-FOBT. The authors evaluated a total of 78 patients over a period of 20 mo.

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Laboratory characteristics of recent hepatitis A in Korea: Ongoing epidemiological shift

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Abstract

AIM: To evaluate seroprevalence of hepatitis A virus (HAV) antibody and investigate demographic, clinical, and laboratory features of recent cases in Korea.

METHODS: For the evaluation of hepatitis A seroprevalence, we analyzed the data from 3127 subjects including, healthcare workers and patients who visited Konkuk University Hospital, a secondary referral center, from January to October 2009. The sera with positive IgM were excluded from seroprevalence data for total HAV antibody. We retrospectively reviewed the electronic medical records of 419 patients with HAV, who were diagnosed by the presence of serum IgM antibodies against HAV. All patients presented at Konkuk University Hospital between August 2005 and September 2008.

RESULTS: Among 3127 sera tested, 1428 (45.7%)

were positive for anti-HAV antibody. The seroprevalence was very low in teenagers or those in their twenties, increased in those in their thirties, and was > 90% in older patients. In children younger than 10 years, seroprevalence was increased again. Most patients with HAV hepatitis were in their twenties and thirties. The γ -glutamyl transpeptidase increased with age and was significantly higher in patients older than 30 years. Indicators of severity, such as decreased albumin and increased bilirubin, were also more prominent in the older age group; however, the leukocyte count was higher and the frequency of leukopenia was lower in younger patients than in older adults.

CONCLUSION: There has been an apparent epidemiological shift in HAV seroprevalence and a change in the peak age of HAV hepatitis. This study could provide baseline data of recent hepatitis A in Asia.

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Key words: Hepatitis A virus; Seroprevalence; Epidemiology; Korea

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INTRODUCTION

The hepatitis A virus (HAV) is included in the picornaviridae family of viruses, which are small, non-enveloped,

single-stranded RNA viruses^[1]. They are transmitted almost exclusively by the fecal-oral route, and transmission is enhanced by poor hygiene, overcrowding, and contaminated food or drink^[2]. Infection with HAV is prevalent throughout the world, but it shows diverse epidemiological patterns according to the socioeconomic conditions^[3,4]. A high prevalence of this virus is associated with poor hygiene and living standards^[5]. In hyperendemic areas, almost all people have protective anti-HAV antibodies as a result of subclinical exposure during childhood^[2]; however, improved environmental conditions have resulted in fewer subclinical infections and a subsequent increase in the non-immune population. In Korea, there has been a rapid epidemiological shift in HAV infection as a result of rapid economic development^[3,6,7]. In 1980, most adults had immunity to HAV because of childhood exposure. Currently, less than 10% of people in their teens or twenties have anti-HAV IgG^[6]. Thus, adult cases of acute hepatitis A have increased rapidly during the past 10 years due to the emergence of susceptible adults in Korea^[6,8].

In this study, we evaluated seroprevalence of total HAV antibody and investigated demographic, clinical, and laboratory features of recent cases of hepatitis A in Korea, where there is an ongoing epidemiological shift. Moreover, we compared laboratory characteristics of HAV according to patient age before and after the epidemiological shift.

MATERIALS AND METHODS

Study populations

For the evaluation of hepatitis A seroprevalence, we analyzed the data from 3127 subjects, including healthcare workers and patients, who visited Konkuk University Hospital, a secondary referral center, from January to October 2009. The sera with positive IgM were excluded from the seroprevalence data for total HAV antibody. We retrospectively reviewed the electronic medical records of 419 patients with HAV, who were diagnosed by the presence of serum IgM against HAV. All patients presented at Konkuk University Hospital between August 2005 and September 2008. We investigated the clinical, laboratory, and epidemiological features of these patients and analyzed age-specific characteristics. We compared laboratory findings of patients who are younger and older than 30 years of age because these groups were considered to have been exposed to different epidemiological environments, based on the results of total HAV antibody seroprevalence in other recent studies^[6,7].

Laboratory tests

A routine chemistry test (Neo-200FR autoanalyzer; Toshiba Medical Systems Co., Tokyo, Japan), complete blood count, and coagulation studies (Sysmex Co., Kobe, Japan) were performed. We tested serum total HAV antibody using the HAV Total immunoassay kit (Siemens Healthcare Diagnostics, Tarrytown, NY, USA). To detect serum anti-HAV IgM, we used commercially available

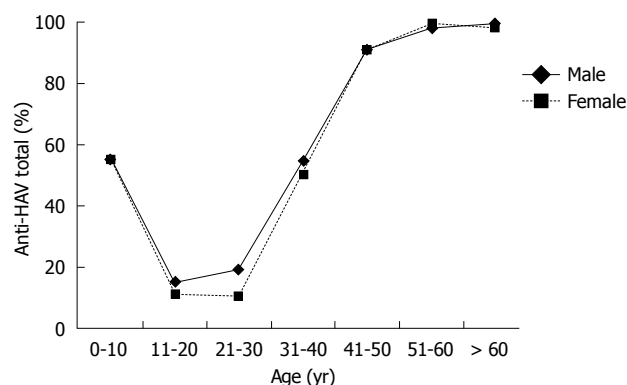


Figure 1 Age-specific seroprevalence of total anti-hepatitis A virus (HAV) antibody in Korea. The seroprevalence was very low in patients in their teens or twenties, increased in those in their thirties, and was > 90% in older patients. In children younger than 10 years, seroprevalence was increased ($n = 3127$).

electrochemiluminescence immunoassay kits (Roche Diagnostics, Mannheim, Germany).

Statistical analysis

Relationships between variables were analyzed using the Fisher exact test for categorical variables and Student's *t* test for continuous variables. Statistical analysis was performed using SPSS software, version 11.0 (SPSS Inc., Chicago, IL, USA). $P < 0.05$ was considered statistically significant.

RESULTS

Age-specific seroprevalence of total anti-HAV antibody

The median age of population tested was 31 years and 43.2% were male. Among 3127 sera tested, 1428 (45.7%) were positive for total anti-HAV antibody. The seroprevalence was different depending on age and sex (Figure 1). The seroprevalence was very low in patients in their teens or twenties, increased in the those in their thirties, and was > 90% in older patients. In children younger than 10 years, seroprevalence was increased.

Characteristics of hepatitis A cases in Korea

The median age of 419 patients with HAV hepatitis was 29 years and 62% were male. Aspartate aminotransferase (AST) and alanine aminotransferase (ALT) showed a marked increase in almost all patients, and ALT was higher in most cases (median AST/ALT ratio 0.7). Variable elevations of alkaline phosphatase (ALP), γ -glutamyl transpeptidase (GGT), and total bilirubin (TB) were evident in most patients. Leukopenia, thrombocytopenia, and prolonged prothrombin times (PT) were common findings. Among 419 cases, 352 patients (84%) needed hospital admission, and the median hospitalization period was 6 d (range: 1-31 d). Frequent symptoms were nausea/vomiting (55%), fever (49%), anorexia (34%), myalgia (32%), abdominal pain (23%), and dark urine (20%).

Twenty-one patients (5%) were chronic hepatitis B carriers. There were no cases of fulminant hepatitis or death in the study period.

Table 1 Age distribution and age-specific laboratory findings of Korean hepatitis A cases

	Age (yr)			
	≤ 20	21-30	31-40	> 40
<i>n</i> (%)	22 (5.2)	241 (57.5)	132 (31.5)	24 (5.7)
Male:female (ratio)	13:9 (1.4)	141:100 (1.4)	93:39 (2.4)	13:11 (1.2)
Laboratory finding [median (range)]				
AST (IU/L)	2130 (105-15390)	2265 (37-16330)	2128 (44-15450)	1661 (19-5980)
ALT (IU/L)	2384 (492-9020)	2601 (15-9820)	2586 (42-12420)	1988 (11-5530)
ALP (IU/L)	254 (124-711)	201 (41-936)	222 (65-883)	252 (138-527)
GGT (IU/L)	302 (128-781)	345 (8-1195)	411 (37-1705)	479 (29-705)
TB (mg/dL)	5.3 (0.3-11.0)	5.0 (0.3-21.6)	6.1 (0.4-40.2)	5.5 (0.4-17.5)

AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; ALP: Alkaline phosphatase; GGT: γ -glutamyl transpeptidase; TB: Total bilirubin.

Table 2 Comparison of laboratory characteristics according to age (mean \pm SD)

	Age (yr)		<i>P</i> value
	≤ 30 (<i>n</i> = 263)	> 30 (<i>n</i> = 156)	
Male:female	154:109	106:50	0.061
Laboratory finding			
AST (IU/L)	2254 \pm 2598	2056 \pm 2542	0.450
ALT (IU/L)	2583 \pm 1999	2494 \pm 2140	0.669
AST/ALT ratio	0.9 \pm 1.3	0.8 \pm 0.7	0.326
ALP (IU/L)	205.8 \pm 109.5	227.1 \pm 117.2	0.062
GGT (IU/L)	342 \pm 197.9	421 \pm 299.6	0.006
TB (mg/dL)	5.0 \pm 4.0	6.0 \pm 6.0	0.051
Albumin (g/dL)	4.0 \pm 0.3	3.8 \pm 0.3	0.000
Globulin (g/dL)	2.9 \pm 0.4	3.1 \pm 0.4	0.012
Leukocyte count ($\times 10^9$ /L)	5.0 \pm 1.9	5.6 \pm 2.5	0.002
Leukopenia (%)	33.3	20.5	0.003
Platelet count ($\times 10^9$ /L)	169 \pm 74	181 \pm 75	0.111
Thrombocytopenia (%)	45.6	39.0	0.218
PT (s)	15.1 \pm 2.5	15.1 \pm 3.3	0.978
Prolonged PT (%)	43.5	38.7	0.356
aPTT (s)	41.7 \pm 5.3	41.1 \pm 5.6	0.302
Prolonged aPTT (%)	24.0	17.6	0.173
Hospital stay (d)	6.5 \pm 4.6	6.7 \pm 7.4	0.771

PT: Prothrombin times; aPTT: Activated partial thromboplastin time.

Age distribution of recent hepatitis A in Korea and age-specific laboratory findings

Patients with HAV were mostly in their twenties (57.5%) and thirties (31.5%) (Table 1). The number of patients in the other age groups (≤ 20 or > 40 years) were relatively few (5.2% and 5.7%). There was only one patient younger than 10 years old. Men were more prevalent in all age groups, and this tendency was more pronounced for men in their thirties (M/F ratio 2.4). GGT increased with age ($P = 0.011$), but other laboratory findings were not significantly different between age groups.

We compared laboratory findings of the patients who are younger and older than 30 years old (Table 2). Levels of the enzymes AST, ALT, and ALP were not significantly different between the two groups; however, the older age group showed a significant increase in GGT, decrease in albumin, and increase in globulin (decreased A/G ratio) ($P = 0.006$, $P = 0.000$, and $P = 0.012$, respectively). TB was also increased in the older group,

although this was not statistically significant ($P = 0.051$). In contrast, abnormalities in hematological parameters were more severe in the younger group, especially with regard to decreased leukocyte count. The results of coagulation studies and duration of hospital stay were not significantly different.

DISCUSSION

In highly endemic areas with poor socioeconomic conditions, HAV infection occurs in young children, possibly due to a lack of transmission control and the ubiquitous presence of HAV in the environment^[5,9]. In contrast, HAV exposure is relatively rare in developed countries such as the United States, Canada, and countries in Western Europe because possible transmission factors are controlled^[2,4,10,11]. The majority of symptomatic cases occur in adolescents, adults, and people with risk factors^[4]. HAV infection in non-immune adults can cause severe hepatitis^[12-14]. Complications include coagulopathy, encephalopathy, renal failure, prolonged disease, and disease relapses^[9]. In the past in South Korea (1980s), HAV was endemic, and most adults acquired natural immunity through asymptomatic infection^[7,15]. Due to the rapid improvement of socioeconomic conditions, the childhood incidence of HAV infection and the immune population have decreased, thus, the incidence of adult infection has increased steadily due to a lack of natural immunity^[7,8,16,17]. After 2000, symptomatic cases of HAV infection increased markedly and the age of affected patients was significantly higher before 2000 than after 2000^[7]. This transitional pattern also has occurred in other Asian populations that have similar environmental conditions^[2,3]. In our study, seroprevalence increased with age and showed an abrupt rise in patients older than 30 years of age compared to those younger than 30 years of age. These results are consistent with other recent studies in Korea^[6,7]. This is thought to be due to different epidemiological and socioeconomic environments during the economic development of Korea. Of note, with the recent increase of HAV vaccination in childhood, seroprevalence has increased in children younger than 10 years (Figure 1).

We evaluated the features of recent hepatitis A cases at a single center where we have observed a rapid

increase in the number of cases in recent years. These cases were analyzed for age-specific laboratory characteristics. In our study, most patients with HAV were in their twenties and thirties and most were male. The male predominance was especially true of patients in their thirties, which may be due to more exposure to the virus in men of that age. Besides markedly increased levels of enzymes related to acute hepatitis, most patients also showed increased TB levels and PT. Hematological abnormalities such as leukopenia and thrombocytopenia were noted in about one third of patients with hepatitis A (Table 2). These findings reflect more severe manifestations of acute hepatitis. Eighty-four percent of patients required hospitalization, which is much more than previously reported in the United States^[4,9], but similar to other recent reports in Korea^[6,7].

Although other enzymes were not significantly different between age groups, GGT increased with age and was significantly higher in patients older than 30 years ($P = 0.006$). Indicators of severity, including decreased albumin and increased TB^[1,18], were also more predominant in the older group ($P = 0.000$ and 0.051 , respectively) (Table 2). However, the leukocyte count was higher and the frequency of leukopenia was lower in younger patients than older adults ($P = 0.002$ and 0.003 , respectively).

In conclusion, we investigated age-specific seroprevalence and laboratory characteristics of Korean patients recently infected with HAV. There has been an apparent epidemiological shift and change in the peak age of infection. This study could provide valuable baseline data of recent hepatitis A in an Asian area with an epidemiological shift.

COMMENTS

Background

In Korea, there has been a rapid epidemiological shift in hepatitis A virus (HAV) infection over the past decade as a result of rapid economic development. Adult cases of acute HAV have increased rapidly during the past 10 years due to the emergence of susceptible adults in Korea.

Research frontiers

The transitional pattern of HAV epidemiology has occurred in Korea and other Asian populations that have similar environmental conditions. The large scale data from various populations regarding this issue would be valuable for establishing recent epidemiological features of HAV infection.

Innovations and breakthroughs

In 1980, most adults had had immunity to HAV because of childhood exposure. Currently, less than 10% of people in their teens or twenties have anti-HAV IgG. In this study, the authors evaluated seroprevalence of total HAV antibody, with large-scale data, and investigated demographic, clinical, and laboratory features of recent cases of HAV in Korea, where there is an ongoing epidemiological shift.

Applications

There has been an apparent epidemiological shift in HAV seroprevalence and a change in the peak age of HAV hepatitis. Overall, these cases have revealed a severe pattern of acute hepatitis. This study could provide valuable baseline data of recent hepatitis A in an Asian area with an epidemiological shift.

Peer review

This manuscript is of interest.

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Colonoscopy in Hong Kong Chinese children

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Abstract

AIM: To investigate the safety and diagnostic yield of colonoscopy in Chinese children in whom the procedure is not often done.

METHODS: We conducted a retrospective review of all colonoscopies in consecutive children who underwent their first diagnostic colonoscopy from Jan 2003 to 2008.

RESULTS: Seventy-nine children (48 boys, 31 girls; mean age 9.2 ± 4.2 years) were identified and reviewed with a total of 82 colonoscopies performed. Successful caecal and ileal intubation rates were 97.6% and 75.6% respectively. Forty patients (50.6%) had a positive diagnosis made in colonoscopy and that included colonic polyps (23), Crohn's disease (12), ulcerative colitis (1), and miscellaneous causes (4). 80% of polyps were in the rectosigmoid colon. All but one were juvenile hamartomatous polyps. The exception

was an adenomatous polyp. The mean ages for children with inflammatory bowel disease (IBD) and polyps were 11.3 and 4.3 years respectively. There was no procedure-related complication.

CONCLUSION: Colonoscopy is a safe procedure in our Chinese children. The increasing diagnosis of IBD in recent decades may reflect a rising incidence of the disease in our children.

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Key words: Colonoscopy; Children; Inflammatory bowel disease; Colonic polyps

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INTRODUCTION

The safety and effectiveness of colonoscopy in the investigation of lower gastrointestinal tract pathology in children has been established for more than 2 decades^[1]. It remains to be a procedure that mandates highly specialized expertise and is usually performed in a tertiary referral center. Investigation of inflammatory bowel disease (IBD), whether for diagnosis or follow-up evaluation, and suspected colonic polyps are the most common indications for pediatric colonoscopy in European and North American society^[2-5]. Population-based studies suggest

the uneven distribution of IBD throughout the world with the highest disease rates in Caucasian countries^[6]. It has been believed that the incidence of IBD in Chinese children is low and as a result pediatric colonoscopy is not as commonly done in the Chinese population as in Western countries. Recent reports suggested an increasing incidence of pediatric IBD in Western countries while it is unknown whether a similar increase happens in Chinese children as well^[7-9]. In this study, we aimed to investigate the diagnostic yield and safety of colonoscopy, and the pathology pattern of our Chinese children who underwent this procedure.

MATERIALS AND METHODS

A retrospective review was conducted on all consecutive pediatric patients who underwent their first diagnostic colonoscopy during the period from January 2003 to 2008 in a tertiary referral center serving a population of 1.32 million. The data were collected from the hospital electronic database system which required prospective entry of all the patients who underwent endoscopic procedures. Our institution was the only center that provided a pediatric colonoscopy service in the region.

Demographic data, indications for colonoscopy, endoscopic findings, extent of the procedure and follow-up progress were analyzed. All the colonoscopy procedures were performed by a single endoscopist (Tam YH) using a pediatric videoscope (Olympus PCF 200, Tokyo, Japan) in the presence of an anesthetist. Each patient had bowel preparation of low residual diet for 3 d prior to examination followed by admission to our hospital one day before the procedure for anesthetic assessment and further bowel cleansing with either oral sodium phosphate or polyethylene glycol-based solution. The endoscopy suite was equipped with an anesthetic machine and the majority of pediatric patients had the procedure done under general anesthesia by orotracheal intubation with inhalation anesthetic agents. A few exceptions, usually being older children, were put under conscious sedation by anesthetists using a combination of agents including ketamine, midazolam and propofol. Mucosal biopsies were taken whenever indicated and polypectomy were performed by snare with electrocautery.

RESULTS

Seventy-nine children, being all ethnic Chinese, were identified and reviewed. These included 48 boys (61%) and 31 girls, aged from 21 mo to 16 years (mean = 9.2 ± 4.2 years, median = 10 years). The two most common indications were painless per rectal bleeding ($n = 46$, 58%) and suspected IBD ($n = 23$, 29.1%). Other miscellaneous indications included investigation for protein losing enteropathy, enteritis in immunocompromised patients, Graft-versus-host diseases after bone marrow transplant, iron deficiency anemia, change of bowel habit and strong family history of carcinoma of the colon with parental anxiety.

A total of 82 colonoscopies were performed in the

Table 1 Indications and diagnoses of 79 children after colonoscopy

Indications	Diagnoses	<i>n</i>
Painless per rectal bleeding ($n = 46$)	Colonic polyps	23
	Normal findings	23
Suspected IBD ($n = 23$)	Crohn's disease	12
	Ulcerative colitis	1
	Normal findings	10
Miscellaneous ($n = 10$)	Eosinophilic enteritis	1
	CMV enteritis	1
	GVHD	1
	Multiple colonic haemangioma	1
	Normal findings	6

IBD: Inflammatory bowel disease; CMV: Cytomegalovirus; GVHD: Graft-versus-host disease.

Table 2 Locations of the 25 colonic polyps in 23 children

Locations	<i>n</i> (%)
Rectum	11 (44)
Sigmoid colon	9 (36)
Descending colon	3 (12)
Transverse colon	1 (4)
Ascending colon	1 (4)

79 patients during the study period. All but 7 of the procedures were done under general anesthesia. Complete colonoscopy was successful in 80 procedures (97.6%). Ileal intubation was routinely attempted in patients with suspected IBD and was successfully performed in 22 out of 23 children (95.7%) investigated for IBD. Ileal intubation was attempted but failed in one patient with Crohn's disease (CD) because of an edematous ileocaecal valve. Overall, ileal intubation was performed in 62 out of the total of 82 colonoscopies (75.6%). Three children had a repeat colonoscopy during the study period for recurrent colonic polyp, follow-up progress of CD and argon beam coagulation for multiple colonic haemangioma respectively.

Forty out of 79 patients (50.6%) had a positive diagnosis made in colonoscopy with or without biopsy. These included colonic polyps ($n = 23$), CD ($n = 12$), ulcerative colitis (UC) ($n = 1$) and other miscellaneous pathology (Table 1). Children diagnosed to have IBD were found to be significantly older than children with colonic polyps (11.3 ± 3.7 years *vs* 4.3 ± 1.9 years; mean age difference = 7.1 years; 95% CI: 5.2-9.0). Of the 12 patients diagnosed with CD, there were 7 girls and 5 boys. The only patient with UC was a girl.

Among the 23 children with polyps, there were 14 males and 9 females. Twenty two of them had solitary colonic polyp while 1 patient had 3 synchronous polyps at multiple sites. Of the 25 polyps, 20 (80%) were located in the rectosigmoid colon (Table 2). Therapeutic colonoscopic polypectomy was successful in all but 1 patient who had a huge polyp with a broad base at the transverse colon and partial resection was achieved during colonoscopy. The patient subsequently underwent elective laparoscopic-assisted bowel resection and pathology was confirmed to

be juvenile polyp. Histological examination revealed all the polyps removed to be juvenile hamartomatous polyps except one which was tubular adenoma that developed in a child who presented with painless per rectal bleeding without any family history of colonic polyp or cancer. One child developed recurrent juvenile polyp 1 year afterwards and required a second colonoscopic polypectomy. There were no procedure-related complications including perforation, bleeding that required re-examination or checking of hemoglobin after procedure, and cardiopulmonary problems caused by general anesthesia or sedation. At a mean follow-up of 22 ± 16 mo, 2 patients died of complications after bone marrow transplant. None of the patients with normal colonoscopy findings had persistent symptoms that required a repeat colonoscopy or further radiological imaging to exclude any pathological conditions.

DISCUSSION

Reports in Western countries have suggested a significant increase in the incidence of IBD in children over the last few decades^[7-9]. The annual incidence rates in United Kingdom and North America were estimated to be 5.2 to 7.05 per 100 000 pediatric populations in recent reports^[10,11]. IBD has been believed to occur rarely in Chinese children. However, a recent population-based study in North America did not find any difference in the incidence between Caucasian children and other racial groups including Asians^[11]. The authors suggested changing environmental factors had played an increasingly important role in the pathogenesis of pediatric IBD.

Our institution serves a population of 1.32 million and is the only center to provide a pediatric colonoscopy service within the region. If we could assume all the newly diagnosed cases of childhood IBD in our region were captured by our study during the review period and based on the population census report of Hong Kong in 2006 that children aged 18 or less accounted for 20.3% population, we could estimate the annual incidence rate in our children to be 7- to 8-fold less than the figures in United Kingdom and North America. Epidemiological data of IBD in Chinese children is scarce. A study conducted two decades ago reported only 1 case of IBD out of 65 colonoscopies in our Chinese children over a six-year period^[12]. We have previously reported 9 children (CD = 7; UC = 2) who were diagnosed and treated in our institution in 10 years from 1992 to 2002^[13], while 13 cases (CD = 12; UC = 1) were diagnosed within 6 years in this study. The increase in diagnosis of childhood IBD in the most recent decade compared with our local data in the 80s and 90s may reflect a possible increase in incidence of the disease although attribution to changes in referral patterns cannot be excluded. A central register of pediatric IBD, which is lacking currently in our locality, is deemed necessary to accurately monitor the incidence of the disease in our children^[14].

We reported a mean age of 11.3 years at the time of diagnosis and a higher incidence of CD over UC in our

childhood IBD. The results are in keeping with data reported in Western countries^[10,11,14-16]. Our finding of more instances of CD in female children was in contrast to the consistent findings of male predominance in Caucasian populations^[10,11,14-16]. Female predominance of CD is noted in the adult population^[17]. However, our small sample size does not allow us to make any conclusion about the gender predominance of IBD in Chinese children.

Our results of male predominance and mean age of 4.3 years in children with colonic polyps are in keeping with previous reports^[18-22]. About 80% of the polyps were found in the rectosigmoid colon of our children and only 8% were proximal to splenic flexure. Our findings are similar to those reported from India and Netherlands^[4,20,22]. Recent data in North America found more proximal polyps with 54% in the rectosigmoid colon and 32% proximal to the splenic flexure^[21]. Genetic and environmental factors may likely account for the difference between races. Despite our small series, there was one case of adenomatous polyp in a child with a solitary polyp in the sigmoid colon. This finding agrees with others that adenomatous polyps do occur in children, though infrequently, and pancolonoscopy is indicated as the initial procedure for investigation of suspected colonic polyps in children in view of the neoplastic potential of an unidentified polyp^[18,20,21].

Despite the small case volume, our reported rates of 97% and 75.6% in caecal and ileal intubation respectively compare well with other series^[1-4]. We did not encounter any complications related to the procedure, in accordance with the universally low complication rates reported in pediatric colonoscopy^[23]. All of our procedures were done by a single endoscopist. Concentration of expertise and experience helps to promote good clinical results. However, the small case volume remains to be a major limitation to training of next generations. In a prospective survey in United Kingdom, specialized pediatric centers perform a mean of 24 colonoscopies over a 4-mo period, which is over 5-fold of our volume^[24]. Pediatric guidelines have been issued in North America to recommend the minimum number of procedures to be performed to acquire competency of skills^[25]. Recommendations in Western countries may not be applicable in our locality. Rotation training in adult colonoscopy and virtual endoscopy training^[26] may provide an alternative way of providing training in pediatric colonoscopy in Hong Kong.

COMMENTS

Background

Colonoscopy in children is a highly specialized endoscopic procedure that is not often performed in the Chinese population as inflammatory bowel disease (IBD), which is an important indication for this procedure, is quite rare in Chinese children. Data of the safety, indications and diagnostic yield of colonoscopy in Chinese children is rarely reported. In this study, the authors retrospectively reviewed all the Chinese children who underwent their first diagnostic colonoscopy in a tertiary referral center within a 6-year period.

Research frontiers

There were 79 children who underwent their first colonoscopy over 6 years in the authors' institution that serves a population of 1.32 million and is the only

center that provides this service in the region. The procedure was successfully completed in 97.6% of cases without any complications. Pathological conditions that could account for the symptoms were identified in 50% of cases by colonoscopy. The two most common positive diagnoses were colonic polyps in 23 children and IBD in another 13 patients.

Innovations and breakthroughs

The results found that there was an increase in the diagnosis of IBD by colonoscopy in the recent decade compared with our local data in the 1980s and 1990s. Data in Western countries have recently suggested an increase in the incidence of IBD in children. The authors' findings suggest the possibility of a similar increase in the occurrence of this disease in Hong Kong Chinese children in whom the disease has been believed to be extremely uncommon for many years.

Applications

The findings suggested that establishment of a central registry for childhood IBD is strongly indicated to monitor its incidence in Chinese children.

Peer review

This is a retrospective cohort study of all pediatric patients undergoing colonoscopy at a tertiary centre over a 6 year period. Though it is a simple observational study of workload undertaken during this period, it does add background value with respect to identified pathologies in a predominantly Asian pediatric population.

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Prognostic factors in the surgical treatment of caudate lobe hepatocellular carcinoma

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Abstract

AIM: To evaluate the short- and long-term outcomes of liver resection for caudate lobe hepatocellular carcinoma (HCC).

METHODS: We retrospectively analyzed 114 consecutive patients with HCC, originating from the caudate lobe, who underwent resection between January 2001 and January 2007. Univariate and multivariate analyses were performed on several clinicopathologic variables to determine the factors affecting long-term outcome and intrahepatic recurrence.

RESULTS: Overall mortality and morbidity were 0% and 18%, respectively. After a median follow-up of 31 mo (interquartile range, 11-66 mo), tumor recurrence had occurred in 76 patients (66.7%). The 1-, 3-, and 5-year disease-free survival rates were 65.7%, 38.1%, and 18.4%, respectively. The 1-, 3-, and 5-year overall

survival rates were 76.1%, 54.7%, and 31.8%, respectively. Univariate analysis showed that subsegmental location of the tumor (45.7% vs 16.2%, $P = 0.01$), liver cirrhosis (12.3% vs 47.9%, $P = 0.03$), surgical margin (18.5% vs 54.6%, $P = 0.04$), vascular invasion (37.9% vs 23.2%, $P = 0.04$) and extended caudate resection (42.1% vs 15.4%, $P = 0.04$) were related to poorer long-term survival. Multivariate analysis showed that only subsegmental location of the tumor, liver cirrhosis and surgical margin were significant independent prognostic factors.

CONCLUSION: Hepatectomy was an effective treatment for HCC in the caudate lobe. The subsegmental location of the tumor, liver cirrhosis and surgical margin affected long-term survival.

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Key words: Hepatectomy; Hepatocellular carcinoma; Caudate lobe; Prognostic factors

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INTRODUCTION

Refined surgical and anesthetic techniques, a better understanding of liver anatomy^[1,2], and improvements in postoperative management have increased the indications for hepatectomy in patients with hepatocellular carcinoma (HCC) in the caudate lobe. Hepatic resections can now

be performed in high-volume centers with an acceptable morbidity of 50%-60% and no mortality^[3,4]. Caudate HCC has a poorer prognosis than HCC originating from other lobes due to its proximity to the portal trunk and inferior vena cava, which facilitates intrahepatic and systemic spread early in the disease^[5,6]. Although some surgeons have reported successful surgical treatment of caudate HCC with transarterial chemoembolization (TACE)^[7,8] or local ablation^[9-11], hepatic resection has been considered to be the treatment of first choice^[12-14]. The most important factor currently limiting the disease-free interval is the high rate of tumor recurrence, which ranges from 50%-60% at 3 years^[15,16]. In order to improve surgical outcome, it is necessary to evaluate the potential risk factors for long-term survival and to establish guidelines for the appropriate use of hepatectomy for caudate lobectomy. We therefore retrospectively evaluated 114 consecutive patients who underwent hepatic resection for HCC originating in the caudate lobe, in order to assess the influence of common clinicopathologic variables on recurrence and long-term survival.

MATERIALS AND METHODS

Between January 2001 and January 2007, 114 consecutive patients with HCC in the caudate lobe underwent hepatic resection at the Eastern Hepatobiliary Surgery Hospital, Second Military Medical University. Computed tomography (spiral-CT), detection of serum α -fetoprotein level (AFP), and hepatic ultrasound-guided fine-needle biopsy were used for preoperative diagnosis of HCC. Needle biopsy was not performed in patients with an elevated serum level of AFP and typical imaging of HCC, to avoid needle tract seeding of tumor cells. Clinicopathologic and follow-up data for each patient were recorded in a computerized database, regularly updated for tumor recurrence and survival status. Resection was considered "extended" if the caudate lobe as well as other lobes or segments were removed, according to Couinaud's classification. Intraoperative ultrasound was routinely performed in all patients, in order to detect tumor invasion into the major branches of the portal vein and hepatic veins, or the presence of lesions in the contralateral lobe. Tumor clearance at the resection margins of at least 5 mm was considered adequate to define the surgical procedures as curative (R0)^[17]. Hospital mortality was defined as death within 30 d after operation, including operative deaths. Tumor recurrence was considered as evidence of hepatic tumoral lesions after a curative resection. All patients discharged were followed-up at our department every 3 mo in the first year, every 6 mo in the second year, and every 6 mo thereafter. The follow-up consisted of physical examination, blood tests, serologic liver function test, detection of serum AFP level and liver ultrasound or CT scan.

Liver resection was carried out using a clamp-crushing technique in all patients. Intraoperative ultrasonography was routinely used to locate the carcinoma, exclude daughter nodules, and identify the relationship of the tumor with the major vessels, so minimizing blood loss and avoiding injury to the main trunk vessels. We used

multiple occlusion techniques, including continuous or intermittent Pringle maneuver, hemihepatic vascular clamping, or total hepatic vascular exclusion, determined on a case-by-case basis.

Statistical analysis

Continuous data were expressed as medians and inter-quartile ranges. Survival curves were calculated using the Kaplan-Meier method and compared using the log-rank test. For comparison of survival, continuous variables were dichotomized using the respective medians as the cut-off values. Only the variables that were significant in univariate analysis were entered into a Cox regression model to identify the clinicopathologic factors with independent prognostic significance. Patients with hospital mortality (within 30 d) were excluded from the evaluation of these factors with regard to long-term and disease-free survival. Statistical analysis was carried out using SPSS computer software (SPSS Inc., Chicago, IL, USA). Differences were considered significant if the *P* value was < 0.05.

RESULTS

The clinicopathologic characteristics of the 114 resected patients are shown in Table 1. There were 87 males (76%) and 27 females (24%). The median age was 49 years. The Child-Pugh grading system for the prognosis of liver cirrhosis was applied in all patients. All patients were classified as Child-Pugh grade A. Data regarding serum AFP levels were available for all patients, and 90 patients (79%) were AFP-positive. The median preoperative AFP level was 195 ng/mL. Seventy-eight (68.4%) patients had undergone isolated caudate lobectomy and 36 (31.6%) had undergone extended caudate lobectomy (Table 2).

A curative resection (surgical margin > 5 mm) was achieved in 59% of cases (67 patients). Histopathologic examination showed that 84% of patients had a differentiated tumor (74% trabecular type, 1% fibrolamellar type, and 9% mixed type), while 16% had undifferentiated tumors. Vascular invasion was found in 38% of patients. There was no postoperative mortality. The hospital morbidity rate was 18% (21 patients). The most frequent complications were infections (abdominal abscess, pleural effusion and bronchopneumonia), liver failure, hemorrhage, ascites and mild lower limb edema.

The median follow-up period was 31 mo (interquartile range, 11-66 mo). A total of 65 patients (57.0%) died during the follow-up period. Eight patients (7.0%) were lost to follow-up at 4, 7, 9, 18, 30, 42, 50 and 54 mo. Up to the last follow-up date (January 2007), 41 patients (36.0%) were alive, of whom, 21 were disease-free (18.4%). Tumor recurrence occurred in 49 patients (75.4%), and disease progression was the leading cause of death in 65 patients (57.0%). The 1-, 3-, and 5-year disease-free survival rates were 65.7%, 38.7%, and 18.8%, respectively. The 1-, 3-, and 5-year overall survival rates were 76.1%, 54.7%, and 31.8%, respectively (Figure 1).

Statistical analysis

The prognostic influences of the clinicopathologic char-

Table 1 Clinical and pathologic characteristics of 114 HCC patients

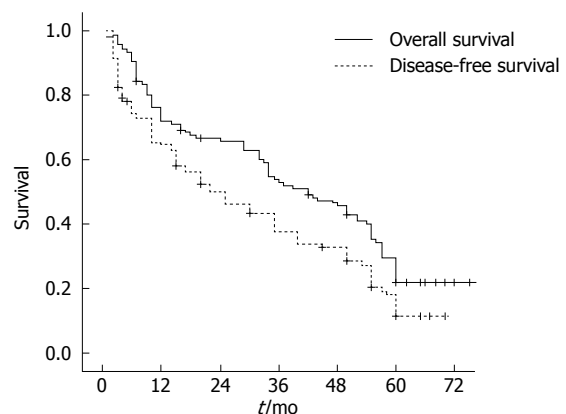
Characteristics	n (%)
Age (yr)	
≤ 65	89 (77)
> 65	25 (23)
Gender	
Male	87 (76)
Female	27 (24)
Cirrhotic liver	
No	36 (32)
Yes	78 (68)
Tumor size (cm)	
≤ 5	50 (44)
> 5	64 (56)
Serum AFP (ng/mL)	
≤ 200	69 (61)
> 200	45 (39)
Subsegmental location	
Spiegel lobe	39 (34)
Paracaval portion	54 (47)
Caudate process	21 (19)
Tumor number	
Solitary	91 (80)
Multiple	23 (20)
Pringle maneuver	
No	34 (30)
Yes	80 (70)
Surgical treatment	
Isolated caudate lobectomy	78 (68)
Extended hepatectomy	36 (32)
Surgical margin (mm)	
≤ 5	47 (41)
> 5	67 (59)
Histologic grading	
Differentiated	96 (84)
Undifferentiated	18 (16)
Capsule	
No	95 (93)
Yes	19 (17)
Vascular invasion	
No	71 (81)
Yes	43 (38)
Daughter nodules	
No	83 (73)
Yes	31 (27)

HCC: Hepatocellular carcinoma; AFP: α -fetoprotein.

acteristics are shown in Table 3. There were no associations between age or sex and survival rate. No significant differences in survival rate were noted between patients with AFP levels $>$ or \leq 200 ng/dL, with tumor sizes \leq or $>$ 5 cm, with or without capsulated tumors, or among patients with well-differentiated or poorly-differentiated HCC. The extent of the hepatic resection (isolated *vs* extended) did not influence the long-term survival. The effect of tumor subsegmental location was also investigated and it was shown that there was a significant survival difference between patients with tumors in the Spiegel lobe compared to the paracaval portion and caudate process ($P < 0.01$). There was no significant difference in 3-year survival between solitary and multiple tumors, but a significant difference in overall survival was observed between patients with or without liver cirrhosis. The 5-year survival rate of patients without cirrhosis was

Table 2 Hepatectomy for caudate lobe hepatocellular carcinoma

Operations	n (%)
Isolated caudate lobectomy	78 (68.4)
Spiegel lobe	27
Paracaval portion and caudate process	31
Spiegel and paracaval portion	2
Paracaval portion	7
Caudate process	4
Complete caudate lobectomy	7
Extended hepatectomy	36 (31.6)
Spiegel and left hemihepatectomy	5
Spiegel and segment IV	2
Spiegel and II, III	3
Spiegel and VI, VII	1
Spiegel and VII	1
Paracaval portion and caudate process and right hemihepatectomy	4
Paracaval portion and caudate process and segment IV	2
Paracaval portion and caudate process and VII	1
Paracaval portion and caudate process and segment II, III	3
Paracaval portion and caudate process and V, VI	2
Paracaval portion and segment II, III	3
Paracaval portion and segment VI, VII	1
Complete caudate lobe and right hemihepatectomy	1
Complete caudate lobe and segment IV	1
Complete caudate lobe and left hemihepatectomy	4
Complete caudate lobe and segment II, III	1
Complete caudate lobe and segment VI	1

**Figure 1** Disease-free and overall survival curves.

significantly higher than in patients with viral cirrhosis (47.9% *vs* 12.3%, $P = 0.03$). The presence or absence of vascular invasion was also a significant prognostic factor for survival; patients without vascular invasion had significantly higher 5-year survival rates than those with vascular invasion (37.9% *vs* 23.2%, $P = 0.05$).

In multivariate analysis, only subsegmental location, liver cirrhosis and surgical margin were confirmed as independent prognostic factors for overall survival (Table 4). None of the clinicopathologic factors analyzed were significantly correlated with disease-free survival (Table 5).

DISCUSSION

Although HCC arising from the caudate lobe has been reported to be relatively rare, its surgical treatment

Table 3 Overall survival: univariate analysis of prognostic clinicopathologic factors

	3-yr survival (%)	5-yr survival (%)	P-value
Age (yr)			
≤ 65	52.8	34.2	0.24
> 65	63.1	52.0	
Gender			
Male	56.1	38.4	0.41
Female	60.6	50.0	
Cirrhosis			
Yes	35.0	12.3	0.03
No	60.6	47.9	
Serum AFP (ng/mL)			
≤ 200	55.9	34.6	0.57
> 200	55.4	45.5	
Tumor location			
Spiegel lobe	63.1	45.7	0.01
Paracaval portion	22.9	16.2	
Caudate process	25.2	14.9	
Tumor size (cm)			
≤ 5	58.4	43.3	0.48
> 5	57.0	39.4	
Pringle maneuver			
No	67.7	42.1	0.73
Yes	62.5	37.9	
Surgical margin (mm)			
≤ 5	20.4	18.5	0.02
> 5	60.7	54.6	
Surgical treatment			
Isolated caudate lobectomy	52.1	42.1	0.04
Extended hepatectomy	27.9	15.4	
Histologic grading			
Differentiated	57.7	33.6	0.79
Undifferentiated	52.2	29.4	
Capsule			
No	65.3	41.5	0.70
Yes	56.1	35.9	
Vascular invasion			
No	52.4	37.9	0.05
Yes	29.2	23.2	
Daughter nodules			
No	56.1	36.8	0.38
Yes	45.3	25.0	

Table 4 Overall survival: multivariate analysis of prognostic clinicopathologic factors

	Hazard ratio	95% CI	P-value
Location of tumor	0.176	0.046-0.701	0.02
Liver cirrhosis	4.874	1.107-19.339	0.04
Surgical margin	1.36	0.210-2.375	0.04

presents a challenge and is associated with high risks for the surgeon, due to its unique anatomic location and its complicated relationship with the major vasculature. To the best of our knowledge, the tumor with the largest reported volume was a HCC in the caudate lobe. Local ablation of HCC in the caudate lobe has been reported and evaluated^[12-14], but hepatic resection remains the mainstay for the treatment of HCCs, and is the only approach that provides consistent, long-term survival^[18-21].

Due to the lack of large series of patients with HCC in the caudate lobe, studies have produced conflicting

Table 5 Disease-free survival: univariate analysis of prognostic clinicopathologic factors

	3-yr survival (%)	5-yr survival (%)	P-value
Age (yr)			
≤ 62	46.7	21.7	0.76
> 62	38.2	31.4	
Gender			
Male	49.4	26.4	0.43
Female	25.1	20.7	
Cirrhosis			
Yes	42.6	37.2	0.19
No	51.9	29.3	
Serum AFP (ng/mL)			
≤ 200	36.5	20.1	0.66
> 200	32.4	29.7	
Tumor location			
Spiegel lobe	50.5	34.4	0.07
Paracaval portion	36.9	20.6	
Caudate process	41.0	26.2	
Tumor size (cm)			
≤ 5	40.6	23.0	0.69
> 5	44.0	26.4	
Pringle maneuver			
No	40.4	22.6	0.66
Yes	36.1	19.8	
Surgical margin			
Positive	42.6	22.8	0.08
Negative	55.2	30.7	
Surgical treatment			
Isolated caudate lobectomy	43.4	24.0	0.35
Extended hepatectomy	41.7	35.8	
Histologic grading			
Differentiated	42.1	24.2	0.42
Undifferentiated	33.9	28.7	
Capsule			
No	36.3	19.0	0.65
Yes	43.8	26.3	
Vascular invasion			
No	45.0	29.7	0.25
Yes	34.0	21.3	
Daughter nodules			
No	56.1	36.8	0.09
Yes	45.3	25.0	

reports on the effect of this surgery and prognosis following liver resection. During the late 1980s to early 1990s, several groups^[5,12,17] reported that HCCs originating in the caudate lobe easily produced intrahepatic metastases because of the corresponding short portal veins, giving these patients a poor prognosis. Others during the late 1990s^[6,16,22], however, reported comparable survival rates for patients with HCCs in the caudate lobe and those with HCCs in other parts of the liver. Our results were in accord with the former findings. In this study, we performed multivariate analysis and calculated survival rates for patients with caudate HCC after resection, in relation to clinicopathologic factors. Our data showed that long-term survival of patients with HCC after hepatectomy depended on the background of cirrhosis, subsegmental location of HCC, surgical resection margin, and extended caudate resection. The overall survival rates in our group after resection of HCC of the caudate lobe were 76.1% at 1 year, 54.7% at 3 years and 31.8% at 5 years. The disease-free survival rates were

65.7% at 1 year, 36.1% at 3 years and 18.8% at 5 years. The results showed poorer overall survival at 5 years than that reported by Ikegami *et al*^[16] (66.7%). However, the diameter of the tumors in most of the patients in this earlier study was < 5 cm, which was the reason for the limited hepatic resection performed. In contrast, the median tumor diameter in our series was 5.7 cm, which could account for the poorer prognosis. Overall survival rates of 85.3% at 1 year, 67.0% at 3 years and 50.5% at 5 years were reported in a series of 12 118 patients after resection of HCC by Ikai *et al*^[23]. From this point of view, the overall survival of patients with HCC of the caudate lobe after resection was poorer than that of patients with HCCs of other lobes. This survival difference may be related to the anatomy and characteristics of the caudate lobe.

Univariate and multivariate analyses showed significant differences in overall survival depending on subsegmental location of HCC. The results of the current study showed that patients with tumors located in the Spiegel lobe had a better prognosis than those whose tumors were located in the paracaval portion. HCC located in the paracaval portion is contiguous with the major vessels and adequate surgical tumor margins cannot be obtained, especially in the case of liver cirrhosis, making expanded hepatic resection impossible. Asahara *et al*^[24] suggested that caudate lobectomy should extend past the right border of the inferior vena cava for adequate resection of the paracaval portion. Counterstaining can be used to identify the border between the paracaval portion and the posterior segments, but although this can demonstrate the border at the liver surface, it is still difficult to accurately identify the border deep within the liver parenchyma. Exposure of the paracaval portion is also difficult due to its deep location, and the greater manipulation required increases the risk of intrahepatic metastasis through the portal vein to the remnant liver.

The importance of chronic liver disease in the prognosis of patients undergoing resection for HCC is well known^[23,25-27], and patients with hepatitis C- or hepatitis B-related cirrhosis have poorer prognoses than those with cirrhosis due to other causes^[28]. In our study, the cumulative survival rates at 3 and 5 years were 60.6% and 47.9% in non-cirrhotic patients, and 35.0% and 12.3% in cirrhotic patients, respectively. After liver resection for HCC, some reports found that chronic active hepatitis and cirrhosis were the most significant risk factors for intrahepatic recurrence through multicentric carcinogenesis, so-called “multicentric occurrence”. This can be explained by the fact that repeated inflammation and cellular necrosis in patients with chronic hepatitis or cirrhosis enhance proliferation and accelerate the development of new foci of HCC, associated with an increased rate of random mutations and promotion due to gene instability^[23,26].

A surgical margin of < 5 mm was also identified as an independent risk factor for poor survival following resection of caudate HCC. Although the importance of the size of the surgical margin is controversial, particularly in large tumors, and although satellite nodules have been found at some distance from the tumor, it is usually

believed that the risk of recurrence is lower when the surgical margin is large^[29-32]. However, obtaining a negative margin may be difficult, particularly in large HCCs, and especially in those located in the caudate lobe. Although we aimed to preserve a wide margin where possible in the current series, it measured < 5 mm in 44% of patients. After analysis of 209 consecutive liver resections in patients with HCC, Tralhão *et al*^[33] failed to identify any factors significantly predictive of a thin surgical margin, but thin margins were more common in patients with large tumors, and particularly in those with centrally located tumors. Even in patients with small but centrally located tumors, surgical margins of > 10 mm were infrequent. In our study, 64% of tumors were > 5 cm and related to the paracaval portion, which may be the main reason for the lower incidence of negative margins. Although extensive hepatectomy can obtain a high negative margin rate, only 32% of patients in this study underwent extended caudate lobectomy due to the presence of liver cirrhosis and underlying hepatitis. These results support the prognostic significance of surgical margin and extended resection for overall survival, and suggest that efforts should be made to increase the tumor-free margin. Extended caudate lobectomy is recommended, so long as acceptable liver function is maintained. It has been suggested that adjuvant intraarterial chemotherapy and/or preoperative TACE may reduce the risk of tumor recurrence and improve long-term survival following liver resection for caudate HCC^[7]. However, this was not confirmed in our study. Prospective, randomized clinical trials are needed to investigate the role of perioperative TACE for caudate HCC.

In conclusion, hepatectomy was an effective treatment for HCC in the caudate lobe. The subsegmental location of the tumor, presence of liver cirrhosis and the surgical margin affected long-term survival.

COMMENTS

Background

Hepatic resection is considered, in principle, to be the first choice for treatment of hepatocellular carcinoma (HCC) in the caudate lobe. However, the surgical treatment for HCC in the caudate lobe presents a major challenge and is associated with high risks for the surgeon, due to its unique anatomic location and complicated relationship with the major vasculature. Until now, the prognosis for patients following resection of caudate lobe HCC has not been fully determined.

Research frontiers

Caudate HCC has a poorer prognosis than HCC originating from other lobes, due to its proximity to the portal trunk and inferior vena cava, which facilitate its intrahepatic and systemic spread early in the disease. A large number of studies have confirmed that liver disease, tumor grade, tumor size, tumor margin, blood loss and other factors influence the incidence of tumor recurrence and long-term survival after resection of HCC.

Innovations and breakthroughs

Hepatectomy was an effective treatment for HCC in the caudate lobe. The subsegmental location of the tumor, presence of liver cirrhosis and surgical margin affected long-term survival.

Applications

The results of this research suggest that surgeons should make every effort to increase the tumor-free margin. Extended caudate lobectomy is recommended, so long as liver function can be maintained.

Terminology

The caudate lobe is generally divided into three regions: the left Spiegel, the

process portion, and the paracaval portion. Isolated caudate lobectomy involves the removal of all or part of the caudate lobe, and extended hepatectomy involves resection of all or part of the caudate lobe, in conjunction with other lobes of the liver.

Peer review

The authors report a series of 114 liver resections in patients with HCC in the caudate lobe. The perioperative data, complications and outcome were analyzed. This study addressed an important and interesting issue.

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Alterations of tumor-related genes do not exactly match the histopathological grade in gastric adenocarcinomas

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oligonucleotide and locked nucleic acid modifying probe within the tissue array. The data obtained were processed by support vector machines by four different feature selection methods to discover the respective critical gene/gene subsets contributing to the GA activities of different pathological gradings.

RESULTS: In comparison of poorly differentiated GA with normal tissues, tumor-related gene *TP53* plays a key role, although other six tumor-related genes could also achieve the Area Under Curve (AUC) of the receiver operating characteristic independently by more than 80%. Comparing the well differentiated GA with normal tissues, we found that 11 tumor-related genes could independently obtain the AUC by more than 80%, but only the gene subsets, *TP53*, *RB* and *PTEN*, play a key role. Only the gene subsets, *Bcl10*, *UVRAG*, *APC*, *Beclin1*, *NM23*, *PTEN* and *RB* could distinguish between the poorly differentiated and well differentiated GA. None of a single gene could obtain a valid distinction.

CONCLUSION: Different from the traditional point of view, the well differentiated cancer tissues have more alterations of important tumor-related genes than the poorly differentiated cancer tissues.

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Key words: Pathological grading; Gastric adenocarcinoma; Tumor-related gene; Support vector machine; RNA *in situ* hybridization

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Abstract

AIM: To investigate the diverse characteristics of different pathological gradings of gastric adenocarcinoma (GA) using tumor-related genes.

METHODS: GA tissues in different pathological gradings and normal tissues were subjected to tissue arrays. Expressions of 15 major tumor-related genes were detected by RNA *in situ* hybridization along with 3' terminal digoxin-labeled anti-sense single stranded

Liu GY, Liu KH, Zhang Y, Wang YZ, Wu XH, Lu YZ, Pan C, Yin P, Liao HF, Su JQ, Ge Q, Luo Q, Xiong B. Alterations of tumor-related genes do not exactly match the histopathological grade in gastric adenocarcinomas. *World J Gastroenterol* 2010;

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INTRODUCTION

Malignant tumor is one of the leading causes of death, with a mortality of 12.5%^[1]. Stomach adenocarcinoma is one of the major types of gastric cancer which is the second major cause of oncologic death worldwide. Although the incidence and mortality of stomach adenocarcinoma have been obviously declined, it is still one of the most serious health burdens in the world^[2-4], and surgical resection remains the only curative treatment to improve the survival of patients with gastric cancer^[5,6]. Generally, clinicians consider the degree of malignancy based on the histopathological grade of tumors, and predict the prognosis of patients and estimate the survival rates according to the pertinent criteria and the intraoperative findings^[7,8]. Although histological classifications are widely used for gastric adenocarcinoma (GA), their prognostic value is still controversial. The degree of malignancy always implies the early cancer metastasis, invasion and the mortality rates^[9-11]. Because the occurrence of cancer is closely related to the tumor-related genes, we explored the diverse characteristics of different pathological gradings of GA by investigating 15 tumor-related genes, which have been currently proved to be closely related to carcinogenesis. They represent different cancer formation mechanisms and have tight connection and mutual regulation^[12-15]. Among them, the *C-myc*^[16] is an oncogene, Cyclin D1^[17] is a cell cycle protein, *BCL10*^[18] is an anti-apoptotic gene, *KAI1* and *NM23*^[19] are metastatic suppressor genes, *Beclin1*^[20] and *UVRAG*^[21] are cellular autophagy genes, *TP53*^[22], *RB*^[23], *PTEN*^[24], *Ptc1*^[25], *BRCAl*, *BRCAl2*^[26], *FHIT*^[27] and *APC*^[28] are tumor suppressor genes.

MATERIALS AND METHODS

Materials and tissue microarray construction

One hundred and twelve primary samples of GA and normal tissues were snap-frozen and stored at -70°C. There were 40 samples from patients (age range: 42-78 years, 22 males and 18 females) with poorly differentiated GA, 28 samples from patients with well differentiated GA (age range: 50-81 years, 18 males and 10 females), and 44 samples of normal tissues (age range: 38-72 years, 30 males and 14 females). All the patients were Chinese, who underwent operations at the Affiliated Zhongshan Hospital of Xiamen University between 2000-2006.

Tissue blocks measuring approximately 1.5 cm × 1.5 cm × 0.3 cm and non-pathologic organs were fixed in phosphate-buffered saline (PBS) containing 4% paraformaldehyde (1% DEPC, pH 7.4) for 24 h, dehydrated through gradient ethanol, and embedded in paraffin. A hematoxylin and eosin (HE)-stained section was made

from each block to define the representative tumor region. Representative areas in different lesions were carefully selected on HE-stained sections and marked on individual paraffin blocks. Tissue cylinders with a diameter of 1 mm were then punched from tumor areas in each “donor” tissue block and put into a recipient paraffin block using a custom-made precision instrument. Five-mm sections of the resulting multiple tumor tissue microarray (TMA) blocks were transferred to glass slides using the paraffin sectioning aid system [adhesive-coated slides (PSA-CS4x), adhesive tape, and UV lamp; Instrumedics, Inc., Hackensack, NJ], supporting the cohesion of 0.6-mm array elements. The final TMA consisted of cores of 1 mm in diameter each spaced at 0.8 mm between core centers. A section stained with HE was reviewed to confirm the presence of morphologically representative areas in the original lesions.

Preparation of the 15 tumor-related gene probes

Antisense probe perfectly matched to corresponding sequence, Lock nucleic acid modified probe increased the stability of the probe and sensitivity. The type of tumor-related gene and probe sequence are shown in Table 1. All probes were synthesized by Shanghai Sheng Gong Corporation.

RNA *in situ* hybridization

Hybridization procedures were performed based on the instructions of RNA *in situ* hybridization (RISH) kits (Cybrdi, USA) with some modifications. The glassware was washed, rinsed in distilled deionized water, and autoclaved before use. Gloves were worn when the glassware and slides were handled to prevent RNase contamination on the tissues. Because of the differences in tissues and probes, we performed different pilot-experiments to achieve the best results (Table 1). Deparaffinized sections were mounted onto Denhardt-coated glass slides and treated with pepsin (0.25 mg/mL in DEPC H₂O-HCl) for 25-30 min in a 37°C water bath. The treated sections were then processed for *in situ* hybridization at 42-45°C for 36-48 h. The hybridization mixture contained the labeled oligonucleotide probe, 50% formamide, 10 mmol/L Tris-HCl, 1 mmol/L vanadyl-ribonucleoside complex (Sigma 94740), 1 mmol/L CTAB (Sigma 855820, pH 7.0), 0.15 mol/L NaCl, 1 mmol/L EDTA (pH 7.0), 1 × Denhardt's mixture and 10% dextran sulfate. After hybridization, the slides were washed three times, 30 min each time, in 0.1 mol/L TBS at room temperature, then treated with TBS (100 mmol/L Tris, pH 7.5, 150 mmol/L NaCl) containing a 1% blocking reagent (Roche) and 0.03% Triton X-100 for 30 min at room temperature and incubated for 30 min with anti-digoxigenin alkaline phosphatase-conjugated antibodies (Roche) diluted at 1:500 in TBS containing 0.03% Triton X-100 and a 1% blocking reagent. After being washed three times, 15 min each time, in TBS and 0.05% Tween, the slides were rinsed in a DAP-buffer (100 mmol/L Tris, pH 9.5, 100 mmol/L NaCl, 50 mmol/L MgCl₂) and subsequently

Table 1 Different pilot experiments and probe sequence of 15 tumor-related genes

Tumor-related genes	Probe sequence
APC	5-TTGGTTCCAGATGACTTGTGAGCC(T)TCGAGGTGCAGAGTGTGTG CTACTAG-3dig
Bcl10	5-CTGTATCAGGAAGTTCTGTG(T)TTTTCTCGCCGAATAG ATTCAACAAGGGTG-3dig
Beclin1	5-CCAAGCAGCATTAATCTCATTCCA(T)TCCACGGGAACACTGGGCAGGCGACC-3dig
BRCA1	5-CCTCTTTCTTCATCATCTGAAACCAAT(T)CCTTGTCACCTCAGACCAACTCCCT-3dig
BRCA2	5-AAGCGATGATAAGGGCAGAGGAAAAGG(T)CTAGGGTC AGGAAAGAATCCAAGT-3dig
FHIT	5-AGTCCTCCTTGTCTATGTTTCTGGAGC(T)CCTCATAGATGCTGT CATTCTGTG-3dig
KAI1	5-GCAGAAGCCCTTCTCAGAAAGGC(T)GTTGTCCTCT TCCCTTGACTTCGC-3dig
NM23	5-GGAATCCTTTCTGCTCAAAACGC(T)TGATAATCTCTCCACAAGACCCGCTG-3dig
Ptch	5-CGCTTCGTGGTCAGGACAT(T)AGCACCTTCTCTTTAG GGGTCTGTATCAT-3dig
PTEN	5-CCTCTGATATCTCTTTTGTTC(T)GCTAACGATCTCTTTGATGATGGCTG-3dig
RB	5-TGAGCACACGGTCGCTGTACA(T)ACCATCTGATTATTTCTGGAA CTCT-3dig
UVRAG	5-CTCCTTGTCTTGGCTAGGGTGACA(T)TCGCGTGGCCT CCGTTTAAGCTGCCAAC-3dig
TP53	5-CCAGGACAGGCACAAACACGCACCT*CAAAGCTGTTT CGTCCAGTAGATTAC-3dig
Cyclin D1	5-CCTCTCGCACTTCTGTTCTCGCAGACCT*CCAGCATCCAGTGGCGACGATCTTCCG-3dig
C-myc	5-CTTCTCATCTTCTGTCTCTCTCAGAGT*CGCTGCTGGTGGTGGCGGTGTC-3dig

hybridization signals were visualized using nitroblue tetrazolium and 5-brom-4-chlor-3-indolyl phosphate as substrates [DAP-buffer in 10% PVA (Sigma 341584)].

Gene expression analysis

Two techniques for data analysis were adopted: a statistical method used to calculate the *P* values of genes in different samples, and a machine learning method applied to further discover the relationship between genes and corresponding samples.

The significance level of the 15 tumor-related genes were analyzed by Wilcoxon rand sum test, which is an efficient nonparametric statistical method to compare two groups of data and determine their differences. It is important to choose an efficient machine learning method to further explore the connections between genes and different cancers. However, it is hard to decide what kind of functions the 15 tumor-related genes would have for the different types of cases. So it is necessary to separately analyze the effects of both a single gene and different gene groups in different specimens. However, since there are so many ways to construct a gene group within 15 genes, efficient methods are required to shrink the scope of gene group construction. To achieve this, four classical feature selection methods were used to analyze gene expression levels, including: *t* test, entropy, Bhattacharyya and Wilcoxon. All these methods were provided in bioinformatics toolbox embedded in Matlab 7.1. Based on different criteria for feature selection, different methods would result in genes in different order of importance. The genes were classified into different groups. The discrimination ability of the gene groups was measured by support vector machine (SVM). The genes with biological significance were discovered by comparing the results.

There were three steps to analyze gene expressions: firstly, the gene expressions of different specimens were measured with the Wilcoxon rank sum test, so that *P* value of each gene can be calculated, and then used to evaluate the homologous extent of the two specimens. Secondly, the classification ability of each gene was

analyzed singly among different tissues to further assess the importance of each gene in different tissues. Thirdly, the results obtained by the combination of different genes were investigated. The relationship among genes could be discovered in this way. It is easy to evaluate the classification ability of a single gene using SVM with 10-fold cross-validation (CV) directly. However, since there are many ways to select the 15 genes to form a gene group, it is necessary to take a reliable selection method. In our analysis, a gene group starts from an empty one. A filter method was applied to rank the genes, and then a gene was added to the group according to the score of the rank. The gene group was used to discriminate the samples in two types of tissues using SVM by the 10-fold CV method. This process ended when all genes were added to the group. In addition, as the sample sizes varied in different diseases, the Area Under Curve (AUC) of the receiver operating characteristic (ROC) was deployed in our experiments. A ROC curve represents the true positive rate as a function of the corresponding false positive rate, and AUC provides a measure of performance that is sensitive to the distribution of the activity classes in test sets. Finally, the best gene subsets can be found by the highest AUC.

RESULTS

TMA technical adjustment

The tissue micro-array technology was substantially different from the traditional multi-tissue blocks. The most important advantages of TMA technology include increased capacity, negligible damage to the original tissue blocks, precise positioning of tissue specimens, and possibility of automatic construction and analysis of arrays. In this study, we chose 4% paraformaldehyde in PBS (1% DEPC PBS) as a fixation agent, which can decrease the degradation of RNA and yield a good morphology. The analysis of RISH showed that 80%-95% of tumor samples were interpretable. RISH-related weak hybridization, background, and tissue damage were responsible for about one-sixth of the non-informative cases.

Table 2 *P* values of 15 genes in different comparisons

<i>APC</i>	<i>Bcl10</i>	<i>Beclin1</i>	<i>BRCA1</i>	<i>BRCA2</i>	<i>FHIT</i>	<i>KAI1</i>	<i>NM23</i>	<i>Ptch</i>	<i>PTEN</i>	<i>RB</i>	<i>UVRAG</i>	<i>TP53</i>	<i>Cyclin D1</i>	<i>C-myc</i>
0.0523	0.0002	0.0000	0.0000	0.0000	0.0000	0.0000	0.2603	0.0000	0.0006	0.0000	0.5295	0.0000	0.0000	0.0048
0.0000	0.7527	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0001
0.0000	0.0018	0.0001	0.0371	0.0028	0.1999	0.0129	0.0000	0.1142	0.0001	0.0009	0.0000	0.0092	0.3676	0.0158

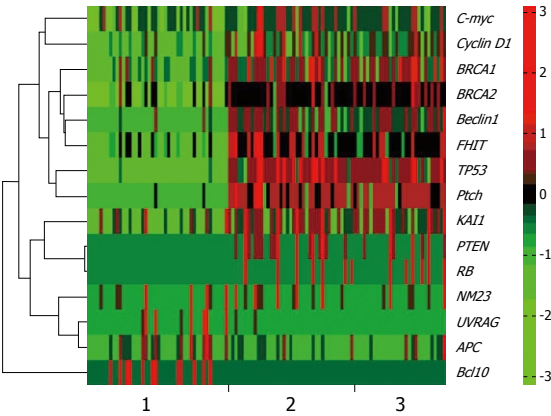


Figure 1 Gene expression levels of three different tissues. 1: Normal tissues; 2: Well differentiated gastric adenocarcinoma (GA); 3: Poorly differentiated GA. X-axis represents samples, and each unit represents a gene expression level of the corresponding sample. Y-axis represents genes, whose name marked at the right side of the figure. Green color indicates the low-expression level of corresponding gene, and red represents over-expression.

Expression of tumor-related genes in different specimens

RISH was used to detect specific RNAs *in situ*. The typical results of ISH were observed as amethyst dots on arrays, RNA analysis and quantification required completely intact, non-degraded RNA samples to produce optimal results. Vanadium oxide ions and formation of complex nucleoside could protect RNA degradation from RNase. Cetyltrimethylammonium bromide could stabilize the Oligo probe and target sequence formation of double-stranded structures, thus improving the reannealing speed. The monomer containing LNA greatly improved the stability and sensitivity of RNA-targeted *in situ* hybridization. According to the results of RISH, positive organizational coloring cell counts were classified under the microscope (Figures 1 and 2).

Analysis of tumor-related gene expressions

Results obtained at the first step: The gene expressions of different specimens were measured with the Wilcoxon rank sum test. Table 2 shows the *P* value obtained in experiments. From the results, we found that the normal tissues were quite different from the cancer tissues. The *P* value of different genes indicated that there were 12 out of 15 genes and 14 out of 15 genes with significant biological difference in comparison of normal tissues with the cancer tissues.

Results obtained at the second step: The classification ability of each gene was analyzed singly among different tissues. From Figure 3, it could be found that

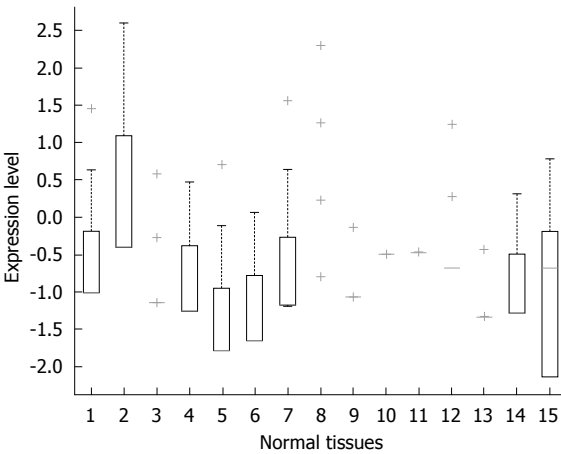
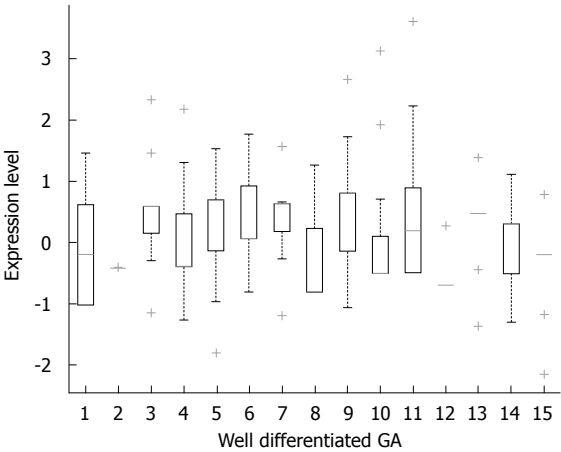
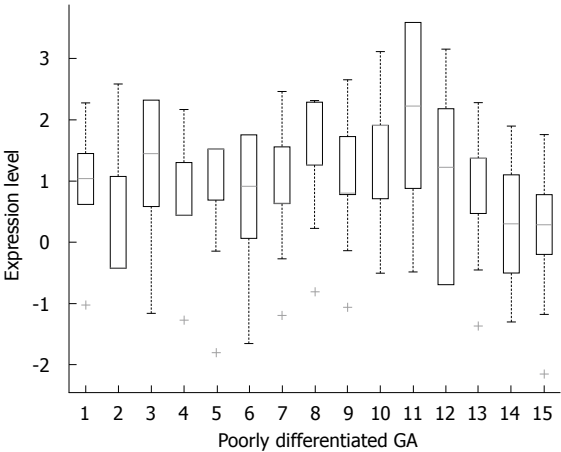


Figure 2 Distribution of gene expression levels. 1: *APC*; 2: *Bcl10*; 3: *Beclin1*; 4: *BRCA1*; 5: *BRCA2*; 6: *FHIT*; 7: *KAI1*; 8: *NM23*; 9: *Ptch*; 10: *PTEN*; 11: *RB*; 12: *UVRAG*; 13: *TP53*; 14: *Cyclin D1*; 15: *C-myc*.

when using a single gene, both well differentiated GA and poorly differentiated GA could be well distinguished

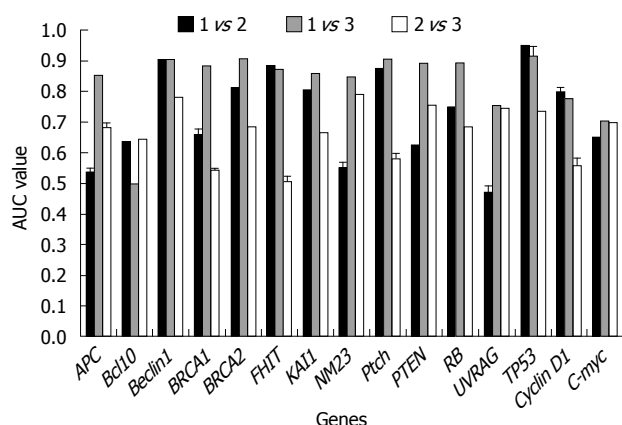


Figure 3 Classification of two sample groups with a single gene. 1: Normal tissues; 2: Well differentiated GA; 3: Poorly differentiated GA. AUC: Area Under Curve.

from the normal tissues. When compared with normal tissues, two different gene subsets were discovered respectively: *Beclin1*, *BRCA2*, *FHIT*, *KAI1*, *Pch*, *TP53*; and *APC*, *Beclin1*, *BRCA1*, *BRCA2*, *FHIT*, *KAI1*, *NM23*, *Pch*, *PTEN*, *RB*, *TP53* in poorly and well differentiated GAs. In comparison of well and poorly differentiated GA, none of the single genes could achieve a high classification performance.

In this analysis, when the AUC results were lower than 80%, the corresponding gene(s) had no classification power under our hypothesis. If a gene can improve the AUC by more than 80%, it can be regarded as a key gene as it can classify two different sample groups.

Results obtained at the third step: The results were analyzed in three aspects: (1) The poorly differentiate GA was compared with normal tissues, and the results indicated that *TP53* was the key gene for distinguishing the two tissues (Figure 4). It was obvious that when *TP53* was used, the AUC results were immediately improved; (2) Well differentiated GA was compared with normal tissues, and the results showed that *TP53*, *RB*, and *PTEN* are the key genes, which had high classification abilities (Figure 5); and (3) We compared the poorly differentiated with well differentiated GA, and seven genes (*Bcl10*, *UVRAG*, *APC*, *Beclin1*, *NM23*, *PTEN*, and *RB*) were found to be closely related to different pathological gradings of gastric cancers (Figure 6).

DISCUSSION

Traditional clinical point of view

Histopathological differentiation level represents the deterioration degree of tumors. The stomach carcinoma is one of the most prevalent cancer types in the world^[1,2]. Only a limited number of biomarkers are available for its detection and prognostic evaluation. Up to now, the clinicians still determine the degree of malignancy by histopathological differentiation method. Two major types of stomach carcinoma are distinguished accord-

ing to their morphological and clinicopathological classifications: well-differentiated/intestinal type and poorly differentiated/diffuse type^[6,29]. Here, the well or poorly differentiated level represents the malignant degree of tumors, and implies different prognosis. The study of Muro-Cacho *et al.*^[30] indicated that only the degree of necrosis and phenotypic differentiation toward smooth muscle were found to be indicators of poor prognosis in the multivariate analysis. Based on their observations, a classification scheme for gastrointestinal stromal tumors was proposed. Lee *et al.*^[31] clarified the importance of the mucin phenotype in clinic. Despite a well-defined correlation between histological differentiation and Lauren's classification of GA, the mucin phenotype was not in agreement entirely with Lauren's classification. Instead of the histologic differentiation and Lauren's classification, I-phenotypic expression was an independently important prognostic factor of gastric cancers. We have often observed that the level of pathological differentiation and prognosis are inconsistent in clinic. It is well known that the higher degree of malignancy, the earlier occurrence of cancer metastasis and invasion, and the more important alterations of tumor-related genes. So our research about the relationship between different histopathological grades and the tumor-related genes in GA is of great clinical significance.

TNM-Gene diagnosis of gastric cancer: A better diagnostic criterion in the future

The characteristics of the two major types of gastric cancer can be attributed to different tumor-related gene activations. A large number of tumor-related genes involved in signal transductions and cell cycle regulation have been implicated in gastric cancer progression. The study of Wu *et al.*^[32] indicates that according to the molecular pathological background, mucinous adenocarcinomas of the stomach consist of at least three subtypes: the mutator-type, the suppressor (*p53*-type) and the unclassified tumors. It would provide clinicians with useful information for clinical diagnosis by further exploration of carcinomas with more detailed morphological and biological phenotyping. Wang *et al.*^[33] indicated that the *MUC1* gene might be an indicator of poor prognosis. Based on these studies, we explored the alteration of tumor-related genes in different pathological differentiation levels to obtain TNM-Gene diagnosis in GA. Although the data of gene expressions are complicated and irregular, we attempted to discover the their correlations using SVM by a 10-fold CV method^[34-36].

Alterations of tumor-related genes hardly well matching the histopathological grades

Tumor grade represents a gestalt of all molecular changes in malignant tumors and reflects their aggressiveness. In addition, it has been proved to enhance prognostic information. Chandler *et al.*^[37] evaluated the degree of inter-observer variation in grading by conducting a nationwide survey of histopathologists, and drew an important

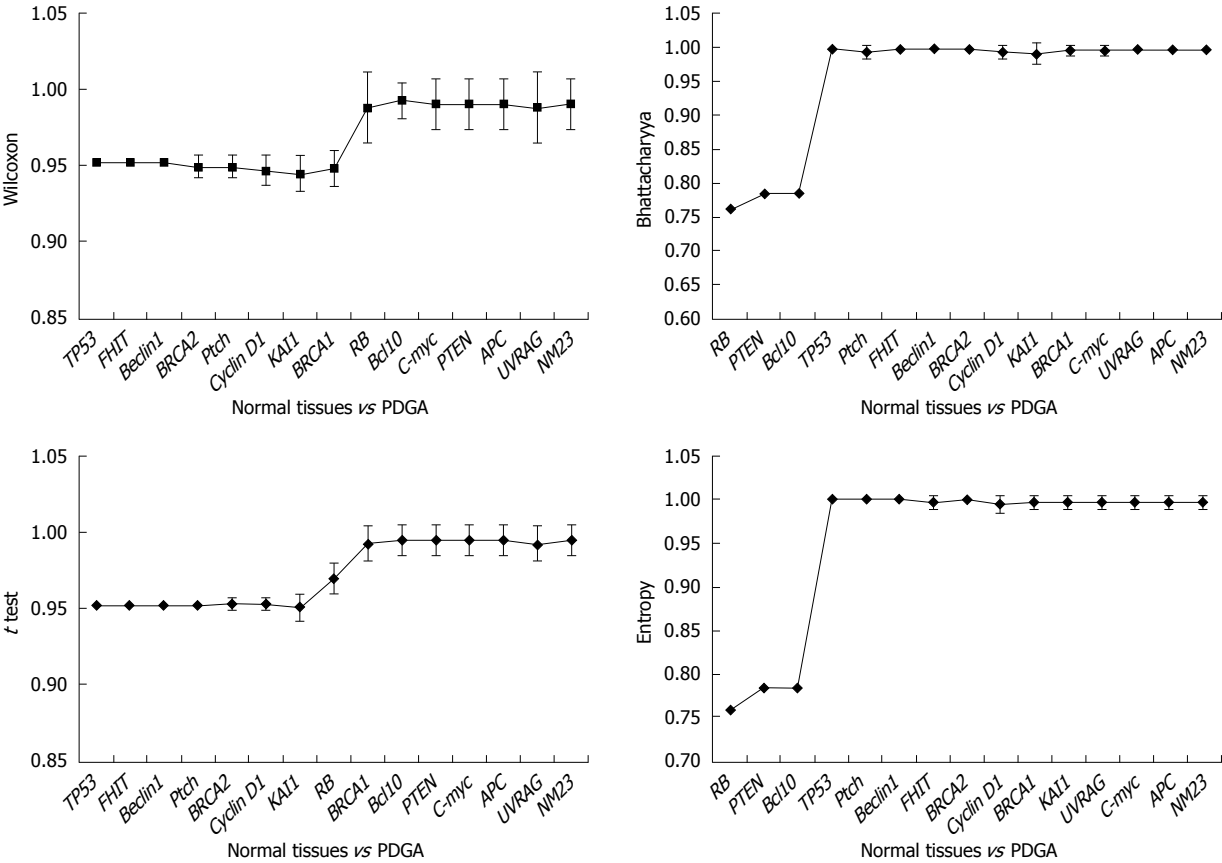


Figure 4 Comparison of normal tissues and poorly differentiated GA (PDGA). X-axis represents the sequence of selected genes, and Y-axis represents the average and standard deviation of the AUC scores obtained in the ten 10-fold cross-validation (CV) with the corresponding gene groups.

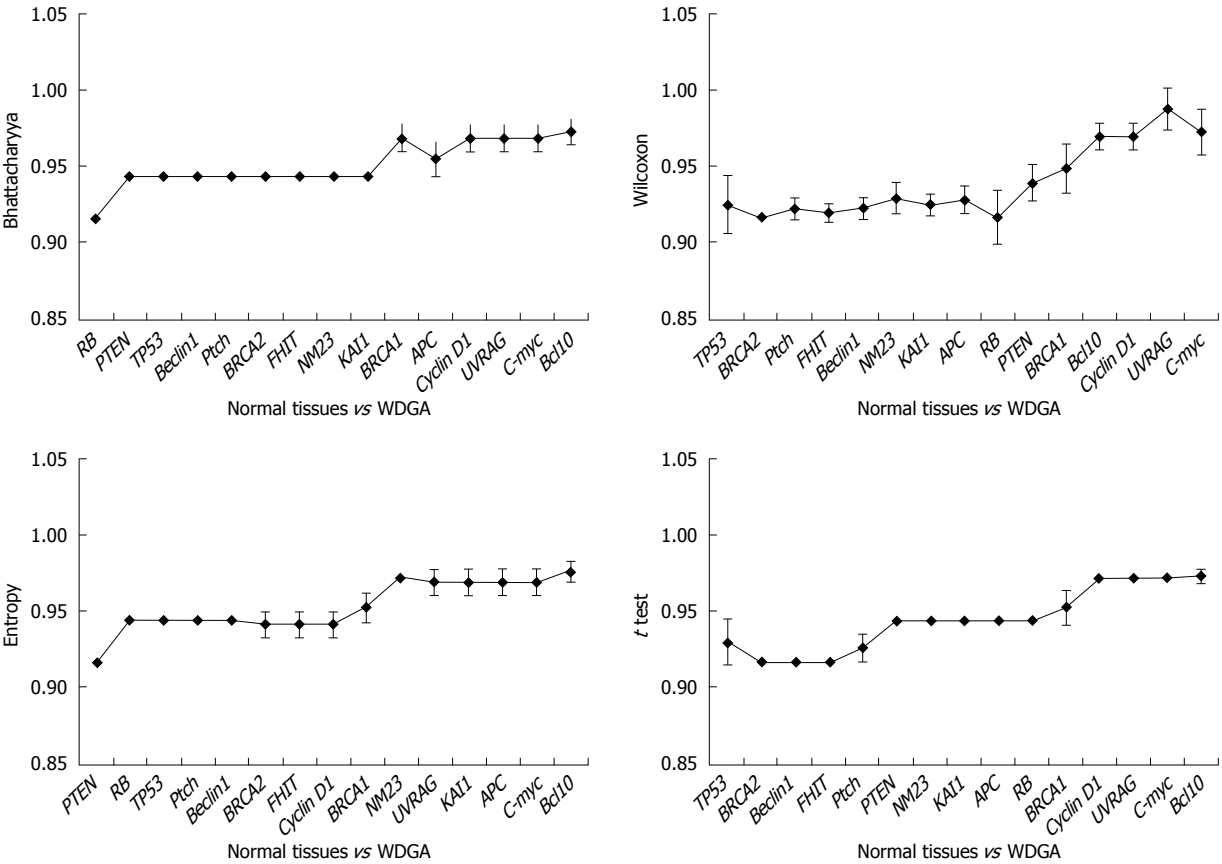


Figure 5 Comparison of normal tissues and well differentiated GA (WDGA).

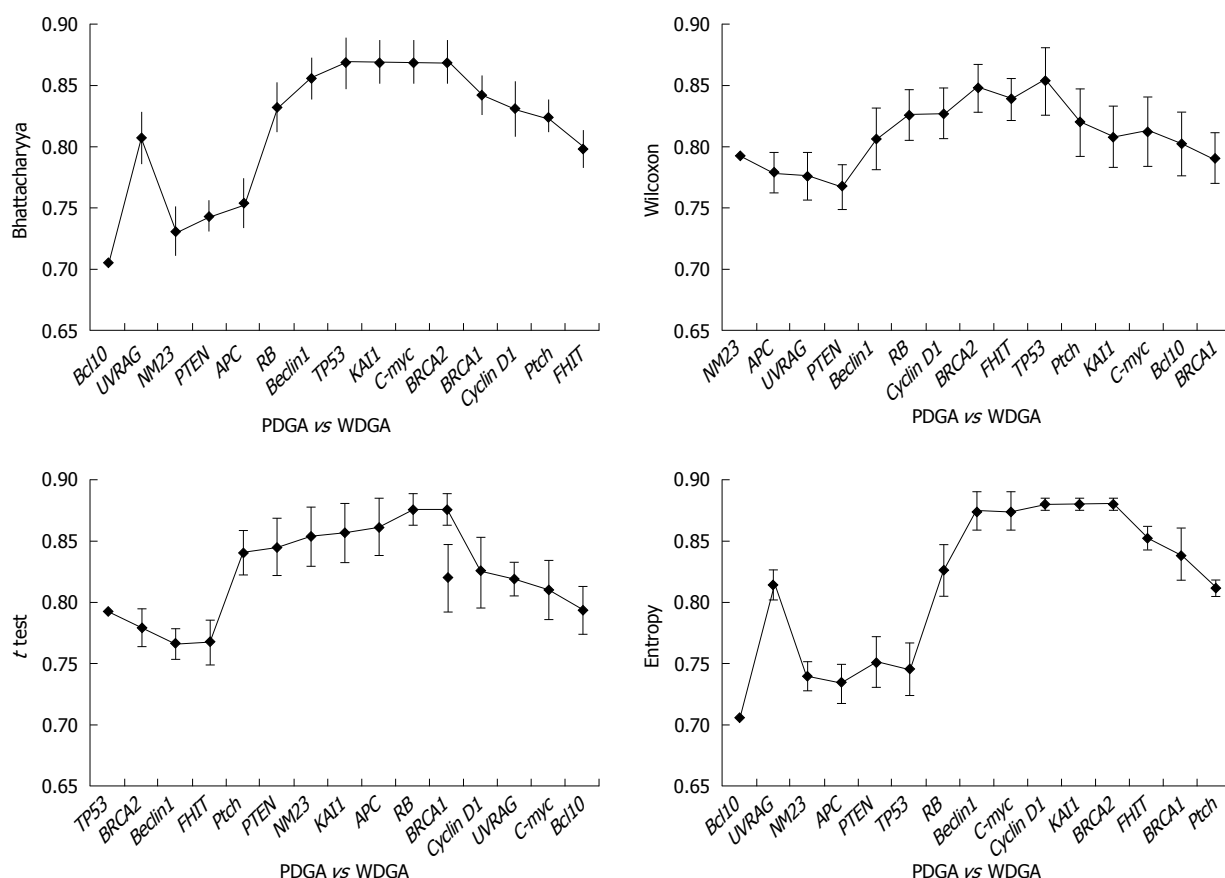


Figure 6 Comparison of WDGA and PDGA.

conclusion: given the fact that the histopathological criteria of stage and grade still provide the mainstay of prognostication and clinical decision-making, we should make more efforts to improve grading criteria and standardize the low- and high-grade categories. However, this conclusion is contradictory to ours. It is usually considered that poorly differentiated GA is a high-grade carcinoma with poor prognosis, and have multiple important alterations of tumor-related genes, and well differentiated GA means low-grade carcinoma with better prognosis, and the reason may be that tumor-related gene alteration was caused by cumulation of injury and repair. But our results indicate that *TP53* is the key tumor-related gene relating closely to the canceration of poorly differentiated GA; and there are multiple tumor-related gene alterations in well differentiated GA. We suggest that the alteration level of tumor-related genes is bound up with grade of malignancy, histopathological grading and prognosis. So we have come up with a conclusion: the alterations of tumor-related genes do not exactly match the histopathological grades. Furthermore, we suspect that histopathological tumor grade does not exactly match the degree of malignancy. There are slight differences between poorly and well differentiated GA. No single tumor-related gene can distinguish the two groups of tumors, and only one gene subset consisting of seven genes can distinguish the two tumors. So we speculate that synergistic actions of multiple genes lead to different specimens.

Outlook and speculation

Compared with general pathological diagnosis, the TNM-Genetic diagnostic methods are more accurate to determine the extent of malignancy of tumors and prognoses. *TP53* is the most important tumor-related gene^[38-45]. Among the 15 genes, only the alteration of *TP53* closely relates to poorly differentiated GA. But besides *TP53*, other ten genes are connected with the well differentiated GA. To sum up the results, *TP53* alters in both groups of tumors, but leading to different Edmonson, we speculate that the alterations of *TP53* may have completely different subtypes, which have the different functions.

COMMENTS

Background

Gastric cancer is the second major cause of oncologic death worldwide. Because the occurrence of cancer is closely related with the tumor-related genes, the authors explored the diverse characteristics of different pathological gradings of gastric adenocarcinomas (GAs) by investigating 15 tumor-related genes. It has been proved that the 15 critical tumor-related genes selected are involved in carcinogenesis, and they represent different formation mechanism of cancers.

Research frontiers

TNM-Genetic diagnosis of gastric cancer is a better diagnostic criterion. The characteristics of two major types of gastric cancer can be attributed to different tumor-related gene activations. A large number of tumor-related genes involved in signal transductions and cell cycle regulation have been implicated in gastric cancer progression. So the authors explored the alteration of tumor-related

genes in different pathological differentiation levels in an attempt to obtain TNM-Genes about different GAs.

Innovations and breakthroughs

Tumor grade represents a gestalt of all molecular changes in malignant tumors and reflects their aggressiveness. The results of this study is different from the traditional opinions as the well differentiated cancer tissues have more alterations of important tumor-related genes than those of the poorly differentiated cancer tissues.

Applications

To sum up the results, *TP53* alters in both groups of tumors, but leading to different Edmonson, the authors speculate that the alterations of *TP53* may have completely different subtypes, which have the different functions. Additionally, there are slight differences among tumor-related genes between poorly differentiated and well differentiated GAs, and the associated alterations of gene subset, *Bcl10*, *UVRAG*, *APC*, *Beclin1*, *NM23*, *PTEN* and *RB*, are closely related to different pathological gradings of GAs.

Terminology

ROC: receiver operating characteristic, a graphical plot of the sensitivity vs (1-specificity); AUC: the area under the ROC curve, reflecting the relationship between sensitivity and specificity for a given test; SVM: support vector machines, a set of related supervised machine learning methods used for classification or regression.

Peer review

The study is aimed to identify biomarkers of poorly and well differentiated GAs. The authors found that among tumor related genes tested only *p53* significantly changed in poorly differentiated GA, whereas well differentiated tumor showed alterations in several tumor-related genes, including *p53*, *Rb* and *PTEN*.

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Systematic review of D2 lymphadenectomy *versus* D2 with para-aortic nodal dissection for advanced gastric cancer

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Author contributions: Wang Z and Chen JQ contributed equally to this article; Wang Z, Chen JQ and Cao YF designed this study; Wang Z and Chen JQ performed this research; Wang Z and Chen JQ analyzed the data; Chen JQ and Cao YF interpreted the results; Wang Z drafted the article; Chen JQ and Cao YF revised the paper.

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Abstract

AIM: To evaluate the feasibility and therapeutic effects of para-aortic nodal dissection (PAND) for advanced gastric cancer.

METHODS: Randomized controlled trials (RCTs) and non-randomized studies comparing D2 + PAND with D2 lymphadenectomy were identified using a pre-defined search strategy. Five-year overall survival rate, post-operative mortality, and wound degree of surgery between the two operations were compared by using the methods provided by the Cochrane Handbook for Systematic Reviews of Interventions.

RESULTS: Four RCTs (1120 patients) and 4 non-randomized studies (901 patients) were identified. Meta-analysis showed that there was no significant difference between these two groups in 5-year overall survival rate [risk ratio (RR) 1.04 (95% CI: 0.93-1.16) for RCTs

and 0.96 (95% CI: 0.83-1.10) for non-randomized studies] and post-operative mortality [RR 0.99 (95% CI: 0.44-2.24) for RCTs and 2.06 (95% CI: 0.69-6.15) for non-randomized studies]. There was a significant difference between these two groups in wound degree of surgery, operation time was significantly longer [weighted mean difference (WMD) 195.32 min (95% CI: 114.59-276.05) for RCTs and 126.07 min (95% CI: 22.09-230.04) for non-randomized studies] and blood loss was significantly greater [WMD 301 mL (95% CI: 151.55-450.45) for RCTs and 302.86 mL (95% CI: 127.89-477.84) for non-randomized studies] in D2 + PAND.

CONCLUSION: D2 + PAND can be performed as safely as standard D2 resection without increasing post-operative mortality but fail to benefit overall survival in patients with advanced gastric cancer.

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Key words: Systematic review; Meta-analysis; Gastric cancer; D2 lymphadenectomy; Para-aortic nodal dissection

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INTRODUCTION

Generally, either incidence or mortality of gastric cancer (GC) has fallen dramatically during the past decades in

European and American countries. Despite its recent decline, GC is still the fourth most common carcinoma and the second leading cause of death by tumor worldwide^[1-3]. GC can be divided into two groups, early GC and advanced GC, and advanced GC accounts for 92%-95% of cases in China, 40%-60% in Japan, and 80%-90% in Europe^[4-6] presently. Up to now, treatments for GC include surgery, chemotherapy, radiotherapy and so on. Despite the poor prognosis, surgery is commonly accepted as the preferred treatment for advanced GC^[7]. Five-year survival rate can achieve 45% or even more in Japan, 40% or so in China, only about 20% in Western countries and as low as 6% in sub-Saharan Africa^[8-11] for advanced GC.

Although surgical operation remains the primary therapeutic modality, views over the optimal resection for patients with GC remain controversial^[12]. In patients with GC, lymph node metastasis can occur during the early stages, and regional lymph node dissection (LND) is regarded as an important part of en bloc resection for GC. However, the extent of lymphadenectomy for the greatest result is controversial, and there is no consensus worldwide. Introduced by Japanese surgeons in 1960s, D2 lymphadenectomy is the recommended standard practice in patients undergoing an operation with a curative intent^[13-16], which required the systematic dissection of lymph nodes in the first tier (perigastric) and the second tier (along the celiac artery and its branches). Compared with traditional D1 lymphadenectomy, D2 doesn't increase the operation morbidity and mortality^[17], and shows a higher 5-year survival rate^[18]. Recently, it was reported that 18%-40% of patients with advanced GC had metastasis present in the para-aortic nodes^[19-21], and more often if the tumor was located at the proximal third of the stomach^[22]. Taking this into consideration, some researchers assumed that removing these lymph nodes could accomplish curative resection (R0) which might improve the clinical outcome of advanced GC patients^[19,23,24]. However, this extended dissection not only can increase the post-operative morbidity and mortality but also can affect the function of near abdominal organs, and its survival benefit is still uncertain. Some studies considered that morbidity after para-aortic nodal dissection (PAND) such as pancreatic fistula and respiratory complications were significantly higher^[25], and these complications were considered to be associated with the surgeons' experience. However, some retrospective reports revealed that there was no significant difference in post-operative complications and mortality between D2 and D2 + PAND; D2 + PAND could be performed safely by well-trained surgeons^[26,27]. With respect to long-term survival after the two operations, there was stormy dispute at the same time^[27-29]. At the moment there is no consensus about whether patients with advanced GC should receive PAND or not, who should accept it, and how to ascertain the metastasis of PAN before operations.

Although a systematic review referring to the extent

of lymphadenectomy for GC has been published, it only valued the short-term outcomes^[30] and the assessment of 5-year survival after PAND in patients with GC was not presented. Five-year survival is considered to be an important outcome in evaluating the therapeutic effects of GC. Therefore a systemic review aiming to evaluate the feasibility and therapeutic effects of PAND is urgently needed. In this study, we assessed literature existing and conducted a meta-analysis for comparing the clinical effectiveness of these two operations.

MATERIALS AND METHODS

Search strategy

The following databases were searched systematically in our study: PUBMED, EMBASE, and the China Biological Medicine Database (CBM-disc), CNKI (China National Knowledge Infrastructure Whole Article Database) from January 1980 to February 2009, as well as the Cochrane Central Register of Controlled Trials (CENTRAL) on The Cochrane Library issue 1, 2009. All controlled trials comparing D2 lymphadenectomy and D2 + PAND in the surgical treatment of GC were identified. In this review, the language of search strategy was not limited, and the following search terms were used: stomach neoplasms, stomach cancer, stomach carcinoma, gastric cancer, gastric carcinoma, gastric neoplasms, D2+, D2 plus, D3, D4, R3, R4 (in some studies, the classification of gastric carcinoma are according to the new edition^[31]; it is also called D3 or R3 in the new edition and called D4 or R4 according to the old edition^[32]), superextended lymphadenectomy, para-aortic lymph nodes dissection, para-aortic nodal dissection. Both free text and MeSH search for keywords were employed.

To identify further potentially relevant studies, reference lists from selected studies through electronic searching were hand searched; furthermore in order to obtain any relevant unpublished materials, we contacted scholars in the field of gastroenterology.

Inclusion and exclusion criteria

Types of studies: Comparative trials with recorded 5-year overall survival rate or secondary outcomes (post-operative morbidity and mortality, wound degree of surgery) were included. Non-comparative studies, case series, case reports and studies using historical controls were excluded.

Types of patients: Trials in which patients were pathologically proved to have gastric adenocarcinoma and qualified for gastrectomy were included. Trials in which patients had distant metastasis, gastric stump cancer, disseminated cancer cells, synchronous malignancy in other organs, serious cardiovascular or respiratory disorders, hepatic or renal failure were excluded.

Types of interventions: Trials comparing the results of

D2 and D2 + PAND were included. Trials comparing the results of these two operations but also with pre-operative or post-operative chemotherapy were excluded.

Types of outcome measures: Primary outcome: 5-year overall survival rate; secondary outcome: post-operative morbidity, mortality and potential risk factors for post-operative morbidity, wound degree of surgery (operation time and blood loss during operation).

Data collection and analysis

Method of agreeing inclusion of studies: This course was performed by two authors (Wang Z and Chen JQ) independently according to preformed inclusion criteria. Titles and abstracts were scanned first to make a list of possibly related literature, and then full texts were obtained for those articles identified as either relevant or not clear, only trials coincident with pre-determined criteria of this review were included. Disagreements were settled by consensus.

Quality assessment: Since there was no consensus about quality assessment of non-randomized studies, in our review, the Cochrane Handbook for Systematic Reviews of Interventions in which criteria for non-randomized studies were the same as randomized controlled trials (RCTs) was used to assess the methodological quality of included studies^[33]. The criteria included six items as following: (1) Adequate sequence generation? (2) Allocation concealment? (3) Blinding? In our review, if result measurements (only wound degree of surgery and post-operative morbidity were included, not considering 5-year survival rate and post-operative mortality) were masked, it would be regarded as low risk of both performance and detection bias; (4) Incomplete outcome data addressed? In our review, we considered the missing outcome data which were less than 10% to be low risk of bias, 10%-15% to be moderate risk of bias, and more than 15% to be high risk of bias; (5) Free of selective reporting? and (6) Free of other bias?

Options for the results of quality assessment: Yes (low risk of bias), probably yes (moderate risk of bias), No (high risk of bias). No rating of the studies was performed; each was accepted or rejected based on the six items noted above. The guidelines in the QUOROM Statement^[34] were consulted by us from the literature search to the presentation of the results and discussion.

Data extraction: Using a pre-defined data extraction form, two reviewers (Wang Z and Chen JQ) extracted data about characteristics of included studies and baseline characteristics of patients independently, which included following items: author, publication date, number of patients, age and sex of patients, serosa and lymph node states, 5-year overall survival rate, post-operative morbidity and mortality, operation time and blood loss during operation. If necessary, the authors

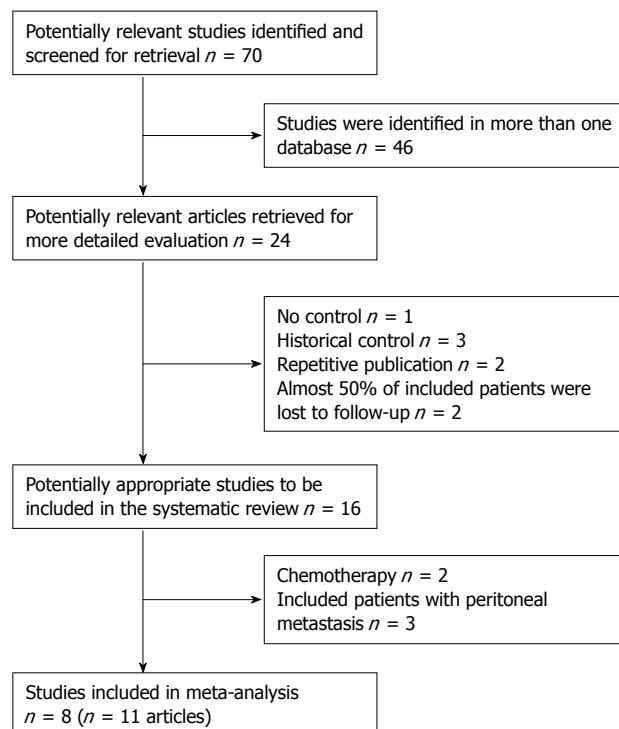


Figure 1 QUORUM flow chart for studies.

of the original articles were contacted for available data. Final agreement was achieved through discussion.

Data synthesis: In our study, the individual and pooled statistics were calculated using the fixed effect model, but a random effect model was used if *P* value of heterogeneity test was less than 0.1. The results were expressed with risk ratios (RR) for dichotomous data and weighted mean differences (WMD) for continuous data^[33], and 95% confidence intervals (CI) were also calculated. Heterogeneity between included studies was tested using χ^2 test. If heterogeneity was present, we would try to check the cause out from aspects of study design and quality, differences in intervention and baseline characteristics of included patients, by using methods of subgroup and sensitivity analysis. Funnel plots were drawn to assess publication bias. The RCTs and non-randomized studies were analyzed separately, and trials with continuous data recorded as the form of median and range were excluded from meta-analysis. Statistical analysis was performed using RevMan 5.0.18, which was provided by the Cochrane Collaboration^[35].

RESULTS

Selection of included studies

According to the search strategy referred to above, a total of 70 studies were yielded: 23 in PubMed, 16 in EMBASE, 11 in CENTRAL, 20 in CBM-disc and CNKI. Many literatures were searched in more than one database; finally 4 RCTs and 4 non-randomized studies (11 articles) were considered eligible for inclusion. The trial selection process was summarised in Figure 1. The reasons for

Table 1 Characteristics of included studies (ITT: intention-to-treat)

Study ID (author, yr)	Design	Methods	Patients	Interventions	Outcomes	Notes
Jiang <i>et al</i> 2000 ^[36]	RCT	Single center, parallel group, not clear about the method of randomization	Histologically proved curable advanced gastric cancer	D2 vs D2+	Post-operative morbidity and mortality; Operation time	Regional lymph nodes were classified according to 1th English edition 1995 ^[32]
Sano <i>et al</i> 2004 ^[37] and Sasako <i>et al</i> 2008 ^[39] and Koderia <i>et al</i> 2005 ^[38]	RCT	Multicenter, parallel group, computer stratified randomization	Histologically proved advanced and curable gastric cancer	D2 vs D2+	Post-operative morbidity and mortality; Operation time; Blood loss during operation; Five-year survival rate	ITT was used in result analysis; Regional lymph nodes were classified according to 1th English edition 1995 ^[32]
Yonemura <i>et al</i> 2006 and 2008 ^[40,41]	RCT	Multicenter, parallel group, computer randomization	Younger than 75-yr old, histologically proved advanced and curable gastric cancer	D2 vs D2+	Post-operative morbidity and mortality; Operation time; Blood loss during operation; Five-year survival rate	Sample size was calculated before trials
Kulig <i>et al</i> 2007 ^[42]	RCT	Multicenter, parallel group, simple computer randomization.	Histologically proved curable gastric adenocarcinoma	D2 vs D2+	Post-operative morbidity and mortality; Operation time; Blood loss during operation	Regional lymph nodes were classified according to 2nd English edition 1998 ^[31] ; ITT was used in result analysis
Maeta <i>et al</i> 1999 ^[43]	non-RCT	Single center, parallel group, without randomization	Histologically proved advanced gastric cancer, younger than 75-yr old	D2 vs D2+	Post-operative morbidity and mortality; Operation time; Blood loss during operation	In D4 group, lymph nodes (a1, a2, b1 and b2) were all removed; Sample size was smaller than that calculated before study
Bostanci <i>et al</i> 2004 ^[44]	non-RCT	Single center, parallel group, prospective study without randomization	Histologically proved gastric cancer	D2 vs D2+	Post-operative morbidity and mortality; Operation time	Surgical procedure was not very clearly described in the article
Kunisaki <i>et al</i> 2006 ^[45]	non-RCT	Multicenter, parallel group, retrospective analysis	Curable advanced gastric cancer proved by histology	D2 vs D2+	Post-operative morbidity and mortality; Operation time; Blood loss during operation; Five-year survival rate	Data about length of post-operative hospital stay were obtained from the author
Hu <i>et al</i> 2009 ^[46]	non-RCT	Single center, parallel group, retrospective analysis	Histologically proved curable gastric cancer	D2 vs D2+	Post-operative morbidity and mortality; Operation time; Five-year survival rate	In D4 group, lymph nodes (a1, a2 and b1) were dissected

RCT: Randomized controlled trial.

excluding studies were as following: study type, baseline characteristics of included patients, repetitive publication, chemotherapy, distant metastasis, confounding allocation and high rate of loss of follow-up.

Description and risk of bias of included studies

Since both RCTs and non-randomized studies were included in our study, we described them both respectively. Additionally, although there was no significant difference in each study, we did not summarize the surgical details as a table in either RCTs or non-randomized included studies since there were different descriptions in different studies. Characteristics of included studies, baseline characteristics of patients and potential bias of included studies were listed in Tables 1-3, respectively.

Description and risk of bias of RCTs

In total 1120 patients in 4 RCTs (7 articles)^[36-42] were eligible for inclusion, 571 patients in the D2 group and 549 patients in the D2 + PAND group, and the sample size of studies varied from 53 to 523. All included RCTs

except one^[36] used computer randomization, but only one trial used a central telephone registration system^[37]; the concealment of allocation was unclear in three trials. Blinding referred to result measurements being blinded in our study, and wasn't clearly described in all trials. Completeness of data was good in 4 included trials. There was no significant difference in baseline characteristics of patients (age, sex, T stage and lymph node states) between D2 and D2 + PAND groups in included trials except the trial reported by Jiang *et al*^[36], in which T stage and lymph node state were not clearly reported. Three articles showed that only surgeons skilled in D2 and D2 + PAND operation participated in their studies^[37,40,42]. Selecting reports and other bias were not clearly recorded in their published reports.

Description and risk bias of non-randomized studies

In total 901 patients in 4 non-randomized studies^[43-46] were included, 620 patients in the D2 group and 281 patients in the D2 + PAND group, and sample size of studies ranged from 70 to 580. No randomization and

Table 2 Baseline characteristics of patients in included studies

Study ID (author, yr, <i>N</i> _{D2} / <i>N</i> _{D2+})	Age (yr) (mean/SD or median/range)			Sex (male/female)			Pathological T stage (serosa negative/positive)			Pathological node status (negative/positive)		
	D2	D2 +	<i>P</i>	D2	D2 +	<i>P</i>	D2	D2 +	<i>P</i>	D2	D2 +	<i>P</i>
Sano <i>et al</i> 2004 ^[37] , Sasako <i>et al</i> 2008 ^[39] (263/260)	60 (25-75)	61 (27-75)	> 0.05	176/87	183/77	> 0.05	134/129	146/114	> 0.05	79/184	96/164	> 0.05
Kulig <i>et al</i> 2007 ^[42] (141/134)	56 (31-81)	54 (34-77)	> 0.05	85/56	83/51	> 0.05	64/77	54/84	> 0.05	50/91	56/78	> 0.05
Yonemura <i>et al</i> 2006 and 2008 ^[40,41] (135/134)	63.8 (9.7)	62.5 (10.2)	> 0.05	90/45	91/43	> 0.05	63/72	61/73	> 0.05	37/98	35/99	> 0.05
Jiang <i>et al</i> 2000 ^[36] (32/21)	61 (46-83)	65 (34-84)	NR	19/13	11/10	NR	NR	NR	NR	NR	NR	NR
Maeta <i>et al</i> 1999 ^[43] (35/35)	60 (11)	59 (9)	> 0.05	20/15	21/14	> 0.05	2/33	6/29	> 0.05	15/20	12/23	> 0.05
Hu <i>et al</i> 2009 ^[46] (55/62)	58.8 (11.4)	54.3 (11.4)	< 0.05	42/13	48/14	> 0.05	13/42	15/47	> 0.05	16/39	23/39	> 0.05
Kunisaki <i>et al</i> 2006 ^[45] (430/150)	62.2 (12.5)	59.3 (10.7)	> 0.05	286/144	109/41	> 0.05	197/233	71/79	> 0.05	126/304	34/116	< 0.05
Bostanci <i>et al</i> 2004 ^[44] (100/34)	58.5 (13)	59 (12.6)	> 0.05	63/37	21/13	> 0.05	98/2	29/35	NR	NR	NR	NR

NR: Not reporting.

Table 3 Potential bias of included studies

Study ID (author, yr)	Adequate sequence generation	Allocation concealment	Blinding	Incomplete outcome data addressed	Free of selective reporting	Free of other bias
Jiang <i>et al</i> 2000 ^[36]	Probably yes	Probably yes	Probably yes	Yes	Probably yes	Probably yes
Sano <i>et al</i> 2004 ^[37] , Sasako <i>et al</i> 2008 ^[39]	Yes	Yes	Probably yes	Yes	Probably yes	Probably yes
Yonemura <i>et al</i> 2006 and 2008 ^[40,41]	Yes	Probably yes	Probably yes	Yes	Probably yes	Probably yes
Kulig <i>et al</i> 2007 ^[42]	Yes	Probably yes	Probably yes	Yes	Probably yes	Probably yes
Maeta <i>et al</i> 1999 ^[43]	No	No	Probably yes	Yes	Probably yes	Probably yes
Bostanci <i>et al</i> 2004 ^[44]	No	No	Probably yes	Yes	Probably yes	Probably yes
Kunisaki <i>et al</i> 2006 ^[45]	No	No	Probably yes	Yes	Probably yes	Probably yes
Hu <i>et al</i> 2009 ^[46]	No	No	Probably yes	Probably yes	Probably yes	Probably yes

concealment of allocation were performed. According to the definition of blinding in our review, blinding was unclear in 4 included studies. Completeness of data was good; the rate of loss of follow-up was less than 15% for all included studies. The description of selective reporting and other bias was not described in detail. Baseline characteristics were similar between D2 and D2 + PAND groups in all included studies.

Effects of interventions (Effectiveness in RCTs)

Five-year overall survival rate: Two RCTs^[39,41] (792 patients) reported 5-year overall survival rate, and the pooled RR was 1.04 (95% CI: 0.93-1.16). There was no significant difference between D2 + PAND and D2 groups. The *P* value of heterogeneity test was 0.88 (Figure 2).

Data from only 1 RCT^[39] could be analyzed, with individual RR 1.14 (95% CI: 1.01-1.29) for the serosa negative subgroup and 0.71 (95% CI: 0.53-0.94) for the serosa positive subgroup. There was a significant difference between these two subgroups. Compared with D2 dissection, five-year overall survival rate for D2 + PAND in the serosa negative subgroup was significantly higher than that in the serosa positive subgroup, but tests for heterogeneity were not applicable in these two subgroups (Figure 3).

Post-operative morbidity and mortality: Since there were different definitions and contents in each study,

post-operative morbidity data couldn't be used in a meta-analysis. All RCTs (1107 patients) reported data on post-operative morbidity, but the results in four included studies were not consistent. Apart from the study reported by Yonemura *et al*^[40] [individual RR 1.52 (95% CI: 1.05-2.18)], three other trials reported there was no significant difference between D2 and D2 + PAND groups (Figure 4A).

The post-operative mortality data of all included RCTs (1107 patients) could be used, and the combined RR was 0.99 (95% CI: 0.44-2.24), which revealed that there was no significant difference between D2 + PAND and D2 groups. The test for heterogeneity was negative with a *P* value of 0.31 (Figure 4B).

Operation time: Data of two RCTs^[36,40] (309 patients) were applicable; the pooled WMD was 195.32 min (95% CI: 114.59-276.05), suggesting there was a significant difference between these two groups. Operation time in the D2 + PAND group was significantly longer than that in the D2 group. The *P* value of the heterogeneity test was 0.24 (Figure 4C).

Blood loss during operation: Only one RCT^[40] (256 patients) was useful for analysis, and individual WMD was 301 mL (95% CI: 151.55-450.45). There was a significant difference between these two groups. Blood loss in the D2 + PAND group was significantly more

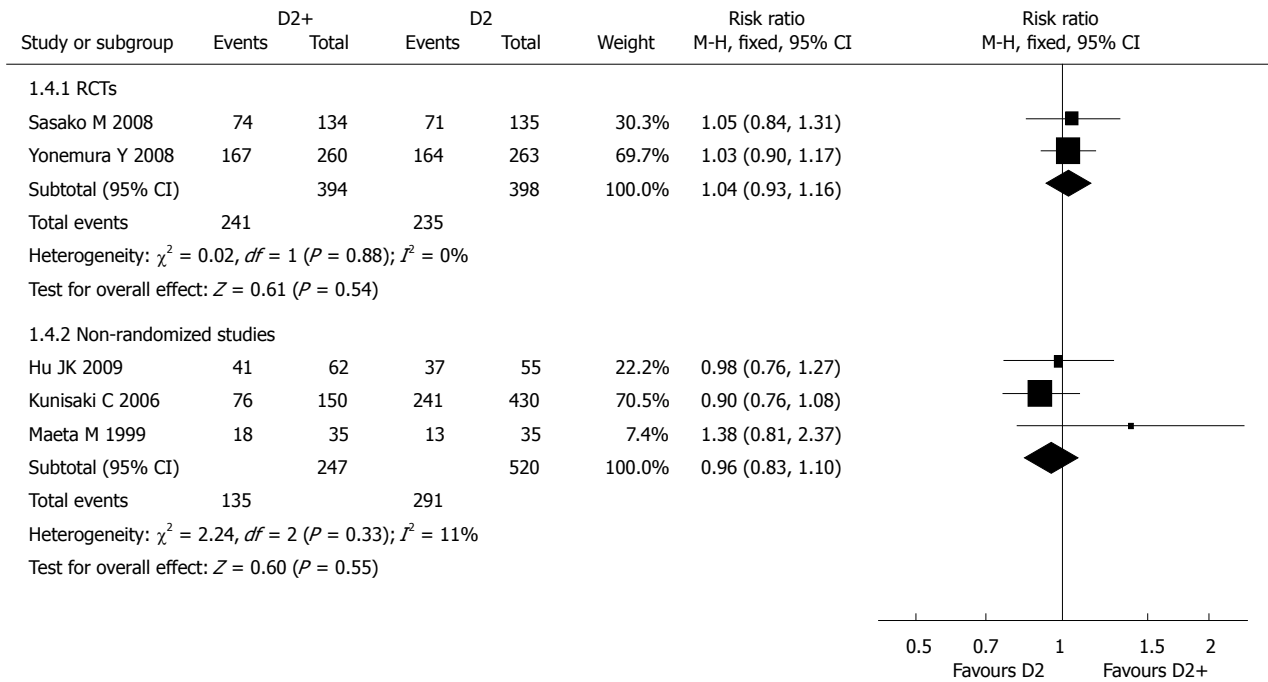


Figure 2 Meta-analysis of 5-year overall survival rate comparing D2 + PAND with D2 gastrectomy.

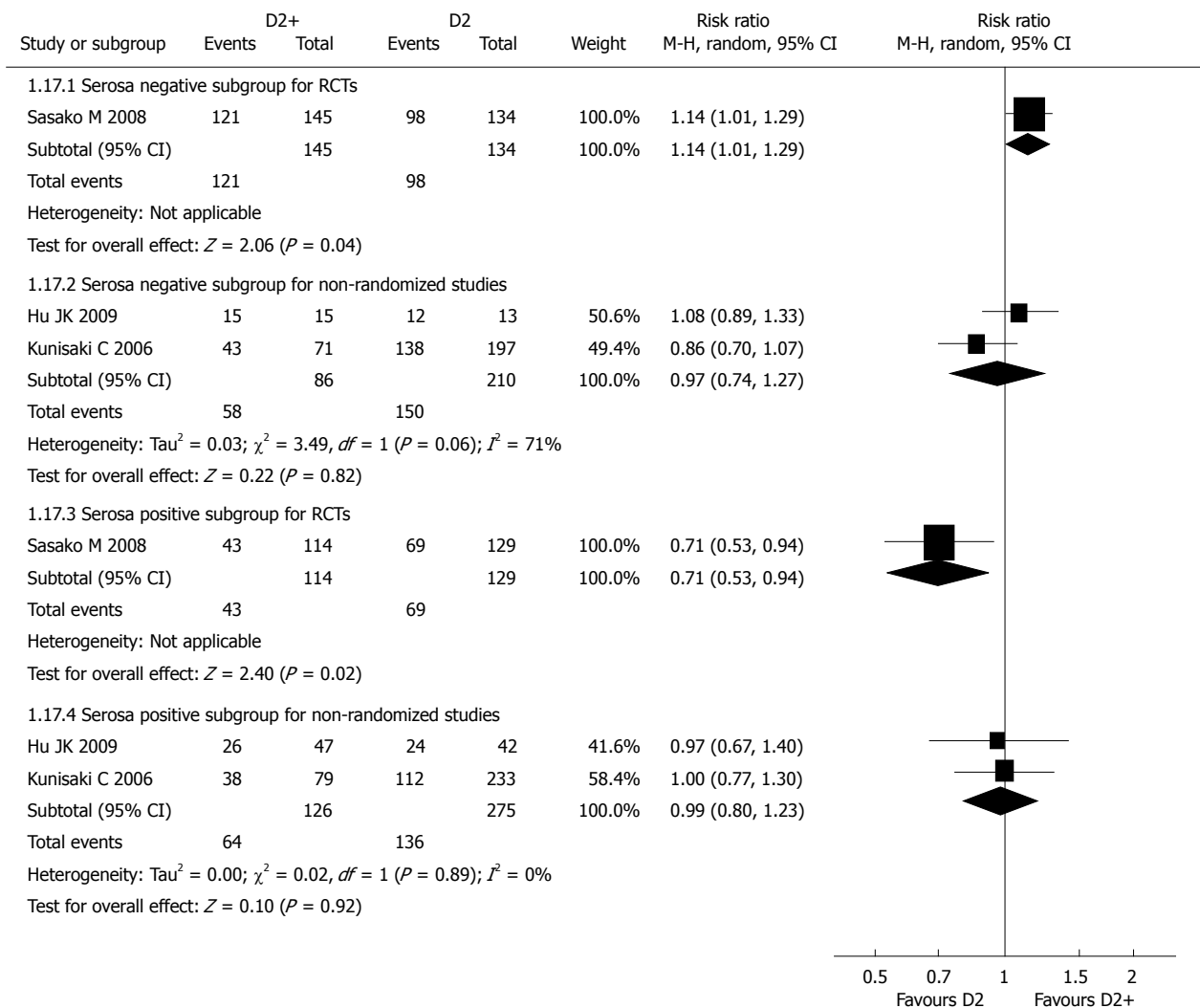
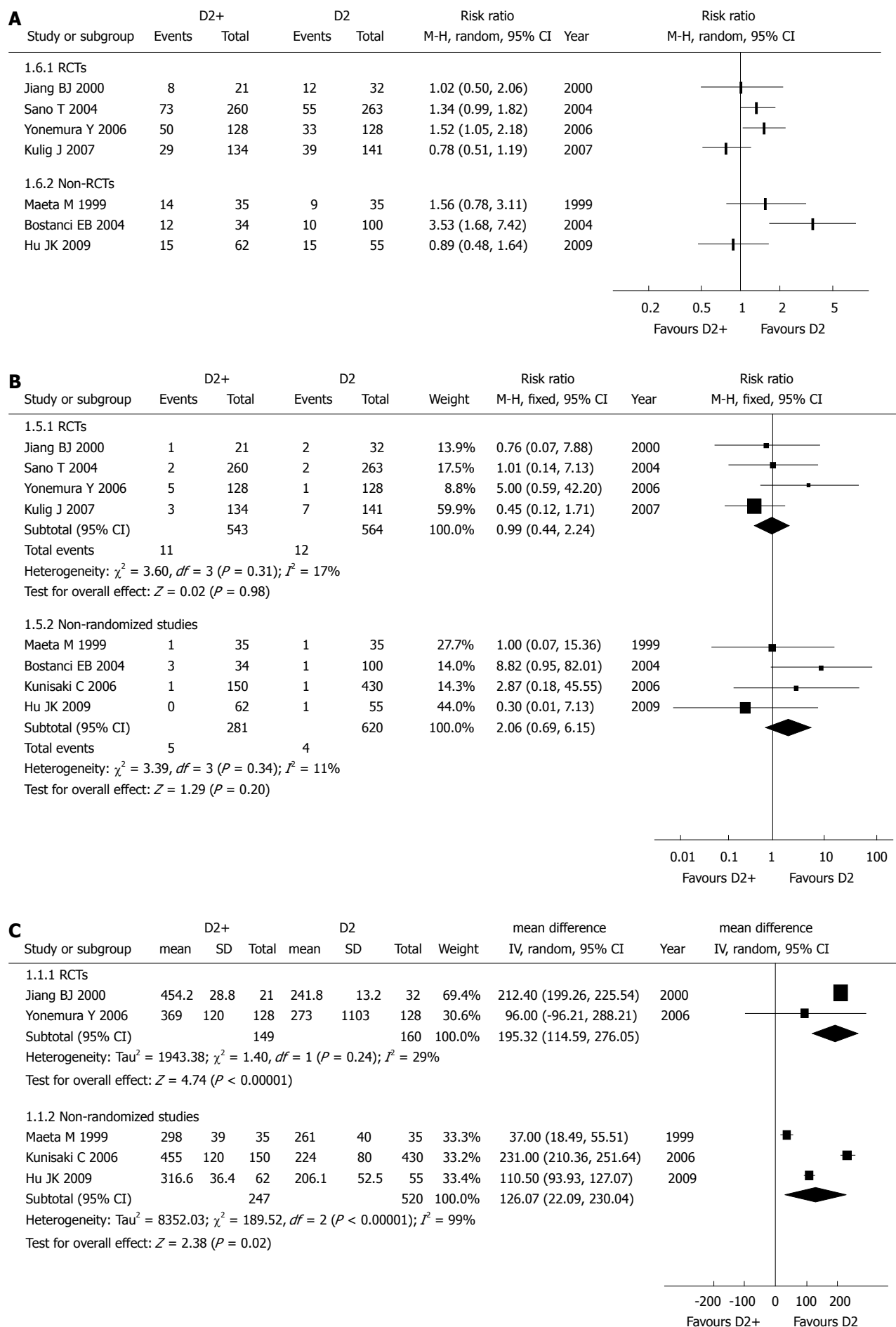


Figure 3 Meta-analysis of 5-year overall survival rate for serosa negative and positive subgroups comparing D2 + PAND with D2 gastrectomy.



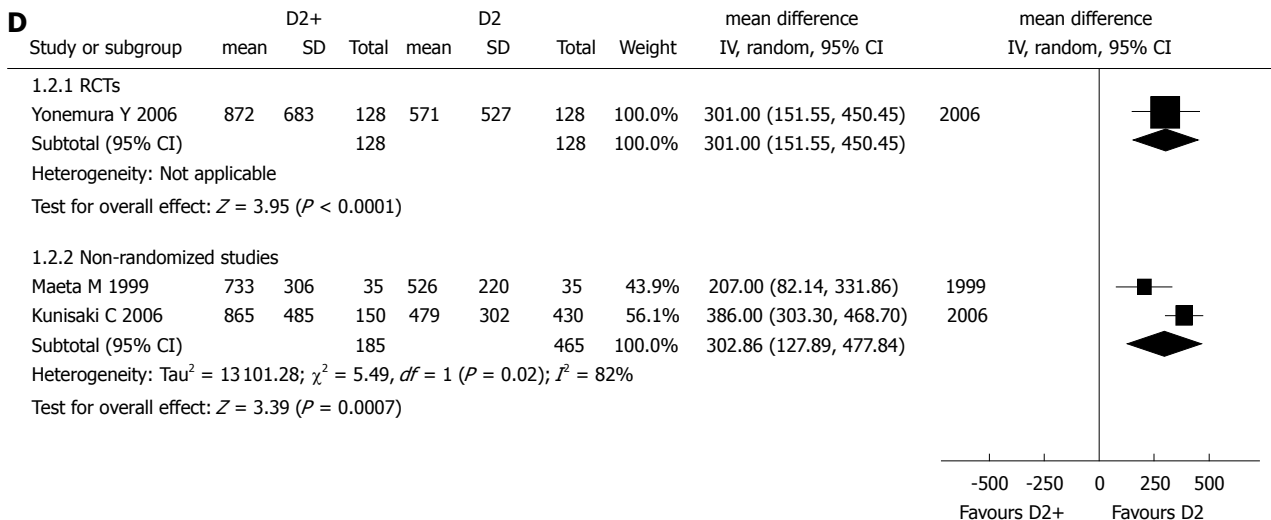


Figure 4 Meta-analysis of post-operative morbidity comparing (A), post-operative mortality (B), operation time (C), blood loss during operation (D) D2 + PAND with D2 gastrectomy.

than that in the D2 group, and the test for heterogeneity was not applicable (Figure 4D).

Effects of interventions (Effects in non-randomized studies)

Five-year survival rate: Three non-randomized studies (767 patients)^[43,45,46] reported 5-year overall survival rate, and the pooled RR was 0.96 (95% CI: 0.83-1.10), suggesting there was no statistical significance between the two groups; the P value of the heterogeneity test was 0.33 (Figure 2).

Two non-randomized studies^[45,46] were included in the analysis. Pooled RR for serosa negative and positive subgroups were 0.97 (95% CI: 0.74-1.27) and 0.99 (95% CI: 0.80-1.23), respectively, revealing there was no significant difference between these two subgroups. Comparing with D2, D2 + PAND didn't improve 5-year overall survival whether in serosa negative or positive subgroups. The P values of heterogeneity tests were 0.06 and 0.89, respectively (Figure 3).

Post-operative morbidity and mortality: For similar reasons as listed for RCTs, the post-operative morbidity data couldn't be combined. Post-operative morbidity was reported in three articles^[43,44,46] (321 patients), but results were not coincident. Higher post-operative morbidity was reported by Bostanci *et al*^[44] (individual RR 3.53 (95% CI: 1.68-7.42); however, two other articles^[43,46] reported there was no significant difference between D2 and D2 + PAND groups (Figure 4A).

For post-operative mortality, all included non-randomized studies (901 patients) could be analyzed, and the combined RR was 2.06 (95% CI: 0.69-6.15), suggesting there was no significant difference between the two groups. This indicated D2 + PAND could be performed without increasing post-operative mortality; the test for heterogeneity was negative with a P value of 0.34 (Figure 4B).

Operation time: Data of three non-randomized studies^[43,45,46] (767 patients) were useful for meta-analysis, and the pooled WMD was 126.07 min (95% CI: 22.09-230.04), suggesting that there was a significant difference between the two groups. The operation time for D2 + PAND was significantly longer than that for D2. The P value of the heterogeneity test was less than 0.1 (Figure 4C), and a sensitivity analysis identified that the study reported by Kunisaki *et al*^[45] was the main source of heterogeneity.

Blood loss during operation: Two non-randomized studies^[43,45] (650 patients) could be used for meta-analysis, and the pooled WMD was 302.86 mL (95% CI: 127.89-477.84), revealing there was a significant difference between the two groups. This suggested that there was more blood loss in D2 + PAND. The test for heterogeneity was positive ($P = 0.02$, Figure 4D).

Potential risk factors for post-operative morbidity

Univariate and multivariate analysis were used to identify the potential risk factors for post-operative morbidity in only one RCT^[38]; multivariate analysis revealed that pancreatectomy [RR 5.62 (95% CI: 1.94-16.27)] was the most important risk factor for overall complications, but meta-analysis wasn't practicable.

Testing for publication bias

Because there were too few included studies, we didn't draw funnel plots in our review.

DISCUSSION

The choice for the extent of lymphadenectomy in GC has been controversial^[18,30]. To perform a comparison of the effectiveness and safety between standard D2 and D2 + PAND, we conducted a systemic review based on current studies using the method provided by Cochrane

Collaboration^[33]. Four RCTs (total 1120 patients) and four non-randomized studies (total 901 patients) were included in our review. The results of meta-analysis showed that the 5-year overall survival rate was similar between the two operations. Compared with that in D2 resection, post-operative morbidity and mortality didn't increase in D2 + PAND, but the operation time was significantly longer and the blood loss was significantly greater in D2 + PAND in RCTs and non-randomized studies.

Minimal heterogeneity was found between the RCTs for all the measured outcomes, which ensured the quality of the meta-analysis; however, this systematic review was not faultless. First, baseline characteristics were statistically similar in included studies, but were not exactly the same among each RCT; for example, T1 patients were included by Kulig *et al.*^[42] but not by Sano *et al.*^[37], which suggested a potential selection risk. Second, regional lymph nodes were classified by different classifications^[31,32] in included studies, thus the station of lymphadenectomy was confounding in D2 and D2 + PAND groups, which led to the mixture of interventions between included studies. Additionally, surgical outcomes were related to experience of the surgeon^[47]; considering classification standards of lymph node and learning curves of surgeons performance bias might be introduced in our study. Third, from Table 3, blinding could be seen to be unclear in included studies, so measurement bias might exist. Although this was a general phenomenon in surgical clinical trials, it might weaken the reliability of our results. Fourth, completeness of data could affect the statistical results; according to reports of primary literature, the loss rate of data in all included studies was less than 15%, however this does not eliminate the possibility of potential bias. Fifth, stage migration, as it was known, might improve pathological stage- and N-stage-specific survival in GC^[48,49]. However, this factor wasn't taken into consideration in our included studies; this might affect the stage-specific survival after D2 and D2 + PAND. Finally, it was impossible for us to identify all relevant literature, even though we made great efforts; moreover, publication bias might exist due to the low number of included studies.

Because there were few RCTs, non-randomized studies were included in our review to obtain as much comprehensive information as possible, and this problem is common in surgical systemic reviews. Considering the potential heterogeneity induced by methodology, we didn't combine the data of RCTs and non-randomized studies together. Additionally, although combined results of the non-randomized studies were affected by considerable bias in view of differences in methodology and baseline characteristics of included patients, the aim of meta-analysis of non-randomized studies was just to offer an overview of the general trend of outcomes in these studies. In addition, results of non-randomized studies were found to be similar to that of RCTs, which increased the reliability of our review to some extent.

Comparing with that of D2 resection, the survival advantage of D2 + PAND was controversial^[27-29]. It was found that patients with positive PAN and stage IIIb patients got survival benefit from D2 + PAND by some researchers^[20,23,50]. In our review, we failed to discover an overall survival benefit for D2 + PAND [RCTs: RR 1.04 (95% CI: 0.93-1.16); non-randomized studies: RR 0.96 (95% CI: 0.83-1.10)]. In order to identify possible survival discrepancy between D2 and D2 + PAND groups, we conducted a subgroup analysis which used serosa invasion as the only factor due to shortage of primary data. Although it was considered to be an important prognostic factor for GC, we could not analyze TNM stages. In RCTs, there was a survival benefit for serosa negative patients [RR 1.14 (95% CI: 1.01-1.29)], but in non-randomized studies, there was no significant difference in 5-year survival rate between the serosa negative subgroup [RR 0.97 (95% CI: 0.74-1.27)] and the serosa positive subgroup [RR 0.99 (95% CI: 0.80-1.23)]. Theoretically, a larger range of surgery options should produce survival benefit in more advanced carcinoma, so the finding in our meta-analysis seems unbelievable, and is the opposite to some studies^[51]. Although definitive survival data of a study by Kulig *et al.*^[42] are still to come, there is little reason to expect dramatic changes. Therefore, potential survival benefit of D2 + PAND compared with D2 gastrectomy for patients in specific tumor stages, as well as a method to identify para-aortic lymph node metastasis pre-operatively, should be urgently researched in future.

Pancreatic fistula and respiratory complications after PAND were significantly more common in a report by Kunisaki *et al.*^[25]; however, some retrospective reports revealed that there was no significant difference in post-operative complications and mortality between D2 and D2 + PAND, and D2 + PAND can be performed safely by well-trained surgeons^[26-27]. Post-operative morbidity data were not combined by us due to different definitions and contents in each included study, but controversy also appeared in different studies. Generally speaking, the extent of lymphadenectomy could influence the morbidity rates of GC patients, and it was reported that the greater the resection of lymph nodes, the greater the probability of complications^[40,44]. But on the contrary, some researchers^[36,37,42,43,46] revealed that there was no significant difference in the postoperative morbidity between D2 and D2 + PAND patients. In our review, post-operative mortality data were consistent in RCTs and non-randomized studies, with pooled RR 0.99 (95% CI: 0.44-2.24) and 2.06 (95% CI: 0.69-6.15), respectively, suggesting that D2 + PAND might be performed as safely as standard D2. To identify potential risk factors for post-operative morbidity, we searched all relevant literature, and found only one RCT data could be used^[38]. Results of multivariate analysis from this only fit RCT showed that pancreatectomy was the most important risk factor for overall complications, with RR of 5.62 (95% CI: 1.94-16.27). This finding was concurrent with some

other studies^[52], and demonstrated that the resection of combined organs should be prudent.

In this review, we found that PAND, whether in RCTs or in non-randomized studies, was correlated with longer operation time [RCTs: WMD 195.32 min (95% CI: 114.59-276.05); non-randomized studies: WMD 126.07 min (95% CI: 22.09-230.04)] and more blood loss [RCTs: WMD 301 mL (95% CI: 151.55-450.45); non-randomized studies: WMD 302.86 mL (95% CI: 127.89-477.84)], which were different from that of a related meta-analysis published recently^[30], in which blood loss during operation wasn't analyzed, but operation time was similar with a WMD - 112.60 min (95% CI: 286.22-61.02) between the two operations; clinically, the operative procedure to eradicate para-aortic lymph nodes is more complicated, so the wound degree of surgery should be heavier in D2 + PAND, thus our results were more credible to some extent. As we know, wound degree of surgery was intensively affected by skills of surgeons, so if we want to reduce the wound degree of surgery for PAND patients, pre-operative training of surgeon is necessary. Furthermore, in order to reduce the publication bias from different authors in different nations, standard operating procedures or rules for PAND should be achieved by consensus in time; otherwise, in the future, it will be also difficult to judge which RCT is more reliable.

Quality of life after surgery, as we know, is an important index to evaluate treatment effectiveness for patients with malignant tumors. Unfortunately, very few studies reported this clinical outcome of patients with advanced GC after PAND; so quality of life after PAND should be taken into consideration in future clinical studies.

In conclusion, D2 + PAND can be performed as safely as standard D2. Compared with standard D2, D2 + PAND doesn't have any overall survival benefit, but its' wound degree of surgery is significantly higher, demonstrated as a longer operation time and greater blood loss. Consequently we feel D2 + PAND should be performed prudently. For reducing wound degree during D2 + PAND, pre-operative training of the surgeon is necessary and standard operating procedures or rules for PAND should be achieved by consensus. Additionally, research on the relationship between prognosis of GC patients and combined organ resection, and potential survival benefit of D2 + PAND for some specific stages of advanced GC are urgently needed in the future.

COMMENTS

Background

Although radical gastrectomy is thought to be an important treatment measure, prognosis of advanced gastric cancer (GC) is still poor. A reasonable operation procedure for advanced GC is an issue worthy of investigating nowadays.

Research frontiers

It is reported that 18%-40% of patients with advanced GC had metastasis present in the para-aortic nodes and additional dissection of para-aortic nodes is considered to be a necessary procedure as well as standard D2 gastrectomy in order to achieve R0 resection. However, the effectiveness of D2 + para-aortic nodal dissection (PAND) is still controversial.

Innovations and breakthroughs

Some researchers assumed that PAND might achieve R0 resection and benefit the survival of patients with advanced GC, despite its high post-operative morbidity and mortality. The present systematic review found that D2 + PAND failed to benefit the overall survival of patients with advanced GC, but it could be performed as safely as standard D2 resection without increasing post-operative mortality.

Applications

D2 + PAND should be performed prudently and its potential survival benefit for patients with specific tumor stages of advanced GC should be urgently researched in the future.

Terminology

Standard D2 lymphadenectomy refers to systematic dissection of lymph nodes in the first tier (perigastric) and the second tier (along the celiac artery and its branches); and D2 + PAND refers to additional dissection of para-aortic lymph nodes.

Peer review

This manuscript is well prepared, study design is reasonable, statistical methods are appropriate, and conclusions are based on the convincing statistical analysis. The results and information conveyed in the article might contribute to the knowledge of gastric cancer management.

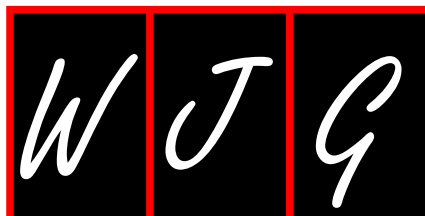
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Hepatic arterial infusion of gemcitabine-oxaliplatin in a large metastasis from colon cancer

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gemcitabine-oxaliplatin should be evaluated in further clinical trials.

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Abstract

Hepatic arterial infusion (HAI) of chemotherapy can be performed in cases of liver-confined metastatic disease, resulting in increased local drug concentrations. Here we report the case of a 61-year-old man who presented with an isolated large unresectable liver metastasis of colon cancer after failure of surgery and multiple administration of systemic chemotherapy. The patient was treated with a combination of gemcitabine and oxaliplatin using HAI. The tolerance was excellent and a radiological complete response was obtained after 8 cycles of HAI. The rationale for the use of gemcitabine and oxaliplatin as well as that for the combination of the 2 drugs is discussed in this paper. HAI of

INTRODUCTION

Hepatic arterial infusion (HAI) of chemotherapy is a treatment option in cases of liver-confined metastatic disease from colorectal carcinoma and results in increased local drug concentrations. Although a recent meta-analysis involving 1277 patients^[1] reported no significant survival benefit of fluoropyrimidine-based HAI in patients with liver metastases from colorectal cancer, the study suggested using novel anticancer agents and/or drug combinations to improve the efficacy of HAI in the future. There is growing evidence for using the combination of gemcitabine and oxaliplatin in different types of malignancies including colorectal cancer.

Here we present a case of radiological complete response after HAI of gemcitabine-oxaliplatin for a large unresectable liver metastasis from a colon cancer.

CASE REPORT

In April 2005, a 61-year-old man was referred to our hospital with rectal bleeding. A colonoscopy revealed a sub-occlusive sigmoid mass 26 cm from the anal margin. A computed tomography (CT) scan performed before surgery revealed one liver metastasis that involved hepatic segment VIII. Carcinoembryonic antigen was not a tumor marker for this patient. The patient underwent sigmoid resection. Pathological examination revealed a sigmoid adenocarcinoma with 7 metastatic regional lymph nodes (pT3 pN2 M1). The patient received 10 cycles of the FOLFOX regimen (fluorouracil + oxaliplatin). The disease was stabilized according to RECIST (Response Evaluation Criteria in Solid Tumours). The patient then underwent segmentectomy of hepatic segment VIII in November 2005. In January 2007, the patient presented with 5 new liver metastases that involved hepatic segments IV and VII. The patient received 12 cycles of the FOLFIRI (fluorouracil + irinotecan) and bevacizumab regimen. A partial response was obtained according to RECIST though the 5 metastases were still present on magnetic resonance imaging (MRI). The patient underwent right hepatectomy with partial resection of hepatic segment IV. This resection was complicated by serious liver failure, from which the patient eventually recovered.

In June 2008, the patient presented with a new liver metastasis measuring 4 cm in diameter. Molecular analysis of the tumor revealed no K-Ras mutation and no B-Raf mutation. The patient was treated with 6 cycles of FOLFIRI + cetuximab. However, in October 2008 the metastasis had progressed and measured 5 cm on the liver MRI (Figure 1). A repeat hepatic resection was contraindicated given the history of serious liver failure and the close proximity of the metastasis to the left portal vein. Therefore, HAI was proposed.

An intrahepatic arterial catheter was percutaneously implanted after hepatic arteriography performed by femoral puncture. The catheter was then connected to a subcutaneous implantable port system, located in the inguinal area. After implantation, CT arteriography and a dynamic contrast-enhanced CT (DCE-CT) scan with injection of 1 mL/s of iodine contrast material (350 mg/mL) through the port were performed to check the absence of misperfusion and to assess tumor perfusion before starting hepatic arterial chemotherapy (Figure 2).

The patient received 2 cycles of gemcitabine and oxaliplatin (1 h infusion of gemcitabine 1000 mg/m² followed by 1 h infusion of oxaliplatin 100 mg/m² every 2 wk. Evaluation by CT arteriography and DCE-CT scan (Figure 2) showed a stable disease according to RECIST, but a dramatic decrease in tumor perfusion. Therefore, the patient received 4 additional cycles. Imaging examinations revealed a partial response (residual tumor diameter: 2.5 cm) as well as an almost complete disappearance of tumor vascularity (Figure 2). After 2 more cycles of arterial chemotherapy, a liver MRI

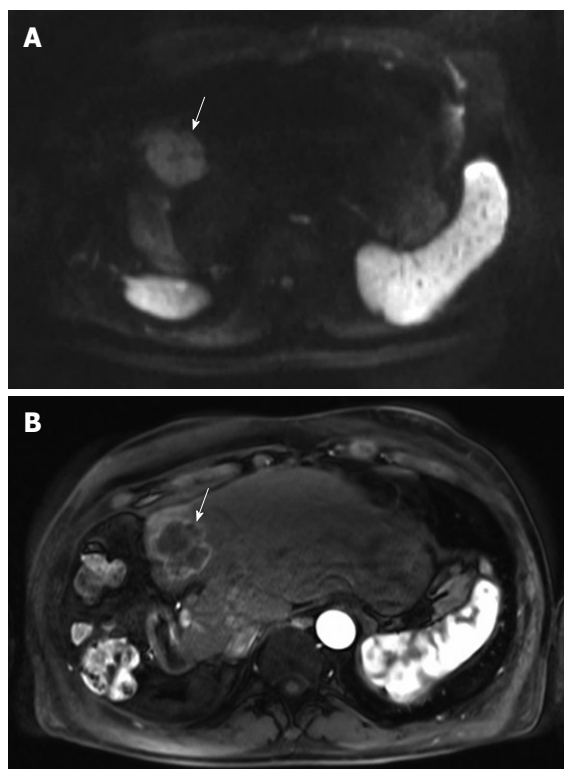


Figure 1 Magnetic resonance imaging (MRI) of the liver before starting hepatic arterial infusion (HAI). A: The hyperintensity of the solitary large (5 cm) metastasis in segment 4 (arrow) on the axial diffusion-weighted image is consistent with malignancy; B: The metastasis is hypervascular (arrow) on the arterial phase after injection of gadolinium chelate.

(Figure 3) showed the absence of tumor hyperintensity on diffusion-weighted imaging and a complete response regarding tumor perfusion. Positron emission tomography-CT with no significant hepatic uptake of ¹⁸F-fluorodeoxyglucose confirmed the absence of a viable macroscopic tumor. Finally, the treatment was completed with radiofrequency ablation of the residual mass. Only mild toxicity was observed, with grade 2 thrombocytopenia and asthenia with no specific toxicity related to the intraarterial procedure. To date, the patient is still in complete remission.

DISCUSSION

HAI of chemotherapy was first described in the early 1960s^[2] and can be proposed in cases of liver-confined metastatic disease. It results in increased local drug concentrations and lower systemic concentrations and hence less toxicity than intravenous administration, provided that the drug is eliminated by hepatic extraction^[3]. The use of HAI is also supported by the fact that, once hepatic metastases grow above 2–3 mm in size, they derive their blood supply from the hepatic artery, while normal hepatocytes are perfused mostly from portal circulation^[4]. Although a recent meta-analysis involving 1277 patients^[1] reported no significant survival benefit of fluoropyrimidine-based HAI in patients with liver

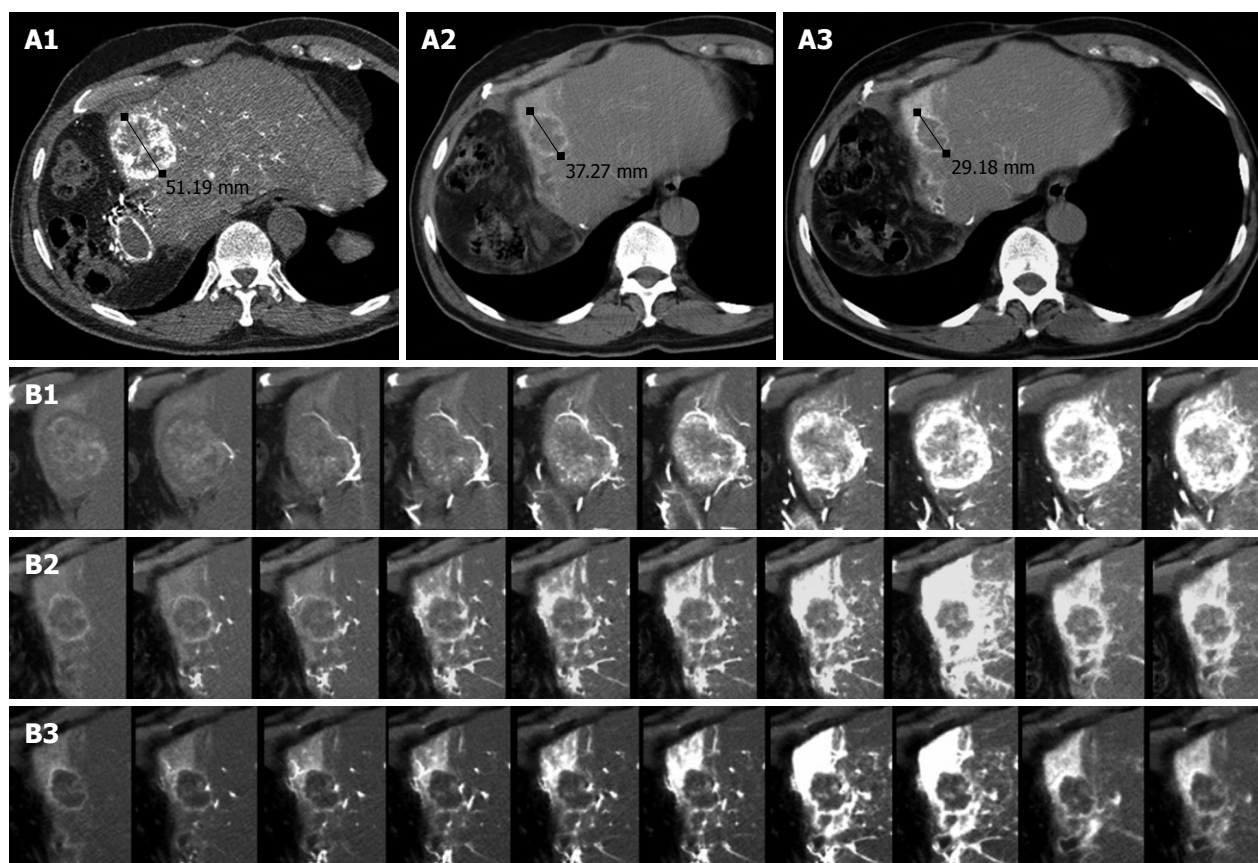


Figure 2 Computed tomography (CT) of liver metastasis. A: CT arteriography at baseline (A1), after 2 cycles (A2) and after 6 cycles (A3) of HAI. The metastasis was hypervascular at baseline. It became shorter after 2 cycles but remained as a stable disease according to RECIST. After 6 cycles, a partial response was observed (43% reduction in diameter); B: Dynamic contrast-enhanced CT (DCE-CT) scan at baseline (B1), after 2 cycles (B2) and after 6 cycles (B3). At baseline, the tumor was progressively and massively enhanced. After 2 cycles, there was a dramatic decrease in tumor perfusion, strongly suggesting the efficacy of HAI. Normal hepatic segment 4 is strongly enhanced. After 6 cycles, only small enhanced areas are visible within the residual tumor.

metastases from colorectal cancer, the study suggested using novel anticancer agents and/or drug combinations to improve the efficacy of HAI in the future. One of the major drawbacks of fluoropyrimidine-based HAI is the prolonged infusion time, which, in addition to causing patient discomfort, is likely to be responsible for chronic hepatobiliary toxicity^[1,5].

To our knowledge, this is the first case to report the combined use of gemcitabine and oxaliplatin for HAI. The addition of oxaliplatin in systemic chemotherapy for metastatic colorectal cancer has resulted in a higher response rate and longer survival than with 5-fluorouracil and leucovorin^[6]. HAI of oxaliplatin in a rabbit VX2 tumor model showed that the drug accumulated in the liver metastases in a 4:3 ratio (tumor/normal liver)^[7]. A high liver extraction ratio has been reported with oxaliplatin administered in HAI^[8], thereby explaining its low systemic bioavailability and thus, the good tolerance^[9]. Human phase I or I / II studies reported a dose-limiting toxicity between 150 mg/m² and 175 mg/m² of oxaliplatin for every 3-wk administration *via* HAI^[10,11]. The 100 mg/m² dose used in our patient was well tolerated and resulted in no specific toxicity. However, since firstly oxaliplatin as a single agent has demonstrated only moderate activity in patients with metastatic colorectal

cancer^[12] and secondly, its combination with fluoropyrimidines would necessitate prolonged infusion with subsequent hepatobiliary toxicity, we needed to use a combination of oxaliplatin with another chemotherapeutic agent.

Gemcitabine is a deoxycytidine analogue which has to be intracellularly phosphorylated to its active forms (gemcitabine diphosphate and triphosphate) by deoxycytidine kinase^[13], increased levels of which are found in liver metastases compared to normal liver tissue^[14]. In addition, an *in vitro* study showed that gemcitabine had concentration- and time-dependent cytotoxic effects on many colon cancer cell lines^[15]. As HAI makes it possible to reach an effective concentration locally, gemcitabine was deemed suitable for HAI. Interestingly, an experimental study conducted by Faivre *et al*^[16] showed, in human colon cancer cell lines, a supra-additive effect of gemcitabine in combination with oxaliplatin, as well as a stronger effect of gemcitabine followed by oxaliplatin than the inverse. In a phase I study by Vogl *et al*^[17], a maximum-tolerated dose of 1400 mg/m² gemcitabine was reached after weekly 20-min HAIs of the drug in patients with cholangiocarcinomas and liver metastases from pancreatic cancer. A recent phase I study reported that gemcitabine given at doses higher or longer than

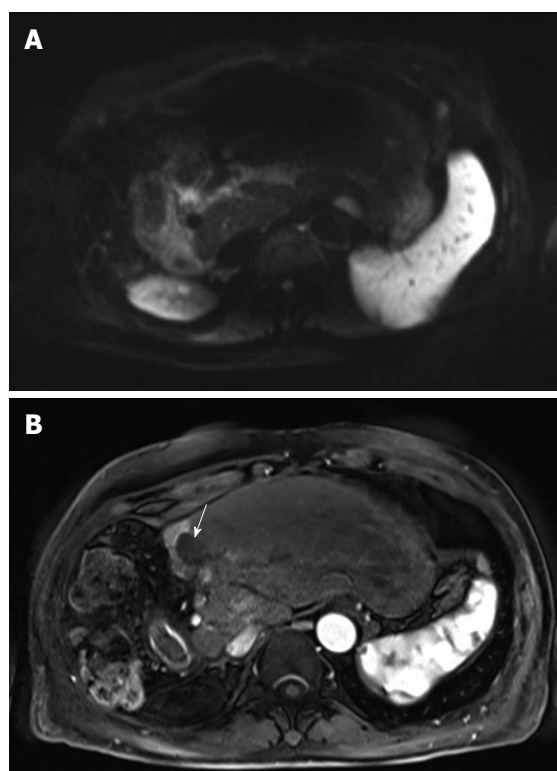


Figure 3 MRI of the liver after 8 cycles of HAI. A: The lesion is isointense to the liver parenchyma on the diffusion-weighted image thereby suggesting the absence of viable tumor; B: The lesion is completely avascular (arrow) during the arterial phase after injection of gadolinium chelate. Note the enhancement of the remnant segment 4.

the recommended systemic dose of 1000 mg/m² over 30 min was well tolerated. All these results are consistent with the excellent tolerance in our patient.

CT arteriography using the implantable port system is useful to detect misperfusion^[18] which may be responsible for undertreatment of liver metastases and/or for inadvertent chemotherapy perfusion of extra-hepatic organs such as the gastroduodenal wall. Early endovascular management prevents gastroduodenal lesions caused by the toxicity of chemotherapeutic drugs and allows treatment by HAI to be continued^[19]. In this case, visual assessment of tumor perfusion on DCE-CT helped us to detect an early response to HAI. Although optimum quantitative perfusion parameters have not yet been determined, visual analysis of DCE-CT may be useful for monitoring the effects of HAI over time.

In conclusion, despite the poor prognosis of colon cancer with synchronous liver metastases, this patient has survived for 52 mo and is to date disease-free. This suggests that further trials involving HAI of oxaliplatin and gemcitabine in metastatic colon cancer are required.

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Splenic lymphangiomas showing rapid growth during lactation: A case report

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INTRODUCTION

Splenic lymphangiomas are a very rare, benign entity that is characterized by the presence of multiple cystic lesions of different sizes that replace all the parenchyma. It is considered to result from developmental failure in the lymphatic system. In the majority of cases, this disease has an asymptomatic course and, despite the use of modern imaging techniques, often makes preoperative diagnosis difficult. Due to the nonspecific features of this disease, splenectomy is needed to make a definitive histological diagnosis and to exclude the possibility of malignant lesions.

CASE REPORT

In January 2006, a 35-year-old woman was admitted to the General Surgery Unit, Department of Surgical and Oncological Sciences, Palermo, Italy after a hematologist (IE) suggested that she undergo splenectomy.

The patient was referred about 5 wk after her second delivery. About 5 mo before, she had noted increasing pain in the left hypochondrium. Abdominal ultrasound showed an increased in spleen volume with a long axis of 10 cm, with no homogeneous ultrasound structure, which indicated the presence of multiple hypoechoic circular masses with a maximum diameter of 1 cm.

Further abdominal ultrasound performed up to 1 wk before delivery showed that the spleen had no additional changes. Immediately after delivery, the patient began to nurse the baby. In particular, this was the first time she had nursed her newborn, because she did not nurse her first child who was born 3 years before.

Due to recurrence of pain in the left hypochondrium, after about 5 wk from delivery, the patient underwent abdominal ultrasound that showed spleen enlarge-

Abstract

Splenic lymphangiomas are a very rare condition that, from 1990 to date, has been described only nine times. In the present report, we describe the first case of splenic lymphangiomas with rapid growth during lactation in a 35-year-old woman. We also underline the difficulty in making an accurate preoperative diagnosis, despite more modern imaging techniques. Total splenectomy was considered to be the treatment needed, both to make a definitive diagnosis and to exclude the presence of malignancy.

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Key words: Lymphangiomas; Spleen; Lactation

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Figure 1 Total body computed tomography. The spleen appeared increased in volume (maximum diameter 18 cm), and it was possible to see multiple low-density lesions with a nodular appearance, which replaced all the parenchyma.

ment, with a long axis that measured up to 18 cm, with a hypoechoic mass that had a diameter of 2.5 cm. Total body computed tomography showed only splenomegaly with multiple low-density lesions with a nodular appearance, which were considered by the radiologist to be features of lymphoma (Figure 1).

There was no fever, nausea or vomiting. Laboratory findings were all within the normal limits. Also, serological screening for hydatid disease was negative.

Due to the rapid increase in spleen volume, the patient suspended lactation and after 2 d, she underwent conventional open splenectomy. During the operation, careful examination of the abdominal cavity was performed to search for the presence of an accessory spleen, which in this case, was not found. The remaining abdominal viscera were normal.

The spleen had an 18-cm long axis, and its macroscopic appearance was characterized by the presence of multiple cystic masses of clear appearance that replaced all the parenchyma, which altered the volume and profile of the spleen (Figure 2). Histological examination revealed various lymphatic space dilatations associated with slow stromal bleeding, which led to a diagnosis of splenic lymphangiomatosis.

Postoperative course was unremarkable and the patient was discharged 6 d after surgery. Additional clinical and instrumental controls did not reveal any further sites of lymphangiomatosis. After a 3-year follow-up, there was no sign of recurrence. The patient did not have a further pregnancy and felt in good health. Ultrasound controls performed on her parents and sons did not show signs of abdominal cystic lesions.

DISCUSSION

Lymphangioma is a cystic, benign, slow-growing tumor and is a very rare condition, usually seen in children, where it is discovered incidentally^[1]. These tumors occur more frequently in females, and 80%-90% are detected before the end of the second year of life. They are generally considered to be a developmental malformation in which obstruction or agenesis of lymphatic tissue results in lymphangiectasia, which is caused by a lack of normal communication of the lymphatic system.

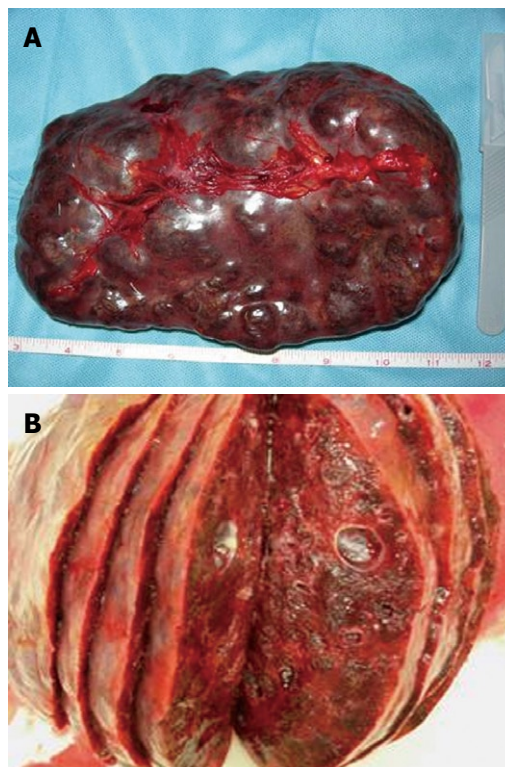


Figure 2 Surgical specimen. A: The spleen was 18 cm on the long axis, and its macroscopic appearance was characterized by the presence of a multiple cystic mass that altered the volume and profile of the spleen; B: A section of the spleen confirmed the presence of a multiple cystic mass with a clear appearance that replaced all the parenchyma.

The neck (75%) and axillary regions (20%) are the most common locations of lymphangioma. However, cases have been reported to originate from any part of the gastrointestinal tract. Lymphangioma can be isolated or diffuse and involve one or more organs. The lymphatic alteration, which involves different organs, is termed systemic lymphangiomas. The spleen can be involved alone or it can be a part of multivisceral involvement.

Isolated splenic lymphangioma can arise with different manifestations^[2]: focal lesions of small size, subcapsular rather than intraparenchymal, with no pathological significance; large cystic lesions that may attain sufficient size to cause significant splenomegaly and left-upper quadrant symptoms; and lymphangiomas in which the spleen is replaced diffusely by expanding lymphangioma, thus leaving very little splenic parenchyma. Isolated splenic lymphangiomas are a rarer form; from 1990 to date, only nine cases have been described in the literature^[1-9]. In this form, the cysts can have different sizes that vary from a few millimeters to two or three centimeters. The spleen, when it is involved, rarely has a normal size, and its volume is frequently greatly increased.

Histologically, lymphangioma is classified as simple, cavernous and cystic, depending on the size of the dilated lymphatics. At gross examination, these cysts have a thick fibrous wall with an internal morphology that is characterized by fibrous trabeculae. Hyalinization and calcification of the fibrous connective tissue may be present. Also, lymphangioma has an endothelial lining, foam cells and a wall that contains lymphatic spaces,

lymphoid tissue and smooth muscle. Histochemical staining of the endothelium demonstrates reactivity with CD31, CD34, factor VIII-related antigen and keratin to varying degrees^[10]. Sexual hormones can influence the growth of lymphangioma. Quack Loetscher *et al*^[11] have described a case of axillary cavernous lymphangioma that rapidly increased in size during pregnancy. They also hypothesized that the possible pathological mechanism for growth of lymphangioma could be related to the overproduction of cytokines such as vascular endothelial growth factor (VEGF)^[11]. Animal studies have shown that prolactin, produced during lactation, stimulates production of VEGF and induces overexpression of its receptor^[12]. In the present case, growth of lymphangiomatosis was observed during the period of lactation.

In the adult population, lymphangioma has an asymptomatic course, and often is found as an occasional feature during instrumental investigations performed for other reasons. When symptoms are present, they are related to the size of the spleen and pain is the most common complaint. Less commonly, patients can present with a palpable mass located close to the hypochondrium.

Due to the rarity of presentation and the asymptomatic course of this disease, it is often difficult to make a correct diagnosis. Imaging studies are useful, both to characterize the splenic malformations and to exclude eventual involvement of other organs. Diagnostic studies of lymphangioma usually begin with ultrasonography, which shows the entire spleen as appearing to be replaced by cystic lesions with an anechoic or hypoechoic pattern^[13]. Color-Doppler integration can demonstrate the vasculature of the mass, including the intrasplenic arteries and veins along the cyst wall, which helps to determine the organ of origin^[13]. Angiography typically reveals an avascular mass. The parenchymogram generally shows multiple focal lucencies of various sizes, the so-called "Swiss-cheese" appearance. Computed tomography demonstrates multiple low-attenuation masses with sharp margins that are typically subcapsular and do not enhance^[14]. Upon magnetic resonance, generally lymphatic malformations appear as hyperdense on T2 imaging, whereas T1 imaging is only slightly increased^[15]. Fine needle aspiration biopsy in splenic lymphangiomatosis is contraindicated because of the bleeding risk and limited amount of tissue for accurate diagnosis^[16]. The differential diagnoses includes malignant (i.e. angiosarcoma or lymphoma) and benign (i.e. hemangioma, littoral cell angioma, and peliosis) splenic vascular proliferation. The instrumental investigations alone are not always useful to make a correct differential diagnosis. This is caused by the rarity of vascular tumors of the spleen and the absence of pathognomonic signs. For these reasons, usually, the correct and definitive diagnosis can be obtained only with histological confirmation; histochemical investigations are needed to make a differential diagnosis among different vascular tumors.

Lymphangiomatosis involves all the splenic parenchyma, therefore, a total splenectomy is the treatment needed. Laparoscopic splenectomy is considered the procedure of choice for normal or moderately enlarged spleens, whereas in the case of severe splenomegaly, open splenec-

tomy is preferred^[17]. During surgery, both open and laparoscopic, the search for accessory spleens is an important step. These must be removed even if they appear macroscopically normal, because they could be involved in the pathological process^[7,10].

Although the development of lymphangioma in pregnancy has been described in the literature^[11,18], the main interest of this case was the rapid growth of splenic lymphangiomatosis, with an increase from 10 to 18 cm in 5 wk during lactation. The rapid growth of the spleen and the suspicion of malignancy led us to perform an open splenectomy.

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Transjugular intrahepatic portosystemic shunt with accidental diagnosis of persistence of the left superior vena cava

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impossible. This report describes a rare vascular malformation characterized by the absence of the right superior vena cava and persistence of the left superior vena cava in a patient with a diagnosis of advanced liver cirrhosis who needed a TIPSS placement in order to control refractory ascites.

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Key words: Left superior vena cava persistence; Liver cirrhosis; Refractory ascites; Transjugular intrahepatic portosystemic shunt

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Abstract

Transjugular intrahepatic portosystemic shunt (TIPSS) is considered a valid therapeutic option for the treatment of portal hypertension and its complications. The guidelines for this procedure have already been established on the basis of the normal vascular anatomy and of various technical radiological aspects. In some few rare cases, diagnosis of a congenital vascular anomaly can be made accidentally by interventional radiologists, making the procedure of the TIPSS placement extremely difficult or in some cases technically

INTRODUCTION

The transjugular intrahepatic portosystemic shunt (TIPSS) is a well known life saving procedure that is performed in patients with complications of portal hypertension, such as variceal bleeding and refractory ascites^[1-5]. The standard technique for this treatment is the right internal jugular vein (RIJV) approach, although some authors have reported the left internal jugular vein (LIJV) approach when the RIJV is not available or cannot be used, due, for example, to vein thrombosis^[6]. Few cases of patients with the absence of the right superior vena cava (RSVC) and the persistence of the

left superior vena cava (LSVC) have been described in the literature^[7]. Even if this is an extremely rare situation, it can create problems during the interventional procedure. We briefly report a case in which a TIPSS was successfully created in a patient with absence of RSVC and persistence of the LSVC.

CASE REPORT

A 69-year-old female with a diagnosis of hepatitis C virus-related cirrhosis was referred to our institute in order to be evaluated for a TIPSS placement because of refractory ascites. In the physical examination the patient had tense ascites despite a consistent use of high dose diuretics and periodic paracentesis (every 1-2 wk). She was well oriented to time and to space and had no episodes of portosystemic encephalopathy in the past. An abdominal computed tomography (CT) scan of the abdomen and a liver Doppler sonography (US) were performed excluding presence of hypervascular focal lesions in the liver and showing patency of the portal vein (PV). The CT scan also showed signs of portal hypertension such as tense ascites, presence of splenogastrorenal shunt, large esophageal and perigastric varices and patency of the umbilical vein. A large volume paracentesis was performed at admission with removal of almost 15 liters of clear ascites. No evidence of infection was found in the fluid (white blood cell count, Gram stain, and culture were negative).

The patient was hemodynamically stable with no need of inotropic drugs. Child-Pugh score was B-8 with a Meld score of 10. She had mild coagulopathy (international normalized ratio 1.4, platelet count 56 000/ μ L), but with good liver function tests (LFTs) (total bilirubin 1.29 mg/dL, albumin 3.4 g/dL). A transthoracic echocardiography showed no evidence of valvular heart disease, and the diameters of the cardiac chambers were within normal limits. Systolic and diastolic functions of the left ventricle were in the normal range. Estimated pulmonary vein pressure was 35 mmHg.

As a result, we decided to perform a TIPSS procedure in order to treat portal hypertension. The procedure was carried out in an angiographic suite under monitored anesthesia care. The right femoral artery was prepared, and an angiogram revealed patency of the superior mesenteric artery. In the venous phase the superior mesenteric and main portal vein were visualized. After sonographic guidance, the RIJV was cannulated, and under fluoroscopic guidance the metallic flexible guidewire was advanced and seen to curve to the left, until reaching the right atrium (Figure 1). A 5 French (F) pig tail catheter was advanced in the right atrium and a venogram revealed a patent LSVC that drained in the coronary sinus and an absent RSVC but in the presence of a viscerocranial situs solitus (Figure 2). Because of the excessive curvature, which made insertion of the Colapinto needle impossible, we decided to use the LIJV and perform a new venogram. Under sonographic guidance, a new catheter was advanced through the LSVC

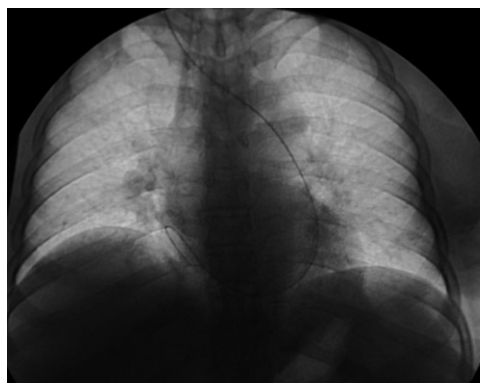


Figure 1 Digital venogram showing the curve of the metallic flexible guidewire from the left to the right atrium.

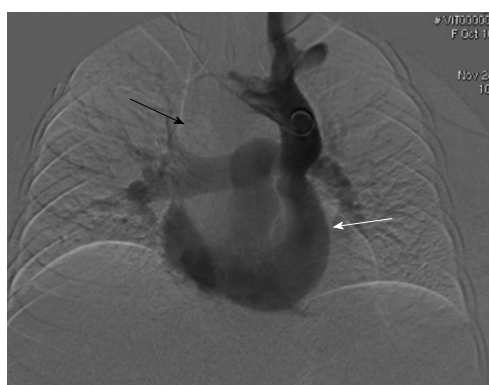


Figure 2 Digital venogram, performed with a 5F pigtail catheter, showing the absence of the right superior vena cava (RSVC) (black arrow) and presence of contrast dye in the left superior vena cava (LSVC) that is draining in the coronary sinus (white arrow).

and the coronary sinus to the right atrium and from the inferior vena cava to the right hepatic vein. The Colapinto coaxial system was applied. The right branch of the main PV was punctured, under fluoroscopic guidance, and a 5F hydrophilic catheter was advanced over a hydrophilic guidewire. Portal hemodynamic measurements were performed and showed free hepatic vein pressure (FHVP) of 12.5 mmHg, right atrial pressure (RAP) of 12 mmHg, and portal vein pressure (PVP) of 23.5 mmHg. A shunt was created employing an 8-mm diameter Viator covered stent which was dilated with a 6-mm angioplasty balloon. New hemodynamic measurements showed FHVP of 14.5 mmHg, RAP of 14 mmHg and PVP of 19 mmHg. A venogram performed after the TIPSS placement showed patency of the stent (Figures 3 and 4) with reduction of the gradient from the portal vein to the right atrium from 11.5 mmHg before stent placement to 5 mmHg after stent placement.

A Doppler US control was performed the day after the TIPSS placement showing good patency and blood flow within the stent. The patient had a good post-procedure course with no complications and was discharged 48 h after the TIPSS creation.

Since then, the patient has had regular clinical and radiological follow-up, having a US exam of the TIPSS



Figure 3 Digital portogram performed after transjugular intrahepatic portosystemic placement showing patency of the stent with good portal flow (white arrow).

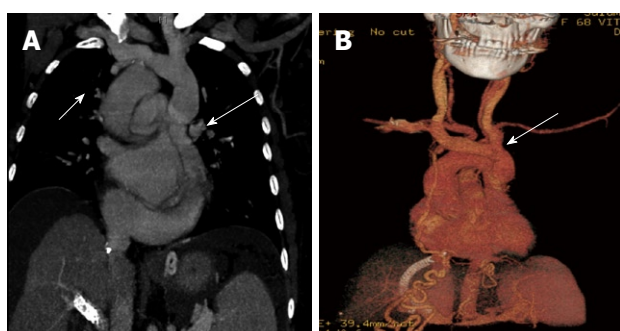


Figure 4 Maximum intensity projection reconstruction in coronal plane (A) and volume rendering reconstruction (B). Absence of the right vena cava (short arrow) with the brachio-cephalic venous trunk, the left jugular vein and the left subclavian vein draining into the left superior vena cava (long arrows). Left SCV is draining into the coronary sinus.

and laboratory tests every 3 mo. The last US exam was carried out 19 mo post-TIPSS creation showing patency of the TIPSS and absence of ascites. Currently the patient is doing well, her Child-Pugh score is B-7, with stable LFTx and no signs of hepatic decompensation.

DISCUSSION

Absence of the right SVC with persistent left SVC in viscerocranial situs solitus is a rare congenital cardiovascular malformation and one of the most common rare thoracic vein anomalies. It is found in < 1% of the general population and in up to 10% of patients with congenital heart disease^[8,9]. The first case was described in 1862 by Halbertsma^[10].

This anomaly is usually diagnosed accidentally, for example, during anesthesiological procedures, such as central vein placement^[7] or, more frequently, during diagnostic and therapeutic cardiovascular procedures, such as right heart catheterization or pacemaker placement^[11,12]. Moreover, it may also emerge during cardiac procedures

in pediatric patients (foramen ovale or atrial septal defect transcatheter closure).

Nowadays, TIPSS is considered to be a valid procedure for the treatment of complications of portal hypertension such as refractory ascites and variceal bleeding. The left internal jugular approach has been reported in previous studies^[6]. To our knowledge, there are a few cases reported in the literature of successful TIPSS placement through the LSVC approach^[13], some of them in patients with a diagnosis of “situs inversus totalis”^[14,15]. Interventional radiologists should be aware of this rare, but possible, vascular anatomy, and understand that it may not represent an absolute contraindication for TIPSS insertion.

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Simultaneous surgery for critical aortic stenosis and gastric cancer: A case report

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INTRODUCTION

Cardiovascular and neoplastic diseases are the main causes of death in Europe. Aortic stenosis is currently the third most common heart disease in Europe and North America. It is mainly caused by degenerative processes, with secondary calcification of the valve. Moderate stenosis is observed in 5% of the population over the age of 75 years, while critical stenosis occurs in 3% of the population, with half of these cases being asymptomatic. Diagnosis is based on preoperative echocardiographic examination. Severe stenosis can be managed with one of the following procedures: valve replacement, transcatheter aortic valve implantation or, in some cases, percutaneous valvulotomy with Inoue balloon^[1].

Gastric cancer remains one of the most common neoplastic diseases in the world, with fourth highest morbidity. Diagnosis is based on pathologic examination of a specimen obtained at gastroscopy. Infiltration depth is evaluated with endoscopic ultrasound. Computed tomography (CT) enables assessment of regional lymph nodes and presence of remote metastases^[2]. Surgical treatment remains a therapy of choice for gastric cancer; however there are no standard procedures. In

Abstract

We describe simultaneous surgery performed on a 71-year-old woman with critical aortic stenosis and gastric cancer that were diagnosed at the same time. The patient qualified for simultaneous surgery for both these diseases. Good early outcome was achieved. There is a lack of standards for treatment of patients with coexistence of two life-threatening conditions. We discuss surgical tactics and potential benefits of such management.

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Key words: Aortic stenosis; Aortic valve replacement; Gastric cancer; Gastric resection

Europe, 5-year survival following R0 gastric resection is 33%-35%^[3].

We present the following case of a patient who underwent simultaneous surgical treatment of gastric cancer and asymptomatic critical aortic stenosis.

CASE REPORT

A 71-year-old female patient (weight 56 kg, height 164 cm) was admitted to the Department of Surgical Oncology because of gastric tumor. She had a history of non-specific epigastric pain and appetite loss for the past 2 mo. Weight loss was 12 kg over 6 mo. Laboratory tests revealed secondary anemia (Hb 10.8 g/dL) and lowered blood ferritin level (5 ng/mL). The patient had a history of melena and loose stool prior to admission to the hospital. She had never been treated for gastrointestinal tract diseases.

Because of non-specific abdominal symptoms and anemia, the patient was referred for endoscopic examination of the upper gastrointestinal tract. Gastroscopy revealed an ulcerated tumor in the upper third of the gastric corpus, reaching over half of its circumference. Pathologic examination of the biopsy specimen revealed gastric adenocarcinoma (adenocarcinoma tubulare G1 exulcerans, intestinal type according to Lauren's classification). Before admission, the patient had never been treated for cardiac or vascular diseases. Cardiac efficiency was classified grade II NYHA (New York Heart Association) scale^[4]. The patient had a history of decreased arterial blood pressure over the past 10 years.

At the age of 38 years, the patient was diagnosed with primary adrenal insufficiency (Addison's disease), treated with hydrocortisone (30 mg orally). One year before admission, after bone marrow biopsy and laboratory findings, the patient was diagnosed with chronic lymphocytic leukemia, requiring only regular control in the hematology out-patient clinic.

At the age of 50 years, the patient underwent two surgeries: left thyroid lobectomy because of nodular goiter (currently in euthyrosis) and left breast amputation because of breast cancer (currently under oncological observation). Allergic history comprised of a pollen allergy.

Electrocardiogram examination revealed regular sinus rhythm with heart rate 71 bpm, PR 156 ms, QT 390 ms, QTc 0.45 ms. Chest X-ray did not show any lung pathology. Sclerotic aorta with typical poststenotic dilation of ascending aorta and hypertrophy of left ventricle were described. Echocardiographic examination revealed critical aortic stenosis with maximal transvalvular pressure gradient of 135 mmHg; mean gradient was 95 mmHg. Valve surface area was estimated to be 0.3 cm². Interventricular septum diameter was 16 mm, and left ventricular ejection fraction was 45%-50%. Additionally, significant calcification of the aortic bulb and aortic valve cusps was found. Subsequently, a coronarography was performed, which showed no significant stenosis in the coronary vessels.

No enlarged lymph nodes in the posterior mediastinum were observed on thoracic CT. On the other hand, on abdominal CT numerous non-characteristic enlarged lymph nodes were observed in the retroperitoneum along the abdominal aorta. This included enlarged lymph nodes surrounding the celiac trunk and in the mesentery of the small intestine. It was not clear, however, whether lymphadenopathy resulted from the chronic lymphocytic leukemia or from metastases of the gastric cancer.

Additionally, gallstones 24 mm in size were found. An anterior gastric wall thickening in the region of lesser curvature was observed. In view of the clinical examination and the results of image studies the patient was classified as having no sign of metastatic disease. During preoperative diagnostic staging, laparoscopy was considered among other available procedures, however, after presenting the case to cardiac surgeons and anesthesiologists, this was called off due to the relatively high risk of sudden cardiac death^[4].

Anesthesiologist consultation assessed the patient's cardiac efficiency as grade II NYHA (New York Heart Association) scale and grade III ASA (American Society of Anesthesiologists) physical status classification^[5,6].

The operation was performed *via* sternotomy and median laparotomy by two surgical teams. There were some doubts about operability of the stomach cancer, therefore the oncological surgeon performed a laparotomy to assess the possibility of radical resection. Subsequently, the thorax was opened by median sternotomy. Massive calcifications in the ascending aorta (porcelain aorta) were found and the cardiothoracic surgeon considered discontinuation (Figure 1A). Ultimately, the decision to proceed with the operation was made. Aortic valve replacement (biological prosthesis Medtronic® Hancock II size 21) with implantation of an ascending aorta prosthesis in the supracoronary position (Vascutek® prosthesis size 26) with extracorporeal circulation (ECC) was performed (Figure 1B). Time of ECC was 148 min. The patient returned to normal circulation without complications. After weaning from ECC, protamine sulfate was administered to reverse the heparin action necessary during cardiopulmonary by-pass. Fresh frozen plasma and platelets were supplemented additionally, to enable the oncological surgeon to perform his part of the operation without disturbance of hemostasis. In the second stage, the oncological team performed total gastrectomy with D1 lymphangiectomy and Roux-en-Y anastomosis (Figure 1C). The procedure was supplemented with feeding jejunostomy. The spleen was removed for technical reasons. Additionally, cholecystectomy was performed because of cholelithiasis. The entire operation finished without complication in a total time of 320 min. Throughout the operation the patient received 4 units of blood, 6 units of fresh frozen plasma, and 2 units of platelet concentrate. The patient was extubated 33 h after the operation. Immediately after the surgery, the patient was admitted to the Intensive Care Unit, staying there for 6 d. She did not

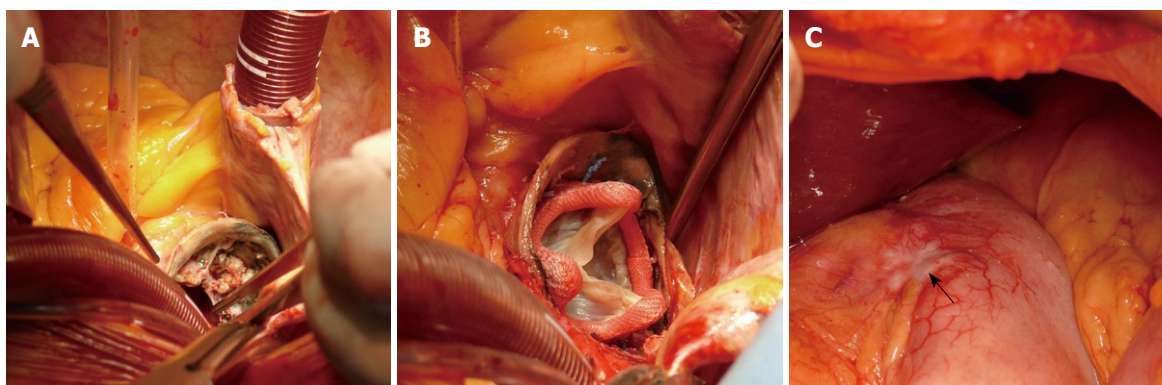


Figure 1 Steps taken in the simultaneous surgery: cardiac (A, B) and oncological (C). A: Excision of the calcified aortic valve; B: Implanted bioprosthesis of the aortic valve; C: Gastric cancer (arrow) seen on gastric wall.

present any abdominal complications. Pericardial drainage was removed on postoperative day 3, and abdominal drainage on day 5. Apart from postoperative depression and slight disorientation, the postoperative course was without problems.

On postoperative day 7, the patient was transferred to the Department of Surgical Oncology for further postoperative care. Psychological care was introduced. Oral feeding was gradually introduced during this period. Postoperative pathological examination of the tumor revealed adenocarcinoma, intestinal type according to Lauren's classification. The subserosal layer was infiltrated (pT2b), and metastases were found in 2 of 8 lymph nodes (pN1) (stage II gastric cancer).

The patient has been followed up for a total of 6 mo to date, with no complications noted.

DISCUSSION

This case is worth presenting because it highlights a number of important issues. Firstly, it is crucial to prepare the patient for surgery thoroughly. This includes taking the patient's history and performing all necessary specialist consultations, bearing in mind various co-existing diseases. Once this has been executed, it becomes possible to plan the optimal treatment. In 2002, recommendations for the management of asymptomatic patients with severe valvular heart disease were established by a European workgroup^[1]. These stated that management of asymptomatic patients with severe valvular heart disease should be based on individual assessment of the risk to benefit ratio. There is a strong need to introduce standards for the diagnosis and management of cardiological diseases in order to decrease the number of cardiac complications in the perioperative period.

Secondly, the existence of highly specialized Departments (Cardiological, Cardiosurgical, Oncological, Endocrinological, *etc.*) within one unit enabled efficient diagnostics, decision making, simultaneous surgical procedures and safe postoperative care in the Intensive Care Unit. This case shows that complex procedures should be performed in specialist units which should be fully

staffed and resourced, to provide optimal investigation and treatment for patients with complex acquired heart disease.

Thirdly, this case proves that a thoroughly executed taking of the patient's history and physical examination helps to prevent incidental omission of important, potentially life-threatening diseases. Cardiological consultations ordered more frequently could lead to increased detection of asymptomatic cardiac diseases and help to avoid later cardiovascular complications. The systematic increasing age of oncological patients has led to the development of a new branch of surgical oncology, called geronto-oncology. This approach may facilitate more individualized treatment, taking into account patient's co-existing diseases.

To optimize the outcome in this case, the decision to perform simultaneous surgical treatment was made. It appeared that there were more arguments supporting the decision to perform simultaneous surgery (even though such a *modus operandi* is rather rare) than those backing the alternative, i.e. two consecutive surgeries^[7,8]. The grounds for simultaneous surgery were: (1) extension of the sternotomy incision through mini-laparotomy is a standard procedure in cardiac surgery and in coronary artery bypass surgery (assessment of the gastropiploic artery); (2) such a procedure does not increase the postoperative risk; and (3) performance of a mini-laparotomy allowed for evaluation of the extent of the neoplastic process in the abdomen and consequently for deciding whether the cancer was operable and whether its simultaneous resection was feasible. If an extensive spread of the disease had been noted, radical surgery could have not been performed or a two-step surgery might have been necessary after 3–4 wk.

The first step was undertaken by an oncological surgeon; laparotomy was performed to assess the possibility of radical resection of gastric cancer. Then the chest was opened and cardiac surgery performed, and after finishing the valve replacement and closure of the chest, the oncological team did their work. In our opinion this was the safest way, which allowed the performance of the second part of the operation without hemostatic

problems that could occur after high dose heparin infusion necessary for the ECC. A different approach could be considered if there was a risk of serious bleeding from the resection lines or anastomoses during the heart-lung machine working time. Additionally, this sequence is important to reduce the risk of mediastinitis. Potential disadvantages of the simultaneous surgery were: (1) increased surgical risk resulting from performing the oncological step after protamine sulfate was administered; and (2) limited operation field allowing for resection of regional perigastric lymph nodes only (i.e. lymphadenectomy type D1).

In a case such as the one we have described here, performing oncological surgery without diagnosing critical aortic stenosis should be treated as malpractice since this scenario could potentially lead to the patient's death.

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Ileocolonic mucormycosis in adult immunocompromised patients: A surgeon's perspective

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Abstract

We report three cases of ileocolic mucormycosis in adult immunocompromised patients presenting as acute abdomen. All patients underwent laparotomy but two of them died from multiorgan failure before the diagnoses were confirmed. The diagnosis of gastrointestinal mucormycosis is rarely suspected, and ante-mortem diagnosis is made in only 25%-50% of cases. These cases illustrate the difficulty encountered by surgeons in managing acute abdomen in neutropenic patients with hematological malignancy. The management of colonic mucormycosis in the published literature is also reviewed.

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Key words: Gastrointestinal mucormycosis; Colon; Immunocompromised; Surgery

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INTRODUCTION

Neutropenic patients can present with acute abdominal catastrophes of various causes and surgical intervention is required. However, surgery is usually associated with a high mortality and morbidity in the immunocompromised host. With the rising incidence of invasive fungal infection in patients with hematological malignancy and transplant recipients, mucormycosis has been reported to be the third most common cause of fungal infection after candidiasis and aspergillosis^[1,2]. Mucormycosis refers to several different diseases caused by *Mucorales*^[3,4]. These rare infections usually only occur in individuals with impaired immunity, having associations with diabetes mellitus, hematological or solid-organ malignancies, transplantation, neutropenia and steroid therapy. The diagnosis of mucormycosis is rarely suspected and ante-mortem diagnosis is made in only 25%-50% of cases^[5].

CASE REPORT

Case 1

A 42-year-old lady received chemotherapy for NK cell lymphoma and, concomitant with disease progression, she presented with ascites and pleural effusion. She complained of abdominal pain and fever in February 2009. Physical examination showed generalized tenderness and the aspirated peritoneal fluid was turbid. Laparotomy was undertaken for suspected perforated viscus. On laparotomy, the ascitic fluid was grossly turbid. The cecum was grossly dilated but no perforation was identified. The rest of the laparotomy was grossly normal except for the in-



Figure 1 Dermal mucormycosis around ileostomy and laparotomy wound.



Figure 2 Fungal colonies over omentum and small bowel surface.



Figure 3 Black ulcer with full bowel infarction at colon on exposed specimen.

flamed omentum which was densely adhered to the pelvic cavity. Peritoneal lavage with saline was the only procedure performed. Postoperatively, Tazocin, amikacin and voriconazole were given as *Klebsiella*, *Escherichia coli* and *Candida albicans* were identified from the peritoneal fluid. However, the patient's condition rapidly deteriorated and she died two weeks after operation. The autopsy showed extensive involvement of fungal elements inside the abdominal cavity. Also, branching septate fungi were isolated in one of the peritoneal fluid cultures after the patient succumbed and later confirmed to be *Rhizopus* species.

Case 2

A 57-year-old man had relapsed diffuse large B-cell lymphoma and underwent palliative chemotherapy and radiotherapy. He presented with peritonitis in September 2008. An urgent computer tomography (CT) scan of the abdomen showed free intraperitoneal gas; emergency laparotomy was undertaken and two 1-cm perforations with indurated edges were identified over the cecum. Ileocecal resection and end ileostomy were performed in view of gross peritoneal contamination. Histological examination showed lymphoma involving the colonic wall and causing the perforations. Broad-spectrum antibiotics (meropenem and vancomycin) and prophylactic fluconazole were given in view of multiple bacteria shown in peritoneal fluid cultures. A well-demarcated gangrenous skin necrosis appeared over the peristomal area and at the upper midline

wound on day 8 (Figure 1). Finally, the patient died of multiorgan failure two days later. Subsequent wound swab culture revealed *Rhizopus* species. No autopsy was performed because of refusal by the family.

Case 3

A 38-year-old male, with persistent precursor B cell acute lymphoblastic leukemia, developed abdominal pain and fever on the eighth day after receiving clofarabine therapy in February 2009. An urgent abdominal CT scan showed features suggestive of appendiceal abscess with suspected pneumoperitoneum. He underwent emergency laparotomy and was found to have gangrenous appendicitis with localized retrocecal abscess. Ileocecal resection and primary anastomosis were performed. Postoperatively, liposomal amphotericin, caspofungin and posaconazole were started immediately as the microbiologist identified *Rhizopus* species in the specimen. Granulocyte colony-stimulating factor was also prescribed for severe neutropenia. The patient recovered well and was transferred back to the general ward. One week later, he still had persistent abdominal distension and developed deep venous thrombosis of the right lower limb. A abdominal CT scan showed that there was no contrast opacification at the right external iliac vessels and impaired contrast enhancement at the terminal ileum. Exploratory laparotomy was undertaken on day 12 after the first operation and multiple hardened fungal colonies were noted over the omentum and small bowel (Figure 2). An inflammatory mass compressed the right iliac vessels, causing decreased venous blood flow. A 20-cm segment of small bowel proximal to the previous ileocolic anastomosis densely adhered to the previous operative site and was ischemic. Small bowel resection, end ileostomy and omentectomy were performed. Multiple patches of full-thickness bowel ischemia were seen over the colonic wall (Figure 3) and mucormycosis was confirmed on histological examination. Though extensive surgical debridement and maximal medical support were administered, the patient died four days after the second laparotomy.

DISCUSSION

Mucormycosis is an opportunistic fungal infection

Table 1 Taxonomic classification of the Zygomycetes

Class	Order	Family	Genus	Species causing human diseases
Zygomycetes	Mucorales	Mucoraceae	<i>Rhizopus</i>	<i>R. oryzae</i> ^{1,2} <i>R. microsporus</i> var. <i>rhizopodiformis</i> ^{1,2} <i>R. pusillus</i> ^{1,2}
			<i>Rhizomucor</i>	
			<i>Mucor</i>	<i>M. circinelloides</i> ^{1,2}
			<i>Absidia</i>	<i>A. corymbifera</i> ^{1,2}
			<i>Apophysomyces</i>	<i>A. elegans</i> ^{1,2}
			<i>Cunninghamella</i>	<i>C. bertholletiae</i> ^{1,2}
	Entomophthorales	Cunninghamellaceae		
		Ancylistaceae	<i>Conidiobolus</i>	<i>C. coronatus</i> ²
		Basidiobolaceae	<i>Basidiobolus</i>	<i>B. ranarum</i> ²

¹Mucormycosis: Infections caused by Mucorales; ²Zygomycosis: Infections caused by Zygomycetes (Mucorales & Entomophthorales).

caused by fungi of the order Mucorales^[4]. However, it is often inappropriately interchanged with the term zygomycosis (infection caused by the class Zygomycetes) in medical literature. The Zygomycetes are divided into two orders: the Entomophthorales, containing very rare pathogenic species; and the Mucorales, which contain the most common human pathogens, including *Rhizopus*, *Mucor*, *Absidia*, and *Cunninghamellaceae* (Table 1)^[3,4]. Differentiation among these species is based on the morphology of the asexual cycle, physiologic characteristics, and zygospore production. Nonetheless, they rarely cause disease because of the low virulence of the organisms and thus they mainly affect immunocompromised individuals. Novel immunosuppressive therapies and other advances in the understanding of disease pathology and diagnosis have contributed to patients living longer with previously debilitating medical problems and impaired immune systems.

The most common agent of mucormycosis is the *Rhizopus* species, which is considerably more virulent than other fungi in the order. Similar to *Aspergillus*, the pathogenesis may be attributed to the tendency to be angioinvasive (vasculotropism) as blood vessels are the best source of oxygen, resulting in local ischemia, necrosis, and tissue infarction as well as providing the nidus for hematogenous dissemination^[3]. Though being less clearly understood than for aspergillosis, several risk factors or predisposing conditions have been well described in the literature. In a large review of 929 patients with zygomycosis^[6], diabetes was the most common underlying risk factor (36%), followed by malignancy (17%), solid organ transplantation (7%), desferrioxamine therapy (6%), and bone marrow transplantation (5%). Most of these conditions are associated with impairment of normal leukocyte immune function. Impairment of this function or a critical decline in white cell number is associated with increased risk for invasive fungal infections. Apart from differences in environmental factors, changes in transplantation procedures, new use of immunosuppressives and the use of voriconazole for the prophylaxis of opportunistic fungal infections have been proposed as risk factors for developing zygomycosis^[7,8]. Voriconazole is not active against Zygomycetes and subsequently may provide selective growth of these fungi. Breakthrough zygomycosis after voriconazole treatment has been re-

ported but the association is still uncertain^[7,9].

Mucormycosis can manifest as different clinical forms, namely rhinocerebral, pulmonary, cutaneous, gastrointestinal, central nervous system, and disseminated/miscellaneous. Gastrointestinal mucormycosis is the rarest form and constitutes only 7% of all mucormycosis cases^[6,10]. It most commonly involves the stomach (57.5%), followed by colon (32.3%) and ileum (6.9%)^[10]. This rare and opportunistic infection has been reported in neonates, probably due to their immature immunity, presenting as necrotizing enterocolitis. In published literature, less than 20 cases of gastrointestinal mucormycosis with colon involvement in adult patients have been published in the last two decades (Table 2)^[10-22]. It also occurs in those with severe malnutrition and intrinsic abnormalities of the gastrointestinal tract, including amoebic colitis, typhoid, pellagra and kwashiorkor^[10]. The infection may arise from ingestion of fungal spores on food or contaminated sputum. Actually, the three patients we have described here were also reported in an outbreak of intestinal infection in our hospital^[23]. They had sole intestinal involvement, likely due to the intake of contaminated allopurinol tablets and commercially packaged food items^[23].

A typical gastrointestinal lesion consists of a dark ulcer with sharply demarcated edges and with necrosis and thrombosis in adjacent vessels. The infection can extend from the lumen of the gut and may cause obstruction, perforation or bleeding. Initial presentations may be abdominal pain and distension, fever, and diarrhea. If there is extensive bowel involvement with multiple ulcers caused by the fungal infection, it may present with gastrointestinal bleeding or even visceral perforation at late presentation^[10]. Consequently, neutropenic fever can be a common presenting feature of mucormycosis, as in our patients. A high degree of clinical suspicion is needed to diagnose this rare condition. Therefore, persistent severe abdominal pain in a patient with neutropenia should alert the clinicians to the possibility of this invasive fungal infection.

Diagnosis depends on histological examination for the presence of predominantly aseptate wide hyphae with focal bulbous and non dichotomous branching occasionally at right angles^[4]. Over 94% of sampled tissues also show infarction and angioinvasion on histology

Table 2 Summary of colonic mucormycosis cases reported in the recent medical literature

Age (yr)/Sex	Morbidity	Location	Operation	Medications	Outcome	Ref.	Yr
21/F	APL	Cecum	Colectomy	Amp B	Survived	[11]	1985
21/F	AML	Descending	Hartmann	Amp B	Survived	[12]	1986
54/F	ALL	Cecum	Right hemicolectomy	Amp B	Dead	[13]	1998
42/F	SLE	Left colon + stomach	Subtotal colectomy	Amp B	Dead	[14]	1998
35/M	Liver Tx	Cecum + liver	Primary repair	-	Dead	[15]	1999
53/F	NHL	Sigmoid + transverse	Hartmann + right hemicolectomy	Amp B → Lip Amp + GMCSF	Survived	[16]	2000
48/F	ALL	Ileocolic + liver	Bowel resection	Amp B → Lip Amp	Dead	[17]	2000
33/M	Renal Tx	Right colon + esophagus	Graft nephrectomy	Amp B + GMCSF	Survived	[18]	2001
65/M	COAD	Right colon	Right hemicolectomy	Amp B	Survived	[19]	2004
56/F	ALL	Ileocolic	Right hemicolectomy	Lip Amp	Dead	[20]	2005
43/F	Renal Tx	Cecum	Right hemicolectomy	Lip Amp	Survived	[21]	2005
58/?	MS	Sigmoid	Hartmann	-	Dead	[22]	2006

APL: Acute progranulocytic leukemia; AML: Acute myeloid leukemia; ALL: Acute lymphocytic leukemia; COAD: Chronic obstructive airways disease; SLE: Systemic lupus erythematosus; Liver Tx: Liver transplant; Renal Tx: Renal transplant; NHL: Non-Hodgkin's lymphoma; MS: Multiple sclerosis; Amp B: Amphotericin B; Lip Amp: Liposomal amphotericin preparation; GMCSF: Granulocyte macrophage-colony stimulating factor.

examination^[24]. Though culture remains the predominant way in which one can identify fungal species, it is positive in only 52% of autopsy cases and only 30% of surgical specimens^[25]. This is because the infection may be localized and cannot be detected in all portions of the specimen submitted for culture. Nowadays there are no reliable serologic or skin tests for mucormycosis. Recently, some have attempted to improve the diagnosis by detecting fungal nucleic acid in the serum using polymerase chain reaction (PCR) or in situ hybridization techniques^[3]. As an adjuvant diagnostic tool, this can be used for confirming the presence of presumptive organisms when histology is positive and cultures are negative. It may provide some guidance in selecting appropriate antifungal therapy when the histological diagnosis is undetermined. Besides pathological and microbiological diagnosis, CT/MRI scan^[26] and endoscopy examination^[11,14] have been described in the literature. Endoscopic features of colonic mucormycosis have been reported as hemorrhagic, edematous mucosa with erosions, resembling endoscopic features of ischemic colitis. A smooth, mushroom-like greenish fungal mass with a small base of attachment to the bowel wall may be seen. Occasionally a black crust overlies the ulcerated area^[11].

From a surgeon's point of view, the principal of management of colonic mucormycosis in these severely immunocompromised patients largely depends on timely diagnosis, reversal of the underlying predisposing conditions, early surgical debridement, and rapid initiation of effective systemic antifungal therapy^[3]. As neutropenic enterocolitis is the commonest cause in the neutropenic cancer patient, careful serial clinical observation with liberal use of CT scanning is suggested^[27,28]. Contrast CT scans may show a thickened colonic wall with decreased attenuation due to edema, necrosis, or a collection of extraluminal fluid^[28]. Also, the scan may detect the presence of a small amount of free gas or pneumatosis intestinalis not visible on plain films, suggestive of bowel perforation. If the initial conservative treatment is not

successful, including bowel rest, hydration, and broad-spectrum antibiotics, some have advocated a more aggressive surgical intervention^[29-31]. As for our patients, they were initially managed in their medical wards for chemotherapy-induced neutropenic colitis; however, our surgical team was consulted after failed conservative treatment.

In the first patient of our series, exploratory laparotomy was selected for investigating suspected bowel perforation in view of persistent peritoneal signs and turbid peritoneal fluid aspirated. However, only dilated cecum with edematous wall but no perforation were identified; the intraoperative diagnosis of neutropenic enterocolitis was compatible with this finding, so that only peritoneal lavage was performed and conservative management was continued afterwards. Although the patient's condition was rapidly deteriorating, no surgical intervention was considered in view of multiple organ failure.

Continuing aggressive medical treatment and supportive measures after surgery for neutropenic enterocolitis have been reported, with high mortality^[28]. Fungal neutropenic enterocolitis has been reported as fatal^[32] as the mucormycosis is a highly angioinvasive infection, resulting in extensive thrombosis and tissue necrosis, and antifungal agents often display poor penetration at the site of infection. Removal of as much of the infected or devitalized tissue as possible while the infection is localized provides the greatest benefit. In the study by Roden *et al*^[6], the survival rate was 57% (51/90) for those treated with surgery alone and 62% (369/596) for those treated with some form of antifungal therapy alone. However, the survival increased to 70% (328/470) for those treated with combined surgery and antifungal therapy. In our third patient, the diagnosis of mucormycosis was confirmed pathologically soon after the first operation, and he began antifungal therapy immediately. Currently, the recommended antifungal therapy for mucormycosis includes amphotericin B and its liposomal preparation which can be delivered with reduced nephrotoxicity^[3]. The common prophylactic antifungal agents used in neu-

tropenic patients (such as fluconazole and itraconazole) or even new agents (voriconazole and caspofungin) are not active against the Mucorales order in clinical and *in vitro* studies. An orally available broad-spectrum investigational triazole, posaconazole, seems to possess activity against this fungus and improves patients' survival in refractory cases^[33]. Furthermore, in addition to posaconazole treatment, another exploratory laparotomy with extensive small bowel resection and omentectomy was performed in our patient, in order to debride all infection foci. This approach has been reported as providing a survival advantage in the published literature^[16,19,21].

Apart from early diagnosis, as mentioned before, correction of any reversible predisposing factors, such as by rectifying diabetic ketoacidosis, withdrawing desferrioxamine therapy or reducing the level of immunosuppression, is an important consideration in patient management. In neutropenic patients, resolution of neutropenia is directly correlated with clinical improvement and better outcomes^[34]. Without neutrophil recovery, antifungal drugs are ineffective. Cytokines, such as interferon- γ and granulocyte macrophage-colony stimulating factor (GMC-SF), can reduce the degree and duration of neutropenia and subsequent infections in these patients. Other non-medication-based interventions, such as hyperbaric oxygen therapy^[35] and novel iron chelators^[36], have found limited success as an adjunctive treatment.

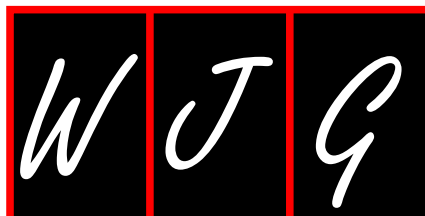
In summary, we report three cases of ileocolic mucormycosis presenting as acute abdomen in neutropenic patients which failed to respond to combined surgical and medical treatment. Though the new antifungal agent posaconazole is effective against the Mucorales order of fungi, timely diagnosis and adequate surgical debridement are essential for the successful management of colonic mucormycosis.

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Conference

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Carolina, United States
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Meeting

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2010

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- 3 **Tian D**, Araki H, Stahl E, Bergelson J, Kreitman M. Signature of balancing selection in Arabidopsis. *Proc Natl Acad Sci USA* 2006; In press

Organization as author

- 4 **Diabetes Prevention Program Research Group**. Hypertension, insulin, and proinsulin in participants with impaired glucose tolerance. *Hypertension* 2002; **40**: 679-686 [PMID: 12411462 PMID:2516377 DOI:10.1161/01.HYP.0000035706.28494.09]

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- 5 **Vallancien G**, Emberton M, Harving N, van Moorselaar RJ; Alf-One Study Group. Sexual dysfunction in 1, 274 European men suffering from lower urinary tract symptoms. *J Urol* 2003; **169**: 2257-2261 [PMID: 12771764 DOI:10.1097/01.ju.0000067940.76090.73]

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- 13 **Harnden P**, Joffe JK, Jones WG, editors. Germ cell tumours V. Proceedings of the 5th Germ cell tumours Conference; 2001 Sep 13-15; Leeds, UK. New York: Springer, 2002: 30-56

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- 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/eid.htm>

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

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Steatosis as a co-factor in chronic liver diseases

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INTRODUCTION

Lipid accumulation in the liver, so-called hepatic steatosis or non-alcoholic fatty liver disease (NAFLD), is a common condition frequently found in healthy subjects not affected by any other liver disease and not drinking alcohol. Hepatic steatosis prevalence has been estimated by magnetic resonance studies to be 35% in the general population and to be 75% in obese persons, and these figures seem to be increasing over time^[1,2]. Nevertheless, its presence generates liver damage in only a small percentage of subjects not affected by other liver diseases. In fact, in only 2% of the general population does hepatic steatosis constitute a real hepatic disease: non-alcoholic steatohepatitis (NASH) with deranged aminotransferases and fibrosis.

Steatosis is also frequently found in liver specimens of patients who have undergone liver biopsy for all the hepatic diseases [hepatitis C virus (HCV), hepatitis B virus (HBV), alcoholism, hemochromatosis *etc.*] and, in some of them (12%), its presence represents an associated disease: steatohepatitis^[3]. Moreover, it is well known that a fatty liver is less protected from the mechanisms of inflammation and fibrosis^[4].

Data reported in the literature suggest that hepatic steatosis can have different influences on a liver affected by other diseases. Therefore, it cannot always be considered as a "benign" condition and simply ignored. On the contrary, it has to be recognized as a "co-factor" capable of affecting the gravity and progression, and also therapeutic perspectives, of liver diseases.

Abstract

The finding of lipid accumulation in the liver, so-called hepatic steatosis or non-alcoholic fatty liver disease, is a common condition frequently found in healthy subjects. Its prevalence, in fact, has been estimated by magnetic resonance studies to be about 35% in the general population and 75% in obese persons. Nevertheless, its presence generates liver damage only in a small percentage of subjects not affected by other liver diseases. It should be defined as a "co-factor" capable of affecting severity and progression, and also therapeutic perspectives, of liver diseases to which it is associated. Herein we will evaluate the impact of hepatic steatosis and obesity on the most common liver diseases: chronic viral hepatitis C and B, and alcoholic liver disease.

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Key words: Liver; Steatosis; Non-alcoholic fatty liver disease

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Herein we will evaluate the impact of hepatic steatosis and obesity on the most common liver diseases: chronic viral hepatitis C and B, and alcoholic liver disease.

HCV AND STEATOSIS

Hepatic steatosis is present in about 50% of liver specimens taken from subjects affected by chronic hepatitis C^[5]. This prevalence is higher than that reported in the general population from magnetic resonance studies, and there are findings that suggest a possible direct role of the HCV on its development^[6]. This hypothesis is supported by a large number of studies that link steatosis to both host characteristics [i.e. body mass index (BMI), waist circumference (WC), insulin resistance (IR)] and viral factors (genotype, viral RNA load).

Host factors

Several risk factors for liver disease progression are known for both chronic hepatitis C (CHC) and NAFLD. These factors are: high BMI, type 2 diabetes mellitus, older age and alcohol consumption and these can also affect the presence and the gravity of hepatic steatosis^[7]. In fact, overweight patients with HCV have more steatosis than lean subjects with CHC and this data has statistical relevance independently from the HCV genotype^[8].

Viral factors and genotypes

It is well known that the presence of steatosis in the liver of CHC patients seems to be related to the presence of virus itself, with a distinctive genotype specificity^[8]. Six different HCV genotypes were identified according to Simmonds *et al.*^[9], characterized by different epidemiological and clinical peculiarities. In particular, the genotypes 1 and 3, the most prevalent in the western world, were found related to steatosis^[6]. HCV virus has, in fact, demonstrated a direct steatogenic effect in cell cultures and transgenic mice^[10,11]. This seems to be confirmed in human studies: there is considerable evidence that in HCV-infected subjects (particularly genotype 3-infected), the grade of hepatic steatosis seems to be related to viral load^[12]. Moreover, there has been evaluation of steatosis disappearing in response to antiviral therapy and its recurrence in the case of relapse with virus reappearance in the liver^[13]. However, “non-3 genotypes” (particularly genotype 1b) also show a distinct association with steatosis, which has encouraged some authors to coin a new definition: virus-associated steatohepatitis (VASH)^[14,15].

Even if the recent literature reports steatosis as “viral” only in genotype 3-infected patients and as “metabolic” (due to host characteristics) in non-3 genotype-infected patients, it should be more accurate to talk about a combination of these two factors. In fact, some studies observed that in non-3 genotype-infected patients who achieved sustained virological response (SVR) there was a reduction or a total disappearance of liver steatosis. In

particular, one study reported a reduction of steatosis in about 46% of such patients and its disappearance in 29%. Moreover, in the same study the genotype 3 patients markedly reduced their liver steatosis after weight loss, confirming that host factors could also be actively involved in generating steatosis in these patients^[16]. To complete this topic, we should also consider the difficulty in obtaining an accurate evaluation of alcohol consumption history in these patients, as documented by the discordant data with regard to the contribution of alcohol intake to steatosis development in HCV patients^[17-19].

Physiopathology

The physiopathological mechanisms associated with steatosis and obesity leading to liver disease worsening are still under debate. Nevertheless, some plausible assumptions have been made and they seem to be supported by experimental evidence from cellular and animal models. Some of these involve oxidative stress, subsinusoidal stellate cell activation, higher apoptosis susceptibility and altered response to cellular damage. Furthermore, fibrogenesis could be exacerbated by other components of metabolic syndrome (MS) such as hyperinsulinemia and hyperglycemia.

Oxidative stress: Fatty liver seems to be more susceptible to the damage induced by such factors that lead to an increase in liver production of oxidant substances. In particular, on the basis of the immune response to HCV, it was postulated that an increase in oxidative stress produces an intensified lipoperoxidation and inflammatory cytokine production leading to programmed cell death, i.e. apoptosis^[20].

Apoptosis: Apoptosis has a central role in liver disease progression and steatosis^[21]. In CHC, a significant increment of this cellular mechanism together with stimulation of fibrosis and inflammatory activity can be observed in liver specimens which also show moderate or severe steatosis, thus suggesting a similar role of apoptosis to that suggested by the histological presentation in NASH^[22]. This mechanism might involve a synergistic effect of apoptosis acting together with other intrinsic HCV factors of liver damage and thus leading to the worsening of fibrosis progression.

Steatohepatitis: 6%^[23] to 18%^[24] of HCV patients with steatosis have an associated steatohepatitis observed in liver specimens. This could be a direct consequence of oxidative stress and lipoperoxidation^[25]. Nevertheless, HCV interference in the production of inflammatory cytokines such as tumor necrosis factor α (TNF- α), transforming growth factor β (TGF- β), interleukin-1 (IL-1) and interleukin-6 (IL-6) might, *per se*, explain the worsening of steatosis and fibrosis in both “viral” and “non viral” steatosis^[26,27]. However, these mechanisms might act in synergism to worsen fibrosis. Indeed, it has

been demonstrated that there is a significant reduction of both steatosis and portal fibrosis after weight loss by diet^[28] or bariatric surgery^[29] in HCV patients.

Insulin resistance: In the field of this research, it has recently been greatly debated as to whether HCV virus exerts its effects on the liver (and on the organism) by involving the intracellular molecular cascade that follows the activation of insulin receptor after its binding with insulin^[30]. HCV virus might interfere with this cascade in a genotype-specific manner^[31-33]. However, this interference (regardless of genotype) could lead to hepatic steatosis (“viral” at least) and worsening of liver fibrosis by direct stimulation resulting from the action of hyperinsulinemia on hepatic stellate sub-sinusoidal cells, with an increase on extracellular matrix production^[34].

The supposed insulin resistance mechanism involves the reduction of expression of insulin receptor substrates 1 and 2 (IRS1 and 2), which are crucial proteins in the post-receptorial cascade of insulin^[35]. The decrease of IRS1 and IRS2 seems to be mediated by a direct over-expression of another intracellular protein: suppressor of cytokine signaling 3 (SOCS3)^[31-33]. This over-expression has been revealed only in genotype 1b patients and it is associated with metabolic syndrome and no response to antiviral therapy^[32]. Recently, this evidence *in vivo* was reproduced *in vitro*. In fact, it was demonstrated that HepG2 cells infected with genotype 1 positive sera had higher SOCS3 levels than those infected with genotype 2 positive sera and that this was associated with a lower IRS1 expression^[36]. Therefore, there is accumulating evidence of a direct “metabolic” effect of the HCV virus on a large number of molecular pathways that lead to hepatic steatosis, IR, MS, and liver fibrosis^[37,38]. TNF- α , an inflammatory cytokine produced by hepatic stellate cells and adipose tissue, is increased in HCV patients with steatosis^[26] and its serum increase, together with the decrease of the adipocytokine, adiponectin, was also demonstrated as being involved in the pathogenesis of NAFLD and IR^[27,39]. The imbalance between TNF- α and adiponectin serum levels in HCV/steatosis patients seems to be genotype-specific and correlated with the severity of liver steatosis^[20] (Figure 1). These findings, taken together, suggest a definite association between IR and steatosis/steatohepatitis with the HCV virus as the “third player”. However, it is still unclear if steatosis represents the first hit to cytokine production leading to IR or the other way around; IR *via* cytokine inflammatory pathways leading to hepatic steatosis. Recent evidence, discriminating between “systemic” and “hepatic” IR, showed that in young, lean, insulin-resistant subjects there was a low prevalence of liver steatosis and no cytokine/adipocytokine changes. This suggests that steatosis and cytokines interact without assuming a primary and independent role in the early stage of IR^[40]. On the other hand, other authors support the idea that hyperinsulinemia is likely to be the consequence rather than the cause of a fatty liver, as suggested by the fact that fatty liver is associated with both hepatic insulin resistance and impaired insulin clearance^[41,42].

From the observations reported above we can confirm that in CHC, hepatic steatosis has to be considered as an important co-factor in the worsening of liver disease. This co-factor should be inhibited by firstly correcting its possible causes, such as being overweight or obese. This suggestion is supported by data from the literature showing that, in patients with MS or IR, a small weight loss (about 4%-5%), in the same way as it acts on blood pressure and glycemic control^[41], can induce steatosis reduction even if BMI is not restored to normal levels^[28,42]. These factors can influence the response to antiviral treatment: it has in fact been demonstrated that the absence or the presence of lower levels of steatosis are positive predictors of achieving SVR in HCV patients^[16,43,44].

STEATOSIS AND HBV

Given the assumed relationship between HCV and steatosis as specified above, we could surmise that chronic hepatitis B (CHB) has an analogous association with steatosis. Only a small number of studies regarding this argument have been published and some of these report steatosis prevalence in CHB^[45,46] while others report a direct comparison between CHC and CHB for the prevalence of MS, obesity and steatosis^[47,48]. These studies report a steatosis prevalence in CHB patients similar to that of the general population. The presence of steatosis correlates with BMI and MS diagnostic criteria (waist circumference, high blood pressure and dyslipidemia), but not with viral genotype or viral load. Moreover, steatosis does not correlate with fibrosis^[45]. Accordingly, these data indicate that the association between steatosis and HCV is specific, whereas this is not the case in HBV-infected patients. Nevertheless, it seems to be appropriate to hypothesize that in HBV-related chronic hepatitis, steatosis is probably also a co-factor of liver disease worsening, particularly if it is characterized as related to a conspicuous entity and/or in a subject with MS and/or if it translates to the real clinical condition of associated steatohepatitis^[49].

STEATOSIS AND ALCOHOL

The typical histological pattern of alcoholic liver disease (defined as drinking more than 40 g a day in males and 30 g in females) is a steatohepatitis^[50]. In fact, a diagnosis of NASH is established when, simultaneous with clinical and histological observations of a hepatic liver disease that is quite indistinguishable from an alcohol-related disease, there is a total assurance of alcoholic abstinence^[51]. Therefore, it is clear that there is a close association between steatosis and alcoholic liver disease, so close that steatosis cannot be considered as a simple co-factor but as part of the histological damage presentation in this liver disease. Nevertheless, nothing prevents us from supposing that obesity might also increase the severity of steatosis in patients with alcoholic liver disease. Indeed, this hypothesis has been confirmed by some literature findings which indicated that being overweight was

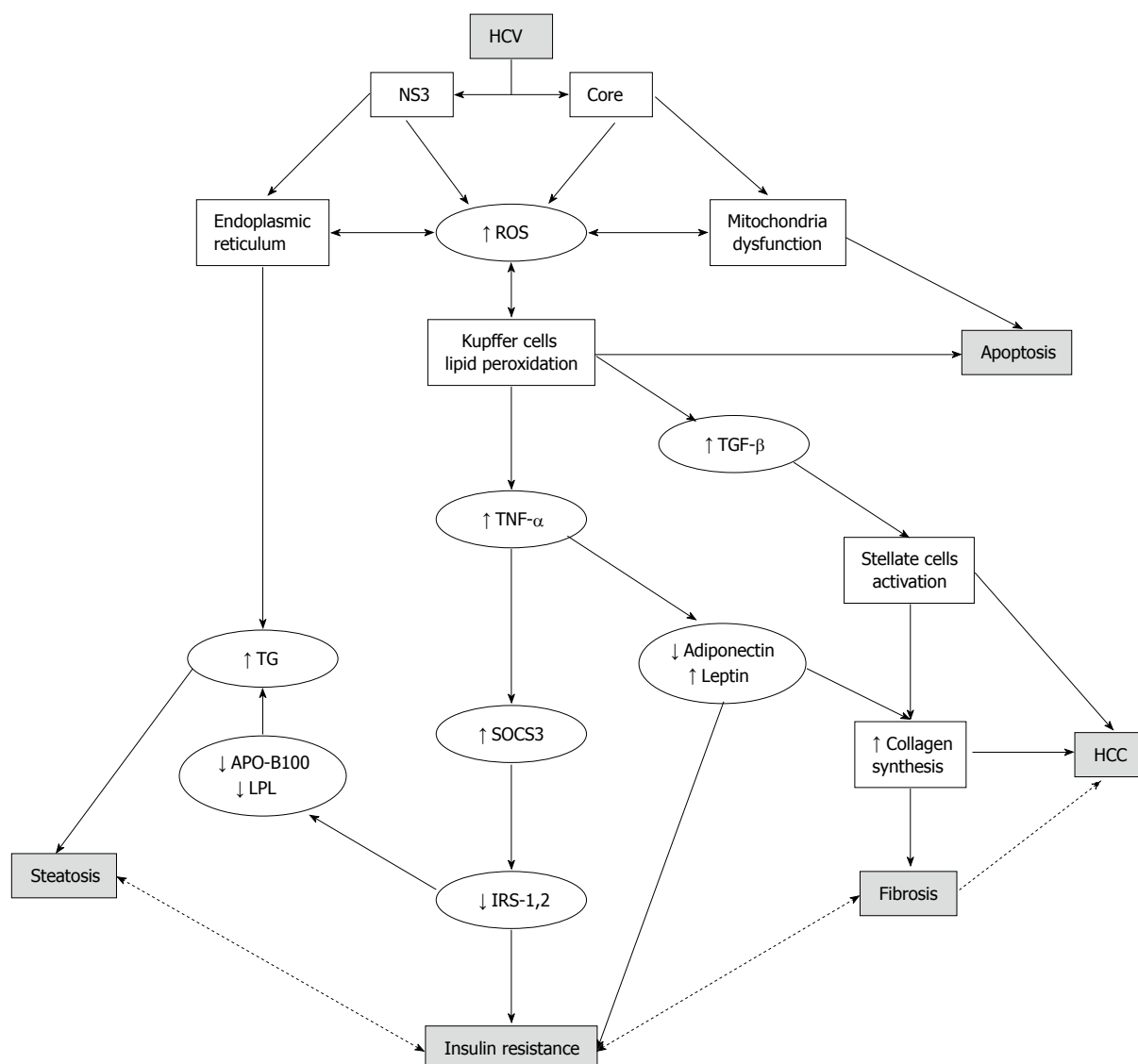


Figure 1 Hypothetical physiopathological pathways leading to insulin resistance, steatosis, fibrosis, hepatocellular carcinoma (HCC), apoptosis and steatosis in hepatitis C virus (HCV) infection. NS3: Non structural HCV protein 3; Core: Core HCV protein; ROS: Reactive oxygen species; TNF- α : Tumor necrosis factor α ; TGF- β : Transforming growth factor β ; TG: Triglycerides; LPL: Lipo-protein-lipase; SOCS3: Signaling of cytokine suppressor type 3; IRS 1-2: Insulin receptor substrate type 1 and 2.

associated with extent of steatosis^[52] and that it was a risk factor for progression to cirrhosis and HCC in alcoholic liver disease^[53,54]. Moreover, obesity and steatosis seem to use the same metabolic mechanisms to produce a worsening of liver damage; increased lipoperoxidation and pro-inflammatory cytokine production have been noted in obese alcoholics^[55,56]. On these grounds, steatosis seems to have a double role: as a constantly present histological finding and as a possible co-factor responsible for worsening of histological damage. Thus, correct management of these patients should also include adequate dietetic counseling.

HEPATOCELLULAR CARCINOMA AND STEATOSIS

Further evidence from the literature indicates that

steatosis, together with obesity and type 2 diabetes mellitus, is a probable risk factor for hepatocellular carcinoma (HCC) development. This has been reported in HCV liver disease in both experimental models^[57] and clinical studies^[58]. Steatosis seems to play the same role also in alcoholic liver disease: a large multicenter study conducted in 19 000 liver transplant patients indicates that obesity and steatosis are risk factors for HCC developing in alcoholic liver disease patients^[59].

CONCLUSION

From the data analyzed in this brief review, we can conclude that hepatic steatosis, a risk-free, benign condition in healthy subjects, becomes a dangerous co-factor of disease progression when it is present in patients affected by another liver disease. It affects the response to

antiviral treatment in HCV patients, and it could also be responsible for the development of HCC, in both HCV and alcoholic patients. Therefore, hepatic steatosis must be considered an important disease co-factor to be taken into account in liver disease patients, in order to make a diagnosis and subsequently to correct the condition.

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Mechanisms of resistance to anti-EGFR monoclonal antibody treatment in metastatic colorectal cancer

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mCRC treatment and has given clinicians more rational options for treating this illness.

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Abstract

Metastatic colorectal cancer (mCRC) continues to be counted as a major health problem. The introduction of newer cytotoxics, irinotecan and oxaliplatin, has achieved a significant improvement in survival rates. Novel targeted therapies (bevacizumab, and cetuximab) in combination with most efficient chemotherapy regimens have pushed the median survival beyond the 2-year mark and increased the proportion of patients which could benefit from resection of metastatic lesions. In addition, several studies have proved that the CRC mutation profiles should influence patient selection or stratification in prospective trials. *KRAS* mutational status represents a paradigm for biomarker development in the era of molecular targeted therapies. The present article is an overview of the most important studies in the development of biomarkers for the optimization of anti-epidermal growth factor receptor (anti-EGFR) treatment in mCRC, beyond *KRAS* mutations, which is a work in progress. The aim will be to identify molecular markers that might be used to select patients with a higher probability of response to anti-EGFR monoclonal antibodies. Overall the accumulating evidence of the molecular biology of CRC has substantially changed the approach to

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INTRODUCTION

Now, more than ever, clinical oncologists are struggling to optimize treatment in cancer patients. With the use of molecular targeted agents and the incorporation of pharmacogenetics and pharmacogenomics in basic cancer treatment, a meaningful relationship between genotype (polymorphisms and mutations), gene expression profiles (level of gene expression of all or of target genes in the genome) and phenotype is being established, aimed at interpreting the variability among individuals in terms of response, resistance and toxicity to different drugs^[1,2].

Pharmacogenetics (e.g. toxicity, age, comorbidities) commonly refers to the effects of a limited number of genes most often associated with drug metabolism, whereas pharmacogenomics (e.g. activity/resistance, gene expression level of all or targeted genes) involves

the study of multigene patterns and pathways within the genome^[3]. Genetic polymorphisms (variants in individual genomes, present in more than 1.5% of the population), somatic mutations in key target genes and differences in gene copy numbers may be responsible for different functional molecular roles and contribute to variability in drug pharmacokinetic and pharmacodynamic processes, altered drug metabolism or activation^[4]. In colorectal cancer (CRC), as well as in other types of cancer, it has long been recognized that the same medications cause different responses in different patients. Genetic variations in drug targets and genes affecting target signal transduction can have a profound effect on drug efficacy and toxicity. This information could help to identify patients who are at increased risk of toxicity and select those likely to respond to specific agents, so that a more patient-specific treatment approach can be initiated^[5].

The epidermal growth factor receptor (EGFR) belongs to the erbB receptor tyrosine kinase family which consists of 4 related transmembrane receptors: erbB1 (EGFR or HER1), erbB2 (HER2/*neu*), erbB3 (HER3) and erbB4 (HER4). Upon ligand binding, EGFR homo- or hetero-dimerizes with other erbB family members and initiates signaling through 2 main intracellular cascades which are mostly involved in cell survival, proliferation and motility. On one side, membrane localization of the lipid kinase PIK3CA counteracts PTEN and promotes AKT1 phosphorylation, and on the other, KRAS activates BRAF, which in turn triggers the mitogen-activated protein kinases^[6]. EGFR is found to be overexpressed in various human malignancies, including CRC, lung, head and neck cancers and, as was initially hypothesized, therapeutic strategies designed to disrupt EGFR function could have anti-tumor activity^[7] (Figure 1).

Two monoclonal antibodies (moAbs) targeting EGFR, the chimeric IgG1 moAb cetuximab and the fully humanized IgG2 moAb panitumumab, have recently entered clinical practice in the metastatic CRC (mCRC) setting. Both bind to the extracellular domain of the EGFR, thus leading to inhibition of its downstream signaling and have been found to provide a modest clinical benefit in pretreated patients^[8-10]. Although they were initially registered for patients whose tumors were found to express the EGFR protein in immunohistochemistry, subsequently, it was clearly demonstrated that this methodology was not adequate to predict treatment efficacy^[11]. Only the development of a skin rash was consistently associated with an increased response rate and progression-free survival in patients treated with anti-EGFR moAbs^[8,10].

Although anti-EGFR therapies are active in some patients, the disease eventually becomes refractory to therapy in nearly all patients. As clinical parameters seem to be inadequate for patient selection, a major challenge is the identification of specific biomarkers that are likely to predict which patients will achieve the best response to such a treatment. EGFR gene status, as it is evaluated by fluorescent or chromogenic *in situ* hybridization (FISH or CISH), the absence or presence of mutations in genes downstream of EGFR and the presence of germline polymor-

phisms are implicated in response to anti-EGFR treatment and can independently impair or enhance its efficacy^[12-15]. As most available data has come from retrospective studies, validation in prospective trials is imperative.

MECHANISMS OF RESISTANCE

Mutations

KRAS mutations: *KRAS* proto-oncogene encodes K-ras G-protein which plays a critical key role in the Ras/mitogen-activated protein kinase (MAPK) signaling pathway located downstream of many growth factor receptors including EGFR and which is involved in CRC carcinogenesis. K-ras recruitment by the activated EGFR is responsible for the activation of a cascade of serine-threonine kinases from the cell surface to the nucleus. *KRAS* mutations (in exon 2, codons 12 and 13) are present in more than one third of CRC patients and lead to the activation of one of the most important pathways for cell proliferation, the Ras/MAPK pathway, by inducing cyclin D1 synthesis. Consequently, in the presence of a *KRAS* mutation this pathway activation cannot be significantly inhibited by an anti-EGFR moAb (cetuximab or panitumumab) which acts upstream of the K-ras protein^[13] (Figure 1).

In 2005, Moroni *et al*^[16] assessed, in a small retrospective study, the mutation status of EGFR downstream intracellular effectors *KRAS*, *BRAF* and *PIK3CA*, and for the first time a trend towards higher response was seen in cetuximab-treated CRC patients whose tumors were of wild-type (WT) *KRAS* status. Subsequently, in 2006 in a study by Lièvre *et al*^[13], *KRAS* mutations were found in 13 out of 30 tumors tested (43%) and this finding was significantly associated with the absence of response to cetuximab (*KRAS* mutation in 0% of the 11 responders *vs* 68.4% of the 19 non-responders; $P = 0.0003$). The overall survival (OS) of patients without *KRAS* mutation in their tumor was significantly higher compared with those patients with a mutation in the tumor ($P = 0.016$; median OS, 16.3 mo *vs* 6.9 mo) (Table 1).

When the results of the 2 above-mentioned studies were analyzed together, the predictive value of the *KRAS* mutation remained significant with a *KRAS* mutation frequency of 52.5% in non-responders compared with 9.5% in responders ($P = 0.001$). Thus, the probability of no response to cetuximab was 91.3% in the presence of *KRAS* mutation whereas as in the absence of such a mutation the probability of being a responder was 50%. The relative risk for a response to cetuximab was 10-fold higher for non-mutated patients compared with that of patients with the *KRAS* mutation [hazard ratio (HR), 10.5; 95% CI: 2.1-51.1]. Accordingly, in 2008, 3 studies, one with panitumumab^[14] and 2 with cetuximab^[17,18], confirmed the importance of *KRAS* mutations in the mCRC setting. In the study by Amado *et al*^[12], *KRAS* mutation status was assessed in tumor samples from mCRC patients who were enrolled in the randomized phase III trial comparing panitumumab plus best supportive care (BSC) with BSC only after failure in 5-fluorouracil (5-FU)-, oxaliplatin- and irinotecan-based chemotherapy^[10]. *KRAS* status was ascertained in 427 (92%)

EGFR pathway components and resistance to anti-EGFR moAbs

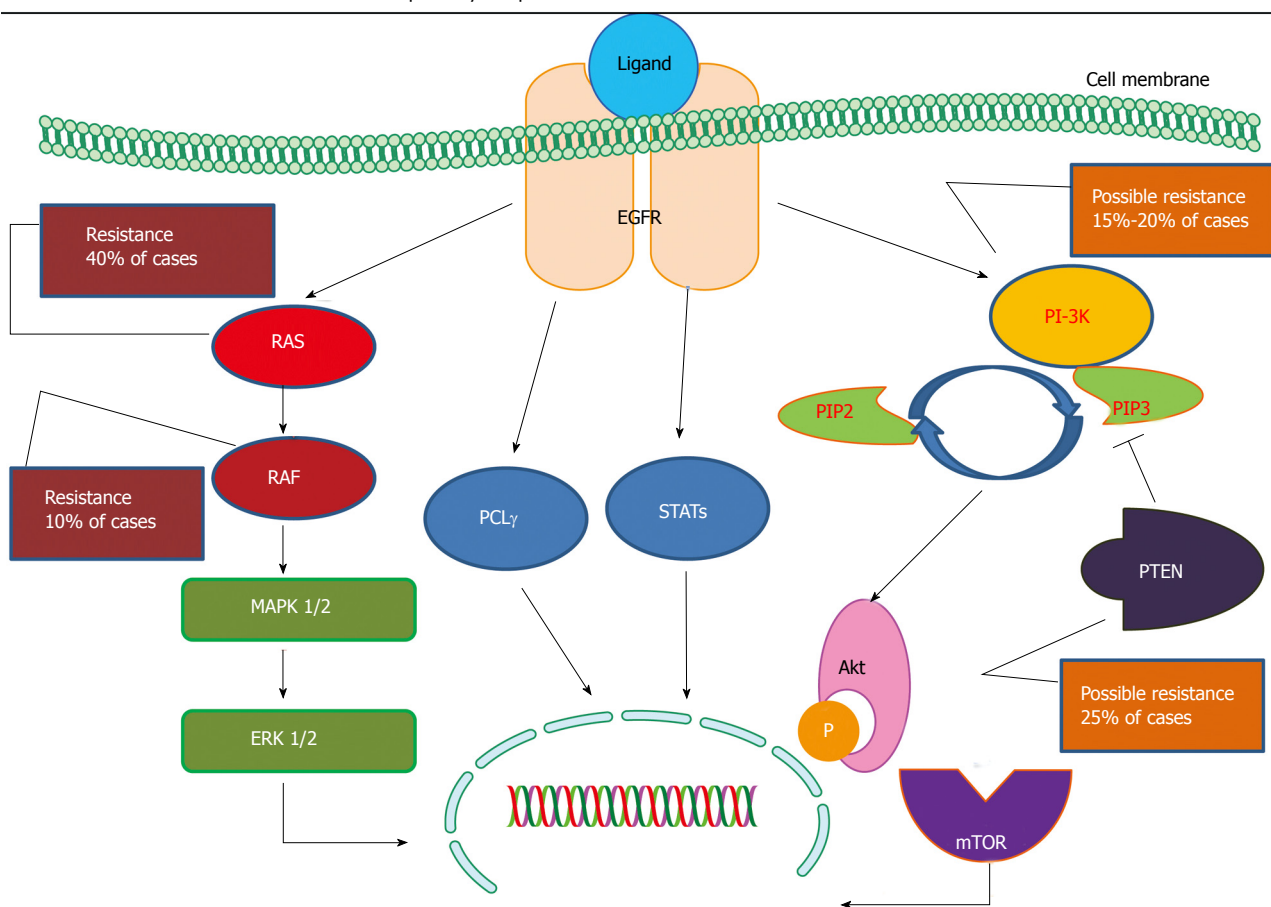


Figure 1 Simplified illustration of the epidermal growth factor receptor (EGFR) pathway with the RAS/MAPK and PIK3CA/PTEN cascades. Specific components of the pathway are correlated with resistance to anti-EGFR monoclonal antibodies (moAbs). As shown, KRAS and BRAF mutations are correlated with resistance to anti-EGFR moAbs, while further evaluation is required for PIK3CA mutations and PTEN loss of expression.

Table 1 Significance of *KRAS* mutations in retrospective single arm studies and randomized prospective trials

Published studies	n	% <i>KRAS</i> mutations	Significant correlations
Retrospective single arm studies			
Moroni <i>et al</i> ^[16]	31	32	None
Lièvre <i>et al</i> ^[15]	30	43	RR, mOS
Lièvre <i>et al</i> ^[18]	89	27	RR, PFS, mOS
De Roock <i>et al</i> ^[17]	66	40	RR, mOS
Randomized prospective trials			
Amado <i>et al</i> ^[12]	427	43	RR, PFS, mOS
Tol <i>et al</i> ^[22]	256	38	RR, PFS
Van Cutsem <i>et al</i> ^[19]	277	38	RR, PFS, mOS
Bokemeyer <i>et al</i> ^[20]	233	42	RR, PFS
Hecht <i>et al</i> ^[21]	865	40	PFS, mOS
Douillard <i>et al</i> ^[23]	1096	40	RR, PFS

RR: Response rate; mOS: Median overall survival; PFS: Progression-free survival.

of 463 patients (208 panitumumab, 219 BSC). *KRAS* mutations were found in 43% of patients. The treatment effect on progression-free survival (PFS) in the WT *KRAS* group (HR, 0.45; 95% CI: 0.34-0.59) was significantly greater ($P = 0.0001$) than in the mutation group (HR, 0.99;

95% CI: 0.73-1.36). Median PFS in the WT *KRAS* group was 12.3 wk for panitumumab and 7.3 wk for BSC. Response rates to panitumumab were 17% and 0%, for the WT and mutant groups, respectively. WT *KRAS* patients had longer overall survival (HR, 0.67; 95% CI: 0.55-0.82; treatment arms combined). No significant differences in toxicity were observed between the WT *KRAS* group and the overall population^[12]. Lièvre *et al*^[18] assessed *KRAS* status by allelic discrimination in 89 mCRC patients treated with cetuximab in 6 different institutions. *KRAS* mutations were present in 27% of the patients and were associated with resistance to cetuximab (0% *vs* 40% of responders among the 24 mutated and 65 nonmutated patients, respectively; $P < 0.001$) and a poorer outcome (median PFS, 10.1 wk *vs* 31.4 wk in patients without mutation; $P = 0.0001$; median OS, 10.1 mo *vs* 14.3 mo in patients without mutation; $P = 0.026$). When these 89 patients were analyzed together with the 30 patients from the previous study^[13], the multivariate analysis showed that *KRAS* status was an independent prognostic factor associated with OS and PFS. In a combined analysis, median OS for patients with 2, one, or no favorable prognostic factors (severe skin toxicity and absence of *KRAS* mutation) was 15.6, 10.7, and 5.6 mo, respectively. Lastly, De Roock *et al*^[17] studied the

KRAS mutation status in 113 irinotecan-refractory mCRC patients treated with cetuximab in 4 institutions and similar results were observed. Objective responses were detected in 27 of 66 WT *KRAS* patients *vs* 0 of 42 *KRAS* mutants. Median OS was significantly better in WT *KRAS* *versus* mutants (43.0 wk *vs* 27.3 wk; $P = 0.020$). In this study an additional association with radiologic response was made and it was found that the benefit was even more pronounced in patients with an early radiological response. The decrease in tumor size was significantly larger at all time points in WT patients. WT *KRAS* patients with an initial relative decrease of tumor size $> 9.66\%$ at week 6 had a significantly better median OS compared with all other patients (74.9 wk *vs* 30.6 wk; $P = 0.0000025$). Among WT *KRAS* patients OS was significantly better in patients with an initial decrease compared with those without (median OS, 74.9 wk *vs* 30.6 wk; $P = 0.00000012$). *KRAS* WT status was associated with survival benefit in cetuximab-treated mCRC^[17]. An objective response was not observed in patients with mutant tumors treated with cetuximab or panitumumab monotherapy.

The predictive significance of *KRAS* mutations was also retrospectively analyzed in 5 prospective randomized trials. The CRYSTAL^[19] trial was the first randomized trial which proved that the addition of cetuximab to a standard chemotherapy regimen (FOLFIRI) improved the response rate and PFS. Despite the statistically significant decrease in the risk of disease progression (HR, 0.85), the absolute benefit was modest (0.9 mo). Subsequently, when a patients' subpopulation was analyzed according to the *KRAS* mutation status, the benefit from the addition of cetuximab was greater (HR, 0.68) in patients with WT primary tumors. In contrast, patients with *KRAS* mutant primary tumors experienced no benefit from the addition of the moAb^[19]. Similar results have been reported from subgroup analysis in 3 other randomized trials, OPUS^[20], PACE^[21] and CAIRO2^[22], in which different combination regimens were used with anti-EGFR moAbs. Finally, the first prospective analysis of a randomized trial (PRIME) has been recently reported^[23]. The patients were randomized to receive FOLFOX4 or FOLFOX4 plus panitumumab and the *KRAS* mutation status was determined in 93% of the enrolled patients. Significant differences were observed in terms of RR (55% *vs* 48%) and PFS (9.6 mo *vs* 8.0 mo; $P = 0.0234$) in favor of the addition of panitumumab in the WT group. In contrast, a detrimental effect was recorded in the *KRAS* mutant group with the addition of the moAb to FOLFOX4 (7.3 mo *vs* 8.8 mo; $P = 0.0227$)^[23] (Table 1). In all these trials, the objective RR were comparable between patients with *KRAS* mutant and *KRAS* WT tumors treated with chemotherapy alone, indicating that *KRAS* mutations are not predictive of the response to chemotherapy. No studies have been published comparing the impact of the 7 specific *KRAS* mutations on the response to anti-EGFR moAbs.

In conclusion, the present data in the international literature suggest that *KRAS* mutations are a predictor of resistance to anti-EGFR moAb therapy and are associated with a worse prognosis and a shorter survival. Ap-

proximately 40% of mCRC patients (those with mutated *KRAS*) could be selected to avoid costly and potentially toxic treatment. WT *KRAS* status identifies mCRC patients who are likely to respond to such a treatment and, thus, have a longer OS. Prospective randomized studies are needed to validate these results which introduce a new era in mCRC targeted therapy.

***BRAF* mutations:** The role of *KRAS* has been extensively analyzed. However, *KRAS* mutations account for only 30%-40% of patients unresponsive to anti-EGFR moAbs treatment, suggesting that additional genetic determinants of resistance must exist. The RAS-RAF-MAPK kinase pathway mediates cellular responses to growth signals (Figure 1). The 3 *RAF* genes encode for cytoplasmic serine-threonine kinases that are the principal effectors of *KRAS* and are regulated by binding to it^[24]. The single substitution missense mutation V600E, located within the kinase domain of *BRAF* (one of the 3 *RAF* genes), is the most common oncogenic mutation in cancer, accounting for more than 80%. The highest frequency is detected in melanomas (about 65%), the *BRAF* V600E mutation is also found at lower frequencies in a wide range of human cancers, such as CRC (10%), gliomas, ovarian and others. The V600E amino acid change results in constitutive activation of the *BRAF* kinase and promotes cell transformation^[25,26]. *KRAS* and *BRAF* mutations are mutually exclusive in CRC^[27,28].

Di Nicolantonio *et al*^[26] retrospectively analyzed 113 mCRC tumors from cetuximab or panitumumab treated patients for *KRAS* and *BRAF* mutations and correlated the results with response, time to progression (TTP) and OS. *KRAS* mutations were present in 30% of the patients and were associated with resistance to cetuximab or panitumumab ($P = 0.011$). The *BRAF* V600E mutation was detected in 11 of 79 patients with WT *KRAS*. None of the *BRAF*-mutated patients responded to treatment, whereas none of the responders carried *BRAF* mutations ($P = 0.029$). *BRAF*-mutated patients had significantly shorter PFS ($P = 0.011$) and OS ($P < 0.0001$) than WT patients, meaning that the *BRAF* V600E mutation was inversely associated with response to anti-EGFR MoAb therapy and correlated with a worse prognosis. In CRC cell lines, the introduction/presence of the *BRAF* V600E allele impaired the therapeutic potential of cetuximab and panitumumab. Pharmacologic inhibition of *BRAF*, as initially hypothesized, restored sensitivity to anti-EGFR MoAbs in the CRC cell lines carrying the *BRAF* V600E mutation. The clinically approved small-molecule kinase *BRAF* inhibitor sorafenib when administered in combination with cetuximab slightly affected proliferation compared with sorafenib alone, whereas it showed a prominent proapoptotic effect. Thus, in the clinic the therapeutic effect of anti-EGFR MoAbs could be restored by 2-hit approaches aimed at blocking the EGFR pathway in multiple locations^[26].

In the same frame as the Di Nicolantonio *et al*^[26] study, Souglakos *et al*^[29] sought to determine retrospectively the predictive value of the *BRAF* (exon 15), *KRAS* (exon 2) and *PIK3CA* (exons 9 and 20) point mutations with re-

spect to clinical outcomes and response to active agents in 168 mCRC patients treated in the USA and Greece with 5-FU-based first-line chemotherapy (71% in combination with oxaliplatin and 34% with irinotecan and 58% with the addition of bevacizumab). In this study population, *KRAS*, *BRAF* and *PIK3CA* mutations were present in 62 (37%), 13 (8%) and 26 (15%) cases, respectively. Multivariate analysis uncovered *BRAF* mutation as an independent prognostic factor for decreased survival (HR, 3.6; 95% CI: 1.7-7.3). However, patients with *BRAF*-mutant tumors had significantly lower PFS (HR, 1.9; 95% CI: 1.03-3.5; $P < 0.0001$) than those whose primary tumors carried only WT *BRAF*. Of 100 patients treated with cetuximab and chemotherapy (8 in first-line and 92 as salvage treatment), the *KRAS* mutation predicted lack of response ($P = 0.001$) and shorter PFS ($P = 0.015$), in accordance with the international literature. *BRAF* mutations also correlated with reduced PFS in response to second-line use of cetuximab ($P < 0.001$). The likelihood of a response between patients with *BRAF*-mutant or *BRAF*-WT tumors was 0% *vs* 17%, and PFS with cetuximab-based therapy was significantly lower when tumors carried mutations in any of the 3 examined genes. *BRAF* mutations conferred a higher risk of relapse (HR, 3.9; $P = 0.0005$) after treatment with cetuximab-containing salvage combinations. These results underscore the potential of mutational profiling to help identify CRCs with different natural history or differential response to particular therapies. Lack of a cetuximab response observed with *KRAS*-mutant tumors may extend to other oncogene mutations, especially *BRAF*. The adverse significance of *BRAF* mutations should guide patient selection and stratification in future clinical trials^[29].

In CRC tumors, *BRAF* mutations are reported to occur more frequently in those cases characterized by the presence of defective DNA mismatch repair (dMMR)^[28]. Although the etiology is still ill-defined, in subsequent studies these mutations were found to occur almost exclusively in tumors showing the involvement of the *hMLH1* gene (one of the genes involved in MMR) due to promoter hypermethylation. Current studies suggest that the *BRAF* V600E mutation occurs in 10% of tumors that are proficient in the MMR pathway (microsatellite stable - MSS/low microsatellite instability - MSI-L) and in > 50% of tumors that have dMMR (high microsatellite instability - MSI-H) due to promoter hypermethylation of the *hMLH1* gene. *BRAF* mutations rarely, if ever, occur in tumors with dMMR because of the presence of germ-line mutations^[30,31]. Thus, *BRAF* V600E is tightly associated with dMMR due to *hMLH1* promoter hypermethylation and not with dMMR due to germ-line alterations.

In conclusion, it seems that the natural history and treatment response of *BRAF*-mutant tumors differ markedly from all others implying that the *BRAF* mutation does not simply substitute for *KRAS* activation in a linear signaling pathway, but likely confers additional or distinct properties, with ominous consequences. The current evidence supports that *KRAS* and *BRAF* mutations are mutually exclusive events. Of course, all these findings need to be formally confirmed prospectively

in randomized clinical trials but if they are, then patients with the *BRAF* V600E mutation might justify foregoing approved treatments in favor of investigational therapy.

***PIK3CA* mutations:** *PIK3CA* is one of the 2 most frequently mutated oncogenes in human tumors. Most of the reported mutations in the *PIK3CA* cluster are in conserved regions within the region coding for the helical and kinase domains of p110 α . These mutations constitutively activate its kinase activity and, thus, make this enzyme an ideal target for drug development^[32].

The *PIK3CA* gene encodes a lipid kinase that regulates alongside *KRAS* signaling pathways downstream of the EGFR. In addition, the p110 α subunit of phosphatidylinositol-3-kinase (PI3K) which is encoded by *PIK3CA*, can be activated by interactions with the RAS proteins^[33] (Figure 1). The *PIK3CA* gene is found mutated in approximately 20% of CRCs and the majority of the relevant mutations are located in the “hotspots” of exon 9 (E542K, E545K) and exon 20 (H1047R)^[34]. PI3K-initiated signaling is normally inhibited by PTEN (phosphate and tensin homologue deleted on chromosome ten). *In vitro* it has been shown that cell lines with activating *PIK3CA* mutations or loss of PTEN expression (PTEN null) were more resistant to cetuximab than WT *PIK3CA*/PTEN-expressing cell lines (14% \pm 5.0% *vs* 38.5% \pm 6.4% growth inhibition, mean \pm SE; $P = 0.008$). Consistently, *PIK3CA* mutant isogenic HCT116 cells showed increased resistance to cetuximab compared with WT *PIK3CA* controls. Furthermore, cell lines that were *PIK3CA* mutant/PTEN null and *RAS*/*BRAF* mutant were highly resistant to cetuximab compared with those without dual mutations/PTEN loss (10.8% \pm 4.3% *vs* 38.8% \pm 5.9% growth inhibition, respectively; $P = 0.002$), indicating that constitutive and simultaneous activation of the RAS and PIK3CA pathways confers maximal resistance to this agent^[35]. In addition, *in vivo*, Frattini *et al*^[36] have shown that loss of PTEN expression, which occurs in approximately 30% of sporadic CRC cases, may be associated with lack of response to cetuximab.

Sartore-Bianchi *et al*^[37] analyzed 110 mCRC patients treated with anti-EGFR MoAbs for mutations of the *PIK3CA* and *KRAS* genes along with PTEN expression. Fifteen *PIK3CA* (13.6%) and 32 *KRAS* (29.0%) mutations were present. *PIK3CA* mutations were significantly associated with clinical resistance to panitumumab or cetuximab. None of the mutated patients achieved an objective response ($P = 0.038$) and when only WT *KRAS* tumors were analyzed, the statistical correlation was even stronger ($P = 0.016$). Patients with *PIK3CA* mutations displayed a worse clinical outcome also in terms of PFS ($P = 0.035$). The authors conclude that these results indicate that *PIK3CA* mutations can independently hamper the therapeutic response to panitumumab or cetuximab in mCRC. When the molecular status of the *PIK3CA*/PTEN and *KRAS* pathways are concomitantly ascertained, up to 70% of mCRC patients unlikely to respond to EGFR MoAbs can be identified^[37]. In the study by Souglakos *et al*^[29], *PIK3CA* mutations were also found to be associated with reduced

PFS ($P = 0.06$), in response to second-line use of cetuximab. In addition, *PIK3CA* mutations conferred a higher risk of relapse (HR, 2.1; $P = 0.01$) after treatment with cetuximab-containing salvage combinations. However, regarding the response to first-line therapy, PFS was similar between patients whose tumors carried mutant or wild-type and *PIK3CA*^[29].

In conclusion, the sum of the published data in the international literature imply that patients with *KRAS*-, *BRAF*- or *PIK3CA*-mutant tumors may all derive little benefit from treatment with anti-EGFR MoAbs. *BRAF* or *PIK3CA* mutations may account for about 1/3 of patients whose WT *KRAS* tumors do not respond to cetuximab^[29]. A priori screening of CRC tumors for *RAS*/*BRAF*/*PIK3CA* mutations could help stratify patients likely to benefit from anti-EGFR MoAbs therapy.

Fcγ-R II a exon 4 131G>A, Fcγ-R III a exon 5 158T>G single nucleotide polymorphisms (SNPs)

As has been shown recently, antibody-dependent cell-mediated cytotoxicity (ADCC) mediated through Fc receptors plays an important role in the anti-tumor activity of IgG1 antibodies. ADCC is an immunological mechanism which involves the interaction between Fc receptors carried on the surface of immune cells such as macrophages and natural killer (NK) cells and the Fc fragment of moAbs which are bound on tumor cells^[38]. This way, moAbs may exert an indirect antitumor activity by recruiting cytotoxic host effector cells, such as monocytes and NK cells^[39]. One group of IgG Fc receptors, FcγRs are expressed on leukocytes and are composed of 3 distinct classes: FcγR I, FcγR II (FcγR IIa and FcγR IIb), FcγR III (FcγR IIIa and FcγR IIIb). The receptors are also distinguished by their affinity for IgG. FcγR I exhibits high affinity for IgG, whereas FcγR II and FcγR III show a weaker affinity. FcγR IIa and FcγR IIIa are activating FcγRs which are expressed on monocytes/macrophages and monocytes/macrophages/NK cells, respectively, and can trigger cytotoxicity of human targets^[39].

Cetuximab, an IgG1 moAb, competes with the natural ligands of EGFR, EGF and transforming growth factor- α (TGF- α). When binding to cancer cells, it inhibits EGFR dimerization and downstream signaling, thus inhibiting proliferation and inducing apoptosis^[40]. In experimental models, it has been shown that another mechanism of action of cetuximab against cancer cells is mediated *via* ADCC. The effectiveness of ADCC may depend on the degree of activation of effector cells after FcγR IIa and FcγR IIIa engagement^[41].

The binding affinity of the FcγRs is under the influence of germline genetic polymorphisms detected on genes encoding for FcγR IIa and FcγR IIIa. The SNP 131G>A (or H131R) in position 131 of exon 4 of *FcγR II a* gene which leads to the substitution of an arginine with an histidine and the SNP 158T>G (or V158F) in position 158 of *FcγR III a* gene, which leads to the substitution of a phenylalanine with a valine, are shown to affect the receptors' affinities for the Fc fragment of antibodies and probably ADCC efficiency^[42,43].

In a publication of Zhang *et al*^[44] in 39 patients with mCRC treated with cetuximab, TTP was statistically significantly better for patients with the FcγR IIa H131R SNP ($P = 0.037$) and for the FcγR IIIa V158F ($P = 0.055$). In a study by Bibeau *et al*^[45], in 69 mCRC patients treated with the combination of irinotecan and cetuximab, it was observed that the patients who were homozygous for the H/H allele of SNP FcγR IIa H131R and/or for the V/V allele of SNP FcγR IIIa V158F had a greater TTP compared with those who carried the R or F alleles (3.2 mo, 2.8 mo, respectively, $P = 0.015$). Nevertheless, the above-mentioned correlations could not be confirmed in other studies^[46,47].

Other polymorphisms

EGFR intron-1 (CA)_n repeat polymorphisms: A highly polymorphic sequence of (CA)_n repeats ($n = 15-22$) is located in intron 1 of the *EGFR* gene. Allele 16 (with 16 CA repeats) is seen more frequently (42%), followed by allele 20 (26%) and 18 (20%)^[48]. *In vivo* and *in vitro* studies have shown that transcriptional activity of the gene is affected, as a result of a variable impact on DNA binding sites, in such a way that the greater number of CA repeats reflects lower EGFR mRNA levels and protein expression^[49,50]. In a study by Amador *et al*^[51], from 19 patients with mCRC treated with gefitinib, those who had a small number of CA repeats more frequently manifested dermatologic toxicity (84% of the patients with ≤ 35 CA repeats and only 33% of those with > 35). In a study by Graziano *et al*^[46] in 110 patients with mCRC who underwent irinotecan-cetuximab salvage therapy after disease progression during or after oxaliplatin-based first-line and irinotecan-based second-line chemotherapy, a small number of intron 1 CA repeats (< 17) was correlated, in a multivariate analysis, with favorable OS (HR, 0.41; 95% CI: 0.21-1.78; $P = 0.006$), treatment response ($P = 0.008$) and more frequent grade 2 and 3 dermatologic toxicity ($P = 0.001$) compared with a larger number of CA repeats (≥ 17). Although the above-mentioned study did not have a control arm and thus the predictive role of the polymorphic repeats could not be determined, it is probable that the higher EGFR expression in patients with a small number of CA repeats could also reflect a greater effect of cetuximab^[46].

EGFR exon 13 497G>A (or R521K) SNP

Another SNP, a G>A substitution (rs11543848) in codon 521 (previously described as codon 497) in exon 13, which encodes a part of the extracellular region of the EGFR, has been described and results in an amino acid substitution of an arginine (R) with a lysine (K). This is located at the boundary between EGFR domain III (the direct interaction site with cetuximab) and domain IV^[52]. This amino acid substitution has been shown to significantly reduce TGF- α binding and ligand-induced EGFR signaling, which could make the cell even more sensitive to targeted receptor inhibition through cetuximab, for example^[52,53].

In a study by Gonçalves *et al*^[52], tumor tissue samples from patients with mCRC treated with irinotecan/ce-

tuximab were analyzed and the EGFR exon 13 variant (R521K) was associated with better PFS and OS. Indeed, the above-mentioned SNP was observed in 11 of the 21 patients who achieved an objective response or stable disease and in only 1 of the 11 patients who had disease progression ($P = 0.02$). In addition, in a third study it has been correlated with longer OS in stage II and III patients after surgery^[54]. Nevertheless, in the study by Graziano *et al.*^[46], in mCRC patients treated with irinotecan/cetuximab, this SNP was not found to be associated with response to treatment or OS.

Epidermal growth factor (EGF) 5'-UTR 61A>G SNP

EGF is one of the natural ligands of EGFR and upon binding it may activate DNA synthesis and cellular proliferation and it has been shown to stimulate mitosis in epidermal cells. The EGF protein is encoded by the *EGF* gene which is located on chromosome 4q25-27 and contains 24 exons^[55]. The only functional polymorphism of the *EGF* gene was identified in 2002 and is located 61 base pairs (bp) downstream of the EGF promoter, in the 5'-untranslated region of the gene. It consists of a substitution of guanine (G) for adenine (A), (61A>G), it modulates the transcription of EGF and it has been correlated *in vitro* and *in vivo* with elevated serum levels^[56,57]. Primarily, it has been studied in patients with melanoma and glioblastoma multiforme, but it has also been detected in 44% of the European white population^[56,57].

In the study by Graziano *et al.*^[46], in 110 patients with mCRC treated with irinotecan and cetuximab, the EGF 61G/G allele was associated with a greater OS (HR, 0.44; 95% CI: 0.23-0.84, $P = 0.01$) but not with greater response rate, PFS and skin toxicity. The exact mechanism by which this SNP is associated with greater survival is not yet known, but, in experimental models and with different concentrations, EGF has been shown to induce apoptosis and growth inhibition rather than the usual growth-promoting effect^[58]. In addition, in another study with 133 mCRC patients treated with cetuximab monotherapy the EGF 61G/G allele was associated with greater PFS ($P = 0.04$)^[47]. In contrast, in the Zhang *et al.*^[59] study with mCRC treated with cetuximab, the EGF 61A/A allele was correlated favorably with an increased OS (median OS 15 mo for EGF 61A/A, 2.3 mo for EGF 61G/G and 5.7 mo for the heterozygote EGF 61A/G).

Cyclin D1 exon 4 870A>G SNP

Cyclin D1 is a cell cycle regulatory protein whose upregulation has been associated with increased proliferation and poor clinical outcome in a number of neoplasms including CRC^[60]. Cyclin D1 is a key element in the downstream EGFR signaling pathway; EGFR inhibition results in cyclin D1 downregulation, leading cells into the G1 phase and subsequently to apoptosis^[61]. The 870A>G SNP in exon 4 of the *cyclin D1* gene (A to G substitution) influences cyclin D1 mRNA splicing in the border between exon 4 and intron 4 resulting in 2 different mRNA transcripts, a and b. The G allele encodes transcript a, whereas the A allele encodes transcript b,

which results in a longer half-life cyclin-D1^[61,62].

Zhang *et al.*^[59] in a pilot study of 39 mCRC patients under treatment with cetuximab monotherapy, found that both the cyclin-D1 870A>G SNP, as well as the EGF 61A>G SNP could be used as predictive molecular markers of cetuximab therapy. More specifically, the cyclin-D1 870A>G SNP was statistically significantly correlated with OS. Patients with the A/A genotype had a very short median OS of 2.3 mo, whereas, patients with at least one G (A/G or G/G) had a median OS of 8.7 mo ($P = 0.019$). Furthermore, when combined with EGF 61A>G, the cyclin-D1 870A>G shows an even further significant association with OS. Patients with the favorable genotypes (at least one A for the first one and one G for the second) had a median OS of 12 mo, in contrast with 4.4 mo in the patients with unfavorable genotypes^[59]. Unfortunately, Nagashima *et al.*^[47] and Graziano *et al.*^[46] did not find similar correlations in their studies.

Rare SNPs

The G765C SNP of cyclooxygenase-2 (COX-2) which has been correlated *in vitro* with reduced promoter activity and the T251A SNP of interleukin-8 which has been correlated with increased interleukin-8 production, have not been associated with response to cetuximab treatment in mCRC patients. However, the COX-2 G765C SNP has been weakly correlated with skin toxicity ($P = 0.15$)^[59]. A schematic representation of the above-mentioned SNPs is shown in Table 2.

Gene copy numbers and EGFR ligands mRNA expression:

As initially reported in a cohort study, the objective tumor response to the EGFR-targeted moAbs, cetuximab and panitumumab, in mCRC occurred in a fraction of patients whose tumors had an increased *EGFR* gene copy number (GCN), as assessed by FISH^[16]. Subsequently, in further studies, the predictive role of *EGFR* GCN was evaluated and an association with objective tumor response^[63] and OS^[63] was demonstrated.

Lièvre *et al.*^[13], using CISH instead of FISH, confirmed the results of Moroni *et al.*^[16], but both studies were inconclusive probably due to the limited number of patients tested and the non-homogeneous treatments they received. In the study by Lenz *et al.*^[63], the *EGFR* GCN was evaluated with polymerase chain reaction (PCR) and although no association was detected with objective responses and PFS, increased GCN was significantly positively correlated with OS. The discrepancies between these studies could be a result of different techniques or sample limitations, but the association of *EGFR* GCN with OS could also reflect its role as an independent prognostic variable^[13,16,63].

In an attempt to test *EGFR* GCN in a larger and more homogeneous patient population and clarify its predictive role in terms of OR, PFS and OS Sartore-Bianchi *et al.*^[14] analyzed mCRC patients' tumors from the randomized phase III trial comparing panitumumab plus BSC with BSC only after failure in 5-FU-, oxaliplatin- and irinotecan-based chemotherapy^[10]. In this study *EGFR*

Table 2 Single nuclear polymorphism (SNP) analysis in patients treated with anti-EGFR moAbs

Publication	SNP	n	Variable	Significance
Zhang <i>et al</i> ^[44]	FcγR II a H131R, V158F	39	TTP	0.037
Bibeau <i>et al</i> ^[45]	FcγR II a H131R, V158F	69	TTP	0.015
Graziano <i>et al</i> ^[46]	FcγR II a H131R, V158F	110	RR, OS	NS
Nagashima <i>et al</i> ^[47]	FcγR II a H131R, V158F	98	PFS	NS
Graziano <i>et al</i> ^[46]	EGFR in1 (CA)n repeats	110	RR, OS	0.006, 0.008
Gonçalves <i>et al</i> ^[52]	EGFR ex 13 R521K	32	PFS	0.020
Graziano <i>et al</i> ^[46]	EGF 61G/G	110	OS	0.001
Zhang <i>et al</i> ^[44]	cyclin-D1 870A>G	39	TTP	0.019
Graziano <i>et al</i> ^[46]	cyclin-D1 870A>G	110	RR, OS	NS
Nagashima <i>et al</i> ^[47]	cyclin-D1 870A>G	98	PFS	NS

anti-EGFR: Anti-epidermal growth factor receptor; NS: Not significant.

GCN was assessed by FISH and its status was evaluated as the mean value of *EGFR* gene copies/nucleus, as the mean value of *EGFR* gene/CEP7 (α -centromeric probe of chromosome 7) and as the percentage of chromosome 7 polysomy (≥ 3 signals per nucleus) scoring 200 tumor cells. A statistically significant positive correlation between increase in mean *EGFR* GCN and probability of response to panitumumab (odds ratio, 5.62; 95% CI: 1.506-20.974) was found with 98.1% specificity (95% CI: 78.5%-96%). In addition, the best cut-off value of mean *EGFR* GCN, ≥ 2.47 *EGFR* gene copies/nucleus, as assessed by ROC analysis, to discriminate responders from nonresponders, had a sensitivity of 100% (95% CI: 54.1%-100%), since no objective response was observed when the *EGFR* GCN was less than this value. Because of the non-homogeneous pattern of *EGFR* GCN in the tumors, the percentage of cells displaying chromosome 7 polysomy and/or *EGFR* gene amplification was also calculated. In the same way, an increase in the percentage of chromosome 7 polysomy was also significantly associated with the probability of objective response (odds ratio, 1.04; 95% CI: 1.007-1.074), with a specificity of 100% (95% CI: 93.2%-100%) and a negative predictive value of 89.7% (95% CI: 78.8%-96.1%). In other words, in patients treated with panitumumab, a mean *EGFR* GCN of less than 2.5/nucleus or less than 40% of tumor cells displaying chromosome 7 polysomy within the tumor predicted a shorter PFS ($P = 0.039$ and $P = 0.029$, respectively) and OS ($P = 0.015$ and $P = 0.014$, respectively), thus generating the hypothesis that these tumors were probably not driven by the *EGFR* pathway. None of the treated patients with mean *EGFR* GCN of less than 2.47/nucleus or less than 43% of tumor cells displaying chromosome 7 polysomy obtained an objective response compared with 6 of 20 and 6 of 19 patients with values greater than these cut-off limits ($P = 0.0009$ and $P = 0.0007$, respectively). Evaluation of BSC-treated patients showed no correlation between *EGFR* GCN or chromosome 7 polysomy status and PFS. Interestingly, in the tumors of patients from the BSC only arm, no correlation was found between *EGFR* GCN and PFS, thus indicating more its predictive, rather than its prognostic role^[14].

In an exploratory clinical trial conducted by Kham-

bata-Ford *et al*^[64], a large prospective human cohort uniformly treated with cetuximab was exploited in an attempt to systematically identify biomarkers associated with disease control to anti-EGFR MoAb treatment. Transcriptional profiling was conducted on RNA from tumor cells in order to identify genes whose expression correlated with best clinical response. *EGFR* GCN was detected at a frequency of 6% by quantitative PCR but its increase within the disease control group was not statistically significant. In addition, gene expression profiles showed that tumors that express high levels of the *EGFR* ligands epiregulin (*EREG*) and amphiregulin (*AREG*) are more likely to show disease control with cetuximab (*EREG*, $P = 0.000015$; *AREG*, $P = 0.000025$) and have significantly longer PFS than patients with low expression (*EREG*: $P = 0.0002$; HR, 0.47; median PFS, 103.5 d *vs* 57 d, respectively; *AREG*: $P = 0.0001$; HR, 0.44; median PFS, 115.5 d *vs* 57 d, respectively)^[64]. Lately, these results were confirmed in another patients' cohort treated with cetuximab and irinotecan in combination^[65]. The authors found that overexpression of *EREG* and *AREG* in *KRAS* WT patients was associated with a decreased risk of progression (HR, 0.41 and 0.43, respectively) and death (HR, 0.42 and 0.4, respectively)^[65].

Lastly, in a study by Scartozzi *et al*^[66], the role of nuclear factor- κ B (NF- κ B) was investigated. NF- κ B plays a role in the activation of the *EGFR* downstream signaling pathway and was shown to be responsible for resistance to antineoplastic agents. *EGFR* can induce NF- κ B, and high levels of *EGFR* expression are essential for *EGFR*-mediated NF- κ B activation^[67]. In the above-mentioned study, NF- κ B and *EGFR* expression were evaluated retrospectively by immunohistochemistry and the results were correlated with response rate, TTP and OS in mCRC patients receiving irinotecan-cetuximab treatment. The response rate was 10% (4 partial responses) *vs* 48% (12 partial responses; $P = 0.0007$) in NF- κ B-positive and NF- κ B-negative tumors, respectively. Median TTP in NF- κ B-positive patients was 3 *vs* 6.4 mo in the remaining patients ($P = 0.021$). Median OS was 9.5 mo *vs* 15.8 mo for NF- κ B-positive and NF- κ B-negative patients, respectively ($P = 0.036$). The difference in median TTP, OS and response rate could mean that NF- κ B

may play a role in predicting the efficacy of irinotecan-cetuximab therapy in the mCRC setting^[66].

CONCLUSION

The CRC mutation profiles should influence patient selection or stratification in prospective trials. *KRAS* mutational status represents a paradigm for biomarker development in the era of molecular targeted therapies. As a result, *KRAS* testing is now mandatory at the presentation of metastatic disease in patients with CRC. In total, almost 50% of mCRC patients' tumors harbor either the *KRAS* (40%) or *BRAF* (10%) mutation and are not candidates for anti-EGFR mAb therapy. Patients with *KRAS* mutations may benefit from combination chemotherapy and anti-vascular endothelial growth factor mAbs (such as bevacizumab). Patients with *KRAS* mutations and resistance or relapse to chemotherapy and/or bevacizumab have limited treatment options and could be candidates for clinical trials with investigational agents, such as mTOR or extracellular-signal-regulated kinase inhibitors. The *BRAF* V600E mutation identifies a subgroup (less than 10%) of patients with an exceptionally unfavorable prognosis. These patients might justify forego approved treatments in favor of investigational therapy, such as molecules that inhibit the WT (sorafenib) and/or the mutant (PXL 4032) *BRAF* allele, either alone or in combination with chemotherapy. In addition, *PIK3CA* mutations appear to be useful predictors for response to anti-EGFR mAbs, but definitive conclusions should be based on the analysis of larger cohorts of patients in randomized trials that include patients who have not been exposed to anti-EGFR targeted therapies.

In addition, the expression of *AREG* and *EREG* was consistently associated with the outcome of cetuximab and panitumumab combination chemotherapy. The results of SNPs and GCN are premature and controversial, and thus need to be explored in a more systematic approach.

The development of biomarkers for the optimization of anti-EGFR treatment in mCRC, beyond *KRAS* mutations, is a work in progress. The aim will be to identify molecular markers that might be used to select patients with a higher probability of response to anti-EGFR mAbs.

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Influence of interleukin polymorphisms on development of gastric cancer and peptic ulcer

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Abstract

Pro-inflammatory cytokines are produced in the gastric mucosa by inflammatory cells activated by chronic *Helicobacter pylori* (*H. pylori*) infection. Polymorphisms of these cytokine genes are associated with individual differences in gastric mucosal cytokine mRNA level, which result in differences in gastric mucosal inflammation, acid inhibition and gastroduodenal disease risk in response to *H. pylori* infection. Although polymorphisms of interleukin (*IL*)-1*B*, *IL*-1*RN* and *TNF*-*A* have been reported to relate well with gastric cancer and peptic ulcer risk, those of *IL*-2, *IL*-4, *IL*-6 and *IL*-8 genes are unclear. In combined analyses using data from previous studies, we found that the risk of gastric non-cardia cancer development was significantly associated with *IL*-4-168 C allele (OR: 0.81, 95% CI: 0.69-1.00) and *IL*-4-590 T allele carrier status (0.61, 0.53-0.73), and *IL*-6-174 G/G genotype (2.02, 1.31-3.10). In peptic ulcer development, *IL*-2-330 G and *IL*-4-590 T allele carriers had a significantly decreased risk (0.37, 0.27-0.50 and 0.58, 0.34-0.99, respectively). Moreover, *IL*-2, *IL*-4, *IL*-6 and *IL*-8 gene genotypes prevalence differs among populations. The inflammatory cytokine gene polymorphisms

(e.g. *IL*-4-590 and *IL*-6-572 for gastric cancer, and *IL*-4-590, *IL*-6-572 and *IL*-8-251 for peptic ulcer) have a more potent influence on development of gastroduodenal diseases in Western than East Asian populations. These cytokine gene polymorphisms, as well as those of *IL*-1*B*, *IL*-1*RN* and *TNF*-*A*, may be used to identify groups at higher risk of gastric cancer and peptic ulcer, and those suitable for their prevention by *H. pylori* eradication therapy in Western populations.

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Key words: *Helicobacter pylori*; Cytokines; Genetic polymorphism; Stomach neoplasms; Peptic ulcer

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INTRODUCTION

Helicobacter pylori (*H. pylori*) infects > 50% of the world's population, and is particularly prevalent in developing countries (> 90%)^[1-3]. Chronic *H. pylori* infection relates not only to the development of upper gastrointestinal diseases, such as peptic ulcer diseases, gastric adenoma, gastric cancer, and gastric mucosa-associated lymphoid tissue lymphoma, but also with some extra-gastrointestinal disorders, such as idiopathic thrombocytopenic purpura, chronic idiopathic urticaria and iron-deficiency anemia^[4-12]. Prevention and treatment of *H. pylori*-related disease has therefore relied on eradication therapy as first-line treatment^[4-12].

The key pathophysiological event in *H. pylori* infection of gastric mucosa is the induction of a gastric mucosal inflammatory response. Following infection, neutrophils and mononuclear cells activated by *H. pylori* and their products infiltrate *H. pylori*-infected gastric mucosa and stimulate the transcription and synthesis of several pro-inflammatory cytokines [e.g. interleukin (IL)-1 β , IL-2, IL-6, IL-8 and tumor necrosis factor (TNF)- α] and anti-inflammatory cytokines (e.g. IL-4 and IL-10)^[13]. The increased production of inflammatory cytokines in response to *H. pylori* infection results in enhanced gastric mucosal inflammation, through binding to specific receptors on target cells.

Most of these inflammatory cytokine genes have genetic variations that influence cytokine levels in the gastric mucosa. Levels of mucosal IL-1 β , for example, the most studied inflammatory cytokine, differ significantly among the different genotypes in three polymorphisms, *IL-1B*-511, -31 and *IL-RN*^[13,14]. Carriers of the *IL-1B*-511 T, -31 C and *IL-RN* *2 alleles have significantly higher IL-1 β levels than those of the other allele^[13]. Consistent with this difference, carriers of the *IL-1B*-511 T, *IL-1B*-31 C alleles and *IL-1RN* *2/*2 (2 repeats of 86 bp) genotype show enhanced suppression of gastric acid secretion, which results in more rapid development of gastric atrophy, and a consequently greater risk of developing gastric cancer than in those with the *IL-1B*-511C, *IL-1B*-31 T and *IL-1RN**1 alleles^[13-18]. However, although IL-2, IL-4, IL-6 and IL-8 levels in gastric mucosa are reported to increase in patients with *H. pylori* infection^[19,20], it remains unknown whether these inflammatory cytokine polymorphisms are associated with gastroduodenal disease development in a similar way as those with *IL-1B* and *TNF-A*. Previously reported associations with disease risk and cytokine gene polymorphisms of *IL-2*, *IL-4*, *IL-6* and *IL-8* are controversial, however, owing to either or both type 2 error and geographical differences (Tables 1-4).

Here, we review differences in the risk of development of peptic ulcer and gastric cancer by different inflammatory cytokine gene polymorphisms of *IL-2*, *IL-4*, *IL-6* and *IL-8*.

IL-2 POLYMORPHISM AND GASTRODUODENAL DISEASES

IL-2, a 15-kDa α -helical cytokine of the Th1 type produced exclusively by activated T cells, promotes the proliferation of lymphocytes, macrophages and NK cells^[21]. IL-2 potently regulates the immune response, and plays important roles in the differentiation of CD41-positive T cells into Th1 and Th2 effector subsets, while inhibiting T-helper 17 differentiation^[22,23]. In T cells, IL-2 binding to the IL-2 receptor activates the Janus kinase (JAK)/signal transducer and activator of transcription (STAT) pathway, as well as mitogen-activated protein kinase (MAPK) and phosphoinositide 3-kinase (PI3K) signaling, which results in the transcription of pro-inflammatory cytokine genes. Through these pathways, IL-2 upregulates the expression of CD25 and IL-2R β , modulates genes involved in cell cycle regulation, and promotes T-cell survival and differ-

entiation into effector and memory cells^[24,25]. IL-2 contributes to the induction and transmission of inflammatory immune responses, including *H. pylori*-induced gastric inflammation.

Two kinds of single nucleotide polymorphism (SNP) occur in *IL-2*-330 and -384 (4q26-q27) of the promoter region, which affect IL-2 production^[26,27]. IL-2 expression level with deletion of the *IL-2*-289 to -361 region was significantly decreased compared with that with the normal gene. *IL-2*-330 polymorphism located in this region is therefore considered to have particular influence on IL-2 levels^[26,27]. In fact, IL-2 production in the *IL-2*-330 G/G genotype is about threefold greater than that of the *IL-2*-330 T/G or T/T genotypes in healthy subjects^[28]. Consistent with this difference, an association between the *IL-2*-330 polymorphism and susceptibility to some inflammatory and immune diseases, such as rheumatoid arthritis, psoriasis and multiple sclerosis, has been reported^[29-31]. IL-2 is therefore also thought to induce *H. pylori*-associated gastroduodenal diseases by regulating Th1 immune responses^[32] and inhibiting gastric acid secretion^[33].

Four studies have investigated the associations with *IL-2*-330 (three studies), +114 (one study) and +384 (one study) polymorphisms and development of atrophic gastritis (one study), peptic ulcer (one study) or gastric cancer (three studies) (Table 1)^[34-37]. With regard to *IL-2*-330 polymorphism, Wu *et al*^[37] have reported that subjects carrying the T allele, a low producer allele, have a significantly reduced risk of gastric cardia cancer (OR: 0.68, 95% CI: 0.46-0.99) compared with those with the G/G genotype. *IL-2*-330 polymorphisms may contribute to the etiology of gastric cardia cancer in Chinese populations^[37]. However, Shin *et al*^[35] failed to demonstrate a significant association with *IL-2*-330 polymorphism and gastric cancer development in the Chinese, while Togawa *et al*^[34] conversely have reported that the *IL-2*-330 T/T genotype increased the risk of gastric cancer-related gastric atrophy (OR: 2.78, 95% CI: 1.26-6.17) in the Japanese. The results for *IL-2*-330 polymorphism are thus controversial. Moreover, no significant association was seen for *IL-2*-384 and +114 polymorphisms and gastric cancer development^[36].

When combined, the results of previous studies of *IL-2*-330 polymorphism^[34,35,37] surprisingly have shown that the risk of peptic ulcer development is 0.57 (95% CI: 0.33-0.98) for the G/G genotype and 0.37 (0.27-0.50) for G allele carriers compared with the T/T genotype (Table 5). However, no association with *IL-2*-330 polymorphism was seen for the risk of gastric non-cardia cancer. This finding is inconsistent with the first hypothesis, which states that patients with the *IL-2* high producer genotype have an increased risk of gastric cancer and gastric ulcer development. Togawa *et al*^[34] have speculated that one possible reason is that a higher IL-2 level is thought to enhance the immune response to eradicate *H. pylori*, and thereby decrease gastric mucosal inflammation. Moreover, an IL-2 promoter construct in a cell line shows higher levels of gene expression with

Table 1 Association of *IL-2* polymorphism and gastroduodenal diseases

Position	Disease	Authors	Year	<i>n</i>					
-330 T/G	GC	Wu <i>et al</i> ^[37]	2009	GC	1026	T/T	T/G	G/G	GCC: T/T: 0.7 (0.4-1.0)
				NUD	1083	491	441	94	
	GC	Shin <i>et al</i> ^[35]	2008	GC	122	516	480	87	NS
				NUD	100	79	35	8	
	PU	Shin <i>et al</i> ^[35]	2008	PU	220	72	16	12	NS
				NUD	100	159	45	16	
-384 G/T	Atrophy	Togawa <i>et al</i> ^[34]	2005	Atrophy	152	72	16	12	T/T: 2.8 (1.3-6.2)
				NUD	443	80	63	9	
						202	196	45	
+114 G/T	GCC	Savage <i>et al</i> ^[36]	2004	GC	87	G/G	G/T	T/T	NS
				NUD	379	16	47	20	
						96	174	109	
	GCC	Savage <i>et al</i> ^[36]	2004	GC	82	G/G	G/T	T/T	NS
				NUD	377	33	35	14	
						149	148	80	

GC: Gastric cancer; GCC: Gastric cardia cancer; PU: Peptic ulcer; IL: Interleukin; NUD: Non-ulcer dyspepsia; NS: Not significant.

Table 2 Association of *IL-4* polymorphism and gastroduodenal diseases

Position	Disease	Authors	Year	<i>n</i>					
-168 T/C	GC	Wu <i>et al</i> ^[37]	2009	GC	1042	T/T	T/C	C/C	C carrier: 0.8 (0.7-1.0)
				NUD	1099	744	271	27	
-590 C/T	GC	Zambon <i>et al</i> ^[57]	2008	GC	40	743	332	24	NS
				NUD	171	C/C	C/T	T/T	
	GC	García-González <i>et al</i> ^[56]	2007	GC	404	32	7	1	NS
				NUD	404	124	43	4	
	GC	Lai <i>et al</i> ^[54]	2005	GC	123	283	107	14	NS
				NUD	162	267	123	14	
	GC	El-Omar <i>et al</i> ^[53]	2003	GC	122	83	38	2	NS
				NUD	209	105	50	7	
	GC	Wu <i>et al</i> ^[52]	2003	GC	220	78	37	7	NS
				NUD	230	153	46	10	
	DU	Zambon <i>et al</i> ^[57]	2008	DU	171	69	5	5	C carrier (diffuse-type): 1.6 (1.0-2.7)
				NUD	107	55	12	12	
-33 C/T	Atrophy	Kato <i>et al</i> ^[51]	2006	Atrophy	788	124	43	4	NS
				Dys	115	79	26	2	
				NUD	1020	506	414	100	
						C/C	C/T	T/T	
984, 2983	GC	Seno <i>et al</i> ^[55]	2007	Atrophy	100	10	70	77	T/C: 2.2 (1.0-4.9)
				NUD	93	42	183	227	
						AA/AA	AA/GC	AA/GA	
	GC			Atrophy	100	52	29	7	AA/GA: 0.3 (0.1-0.9)
				NUD	93	40	27	17	

Although Seno *et al*^[55] investigated nine SNPs (*IL-4*-590, -33, 3437, 3557, 4047, 4144, 4271, 4367 and 8427), data concerning the exclusion of *IL-4*+984 and 2983 were unclear. Dys: Dysplasia; DU: Duodenal ulcer.

the *IL-2*-330 G allele, whereas the transcriptional effect of this polymorphism in lymphocytes shows that the *IL-2*-330 G allele is associated with a lower expression of *IL-2*^[30]. In fact, many studies have shown that the *IL-2*-330 T/T genotype increases the risk of a number of diseases, such as Takayasu's disease^[38], subacute sclerosing panencephalitis^[39] and schizophrenia^[40].

All studies that have investigated the relationship of *IL-2*-330 polymorphism and disease development to date were in Asian populations^[34,35,37]. Further studies, including those in Western populations, will be necessary to solve this discrepancy and establish this relationship.

IL-4 POLYMORPHISM AND GASTRODUODENAL DISEASES

IL-4 is an anti-inflammatory cytokine, which inhibits gastric mucosal *H. pylori*-induced inflammation and atrophy by decreasing interferon γ (IFN- γ), which plays an important role in Th1 immune responses. *IL-4* also plays a central role in the maturation of T-helper cells to the Th2 phenotype. With a shift from a Th1 to a Th2 cell pattern, *IL-4* can enhance the production of anti-inflammatory cytokines (e.g. *IL-10* and *IL-13*), including that of *IL-4*^[41,42], and suppress the production of monocyte-derived pro-

Table 3 Association of *IL-6* polymorphism and gastroduodenal diseases

Position	Disease	Authors	Year	<i>n</i>				
-174 C/G	GC	Gatti <i>et al</i> ^[81]	2007	GC	56	C/C	C/G	G/G
				NUD	112	1	13	42
	GCC	Deans <i>et al</i> ^[82]	2007	GC	197	11	53	48
				NUD	224	43	83	71
	GC	Kamangar <i>et al</i> ^[83]	2006	GC	102	27	54	21
				NUD	152	43	58	51
	GC	El-Omar <i>et al</i> ^[53]	2003	GC	123	16	52	55
				NUD	209	28	98	83
	GC	Hwang <i>et al</i> ^[77]	2003	GC	60	2	9	49
				PU	91	1	18	72
-572 G/C	GC	Kang <i>et al</i> ^[85]	2009	GC	284	C/C	C/G	G/G
				NUD	278	154	113	17
	GC	Hwang <i>et al</i> ^[77]	2003	GC	60	19	29	12
				PU	434	249	167	20
	PU	Chakravorty <i>et al</i> ^[84]	2008	NUD	278	140	123	15
				PU	91	57	27	7
	DU	Hwang <i>et al</i> ^[77]	2003	NUD	62	37	20	5
				DU	60	21	20	19
	GC	Kamangar <i>et al</i> ^[83]	2006	GC	110	G/G	G/A	A/A
				NUD	203	25	59	26
-597 G/A	GC	Hwang <i>et al</i> ^[77]	2003	GC	60	61	86	56
				PU	91	53	29	10
	PU	Chakravorty <i>et al</i> ^[84]	2008	NUD	62	41	16	5
				DU	60	52	8	0
	GC	Hwang <i>et al</i> ^[77]	2003	GC	60	52	8	0
				NUD	211	118	84	9
	GC	Liao <i>et al</i> ^[72]	2008	GC	155	C/C	C/T	T/T
				NUD	211	96	55	4
	GC	Liao <i>et al</i> ^[72]	2008	GC	155	96	55	4
				NUD	211	118	84	9

Although Kang *et al* and Savage *et al* investigated the association with *IL-6*-174 C/G polymorphism and gastric cancer, data were not described in detail (> 99% of patients were of the *IL-6*-174 G/G genotype).

inflammatory cytokines (e.g. *IL-1β*, *IL-6* and *IL-8*)^[42].

IL-4 is overproduced in *H. pylori*-infected gastric mucosa. However, gastric mucosal inflammation has been shown to significantly reduce *IL-4* administration^[19,20], and *IL-4*-deficient mice infected with *H. pylori* show severe gastric inflammation compared with wild-type mice^[43,44]. A balance between Th1 and Th2 cytokines by *IL-4* therefore crucially influences the outcome of *H. pylori* infection. Moreover, *IL-4* is reportedly associated with cancer development *via* its suppression of inflammation, and directly inhibits the growth of human melanoma, renal cell carcinoma and gastric cancer cells^[45].

The family of the *IL-4* gene, which encodes *IL-4*, is located on chromosome 5q31-33, which contains the *IL-3*, *IL-4*, *IL-5*, *IL-9*, *IL-13*, *IL-15* genes as well as the interferon-regulatory factor and granulocyte-macrophage colony-stimulating factor (GM-CSF)^[46]. There are two common polymorphisms in the *IL-4* gene, -590 C/T and a 70-bp sequence variable number tandem repeat at intron 3; and many minor polymorphisms, such as -168, -33, 3437, 3557, 4047, 4144, 4271, 4367, 8427^[47,48]. The *IL-4*-590 polymorphism is located upstream of all known control elements of *IL-4*, such as the negative regulatory element, the NF- κ B recognition sequence, and the TATA box^[49]. Individuals with the *IL-4*-590 T/T genotype can produce *IL-4* at higher levels than those with the C/C

genotype^[48]. *IL-4* polymorphism is reportedly associated with the risk of cancer development (e.g. colorectal cancer^[50]), and the Th2 T-cell response represented by *IL-4* is expected to play a protective role in the development of cancer.

Seven studies have investigated the association of *IL-4*-590 polymorphism and atrophic gastritis (one study^[51]), gastric cancer (five studies^[52-56]), and duodenal ulcer development (one study^[57]) (Table 2). In 2003, Wu *et al*^[52] first reported that a higher prevalence of diffuse-type gastric cancer (OR: 1.64, 95% CI: 1.01-2.67), particularly in gastric cardia cancer (2.44, 1.13-5.27), is observed in *IL-4*-590 C allele carriers, a low producer allele, compared with the *IL-4*-590 T/T genotype, which suggests that low production of *IL-4* is responsible for the development of gastric cancer. However, other studies have failed to demonstrate any significant association of *IL-4* polymorphisms with disease risk^[37,53,54,57,58]. In a combined-analysis of *IL-4*-590 C/T polymorphism^[52-56], however, the risk of gastric non-cardia cancer development was 0.68 (95% CI: 0.57-0.80) for the C/T genotype, 0.36 (0.24-0.53) for the T/T genotype and 0.61 (0.53-0.73) for T allele carriers (Table 6). Moreover, the risk of peptic ulcer development in T allele carriers (0.58, 0.34-0.99) was significantly lower (Table 6). This protective effect of *IL-4*-590 polymorphism is therefore

Table 4 Association of *IL-8* polymorphism and gastroduodenal diseases

	Disease	Authors	Year		<i>n</i>				
-251 A/T						T/T	T/A	A/A	
	GC	Kang <i>et al</i> ^[85]	2009	GC	284	106	136	43	AA: 2.0 (1.2-3.6)
				NUD	275	125	125	25	
	GC	Canedo <i>et al</i> ^[94]	2008	GC	333	111	169	53	NS
				NUD	880	265	445	170	
	GC	Garza-Gonzalez <i>et al</i> ^[58]	2007	GC	78	15	47	16	A carrier: 2.1 (1.1-4.2)
				NUD	230	76	107	47	
	GC	Kamali-Sarvestani <i>et al</i> ^[95]	2006	GC	19	4	6	9	AT: 4.5 (1.5-12.9)
				NUD	153	57	74	22	
	GC	Shirai <i>et al</i> ^[96]	2006	GC	181	83	78	20	MSI (+): TT 5.2 (1.5-18.0)
				NUD	268	211	208	49	
	GC	Savage <i>et al</i> ^[97]	2006	GC	287	71	140	76	NS
				NUD	426	106	205	117	
	GC	Kamangar <i>et al</i> ^[83]	2006	GC	112	42	56	14	NS
				NUD	207	72	111	24	
	GC	Taguchi <i>et al</i> ^[98]	2005	GC	396	161	191	44	AA: 2.2 (1.1-4.6)
				NUD	252	125	105	22	
	GC	Lee <i>et al</i> ^[99]	2005	GC	470	198	213	59	TT: 1.9 (1.3-3.0)
				NUD	308	108	138	62	
	GC	Ohyauchi <i>et al</i> ^[100]	2005	GC	212	93	106	13	A carrier: 1.8 (1.1-2.8)
				NUD	195	106	74	15	
	GCC	Savage <i>et al</i> ^[101]	2004	GC	88	26	39	23	AA: 2.0 (1.0-3.8)
				NUD	429	147	207	75	
	GC	Lu <i>et al</i> ^[102]	2005	GC	250	94	102	54	AA: 1.9 (1.2-3.2)
				NUD	300	119	144	37	
	PU	Kang <i>et al</i> ^[85]	2009	PU	447	160	223	64	GU: AA: 2.7 (1.5-4.8)
				NUD	275	125	125	25	
	PU	Garza-Gonzalez <i>et al</i> ^[58]	2007	PU	29	11	14	4	NS
				NUD	230	76	107	47	
	GU	Kamali-Sarvestani <i>et al</i> ^[95]	2006	GU	61	19	28	14	NS
				NUD	153	57	74	22	
	GU	Ohyauchi <i>et al</i> ^[100]	2005	PU	283	134	127	22	GU:A carrier: 1.8 (1.1-3.0)
				NUD	195	106	74	15	
	PU	Chakravorty <i>et al</i> ^[84]	2008	PU	91	20	46	25	NS
				NUD	62	18	28	16	
	DU	Hofner <i>et al</i> ^[103]	2007	DU	85	15	49	21	AA: 2.3 (1.5-6.4)
				NUD	211	61	106	44	
	DU	Gyulai <i>et al</i> ^[104]	2004	DU	69	11	45	13	A carrier: 4.4 (1.9-10.5)
				NUD	47	21	17	9	
	IM	Leung <i>et al</i> ^[105]	2006	IM	123	23	56	44	NS
			NUD	179	36	92	51		
Atrophy	Taguchi <i>et al</i> ^[98]	2005	Atrophy	215	90	99	26	AA: 2.4 (1.1-4.9)	
			NUD	252	125	105	22		
	NUD	Hamajima <i>et al</i> ^[106]	2003	NUD	448	234	177	37	-
+396 T/G						T/T	T/G	G/G	
	GC	Kamangar <i>et al</i> ^[83]	2006	GC	111	42	55	14	NS
				NUD	208	72	112	24	
	GCC	Savage <i>et al</i> ^[101]	2004	GC	86	29	33	24	GG: 2.1 (1.1-3.9)
			NUD	402	152	181	69		
+781 C/T						C/C	C/T	T/T	
	GC	Kamangar <i>et al</i> ^[83]	2006	GC	111	47	52	12	NS
				NUD	208	81	105	22	
	GCC	Savage <i>et al</i> ^[101]	2004	GC	85	28	41	16	NS
			NUD	406	167	177	62		

Although Seno *et al*^[85] investigated six SNPs (*IL-4*-352, 289, 294, 680, 2217 and 2670), data were not described in detail. IM: Intestinal metaplasia.

significant for gastric non-cardia cancer and peptic ulcer patients with a higher producer genotype.

The prevalence of *IL-4*-590 C/C, C/T and T/T genotypes differs between Western and Asian populations (Table 7). The prevalence of C/C, C/T and T/T genotypes in a Western population with gastric cancer was 69.8% (362/518), 26.1% (135/) and 4.1% (21/), respectively, whereas that in those with non-ulcer dyspepsia

(NUD) was 55.9% (1448/2592), 36.0% (934/) and 8.1% (210/). In a Western population, the risks for gastric non-cardia cancer and peptic ulcer development were 0.55 (95% CI: 0.46-0.67) and 0.55 (0.32-0.94) for T allele carriers, respectively (Table 8). In an Asian population, in contrast, no significant difference was seen between subjects with gastric cancer and NUD. This difference in the influence of *IL-4*-590 polymorphism on disease development

Table 5 ORs for gastric non-cardia cancer and peptic ulcer development in *IL-2-330* polymorphism

	Genotype	¹ NUD (n)	Cancer (n)	OR	95% CI	P value	Ulcer (n)	OR	95% CI	P value
<i>IL-2-330</i>	T/T	870	258	-			159	-		
	G/T	755	209	0.93	0.76-1.15	0.51	45	0.33	0.23-0.46	< 0.01
	G/G	153	35	0.77	0.52-1.14	0.20	16	0.57	0.33-0.98	0.04
	G carrier	908	244	0.91	0.74-1.11	0.33	61	0.37	0.27-0.50	< 0.01

¹NUD includes gastritis without gastric cancer and peptic ulcer, and atrophic gastritis patients. Because we deleted a number of gastric cardia cancer patients, a number of cancer patients shown in this Table do not match that in Table 1.

Table 6 ORs for gastric non-cardia cancer and peptic ulcer development with *IL-4-168* and *-590* polymorphisms

	Genotype	¹ NUD (n)	Cancer (n)	OR	95% CI	P value	Ulcer (n)	OR	95% CI	P value
<i>IL-4-168</i>	T/T	743	744	-						
	T/C	332	271	0.81	0.67-0.98	0.03				
	C/C	24	27	1.12	0.64-2.00	0.70				
	C carrier	356	298	0.81	0.69-1.00	0.05				
<i>IL-4-590</i>	C/C	1716	591	-			46	-		
	C/T	1039	242	0.68	0.57-0.80	< 0.01	18	0.65	0.37-1.10	0.12
	T/T	229	28	0.36	0.24-0.53	< 0.01	2	0.33	0.08-1.35	0.12
	T carrier	1268	270	0.61	0.53-0.73	< 0.01	20	0.58	0.34-0.99	0.05

¹NUD includes patients with gastritis without gastric cancer and peptic ulcer, and atrophic gastritis. Because we deleted a number of gastric cardia cancer patients, the number of cancer patients in this Table does not match that in Table 2.

Table 7 Prevalence of inflammatory cytokine gene genotypes in East Asian and Western populations

Gene	Population	NUD			GC			PU		
<i>IL-2-330</i>		T/T	T/G	G/G	T/T	T/G	G/G	T/T	T/G	G/G
	Asian	870	755	153	258	209	35	159	45	16
<i>IL-4-590</i>		C/C	C/T	T/T	C/C	C/T	T/T	C/C	C/T	T/T
	Asian	268	105	19	229	107	7			
<i>IL-6-174</i>		C/C	C/G	G/G	C/C	C/G	G/G	C/C	C/G	G/G
	Asian	1448	934	210	362	135	21			
<i>IL-6-572</i>		C/C	C/G	G/G	C/C	C/G	G/G	C/C	C/G	G/G
	Asian	126	310	261	34	82	112	1	26	94
<i>IL-6-597</i>		C/C	C/G	G/G	C/C	C/G	G/G	C/C	C/G	G/G
	Asian	140	123	15	170	126	18	165	170	19
<i>IL-8-251</i>		G/G	G/A	A/A	G/G	G/A	A/A	G/G	G/A	A/A
	Asian	37	20	5	3	16	11	62	34	25
<i>IL-8-396</i>		T/T	T/G	G/G	T/T	T/G	G/G	T/T	T/G	G/G
	Asian	102	102	61	45	67	28	75	37	10
<i>IL-8-781</i>		T/T	T/A	A/A	T/T	T/A	A/A	T/T	T/A	A/A
	Asian	1324	1425	443	735	826	233	294	350	86
<i>IL-8+396</i>		T/T	T/G	G/G	T/T	T/G	G/G	T/T	T/G	G/G
	Asian	676	1093	449	243	418	168	76	182	77
<i>IL-8+781</i>		C/C	C/T	T/T	C/C	C/T	T/T	C/C	C/T	T/T
	Asian	152	181	69	27	43	12			
		C/C	C/T	T/T	C/C	C/T	T/T	C/C	C/T	T/T
	Western	72	112	24	29	41	11			
		C/C	C/T	T/T	C/C	C/T	T/T	C/C	C/T	T/T
	Western	167	177	62						
		C/C	C/T	T/T	C/C	C/T	T/T	C/C	C/T	T/T
	Western	81	105	22						

may have a geographic basis, and the effect appears to be stronger in Western populations (Table 8).

With regard to minor polymorphisms of *IL-4*, *IL-4-168*, -33, 984/2983 SNPs have been reported by one study each^[34,37,55]. Compared with the *IL-4-168* C/C high producer genotype^[37], the *IL-4-168* T allele carrier was associated with a significantly decreased gastric cancer risk (OR: 0.83, 95% CI: 0.69-1.00). Further, this significant protective effect was also seen for gastric cardia cancer patients (0.73, 0.56-0.95)^[37].

Thus, a significant protective effect against gastric non-cardia cancer was seen with the higher producer genotype *IL-4-590* and -168 polymorphisms, particularly in Western populations.

IL-6 POLYMORPHISM AND GASTRODUODENAL DISEASES

IL-6, a multifunctional cytokine produced by immune and

Table 8 Comparison of the incidence of gastric non-cardia cancer and peptic ulcer in Asian and Western populations

Disease	Gene	Reference	Genotype	Western			Asian		
				OR	95% CI	P value	OR	95% CI	P value
GC	IL-4-590	C/C	C/T	0.57	0.41-0.72	< 0.01	1.12	0.87-1.65	0.28
			T/T	0.40	0.25-0.64	< 0.01	0.43	0.18-1.04	0.06
			T carrier	0.55	0.46-0.67	< 0.01	1.08	0.79-1.45	0.17
	IL-6-572	C/C	C/G	9.87	2.56-37.98	< 0.01	0.85	0.61-1.19	0.34
			G/G	21.13	5.56-131.98	< 0.01	0.99	0.48-2.03	0.97
			G carrier	13.32	3.64-48.69	< 0.01	0.87	0.63-1.20	0.38
	IL-8-251	T/T	T/A	1.04	0.86-1.25	0.69	1.06	0.93-1.20	0.38
			A/A	1.04	0.83-1.31	0.73	0.95	0.79-1.14	0.56
			A carrier	1.04	0.87-1.24	0.67	1.03	0.92-1.16	0.61
	GU	IL-4-590	C/C	C/T	0.61	0.35-1.05	0.08		
T/T				0.30	0.07-1.24	0.10			
T carrier				0.55	0.32-0.94	0.03			
IL-6-572		C/C	C/G	1.31	0.07-2.56	0.42	0.77	0.55-1.05	0.07
			G/G	2.98	1.05-8.47	0.04	0.67	0.33-1.34	2.66
			G carrier	1.65	0.89-3.04	1.65	0.74	0.55-1.01	0.06
IL-8-251		T/T	T/A	1.48	1.12-1.97	< 0.01	1.11	0.93-1.31	0.25
			A/A	1.53	1.09-2.14	0.01	0.87	0.67-1.14	0.31
			A carrier	1.49	1.14-1.96	< 0.01	1.05	0.89-1.24	0.55

many non-immune cells including monocytes, lymphocytes, macrophages, and endothelial and intestinal epithelial cells, functions as both an inflammatory mediator and endocrine regulator^[59]. IL-6 plays an important role in host defense mechanisms as a messenger between innate and adaptive systems, by stimulating IFN- γ production in T cells and promoting immunoglobulin secretion in activated B cells^[60].

High serum levels of IL-6 family cytokines have been reported in various gastrointestinal cancer cells^[61]. IL-6 and IL-11 belong to the IL-6 cytokines family, which includes ciliary neurotrophic factor, cardiotrophin-1, cardiotrophin-like cytokine, leukemia inhibitory factor, oncostatin M, and IL-27. These act as ligands for the signaling receptor subunit gp130^[62,63]. IL-6 requires specific α receptor subunits and gp130 homodimers of signal transducing receptor^[63]. Recently, mice with a mutation in gp130 (gp130 757^{F/F} mouse) have been established to enhance chronic gastric inflammation and develop gastric neoplasms without *H. pylori* infection, *via* an imbalance between STAT3 and Y-759/SHP-2 signaling^[64]. The presence of the Y757F mutation in the gp130 receptor promotes the failure of SHP-2 phosphorylation and subsequent activation of the pro-apoptotic Ras/Erk and PI3K/AKT pathways, which results in massive STAT3 activation. STAT3 hyperactivity suppresses the cytostatic effect of the stroma on cell proliferation^[65]. Moreover, STAT3 also induces epithelial cell expression of IL-11^[66]. These signaling events promote an oncogenic program in which the expression of anti-apoptotic, pro-angiogenic, and pro-proliferative genes results in inflammation-associated gastric tumorigenesis^[66]. The IL-6 family signaling system is therefore an attractive research target in gastric cancer pathogenesis.

Mucosal IL-6 levels increase in *H. pylori*-associated gastritis^[66,67] and dramatically decrease after eradication of infection^[68]. IL-6 mRNA levels in gastric mucosa correlate with the level of gastric mucosal inflammation^[67,69]. Serum levels of IL-6 are higher in patients with gastric

cancer than gastritis^[70]. IL-6 plays an important role as a prognostic factor in advanced gastric cancer and lymph node metastasis^[71], and a serum IL-6 level > 13 pg/mL correlates with tumor progression and poor survival after resection^[72].

The *IL-6* gene is located on chromosome 7p21 and the SNPs at the 5' flanking region of the *IL-6* promoter have been identified as *IL-6*-174, -572 and -597^[73]. *IL-6*-174 G allele carriers produce higher levels of IL-6 than those with the C/C genotype^[74], and have a higher prevalence of systemic juvenile-onset chronic arthritis, lipid abnormalities^[75] and insulin resistance^[76]. *IL-6*-174 G and -597 G allele carriers are closely linked regardless of ethnic group or disease status^[77]. The *IL-6*-572 G allele is also associated with a higher serum IL-6 level than *IL-6*-572 C/C allele^[78], and is a risk factor for diabetic nephropathy and lung cancer with asthma/atopy^[79,80].

Six studies of the *IL-6*-174 polymorphism^[53,77,81-84], three of *IL-6*-572^[77,84,85], three of *IL-6*-597^[77,83,84] and one of *IL-6*+634^[72] in relation to the development of gastric cancer and peptic ulcer have appeared (Table 3). Gatti *et al*^[81] have reported that the *IL-6*-174 G allele carriers account for a significantly higher incidence of gastric cancer than NUD patients (98.2%, 55/56 and 90.2%, 101/112, respectively). However, Kamangar *et al*^[83] have demonstrated that, compared with G/G genotype IL-6, the low producer genotype *IL-6*-174G/C has an increased risk of gastric cancer, while other studies have shown no significant relationship of *IL-6*-174 polymorphism with gastric diseases. The association of this polymorphism with these conditions thus remains unclear. In contrast, frequencies of the *IL-6*-572 G/G genotype (OR: 0.3, 95% CI: 0.1-0.9) and of G allele carriers (0.5, 0.4-0.8) are lower in *H. pylori*-positive patients with duodenal ulcer than in those with NUD^[85].

In a combined analysis of *IL-6*-174 C/G polymorphism^[13,53,81-84], the risk of gastric non-cardia cancer was 2.02 (1.31-3.10) for the G/G compared with C/C genotype (Table 9). Moreover, the risk of gastric ulcer

Table 9 ORs for the development of gastric non-cardia cancer with the *IL-6-174*, +572, +597 and +634 polymorphisms

	Genotype	¹ NUD (n)	Cancer (n)	OR	95% CI	P value	Ulcer (n)	OR	95% CI	P value
<i>IL-6-174</i>	C/C	126	34	-			1			
	C/G	310	82	0.98	0.63-1.54	0.93	26	10.57	1.42-78.73	0.02
	G/G	261	142	2.02	1.31-3.10	< 0.01	124	59.86	8.27-433.4	< 0.01
	G carrier	571	224	1.45	0.97-2.19	0.06	150	33.10	4.59-238.8	< 0.01
<i>IL-6+572</i>	C/C	177	173	-			327	-		
	C/G	143	143	1.02	0.75-1.40	0.89	214	0.81	0.61-1.07	0.15
	G/G	20	29	1.48	0.81-2.72	0.20	44	1.19	0.68-2.08	0.54
	G carrier	163	172	1.08	0.80-1.46	0.61	258	0.86	0.66-1.12	0.26
<i>IL-6+597</i>	G/G	102	75	-				-		
	G/A	102	67	0.89	0.58-1.37	0.61				
	A/A	61	28	0.62	0.37-1.07	0.09				
	A carrier	163	95	0.79	0.54-1.17	0.24				
<i>IL-6+634</i>	C/C	118	96	-						
	C/T	84	55	0.81	0.52-1.24	0.33				
	T/T	9	4	0.45	0.16-1.83	0.32				
	T carrier	93	59	0.78	0.51-1.12	0.25				

¹NUD includes patients with gastritis without gastric cancer and peptic ulcer, and atrophic gastritis. Because we deleted a number of gastric cardia cancer patients, the number of cancer patients shown in this Table does not match that in Table 3.

was significantly higher with the G/G genotype (58.86, 8.27-433.4) and G allele carriers (33.10, 4.59-233.8) (Table 9). However, the prevalence of *IL-6-174* C/C, C/T and T/T genotypes differs among populations, with the C/C genotype being less common in East Asian, South Asian and Latin American populations (0%-9.8%) than in North American and European populations (13.4%-28.3%)^[13,53,81-84]. In East Asians in particular, the *IL-6-174* polymorphism has a C allele frequency of < 1%, not only in patients with gastric cancer, but also in those with NUD^[36,77,85]. This polymorphism may therefore not be useful in identifying the association with disease development in Asian populations. Although the prevalence of gastric cancer is higher in East Asians and Latin Americans than Caucasians, the difference in *IL-6-174* polymorphism may nevertheless explain the difference in prevalence between Asian and Western countries.

On combined analysis, *IL-6-572*, *IL-6-597* and *IL-6+643* polymorphisms have shown no significant relationship with gastric disease. When patients are divided into Asian and Western populations, however, a clear difference in the prevalence of *IL-6-572* genotypes is seen (Table 7). The risk of gastric non-cardia cancer and peptic ulcer development in Western populations was 21.13 (95% CI: 5.56-131.98) and 2.98 (1.05-8.47) for the *IL-6-572* G/G genotype, respectively (Table 8). In contrast, no significant difference has been seen between Asians with gastric cancer or NUD.

This influence of the *IL-6-174* and *IL-6-572* polymorphisms on disease development may have been due to geographic differences. Furthermore, the influence of *IL-4-590* polymorphism on gastroduodenal diseases is particularly strong in Western populations.

IL-8 POLYMORPHISM AND GASTRODUODENAL DISEASES

IL-8, a member of the CXC chemokine family, which

was originally identified as a potent chemoattractant for neutrophils and lymphocytes, induces not only cell proliferation and migration, but also angiogenesis. IL-8 is produced by gastric epithelial cells during *H. pylori* infection, particularly in the *cag*-pathogenicity-island-positive strain of *H. pylori*, one of the major virulence factors^[86,87]. In addition, IL-8 protein levels are 10-fold higher in gastric cancer than in normal gastric tissue^[68], and directly correlate with the vascularity of the tumors^[88]. The transfection of gastric cancer cells with the *IL-8* gene enhances their tumorigenesis and angiogenesis in the gastric wall of nude mice^[88]. Increased IL-8 levels may amplify the inflammatory response to *H. pylori* by recruiting neutrophils and monocytes, thereby resulting in an advanced degree of gastritis, which ultimately predisposes to the development of gastric cancer.

There are three common polymorphisms in the *IL-8* gene, -251 A/T, 396 T/G and 781 C/T^[47,48]. Of these, *IL-8-251* A allele carrier status is associated with increased IL-8 production^[89]. Consistent with these differences, *IL-8-251* polymorphism influences cancer risk, including that of lung^[90], colorectal^[91], bladder^[92], and prostate cancer^[93].

Seventeen studies of *IL-8-251* polymorphism^[58,83-85,94-106], two of *IL-6+396*^[83,101] and two of *IL-6+781*^[83,101] in relation to the development of gastric cancer and peptic ulcer have appeared. Of these, six studies have shown a significantly increased risk of gastric cancer for the *IL-8-251* A/A high producer genotype or A allele carriers^[58,85,95,98,100-102], while four have shown an increase for peptic ulcer^[85,100,103,104] and one for gastric mucosal atrophy^[98]. The *IL-8-251* A/A genotype is more common in *H. pylori*-positive patients with gastric cancer (OR: 2.0, 95% CI: 1.2-3.6) or gastric ulcer (2.7, 1.5-4.8) than in those with NUD^[85]. In addition, the *IL-8-251* A/A genotype is associated with a higher risk for the intestinal than the diffuse type of gastric cancer^[85]. Moreover, Taguchi *et al.*^[98] have reported that the *IL-8-251* A/A genotype correlates with a higher risk of lymph node and liver metastasis, and is histopathologically

Table 10 ORs for the development of gastric non-cardia cancer with *IL-8*-251, +396 and +781 polymorphisms

	Genotype	NUD (n)	Cancer (n)	OR	95% CI	P value	Ulcer (n)	OR	95% CI	P value
<i>IL-8</i> -251	T/T	2000	978	-			370			
	T/A	2518	1244	1.01	0.91-1.12	0.38	532	1.14	0.99-1.32	0.07
	A/A	892	401	0.92	0.80-1.06	0.24	163	0.99	0.81-1.21	0.90
	A carrier	3410	1645	0.99	0.90-1.09	0.18	695	1.10	0.96-1.26	0.17
<i>IL-8</i> +396	T/T	224	27	-						
	T/G	293	43	1.22	0.73-2.03	0.44				
	G/G	93	12	1.07	0.52-2.20	0.85				
	G carrier	385	55	1.18	0.73-1.93	0.50				
<i>IL-8</i> +781	C/C	248	29	-						
	C/T	282	41	1.24	0.75-2.06	0.39				
	T/T	84	11	1.12	0.54-2.34	0.76				
	T carrier	366	52	1.22	0.75-1.97	0.43				

NUD includes patients with gastritis without gastric cancer and peptic ulcer, and atrophic gastritis. Because we deleted a number of gastric cardia cancer patients, the number of cancer patients shown in this Table does not match that in Table 4.

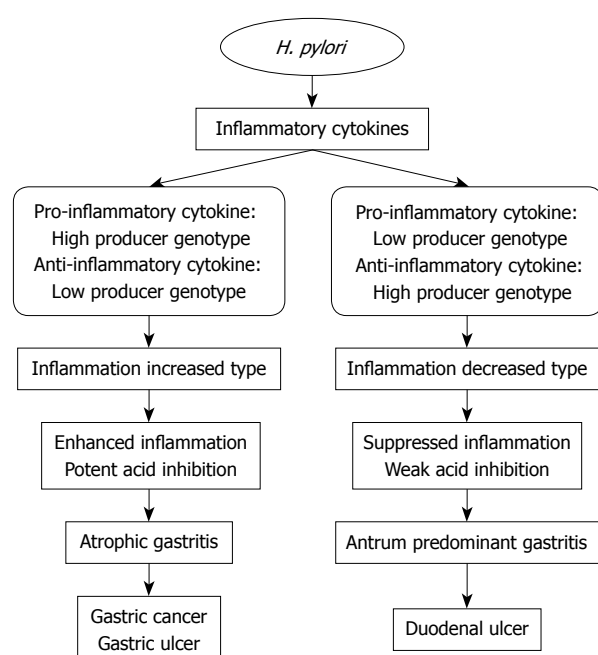


Figure 1 Scheme of the association of inflammatory cytokine polymorphisms and gastroduodenal disease development.

associated with more severe neutrophil infiltration in non-cancerous gastric mucosa adjacent to cancer. These results may be due to the tumorigenic and angiogenic functions of *IL-8* modulating the growth and invasive behavior of malignant tumors by autocrine and paracrine mechanisms, and suggest that genetic variants of *IL-8* potentially affect the prognosis of gastric cancer. Nevertheless, several contrary studies have also appeared. Lee *et al.*^[99], for example, have reported that, compared with the *IL-8*-251 A/A high producer genotype, the A/T genotype had a > 60% risk of gastric cancer (1.62, 1.07-2.46), while the T/T genotype had a > 90% risk (1.93, 1.26-2.95), particularly in the diffuse type; whereas Shirai *et al.*^[96] have reported that the *IL-8*-251 T/T genotype is significantly associated with an increased risk of microsatellite instability (MSI)-high gastric cancer, which is more frequently associated with active *H. pylori* infection than in microsatellite-stable (MSS) cases,

compared to MSI-low/MSS gastric cancer (5.2, 1.5-18.0) and NUD (3.7, 1.1-12.4).

Most studies that have reported positive associations for gastric cancer risk have been conducted in Asian populations^[98,100-102], whereas those that have reported negative findings were conducted in Western populations^[83,97]. In a combined analysis of the *IL-8*-251 polymorphism, no association was seen for the risk of gastric non-cardia cancer development (Table 10). However, the prevalence of *IL-8*-251 genotypes differs between Western and Asian populations (Table 7), and the risk for peptic ulcer in Western populations is higher [1.53 (1.09-2.14) for the *IL-8*-251 A/A genotype and 1.49 (1.14-1.96) for A allele carriers] (Table 8). The *IL-8*-251 polymorphism more potently influences the development of peptic ulcer in Western than East Asian populations.

In combined analysis, the *IL-8*+396 and *IL-8*+781 polymorphisms have no significant relationship with the incidence of gastric disease (Table 10). However, Savage *et al.*^[101] have reported that the *IL-8*-251/+396/+781 AGT/AGC haplotype is associated with a fourfold increased risk of gastric cardia cancer. This haplotypic analysis will help identify groups with a higher risk of disease and should be investigated in a larger study.

Summary of association between *H. pylori*-related diseases and cytokine polymorphisms

In general, gastric mucosal inflammation in *H. pylori* infection of gastric mucosa is exacerbated in patients with high producer alleles of pro-inflammatory cytokines and low producer alleles of anti-inflammatory cytokines, which results in a higher risk for the development of gastric cancer and gastric ulcer (Figure 1). In contrast, low producer allele carriers of pro-inflammatory cytokines and high producer allele carriers of anti-inflammatory cytokines have mild gastric mucosal inflammation (Figure 1). A summary of the association between *H. pylori*-related diseases and cytokine polymorphisms is shown in Table 11. As important points, the prevalence of cytokine gene genotypes differs between Western and Asian populations. Although Asian populations have been reported to be associated

Table 11 Summary of association with inflammatory cytokine genotypes and gastroduodenal diseases in East Asian and Western populations

Gene		Western		Asian	
		PU	GC	PU	GC
<i>IL-2-330</i>	G carrier (<i>vs</i> T)	-	-	↓	NS
<i>IL-4-590</i>	T carrier (<i>vs</i> C)	↓	↓	NS	-
<i>IL-6-174</i>	G carrier (<i>vs</i> C)	↑	↑	-	-
<i>IL-6-572</i>	G carrier (<i>vs</i> C)	NS	↑	NS	NS
<i>IL-6-597</i>	A carrier (<i>vs</i> G)	NS	NS	-	-
<i>IL-8-251</i>	A carrier (<i>vs</i> T)	↑	NS	NS	NS
<i>IL-8+396</i>	G carrier (<i>vs</i> T)	-	NS	-	-
<i>IL-8+781</i>	T carrier (<i>vs</i> C)	-	NS	-	-

↓ decrease in risk of disease; ↑ increase in risk of disease.

with *IL-1B-511*, *IL-10*, *TNF- α* polymorphisms and development of peptic ulcer and gastric cancer^[13,18], the influence of *IL-4*, *-6* and *-8* polymorphisms on the diseases in the current review may be lower. In Western studies, combination analysis of several cytokine gene genotypes is related to development of diseases^[53]. As shown in Table 11, because *IL-4-590*, *IL-6-174*, *IL-6-572* and *IL-8-251* polymorphisms in Western populations relate to development of gastroduodenal diseases, combination analysis including these gene polymorphisms with previously reported *IL-1* and *TNF- α* is expected to increase detection of elevated risk of diseases. These findings should be further evaluated in a larger population.

CONCLUSION

Many genetic factors are associated with the development of *H. pylori*-related diseases. Of these, we have reviewed here the important role of inflammatory cytokines (*IL-2*, *IL-4*, *IL-6* and *IL-8*) and their polymorphisms in *H. pylori*-related diseases. We recommend intensive endoscopic screening and/or eradication therapy for patients at higher risk of gastric cancer based on genetic inflammatory cytokine polymorphisms, albeit that we are unsure whether all factors should be determined. Further data to refine this recommendation are therefore required.

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Methylation of *GATA-4* and *GATA-5* and development of sporadic gastric carcinomas

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Abstract

AIM: To understand the implication of *GATA-4* and *GATA-5* methylation in gastric carcinogenesis.

METHODS: Methylation status of *GATA-4* and *GATA-5* CpG islands in human gastric mucosa samples, including normal gastric biopsies from 45 outpatients, gastric dysplasia [low-grade gastric intraepithelial neoplasia (GIN), $n = 30$; indefinite, $n = 77$], and 80 paired sporadic gastric carcinomas (SGC) as well as the adjacent non-neoplastic gastric tissues was analyzed by methylation specific polymerase chain reaction (MSP) and confirmed by denatured high performance liquid chromatography (DHPLC). Immunohistochemical staining was used to detect protein expression. The correlation between *GATA-4* and *GATA-5* methylation and clinicopathological characteristics of patients including *Helicobacter pylori* (*H. pylori*) infection was analyzed.

RESULTS: *GATA-4* and *GATA-5* methylation was frequently observed in SGCs (53.8% and 61.3%, respectively) and their corresponding normal tissues (41.3% and 46.3%) by MSP. The result of MSP was consistent with that of DHPLC. Loss of both *GATA-4* and *GATA-5* proteins was associated with their methylation in SGCs ($P = 0.01$). Moreover, a high frequency of *GATA-4* and *GATA-5* methylation was found in both gastric low-grade GIN (57.1% and 69.0%) and indefinite for dysplasia (42.9% and 46.7%), respectively. However, *GATA-4* and *GATA-5* methylation was detected only in 4/32 (12.5%) and 3/39 (7.7%) of normal gastric biopsies. *GATA-4* methylation in both normal gastric mucosa and low-grade GIN was also significantly associated with *H. pylori* infection ($P = 0.023$ and 0.027 , two-sides).

CONCLUSION: Epigenetic inactivation of *GATA-4* (and *GATA-5*) by methylation of CpG islands is an early frequent event during gastric carcinogenesis and is significantly correlated with *H. pylori* infection.

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Key words: Dysplasia; Gastric carcinoma; *GATA-4*; *GATA-5*; *Helicobacter pylori*; Methylation

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Wen XZ, Akiyama Y, Pan KF, Liu ZJ, Lu ZM, Zhou J, Gu LK, Dong CX, Zhu BD, Ji JF, You WC, Deng DJ. Methylation of *GATA-4* and *GATA-5* and development of sporadic gastric carcinomas. *World J Gastroenterol* 2010; 16(10): 1201-1208 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v16/i10/1201.htm> DOI: <http://dx.doi.org/10.3748/wjg.v16.i10.1201>

INTRODUCTION

GATA proteins comprise a small family of transcriptional factors defined by a highly conserved DNA-binding domain that interacts specifically with DNA *cis*-elements. Six distinct vertebrate GATA proteins have been characterized and classified into two subfamilies based on their structural and expression patterns. GATA-1, -2 and -3 are important in the development and differentiation of the hematopoietic cell lineage, while GATA-4, -5 and -6 guide development and differentiation in the endoderm-derived organs, and specification of proper gut embryogenesis^[1-3]. GATA-4 deficient cells exhibit an intrinsic defect in gastric epithelial cell differentiation to parietal cells in mice^[4]. Overexpression of GATA-5 induced TFF1 expression^[5]. Thus, GATA-4, -5 and -6 may play a critical role in the regulation of stomach-specific gene expression and cancer development. GATA-6 may function as an oncogene since it is often upregulated in proliferating progenitor cells^[6-8]. In contrast, GATA-4 and GATA-5 may function as tumor suppressors to drive endodermal-specific differentiation because they are potential upregulators of the differentiation-related genes in the endoderm-derived organs^[9].

The endoderm gives rise to the lining epithelium of the gastrointestinal tract. Loss of GATA-4 and GATA-5 might affect gastrointestinal epithelial cell differentiation, leading to tumorigenesis of these cells. In fact, there is growing evidence linking loss of *GATA-4* and *GATA-5* expression to many malignancies, such as primary esophageal, colorectal, gastric and pulmonary cancers^[5,10-12]. Moreover, in addition to genetic inactivation, epigenetic changes such as methylation of CpG islands may play a major role in the loss of *GATA-4* and *GATA-5* function^[5,10,12]. Since methylation of *GATA-4* and *GATA-5* is a frequent event in gastric cancer cell lines^[5], it is interesting to know that methylation silences of *GATA-4* and *GATA-5* during gastric carcinogenesis.

MATERIALS AND METHODS

Gastric tissue samples

Primary sporadic gastric carcinoma (SGC) and the corresponding normal gastric samples: Fresh-frozen surgical SGC and their corresponding adjacent non-neoplastic "normal" samples were from the Tissue Bank at Beijing Cancer Hospital ($n = 80$; 62 males and 18 females, aged 35-81 years, average age 58.5 years). The clinical and histological information for each case was also collected according to approved institutional guidelines. The 1997 UICC-TNM criteria were used for classification of gastric cancers.

Gastric dysplasia samples: Biopsies of gastric dysplasia lesions [low-grade noninvasive gastric intraepithelial neoplasia (GIN), $n = 30$; indefinite for dysplasia, $n = 77$] (51 males and 56 female, aged 41-65 years, average age 51.7 years) were collected from patients without malignant disease enrolled in a gastro-endoscopic survey in Linqu County, a rural area in Shandong Province, China which has one of the world's highest rates of gastric cancer^[13]. Histopathological diagnosis of each case was made by three senior pathologists at the Department of Pathology in Beijing Cancer Hospital, according to the Padova international classification^[14]. Information on *Helicobacter pylori* (*H. pylori*) infection by the ¹³C-urea breath test was also collected^[15].

Gastric biopsies from patients with and without chronic gastritis: Gastric mucosa biopsies were collected from outpatients undergoing gastro-endoscopic examination at Beijing Cancer Hospital. Of 45 gastric biopsies used in the present study, 18 patients were diagnosed with superficial chronic gastritis and 27 without obvious pathological changes (43 males and 2 female, aged 19-47 years, average age 30.7 years).

Informed consent was obtained from all subjects and the institutional review committee approved this study.

Detection of *H. pylori*

H. pylori in the normal or corresponding normal gastric samples was analyzed with a *H. pylori*-specific 23S rRNA-polymerase chain reaction (PCR) assay as described^[16].

Methylation-specific PCR (MSP)

DNA extraction, bisulfite treatment, and MSP were performed as described previously^[17,18]. The MSP primer sequences for *GATA-4* and *GATA-5* were as follows: GATA-4M sense, 5'-GTATAGTTTCGT AGTTTGCGTTTAGC-3'; GATA-4M antisense, 5'-AACTCGCGACTCGAATCCCCG-3'; GATA-4U sense, 5'-TTTGTATAGTTTGTAGTTTGTGTTTAGT-3'; GATA-4U antisense, 5'-CCCAACTCACAACCTCAAATC CCA-3'; GATA-5M sense, 5'-AGTTCGTTTITAGGT TAGTTTTCGGC-3'; GATA-5M antisense, 5'-CCAATA CAACTAAACGAACGAACCG-3'; and GATA-5U sense, 5'-TGGAGTTTGTITTTTAGGTAGTTTITGGT-3'; GATA-5U antisense, 5'-CAAACCAATACAACCTAAACA AACAAACCA-3' as described by Gou *et al.*^[12].

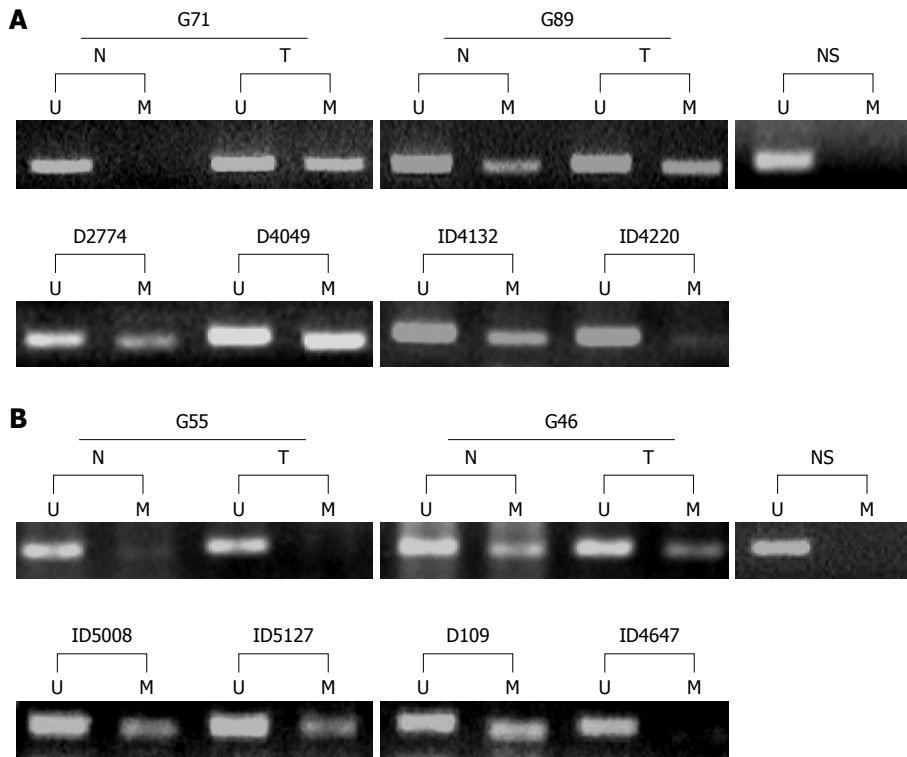


Figure 1 Methylation specific polymerase chain reaction (MSP) analyses of methylation status of the *GATA-4* (A) and *GATA-5* (B) CpG islands in human gastric mucosa samples. The upper panel shows MSP results of gastric cancer tissues (T) and their corresponding non-neoplastic tissues (N). The lower panel shows results of gastric dysplasia. The unmethylated (lanes U) and methylated (lanes M) MSP products were displayed on 2.5% agarose gels. NS: Normal stomach mucosa; D: Definite dysplasia; ID: Indefinite dysplasia.

Sequencing of the MSP products

The MSP products for *GATA-4* and *GATA-5* were sub-cloned with the pEASY-T1 simple clone system (Trans-Gen Biotech Company, Beijing, China), and sequenced on an ABI PRISM 3730 DNA Analyzer.

Separation and quantification of the methylated *GATA-4* by denatured high performance liquid chromatography (DHPLC)^[19,20]

Both methylated and unmethylated *GATA-4* CpG islands were amplified by a universal primer set without CpG (*GATA-4*uni sense 5'-GGAGATTTTAGAGTTT GGAT-3' and antisense 5'-CTCCCACTAACTACCTCT CT-3') under thermal cycle conditions [95°C for 15 min → (95°C for 30 s → 52.8°C for 30 s → 72°C for 50 s) × 40 cycles → 72°C for 10 min]. The 385-bp PCR product of the methylated and unmethylated *GATA-4* were separated by DHPLC at a partial denaturing temperature of 57.1°C and detected by a fluorescence (FL)-detector. The proportion of the methylated *GATA-4* in the testing samples was calculated according to the ratio of the peak area for the methylated *GATA-4* to the total peak area for both the methylated and unmethylated *GATA-4*. Genomic DNA of the HCT116 cell line was used as a *GATA-4* methylation positive control.

Immunohistochemical staining (IHC)

Paraffin sections (4 μm) were dewaxed and rehydrated in xylene and ethanol. For antigen retrieval, the sections were autoclaved in 1.0 mmol/L EDTA for 5 min, followed by

immersion in 3% H₂O₂ methanol for 15 min to block endogenous peroxidase. The sections were then blocked in 15% normal goat serum in phosphate-buffered saline, followed by incubation with polyclonal *GATA-4* or *GATA-5* antibodies (Sigma-Aldrich, Inc., Missouri, USA) (1:100) overnight at 4°C. Then the sections were incubated with biotinylated goat anti-rabbit IgG working solution for 15 min at room temperature. The standard SP (Streptavidin/peroxidase) process was then performed according to the instructions for the HistostainTM-Plus Kits (Beijing Zhongshan Goldenbridge Biotechnology Company, Beijing, China). Diaminobenzidine was used as a chromogen, followed by counterstaining with hematoxylin. Normal gastric mucosa samples were used as a positive control. PBS was used as a negative control to replace *GATA-4/-5* antibody. We regarded *GATA-4* and *GATA-5* expression as positive when 10% or more cancer cells exhibited *GATA-4* and *GATA-5* expression.

RESULTS

Aberrant CpG island methylation of the *GATA-4* and *GATA-5* promoter in primary gastric cancer and their corresponding normal tissues

The promoter methylation status of *GATA-4* and *GATA-5* in eighty SGCs and their corresponding normal samples were analyzed by MSP. *GATA-4* and *GATA-5* methylation was observed in 43 and 49 SGCs (53.8% and 61.3%), respectively (Figure 1, Figure 2A and Table 1). To our surprise, these genes were also fre-



quently methylated in the corresponding non-neoplastic samples, 33 for *GATA-4* (41.3%) and 37 for *GATA-5* (46.3%), which were only slightly lower than those in SGCs (Figure 2A and Table 1).

positive corresponding normal tissues ($1.33\% \pm 0.45\%$ vs $1.10\% \pm 0.56\%$, paired *t*-test, $P = 0.054$). The DHPLC results corresponded well with the above MSP results.

We analyzed the methylation status of these genes in gastric biopsies with low-grade GIN or indefinite for dysplasia in the subjects from Linqu County, a high risk area for stomach cancer in China. Results showed that *GATA4* and *GATA5* were also frequently methylated in these dysplasia lesions, with strong methylated signals in 46 of 98 (46.9%) and 55 of 104 (52.9%), respectively (Figure 2B and Table 2). More *GATA4* and *GATA5* methylation was observed in tissues with GIN (57.1% and 69.0%, respectively) than in indefinite for dysplasia

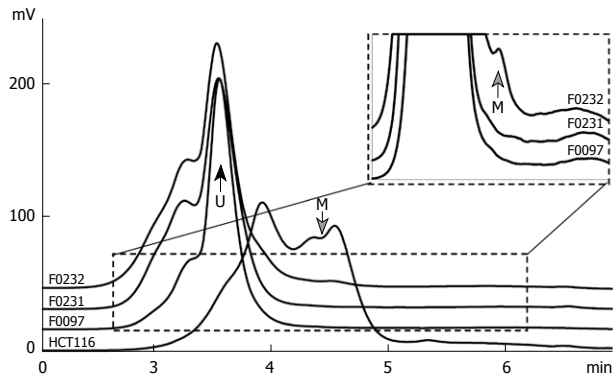


Figure 3 Chromatogram of the methylated and unmethylated *GATA-4* CpG islands by denatured high performance liquid chromatography (DHPLC). The 385-bp PCR product of the methylated and unmethylated *GATA-4* were separated by DHPLC at a partial denaturing temperature 57.1°C and detected by a fluorescence (FL)-detector. The proportion of methylated *GATA-4* in the tested samples was calculated according to the ratio of the peak area for the methylated *GATA-4* to the total peak area for both the methylated and unmethylated *GATA-4*. The gray arrow points to the peak for the methylated *GATA-4* (M) at the retention time 4.5 min; The black arrow points to the peak for the unmethylated *GATA-4* (U) at the retention time 3.6 min. Genomic DNA of HCT116 was used as the *GATA-4* methylation positive control. The inserted chart represents the dashed line surrounding the area. *GATA-4* methylation was detected in the tested sample F0232.

Table 1 Comparison of *GATA-4* and *GATA-5* methylation status with clinicopathological characteristics of SGC

Clinicopathological characteristics	Methylation positive rate (%)			
	<i>GATA-4</i>		<i>GATA-5</i>	
	SGC	N	SGC	N
SGC invasion				
T1&T2 (n = 20)	45.0 ^a	35.0 ^b	45.0	45.0
T3 (n = 50)	54.0	42.0	68.0	48.0
T4 (n = 10)	70.0	50.0	60.0	40.0
Metastasis				
Yes (n = 44)	52.3	31.8	61.4	36.4
No (n = 36)	55.6	52.8	61.1	38.9
<i>H. pylori</i> infection				
Positive (n = 42)	61.9	45.2	64.3	50.0
Negative (n = 34)	41.2	38.2	55.9	44.1
Age (yr)				
≤ 59 (n = 42)	45.2	33.3	57.1	28.6
≥ 60 (n = 38)	63.2	50.0	65.8	65.8 ^c
Sex				
Male (n = 62)	51.6	41.9	61.3	51.6
Female (n = 18)	61.1	38.9	61.1	27.6 ^d
Total n = 80	53.8	41.3	61.3	46.3 ^e

SGC: Sporadic gastric carcinomas; N: Adjacent corresponding non-neoplastic gastric tissue; *H. pylori*: *Helicobacter pylori* detected by the 23S rRNA-PCR assay^[16]; ^{a,b}Analysis for linear trend by EpiInfo 6.0 software, positive rates vs extent of penetration of the stomach wall, $P = 0.198$ and 0.434 , respectively; ^c χ^2 test by EpiInfo 6.0 software, samples from young patients (≤ 59 years old) vs old patients (≥ 60 years old), $P < 0.001$; ^dMale vs female, $P = 0.074$; ^eSGCs vs the normal, $P = 0.057$.

(42.9% and 46.7%), especially for *GATA-5* ($P < 0.05$) (Table 2). *GATA-4* and *GATA-5* methylation in two representative dysplasia samples were confirmed by bisulfite clone sequencing (Figure 4). In contrast, these genes were rarely methylated in the relatively normal gastric biopsies.

Table 2 *GATA-4* and *GATA-5* methylation frequency (%) in gastric dysplasia lesions in subjects from Linqu County, a high risk area for stomach cancer

Classification of gastric dysplasia	<i>GATA-4</i>	<i>GATA-5</i>
Low-grade noninvasive neoplasia	57.1 (16/28)	69.0 (20/29)
<i>Hp</i> -infection		
With	71.4 (15/21)	63.6 (14/22)
Without	14.3 (1/7) ^a	85.7 (6/7)
Indefinite for dysplasia ^b	42.9 (30/70)	46.7 (35/75) ^c
Total	46.9 (46/98)	52.9 (55/104)

^a χ^2 test by EpiInfo 6.0 software, with vs without *Hp*-infection, $P = 0.023$;

^bOnly two cases without *Hp*-infection; ^c χ^2 test by EpiInfo 6.0 software, Low-grade noninvasive neoplasia (GIN) vs indefinite for dysplasia, $P = 0.042$.

Table 3 Correlation of the *GATA-4* and *GATA-5* methylation status with their protein expression in sporadic gastric carcinomas with IHC

Protein expression, by IHC	<i>GATA-4</i> methylation status			<i>GATA-5</i> methylation status		
	M	U	P -value ^a	M	U	P -value
Positive (+ ~ +++)	7	11	0.012	5	9	0.011
Negative (- ~ ±)	11	3		16	3	

IHC: Immunohistochemical staining; + ~ +++: 10% or more cancer cells exhibited a similar positive staining pattern; - or weak: Positive expression in less than 10% cancer cells or a weak staining pattern compared with a non-cancerous area of the same section; M: Methylated; U: Unmethylated; ^aFisher's exact test by EpiInfo 6.0 software.

Among the 45 gastric biopsies (27 normal and 18 superficial chronic gastritis), *GATA-4* and *GATA-5* methylation was only 4/32 (12.5%) and 3/39 (7.7%), respectively (Figure 2C), significantly lower than the methylation in gastric dysplasia and SGCs as well as the adjacent normal samples ($P < 0.001$).

***GATA-4* and *GATA-5* expression was correlated with their methylation status**

We investigated the expression of *GATA-4* and *GATA-5* in normal and gastric carcinoma tissues using IHC. These genes were expressed in the gland region of normal gastric mucosa (Figure 5A and D). In contrast, *GATA-4* and *GATA-5* expression was obviously decreased in many gastric carcinomas (Figure 5B and E, Table 3). A significant inverse relationship was observed between *GATA-4* (or *GATA-5*) methylation and *GATA-4* (or *GATA-5*) protein expression in the gastric samples tested using IHC (Figure 3 and Table 3).

The relationship between methylation frequencies of *GATA-4* and *GATA-5* and clinicopathological parameters including *H. pylori* infection

As shown in Table 1, an increased trend of *GATA-4* methylation was observed in SGCs and the corresponding normal samples with depth of tumor invasion (T1-2, T3, T4), but was not statistically significant. The *GATA-5* methylation positive rate in the corresponding normal tis-

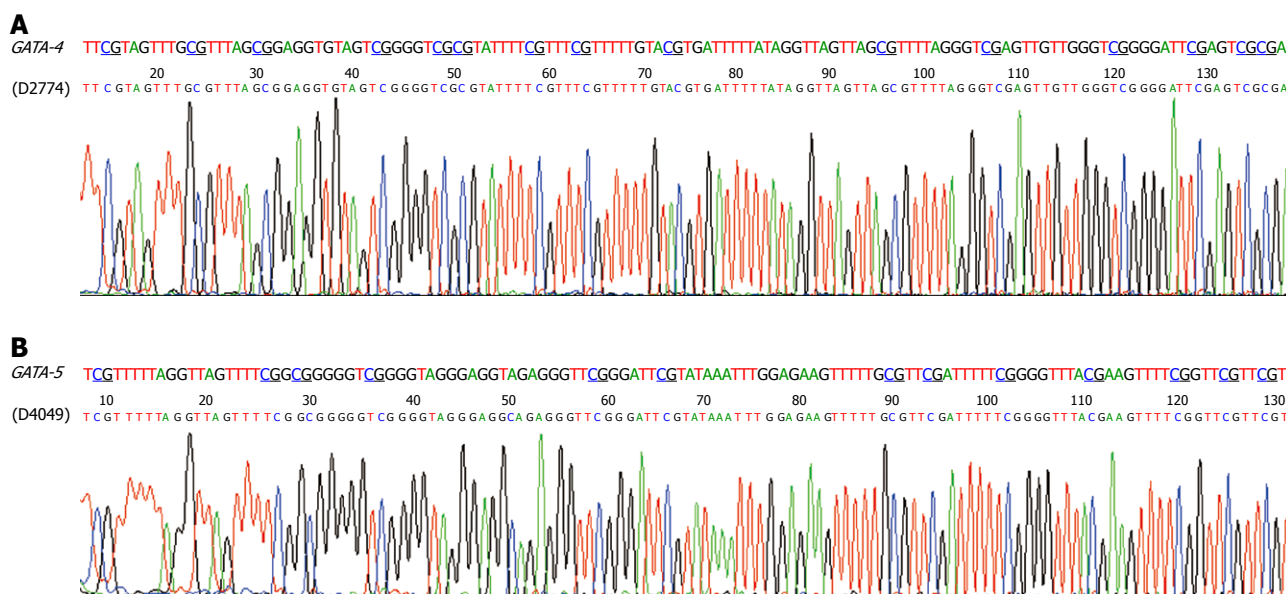


Figure 4 Sequencing of the methylated MSP products for *GATA-4* (A) and *GATA-5* (B) from two representative gastric dysplasia samples. The predicted sequence of bisulfite-modified *GATA-4* and *GATA-5* CpG islands are listed in each panel at the top. The methylated CpG sites are underlined.

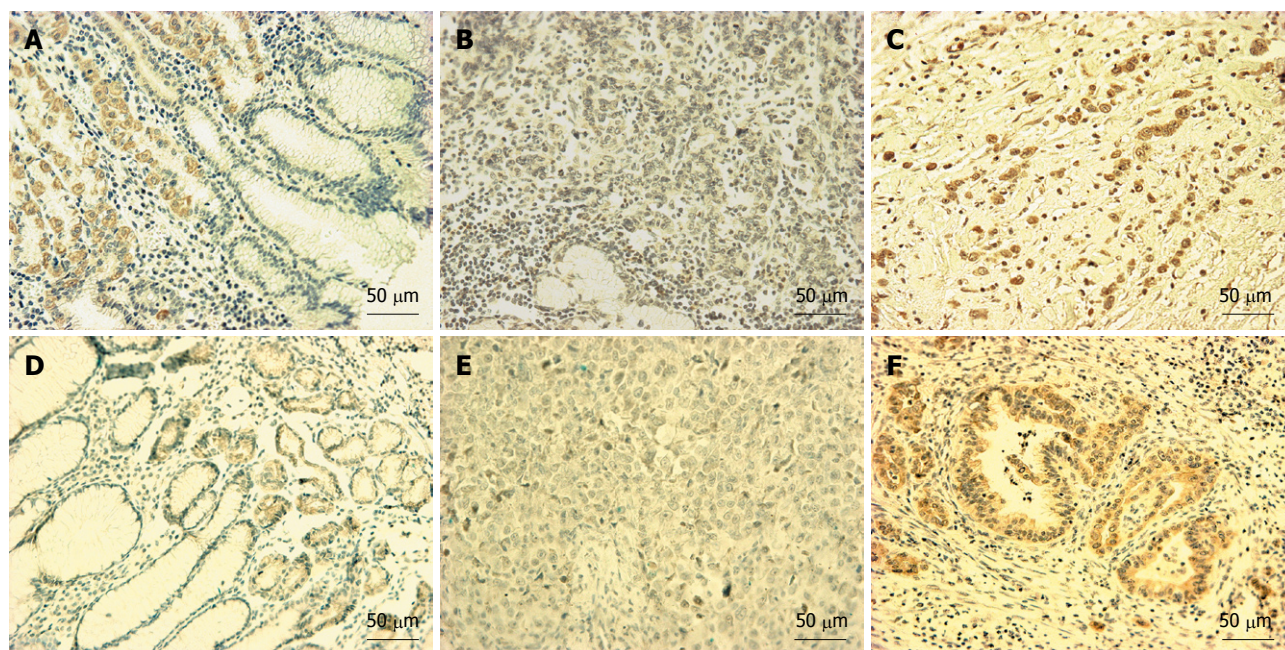


Figure 5 *GATA-4* (A-C) or *GATA-5* (D-F) protein in various gastric mucosa samples by immunohistochemical staining (IHC). A and D: *GATA-4* and *GATA-5* expression were confined to the gland region in the normal gastric mucosa; B and E: Carcinoma cells containing methylated alleles of *GATA-4* (G89) or *GATA-5* (G48) exhibited negative staining; C and F: Unmethylated cancer cells exhibited positive staining in case G27 and G45, respectively. Diaminobenzidine was used as a chromogen, followed by counterstaining with hematoxylin. Black bar, 50 μ m in length.

sues from elderly and male patients was significantly higher than that from young and female patients, respectively (Table 1).

Moreover, a correlation was observed between *GATA-4* (or *GATA-5*) methylation and *H. pylori* infection. More *GATA-4* and *GATA-5* methylation was detected in SGCs and their corresponding normal samples with *H. pylori* infection than in those without infection, but was not statistically significant ($P = 0.071$) (Figure 2B). In the gastric dysplasia tissues, particularly in those with

low-grade GIN, the *GATA-4* methylation positive rate for the patients with *H. pylori* infection was significantly higher than that in patients without *H. pylori* infection (15/21 *vs* 1/7, $P = 0.023$, Table 2).

In addition, all 4 biopsies with *GATA-4* methylation were from *H. pylori* infected patients ($n = 14$). No *GATA-4* methylation was observed in biopsies from subjects without *H. pylori* infection ($n = 18$, $P = 0.027$, two-sides). Of 3 biopsies with *GATA-5* methylation, 2 were from *H. pylori* infected patients (Figure 2C).

DISCUSSION

Epigenetic silencing of *GATA4* and *GATA5* by DNA methylation has been reported in gastrointestinal cancer cell lines and primary carcinomas^[5,10,12]. However, the implication of *GATA4* and *GATA5* methylation in the development of gastric cancer is unclear. Hence, we analyzed the methylation status of *GATA4* and *GATA5* CpG islands in gastric tissues from different kinds of lesions. We found that *GATA4* and *GATA5* methylation was detected in 53.8% and 61.3% of SGCs by MSP, respectively. These results were confirmed by the quantitative DHPLC assay. Furthermore, a significant inverse relationship was observed between methylation status and their protein expression in the gastric samples tested using IHC. These results indicate that *GATA4* and *GATA5* may be frequently inactivated in SGCs by DNA methylation. Moreover, the high prevalence of *GATA4* and *GATA5* methylation in the corresponding normal tissues suggests that these aberrant methylations may be a gastric field-effect, a phenomenon which happens within the whole target regions during carcinogenesis^[21].

GATA4 and *GATA5* were also methylated in 46.9% and 52.9%, respectively, of gastric dysplasia, a precancerous lesion of gastric carcinomas. However, both of these genes were seldom methylated in gastric tissues with or without chronic gastritis [4/32 (12.5%) and 3/39 (7.7%), respectively]. These results indicate that *GATA4* and *GATA5* methylation is an early frequent event during the development of gastric carcinomas.

In addition, we found that the *GATA5* methylation positive rate in the corresponding normal tissues from elderly and male patients was significantly higher than that from young and female patients, respectively. These results are consistent with the higher incidence of gastric carcinoma in males than in females, and the increasing prevalence of gastric carcinomas among elderly subjects.

H. pylori infection is the main cause of chronic atrophic gastritis, which may play an important role in gastric carcinogenesis. A number of tumor-related genes such as *p16* could be inactivated by DNA methylation in gastric mucosa lesions with *H. pylori* infection^[22,23]. In the present study, we found that more *GATA4* and *GATA5* methylation in gastric samples from patients with *H. pylori* infection were detected than in those without *H. pylori* infection, especially for *GATA4* methylation. The *GATA4* methylation positive rate in the low-grade GIN patients with *H. pylori* infection was significantly higher than that in patients without *H. pylori* infection (15/21 *vs* 1/7, $P = 0.023$). These results suggest that *GATA4* and *GATA5* methylation could be initiated in the precancerous stage by *H. pylori* infection.

In addition, among gastric tissues with or without chronic gastritis, all 4 biopsies with *GATA4* methylation were from *H. pylori* infected patients ($n = 14$), no *GATA4* methylation was observed in biopsies from subjects without *H. pylori* infection ($n = 18$; $P = 0.027$, two-sides), and of 3 biopsies with *GATA5* methylation, 2 were from *H. pylori* infected patients. These results

again support the hypothesis that *H. pylori* infection may contribute to epigenetic inactivation of these genes in gastric mucosa.

In conclusion, *GATA4* and *GATA5* methylation was an early frequent field-effect and significantly correlated with the severity of pathological changes during gastric carcinogenesis. *H. pylori* infection may contribute to *GATA4* and *GATA5* methylation in the human stomach.

COMMENTS

Background

Tumor suppressor genes *GATA4* and *GATA5* are important for development of the stomach during embryogenesis. Epigenetic inactivation of these genes by DNA hypermethylation was previously reported in esophageal and lung cancer.

Innovations and breakthroughs

In the present study, Dr. Wen *et al* found that aberrant *GATA4* and *GATA5* methylation was also a frequent event in gastric carcinomas and their adjacent tissues. Interestingly, their work demonstrated that epigenetic inactivation of *GATA4* and *GATA5* was observed in about 50% of gastric mucosa samples with epithelial dysplasia, a precancerous lesion in the stomach. However, such a phenomenon was very rare in gastric mucosa biopsies from healthy subjects or patients with chronic gastritis. They also observed that *Helicobacter pylori* (*H. pylori*) infection correlated well with *GATA4* and *GATA5* methylation. These results indicate that epigenetic inactivation of *GATA4* and *GATA5* is an early frequent event during gastric carcinogenesis by *H. pylori* and might be used to screen patients with a high risk of stomach cancer.

Peer review

The authors have tried to show that methylation of *GATA4* and *GATA5* could be important in the oncogenesis of gastric mucosa in patients coming from a Chinese area where gastric cancer is common. They showed that in cancer and adjacent mucosa methylation of these two antioncogenes is common and apparently related mostly with chronic *H. pylori* infection.

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Solid pseudopapillary tumor of the pancreas: A review of 553 cases in Chinese literature

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tail (19.5%). Forty-five patients (9.2%) were diagnosed as malignant SPT with metastasis or invasion. None of the clinical factors was closely related to the malignant potential of SPT. Surgery was the main therapeutic modality for SPT. Local resection, distal pancreatectomy and pancreatoduodenectomy were the most common surgical procedures. Local recurrence and hepatic metastasis were found in 11 and 2 patients, respectively, after radical resection. Four patients died of tumor progression within 4 years after palliative resection of SPT. The prognosis of SPT patients was good with a 5-year survival rate of 96.9%.

CONCLUSION: SPT of the pancreas is a rare indolent neoplasm that typically occurs in young females. It is a low-grade malignancy and can be cured with extended resection. The prognosis of such patients is good although the tumor may recur and metastasize.

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Key words: Pancreatic neoplasm; Solid pseudopapillary tumor; Diagnosis; Treatment; Prognosis

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Abstract

AIM: To sum up the clinical and pathological characteristics of solid pseudopapillary tumor (SPT) and the experience with it.

METHODS: A total of 553 SPT patients reported in Chinese literature between January 1996 and January 2009 were retrospectively reviewed and analyzed.

RESULTS: The mean age of the 553 SPT patients included in this review was 27.2 years, and the male to female ratio was 1:8.37. Their symptoms were non-specific, and nearly one third of the patients were asymptomatic. Computed tomography and ultrasonography were performed to show the nature and location of SPT. Most of the tumors were distributed in the pancreatic head (39.8%), tail (24.1%), body and

INTRODUCTION

Solid pseudopapillary tumor (SPT) of the pancreas, first reported by Frantz in 1959^[1], is an uncommon but distinct pancreatic neoplasm with a low malignancy, accounting for 1%-2% of all pancreatic tumors^[2]. Different names of this tumor were reported until it was defined by the World Health Organization (WHO) in 1996 as a “solid pseudopapillary tumor” of the pancreas^[3]. SPT occurs mainly in young women and can be treated with surgical resection. SPT patients have a relatively favorable prognosis after surgical resection. With the widespread availability of high-quality imaging systems and a better understanding of its pathology, the number of such patients reported in the literature has been steadily increased. Papavramidis has summarized 718 SPT patients in English literature, mainly from USA, Europe, and Japan^[4]. In this study, the cases of SPT reported in current Chinese literature were reviewed and analyzed.

MATERIALS AND METHODS

SPT-related literatures covered in the Chinese biology and medicine database (CBMD) and the China hospital knowledge database (CHKD) between January 1996 and January 2009 were retrieved. Indexing terms used for the retrieval were SPT, solid cystic tumor, papillary cystic tumor, papillary epithelial neoplasia, solid and papillary epithelial neoplasia, papillary epithelial tumor, Frantz's tumor, solid and papillary tumor, solid and cystic papillary epithelial neoplasm, benign or malignant papillary tumor of the pancreas. A total of 241 articles were searched. Each article was carefully studied to avoid the repetitive adoption of the saved material. A database of the characteristics of these patients was developed, including age, gender, symptoms, tumor location (data were from radiological investigations or surgical record) and size (data were from radiological investigations or surgical record and finally confirmed by pathology), metastasis or invasion of adjacent tissues (data were from radiological investigations or surgical exploration, and finally was confirmed by pathology), treatment (data were from the record of therapy, including the types of surgery), and follow-up. Each case with at least 6 of these characteristics was defined as well-documented. Thus, a total of 117 articles describing 553 well-documented cases of pancreatic SPT were included in our review and analysis. All statistical analyses were performed using the SPSS 15.0 for Windows (Chicago, Illinois).

RESULTS

Of the 553 patients, 59 were men and 494 were women with a male to female ratio of 1:8.37. Their mean age was 27.2 years (range 6 -71 years).

The features of 473 SPT patients (85.5%) are listed in Table 1. The symptoms were non-specific, and coexistence of two or more symptoms was usually found.

Table 1 Symptoms of SPT patients (*n* = 473)

Symptoms	Patients (<i>n</i>)	%
Abdominal pain	178	37.63
Abdominal mass	170	35.94
Abdominal discomfort	155	32.77
Asymptomatic	150	31.70
Vomiting	25	5.29
Post-trauma	23	4.86
Nausea	19	4.01
Back pain	17	3.59
Jaundice	17	3.59
Anorexia	11	2.33
Weight loss	9	1.90
Fever	7	1.48
Other symptoms	5	1.06

SPT: Solid pseudopapillary tumor.

Nearly one third of the patients were asymptomatic, with the tumor found at routine physical examination. The tumor was localized in 507 patients (91.7%). The most common sites of the tumor were the pancreatic head (39.8%), tail (24.1%), body and tail (19.5%), body (11.2%), and neck (3.6%). SPT was found in extra-pancreatic tissues of retroperitoneum, mesentrium, and left adrenal gland in 9 patients (1.8%). The tumor sizes in 512 cases (92.6%) were provided. The mean diameter of the tumor was 7.87 cm (range 1-25 cm).

CT, US and magnetic resonance imaging (MRI) were performed for the evaluation of 460 (83.2%), 337 (60.9%) and 43 patients (7.8%), respectively. Preoperative fine needle aspiration cytology (FNAC), endoscopic ultrasounds (EUS), and endoscopic retrograde cholangiopancreatography (ERCP) were seldom performed. Of the 325 patients (75.0%) diagnosed before operation, only 77 (23.7%) were diagnosed as or suspected of SPT. The misdiagnosis rate for pancreatic adenocarcinoma, cystadenoma, cystadenocarcinoma, islet cell tumor, pancreatic cyst, neuroendocrine tumor, teratoma, and others was 24.6%, 10.7%, 3%, 13.2%, 7.3%, 8.6%, 1.9%, and 7%, respectively. The masses were shown on cross-sectional imaging as heterogeneous (*n* = 300, 60.12%), cystic (*n* = 78, 15.63%), or solid (*n* = 121, 24.25%).

Of the 491 patients (88.8%) with metastasis or invasion, 45 (9.2%) were diagnosed as malignant SPT. The liver, portal/splenic vein/superior mesenteric vein, spleen, diaphragmatic muscle, omentum or peritoneum, duodenum, stomach, colon, and left kidney were involved in 6, 11, 4, 1, 7, 7, 3, 2 and 1 patients, respectively. Enlarged lymph nodes and lymph node metastasis were detected in 10 and 3 patients, respectively. The level of tumor markers, including α -fetoprotein (AFP), carcinoembryonic antigen (CEA), carbohydrate antigen (CA) 19-9, CA125 and CA242, was slightly increased in 11 patients, but only two were diagnosed as malignant SPT. Other clinical factors, including sex, age, symptoms, and tumor size, location and characteristics, were analyzed in some reports. However, none of the features was found

Table 2 Surgical procedures for 315 SPT patients

Operation	Patients (n)	%
Local resection or enucleation	104	33.02
Distal pancreatectomy	99	31.43
Pancreatoduodenectomy	81	25.71
Central pancreatectomy	19	6.03
Total pancreatectomy	2	0.63
Palliative operation	10	3.17
Portal vein reconstruction	3	0.95

to be closely related with the malignant potential of SPT in our study.

At present, radial resection is the main treatment of choice for pancreatic SPT. Local resection, distal pancreatectomy, and pancreatoduodenectomy were the most commonly used surgical procedures for the 315 patients included in our study (Table 2). Resection of a cuff of the portal vein was performed in 3 patients with a vein graft repair and palliative operation was performed in 10 patients as the tumor was adhered to the adjacent tissues or its metastasis was not resectable. Of the 6 patients with liver metastasis, 1 underwent liver operation, 2 had liver biopsy for the metastasis followed by dehydrated alcohol injection and transarterial chemo-embolization (TACE), and 3 received γ -radiation therapy, chemotherapy and radiofrequency, respectively. The most common complication after operation was pancreatic fistula which occurred in 17 cases (5.4%). Other complications including pancreatitis, steatorrhea, wound infection, biliary fistula, prolonged gastric emptying, gastrointestinal bleeding, diabetes mellitus, and ileus were observed in 3 (0.95%), 3 (0.95%), 3 (0.95%), 2 (0.63%), 2 (0.63%), 2 (0.63%), 1 (0.32%), and 1 (0.32%) patients, respectively.

The data of immunohistochemical profile are listed in Table 3. SPT cells were typically positive for vimentin, a1-antitrypsin, a1-antichymotrypsin, and neuron specific enolase. Focal expression of synaptophysin, cytokeratin and chromogranin A was detected in a few tumors. The positive rate was higher for progesterone receptor than for oestrogen receptor, but usually variable. Immunohistochemical staining of Ki-67 was detected in 12 patients, and 4 of 5 patients positive for Ki-67 immunoreactivity were confirmed to have malignant SPT.

Three hundred and twenty-three patients were followed-up for a mean time of 49.2 mo (range 1-209 mo). Radical resection was performed in 305 patients (94.4%), with a mean disease free-survival time (DFS) of 45.1 mo. Of the 11 patients (3.6%) who had local recurrence, 8 received a second resection with a mean DFS of 23.9 mo. Six patients died after complete resection, including two with unrelated disease. Hepatic metastasis occurred in 2 patients 3 and 46 mo respectively after surgery. One is still alive and the other was dead due to metastasis of the tumor. Of the 10 patients after palliative resection, 4 died with a median survival time of 22.4 mo (range 9-47 mo).

Table 3 Immunohistochemical parameters of SPT

Antigen	Patients (n)	Positivity, n (%)
AAT	276	261 (94.6)
Vim	289	269 (93.1)
AACT	237	215 (90.7)
NSE	221	179 (81.0)
CD56	43	29 (67.4)
CD10	51	33 (64.7)
PR	157	89 (56.7)
Ki67	12	5 (41.7)
Syn	196	83 (42.3)
CK	213	66 (31.0)
CgA	193	45 (23.3)
ER	97	11 (11.3)

AAT: a1-antitrypsin; Vim: Vimentin; AACT: a1-antichymotrypsin; NSE: Neuron specific enolase; PR: Progesterone receptor; Syn: Synaptophysin; CK: Cytokeratin; CgA: Chromogranin A; ER: Estrogen receptor.

The estimated 1-, 3- and 5-year survival rate was 99.4%, 97.5%, and 96.9%, respectively. The clinicopathologic features and outcome of SPT in 5 large series reported in Chinese literature between January 1996 and January 2009 are shown in Table 4^[5-9].

DISCUSSION

SPT of the pancreas is a rare neoplasm with a low malignant potential, usually affecting young women in the second or third decade of life. Its pathogenesis still remains unknown, although its tendency to affect young women is due to the involvement of sex hormones. However, no difference has been found in immunohistochemical staining for sex hormone-receptor proteins or in clinicopathologic characteristics attributable to gender alone^[10]. Sun *et al*^[11] reported that 62.5% of SPT patients are infected with hepatitis B virus (HBV), which can induce over-expression of β -catenin in tumor cells, indicating that HBV infection may be involved in the pathogenesis of SPT, which, however, has not been confirmed.

Although the malignant potential of SPT is low, up to 15% of SPT patients develop metastasis. The most common sites of metastasis are the liver, regional lymph nodes, mesentery, omentum, and peritoneum^[12]. Local invasion of the duodenum, stomach, spleen or major blood vessels may also occur, as reported in our study. Washington *et al*^[13] showed that the clinical and pathologic features of SPT, including diffuse growth, venous invasion, nuclear pleomorphism, mitotic rate, necrosis and dedifferentiation, are related to its aggressive behavior or metastatic potential. Other studies indicate that DNA aneuploidy, double loss of X chromosomes, trisomy for chromosome 3, unbalanced translocation between chromosomes 13 and 17 found in SPT patients are associated with its aggressive behavior and may be indicators of its possible metastatic potential^[14,15]. In our study, the clinical factors, including sex, age, symptoms,

Table 4 Clinicopathological features and outcome of SPT

Author	n	Female/male	Median/mean age(yr)	Size (cm)	Surgical treatment	Median/mean follow-up (mo)	Outcome
Zhang <i>et al</i> ^[5]	29	26/3	26.5	8.7 (2-15)	5 PD, 6 DP+S, 15 LR, 1 ID	2-84	28 alive, 1 dead
Cheng <i>et al</i> ^[6]	22	22/0	27.3 (11-65)	9.5 (2.5-25)	11PD, 2CP, 9LR	NA	22 alive, 0 recurred
Zhong <i>et al</i> ^[7]	20	18/2	25.3 (13-48)	8.2 (3-17)	5PD, 4DP+S, 9EN, 2PO	44.4 (9-120)	14 alive, 3 dead, 3 lost
Wang <i>et al</i> ^[8]	16	15/1	34.6 (15-58)	5.6 (2-15)	6PD, 1LR, 3DP, 6DP+S	34.6	15 alive, 1 lost
Zhu <i>et al</i> ^[9]	14	13/1	11.4 (9-14)	7.0 (3-15)	7PD, 5DP+S, 2LR	46.6 (1-192)	14 alive, 0 recurred

PD: Pancreaticoduodenectomy; DP: Distal pancreatectomy; LR: Local resection; ID: Internal drainage; CP: Central pancreatectomy; EN: Enucleation; PO: Palliative operation; S: Splenectomy.

and tumor size, location and characteristics were not closely related to its malignant potential. Four of the 5 patients positive for Ki-67 immunoreactivity were confirmed to have malignant SPT, which is consistent with the reported findings^[16], suggesting that positive staining of Ki-67 may correlate with the malignant potential and poor outcome of SPT. However, there are some limitations in our study due to the available data, more SPT patients should be studied for Ki-67 and new biomarkers in order to better predict its malignancy.

The clinical presentation of SPT is usually unspecific. Most patients have unclear clinical features including abdominal pain or discomfort, poor appetite and nausea, which are related to tumor compression on adjacent organs. SPT is often diagnosed during complementary imaging investigations such as ultrasound or CT scan of the abdomen, usually showing a well-encapsulated complex mass with both solid and cystic components. Dynamic contrast-enhanced CT can show less enhanced tumor, typical cystic spaces in the center, and enhanced solid areas at its surroundings^[17]. MRI is better than CT in detecting the cystic or solid components of the tumor. If MRI reveals an encapsulated mass with solid and cystic components as well as hemorrhage without obvious internal septum, SPT of the pancreas should be highly suspected^[18]. Percutaneous or EUS-guided FNAC can help to distinguish SPT from other pancreatic tumors. However, reports are also available on seeding of the needle tract by neoplastic cells and complications such as bleeding, pancreatic fistula and biliary fistula during the procedure^[19]. Despite the technological advances, preoperative diagnosis is still difficult and only 77 patients were diagnosed as or suspected of SPT in our series. The results of our study show that CT/MRI scans combined with age and gender should be sufficient for the decision to operate, and diagnostic interventions such as FNAC should be performed when radiology fails in diagnosing it.

At present, radical resection is the treatment of choice for SPT even with metastasis or local recurrence. Local resection or enucleation can be performed for small tumors with complete amacula. Distal pancreatectomy combined with or without splenectomy can be performed for pancreatic body and/or tail tumor, and pancreaticoduodenectomy for pancreatic head tumor. Intra-operative frozen section may help to ascertain

the adequate resection of margins. Extensive lymphatic dissection is not warranted since lymph node metastasis was found in only 3 patients (0.61%) in our study. Surgery should be performed for the tumor with local invasion or metastasis^[20]. Sperti *et al*^[21] have reported 17 patients who underwent vascular resection and reconstruction with no death occurred. In our study, the infiltrated portal veins were reconstructed with vein grafts after *en-bloc* resection and the patients had a long survival time. Martin *et al*^[2] reported that four patients underwent resection of liver metastasis and primary tumor, and two of them survived for at least 6 and 11 years, respectively. Other treatment modalities for liver metastasis, such as alcohol injection, TACE, γ -radiation therapy and even liver transplantation, have been reported^[11,22]. The role of chemotherapy and radiotherapy in treatment of SPT is poorly defined at present, since only few reports are available on them.

Grossly, SPT is well-encapsulated and usually well-demarcated from the pancreas, with large spongy areas of hemorrhage on its cut surface alternating with both solid and cystic degenerations. The tumor contains a mixture of solid, cystic, and pseudopapillary patterns in various proportions. The solid portions of the tumor are composed of uniform and polygonal epithelioid cells with well-vascularized stroma and a discohesive arrangement^[12]. Immunohistochemically, SPT is typically positive for vimentin (Vim), α -1-antitrypsin (AAT), α -1-antichymotrypsin (AACT), and neuron specific enolase (NSE)^[23], which is consistent with the finding in our study. However, the unique immunohistochemical features with expression of CD56 and CD10 have not reached an agreement in a recent study^[24]. SPT cells may also reveal focal immunoreactivity for cytokeratin (CK) and synaptophysin (Syn), abnormal nuclear location of β -catenin, and presence of progesterone receptors (PR), and may express galectin-3, all of which are useful in differentiating SPT from endocrine pancreatic tumor^[24].

The prognosis of SPT patients even with local recurrence and metastasis or invasion is good. SPT is limited to the pancreas in over 95% of its patients and can be radically resected^[25]. Its local recurrence rate is less than 10% and usually occurs within 4 years after surgery^[21]. Recurrence, local invasion, and limited metastasis are not the contraindications for resection,

and some patients with “unresectable” SPT may also have a long survival time^[26]. It has been reported that the overall 5-year survival rate of SPT patients is about 95%^[4]. Due to the favorable prognosis and long survival rate of SPT patients with local recurrence or metastasis, it is difficult to identify the predictive factors for their survival time.

Although this retrospective review has certain limits due to the available data, our results are consistent with the reported findings^[4,11]. Further study should be based on the selective cases with more detailed information.

In conclusion, SPT, an infrequently-encountered tumor, typically affects young women without significant symptoms. Its behavior is relatively indolent and largely benign. Patients may survive a long time after radical resection of the tumor. Whenever possible, surgery is justified for local invasion or metastasis of SPT. The prognosis of SPT patients even with unresectable metastasis is good. The role of chemotherapy and radiotherapy remains to be studied.

COMMENTS

Background

Solid pseudopapillary tumor (SPT) of the pancreas is a rare neoplasm with a low malignant potential, usually affecting young women in the second or third decade of life. With widespread availability of high-quality imaging systems and a better understanding of its pathology, the number of cases reported in the literature has been steadily increased in recent years.

Research frontiers

The clinical and pathological characteristics of SPT are still not fully recognized. Although a variety of radiological investigations have been performed for the evaluation, its misdiagnosis rate is still high. The experience in management of SPT, especially with a malignant potential, is still not adequate enough.

Innovations and breakthroughs

In this study, the authors collected relatively comprehensive data from the published Chinese literature and found that SPT is potentially curable with extended resections. The prognosis of SPT patients even with recurrence or unresectable metastasis is good. In addition, the authors analyzed the potential relation between hepatitis B virus infection and pathogenesis of SPT. The positive staining of Ki-67 may correlate with the malignant potential and poor outcome of SPT.

Applications

The results of this study indicate that the behavior of SPT is relatively indolent and largely benign. However, surgical resection is warranted since patients can survive a long time after it.

Peer review

This is a retrospective study summarizing a considerable number of patients (553 patients) with SPT of the pancreas covered in two Chinese databases. The manuscript is consisted of a good description of the patients with their demographic data, symptoms, imaging studies, location and invasion, surgical data and complications, pathology, immunohistochemical profile and survival time provided.

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Bone marrow cells produce nerve growth factor and promote angiogenesis around transplanted islets

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growth factor (NGF) and glucose tolerance (GTT) up to postoperative day (POD) 14. Histological assessment for insulin, von Willebrand factor (vWF) and NGF was performed at POD 3, 7 and 14.

RESULTS: Blood glucose level was lowest and serum insulin was highest in the islet + bone marrow group. Serum NGF increased in islet, bone marrow, and islet + bone marrow groups after transplantation, and there was a significant difference ($P = 0.0496$, ANOVA) between the bone marrow and sham groups. The number of vessels within the graft area was significantly increased in both the bone marrow and islet + bone marrow groups at POD 14 as compared to the islet alone group (21.2 ± 3.6 in bone marrow, $P = 0.01$, vs islet group, 22.6 ± 1.9 in islet + bone marrow, $P = 0.0003$, vs islet group, 5.3 ± 1.6 in islet-alone transplants). NGF was more strongly expressed in bone marrow cells compared with islets.

CONCLUSION: Bone marrow cells produce NGF and promote angiogenesis. Islet co-transplantation with bone marrow is associated with improvement of islet graft function.

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Key words: Islet transplantation; Bone marrow cells; Nerve growth factor; Angiogenesis; Endothelial precursor cells

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Sakata N, Chan NK, Chrisler J, Obenaus A, Hathout E. Bone marrow cells produce nerve growth factor and promote angiogenesis around transplanted islets. *World J Gastroenterol* 2010; 16(10): 1215-1220 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v16/i10/1215.htm> DOI: <http://dx.doi.org/10.3748/wjg.v16.i10.1215>

Abstract

AIM: To clarify the mechanism by which bone marrow cells promote angiogenesis around transplanted islets.

METHODS: Streptozotocin induced diabetic BALB/c mice were transplanted syngeneically under the kidney capsule with the following: (1) 200 islets (islet group: $n = 12$), (2) $1-5 \times 10^6$ bone marrow cells (bone marrow group: $n = 11$), (3) 200 islets and $1-5 \times 10^6$ bone marrow cells (islet + bone marrow group: $n = 13$), or (4) no cells (sham group: $n = 5$). All mice were evaluated for blood glucose, serum insulin, serum nerve

INTRODUCTION

Islet transplantation is a promising treatment for type 1 diabetes mellitus (T1DM). However, clinical islet transplantation using current protocols has not yet yielded long-term insulin-independence^[1]. One of the hurdles to overcome is the lack of a vascular network to support the newly transplanted islets. Initial avascularity in the period during islet isolation, transplantation^[2,3], and the establishment of a vascular network around islets renders islets vulnerable to severe hypoxia for up to 14 d after transplantation^[4]. Therefore, the promotion of angiogenesis is an important endeavor to prevent islet graft failure.

Recently, bone marrow transplantation as a cell therapy for resolution of clinical diseases has been studied. Endothelial precursor cells (EPCs), a heterogeneous population originating in the hematopoietic compartment of bone marrow, have an important role in the angiogenesis of adult tissues^[5]. Transplanted EPCs induce hypoxia-inducible factor-1 α (HIF-1 α) under hypoxic conditions which leads to upregulation of vascular endothelial growth factor (VEGF) and promotes vascularization^[5-7]. Our previous study revealed that transplanted bone marrow produces VEGF and promotes vascularization around the co-transplanted islets^[8].

Nerve growth factor (NGF), which plays an important role in promoting growth, differentiation and function of nerve cells^[9,10] has been shown to have an important role in angiogenesis by stimulating VEGF^[10,11]. Moreover, NGF is secreted by islets and may have a beneficial effect on islet function^[12]. In this study, we focused on NGF levels and its effects to clarify the mechanism of angiogenesis brought by bone marrow cell transplantation.

MATERIALS AND METHODS

Animals

BALB/c male mice (22-27 g, Charles River Laboratories, Inc., Boston, MA, USA) were used as both donors and recipients. The mice were housed under pathogen-free conditions with a 12-h light cycle and free access to food and water. All animal care and treatment procedures were approved by the Institutional Animal Care Use Committee.

Induction of diabetes in recipient mice

Streptozotocin (STZ, 200 mg/kg per mouse, Sigma-Aldrich, St. Louis, MO, USA) was injected intraperitoneally and blood glucose levels were measured by Accu-Chek Aviva glucose monitors (Roche, Indianapolis, IN, USA). We used the recipient mice once the blood glucose level was greater than 19.3 mmol/L (350 mg/dL).

Islet isolation

Murine islets were isolated by collagenase (collagenase V, Sigma-Aldrich) digestion, and separated by Ficoll (Sigma-Aldrich) discontinuous gradients and purified as previously

described^[13]. Collected islets were 133-200 μ m in size^[14]. Based on previous results^[15], 200 islets were considered a marginal islet mass for restoring normoglycemia in streptozotocin-induced diabetes.

Bone marrow cell isolation

The protocol of bone marrow cell isolation was modified from the Soleimani method^[16]. Under general anesthesia with 2% isoflurane, hind limb extirpations were performed at the hip, knee and ankle joint. Muscle and connective tissue were dissected away from the femur and tibia, the knee joint and both ends of the bones were cut. A thirty gauge needle with a 1 mL syringe was inserted into the bone, and bone marrow was flushed by injection with Hanks balanced salt solution (HBSS) for collection. After a single wash, bone marrow was dispersed by incubation with 0.05% trypsin/0.53 mmol/L EDTA solution (Mediatech Inc., Manassas, VA, USA).

Islet and bone marrow cell transplantation and group classification

Cell transplantation was performed under the left kidney capsule. Recipients were divided into four graft groups: (1) islet-alone (200 syngeneic islets per recipient, $n = 12$), (2) bone marrow ($1-5 \times 10^6$ syngeneic bone marrow cells per recipient, $n = 11$), (3) islet + bone marrow (200 syngeneic islets and $1-5 \times 10^6$ syngeneic bone marrow cells, $n = 13$) and (4) sham (skin and renal capsule incisions with no transplantation, $n = 5$).

Islet function parameters

Blood glucose and serum insulin were measured at postoperative days (POD) 0, 3, 7 and 14. Glucose tolerance was assessed at POD 7 and 14. Achievement of normoglycemia was defined as a non-fasting blood glucose level of ≤ 11 mmol/L (200 mg/dL). Intraperitoneal glucose tolerance tests (GTT) were performed by overnight fasting for 10 h and then injecting mice with a 2.0 g/kg body weight of glucose solution followed by tail vein blood samples at 0, 15, 30, 60, 90 and 120 min after injection. Blood glucose levels were measured by Accu-Chek Aviva glucose monitors and serum insulin was measured with a rat/mouse insulin enzyme-linked immunosorbent assay (ELISA) kit (Linco, MO).

Serum NGF measurement

Blood samples for serum NGF were obtained at POD 0, 3, 7 and 14 as for serum insulin. Serum NGF was measured with a NGF ELISA kit (NGF E_{max}[®] ImmunoAssay System, Promega, Madison, WI, USA).

Correlation between NGF and islet functional parameters

To evaluate whether NGF level affects islet function, simple regression analysis was performed between serum NGF and each of the following parameters: blood glucose, serum insulin and area under the curve (AUC)^[17]. All the data acquired from POD 0 to 14 were applied to the analysis.

Histological assessment

Kidney specimens were obtained from three or more mice at POD 3, 7 and 14 and photographs of the fresh organs were taken to assess the density of new vessels around islets and/or bone marrow. Tissue was then fixed with 10% formalin, embedded in paraffin, and cut into 5 μ m sections. Specimens were stained with hematoxylin and eosin (HE) to identify cellular changes. Apoptosis was detected by the terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-biotin nick end labeling (TUNEL) method using an *in situ* apoptosis detection kit (Promega). Sections were treated with proteinase K and incubated with TdT enzyme for 60 min at 37°C. After washing in Phosphate buffer solution (PBS) the sections were further incubated with streptavidin horseradish peroxidase (HRP) solution and visualized with 3,3'-diaminobenzidine (DAB). Immunohistochemical staining was done for insulin (islets), von Willebrand Factor (vWF, for newly formed blood vessels) and NGF. For vWF, specimens were treated with Proteinase K (Dako, Carpinteria, CA). Primary antibodies were guinea pig anti-insulin antibody (Dako, Carpinteria, CA, USA) diluted 1:100, rabbit anti-vWF (Abcam, Cambridge, MA) diluted with 1:2000 and rabbit anti-NGF (Santa Cruz Biotechnology Inc.) diluted 1:100. After incubating with biotinylated secondary IgG antibody (Vector Laboratories, Burlingame, CA, USA and Santa Cruz Inc.), a peroxidase substrate solution containing DAB (Brown, Dako) or aminoethylcarbazol (AEC, Red for insulin, Dako) was used for visualization and counterstained with hematoxylin.

vWF-positive vessel numbers were calculated from vWF-positive lumens at the transplant site.

Statistical analysis

Data are expressed as mean \pm SE of the mean. All the statistical analyses were performed with JMP 5.0.1J for Macintosh (SAS Institute Inc., Cary, NC, USA). Dunnett *t*-test or analysis of variance (ANOVA) was performed. Significance was designated at $P < 0.05$. Correlation coefficients (R^2) in regression analysis were defined as follows: Very strong correlation as $0.8 < R^2 < 1.0$; strong as $0.5 < R^2 < 0.8$, moderate as $0.25 < R^2 < 0.5$; weak $R^2 < 0.25$. Correlation was designated positive when the R^2 was over 0.25 (moderate).

RESULTS

Blood glucose, serum insulin and GTT

These data were previously published^[8]. In summary, islet co-transplantation with bone marrow yielded lower blood glucose, higher serum insulin, and improved glucose tolerance.

Serum NGF

Serum NGF levels were higher in islet, bone marrow and islet + bone marrow groups than in the sham group, and were higher at all times points relative to pre-transplant levels ($P < 0.05$, Figure 1). The increase was

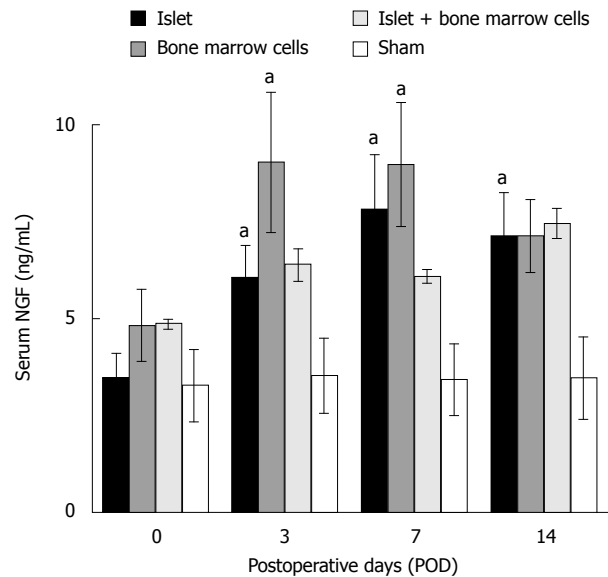


Figure 1 Serum nerve growth factor (NGF) data. Serum NGF levels increased post-transplantation in islet, bone marrow, and islet + bone marrow groups, while there was no increase in the sham group. There was a significant difference between bone marrow and sham group. Statistical analysis was performed by two ways repeated measurement ANOVA and significant difference was $^aP < 0.05$ vs POD 0.

most prominent in the bone marrow *versus* the sham group ($P = 0.0496$, ANOVA).

New vascularization

These data were previously published^[8]. In summary, bone marrow transplantation with or without islets was associated with enhanced angiogenesis which was more prominent in bone marrow alone than in the combined islet-bone marrow group [21.2 ± 3.6 in bone marrow ($P = 0.01$, *vs* islet group), 22.6 ± 1.9 in islet + bone marrow ($P = 0.0003$, *vs* islet group), 5.3 ± 1.6 in islet-alone transplants].

Histological findings

NGF was much more strongly expressed in bone marrow cells as compared to islets at every time point (Figure 2). No apoptotic (TUNEL positive) islets were detected in any of the experimental groups (data not shown).

Correlation between serum NGF and islet function parameters

There were no significant correlations between serum NGF and blood glucose, serum insulin or glucose tolerance (Figure 3).

DISCUSSION

NGF is a neurotrophin that plays a crucial role in promoting growth, differentiation and function of sympathetic nerve cells^[9,10,18]. NGF levels decrease with diabetes and are correlated with neuropathy. Thus a therapeutic trial to increase NGF has been performed to improve diabetic neuropathy^[19]. Recently, some studies

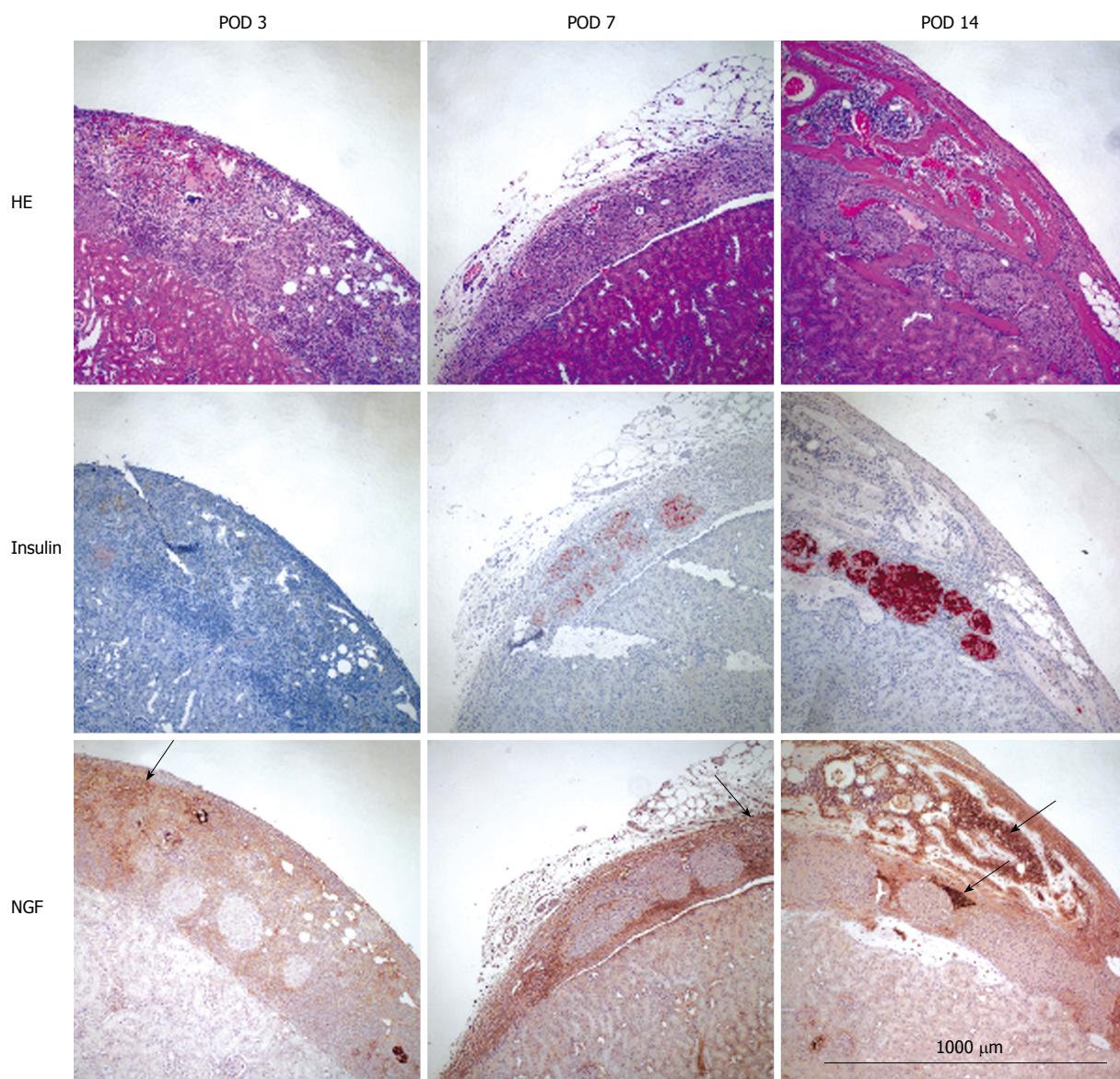


Figure 2 Histological findings. Kidney specimen from islet + bone marrow group stained with HE (top), Insulin (middle) and NGF (bottom) at POD 3, 7 and 14. Bone marrow cells stained strongly for NGF compared to islets at all time points (indicated by arrow). Magnification = 100 ×, Calibration bar = 1000 μm.

have reported the angiogenic effects of NGF^[10,20-25]. Earlier studies showed that NGF promotes the neovascularization of endothelial cells using HUVEC or matrigel assays^[20,24].

In diabetes, NGF has been shown to reverse local tissue hypoxia and endothelial cell impairment^[23,25]. Treatment with NGF prevents apoptosis of endothelial cells related with neovascular formation and progress^[22-25]. Angiogenesis induced by NGF is presumed to help in wound closure^[25] and recovery of ischemia in diabetics^[23], as well as organ remodeling^[26]. NGF may also contribute to the progress, migration and metastasis of tumors^[21,27].

We have previously shown that treatment with NGF improved islet function *in vitro* and *in vivo* and promoted vessel formation around transplanted islets^[12]. NGF may have a role in islet transplantation by promoting angiogenesis and preventing hypoxia at the early post-transplant period. However, this remains to be tested and reproduced

in appropriate trials.

In a previous study, we showed an association between bone marrow cell transplantation and angiogenesis around islets, together with enhanced VEGF expression and improved islet function^[8]. A potential role for NGF in these improvements was the focus of this study in view of reports that NGF is derived from bone marrow stem cells^[28] and stimulates VEGF, promoting angiogenesis^[10,11]. NGF's stronger expression in bone marrow relative to islets in this study was not predicted especially in view of the increase in serum NGF level in the islet group though it may be a function of ambient glucose concentration^[29]. Effects on islet graft function may also be related to the type of NGF receptor activated in the context of bone marrow co-transplantation^[30].

NGF is induced by hypoxia^[23] and also has anti-apoptotic roles^[31], therefore, it has the potential to improve the function and survival of transplanted islets^[12]. No

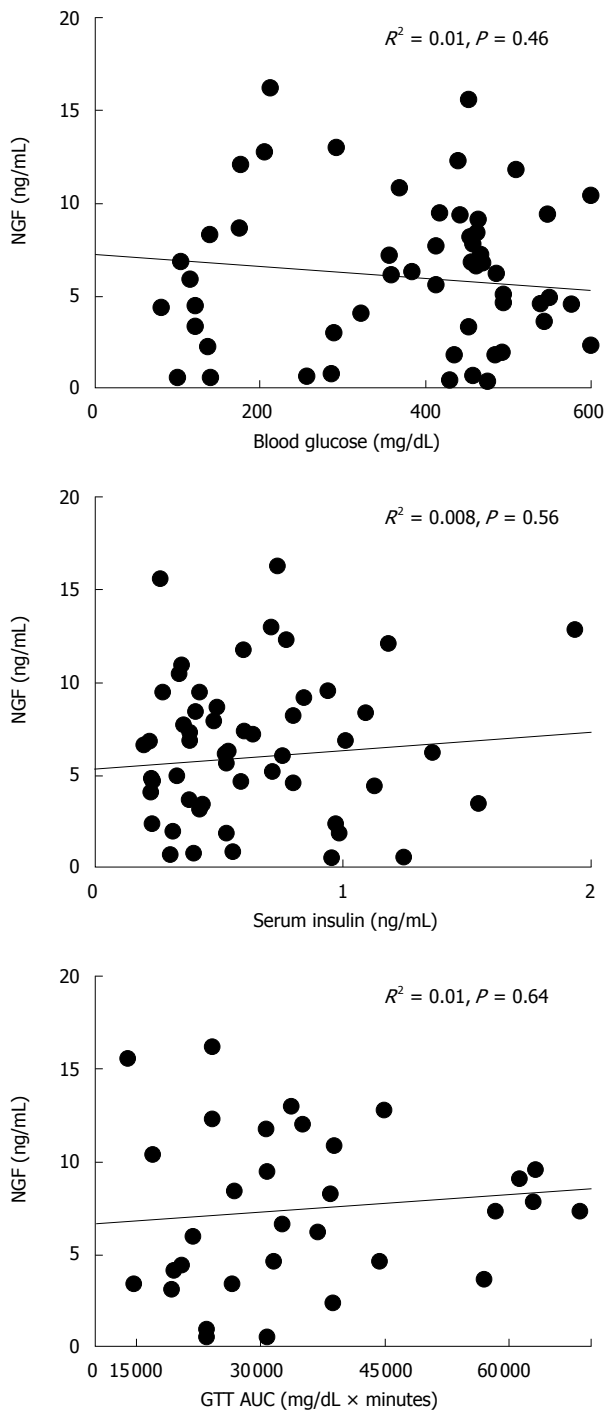


Figure 3 Correlation between serum NGF and blood glucose, serum insulin and GTT AUC. There were no correlation between serum NGF and other islet functional factors. $R^2 > 0.25$ and $P < 0.05$ is significant correlation.

apoptotic islets were detected during the observation span of this study, but renal subcapsular islet transplantation may not uniformly manifest the effects of severe ischemia seen with intraportal islet transplantation^[14]. Therefore, NGF may be particularly beneficial for intraportal islet transplantation.

The lack of correlation between serum NGF and other islet parameters (blood glucose, serum insulin and GTT) remains to be further confirmed. NGF levels vary according to the condition of islets. For example,

one study revealed that STZ stimulation increased NGF secretion^[32] while another showed that NGF is decreased in diabetes-associated conditions^[23]. On the other hand, NGF may reflect the status of islet vascularization rather than its function.

In conclusion, NGF production may underlie the beneficial effect of bone marrow co-transplantation on islet graft function. The mechanism of this potential benefit deserves further investigation.

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COMMENTS

Background

Islet transplantation is a promising treatment for type 1 diabetes mellitus (T1DM). However, clinical islet transplantation using current protocols has not yet yielded long-term insulin-independence. One of the hurdles to overcome is the lack of a vascular network to support the newly transplanted islets. The promotion of angiogenesis is an important endeavor to prevent islet graft failure.

Research frontiers

Nerve growth factor (NGF) is a neurotrophin that plays a crucial role in promoting growth, differentiation and function of sympathetic nerve cells. NGF levels decrease with diabetes and are correlated with neuropathy. Thus a therapeutic trial to increase NGF has been performed to improve diabetic neuropathy. Recently, some studies have reported the angiogenic effects of NGF.

Innovations and breakthroughs

NGF may have a role in islet transplantation by promoting angiogenesis and preventing hypoxia at the early post-transplant period. However, this remains to be tested and reproduced in appropriate trials.

Applications

Bone marrow cells produce NGF and promote angiogenesis. Islet co-transplantation with bone marrow is associated with improvement of islet graft function.

Terminology

Islet transplantation: islet transplantation is one of the therapies for T1DM. Islets are acquired from donor pancreas with the process of islet isolation. Acquired islets are transplanted into liver. Islet transplantation is a possibility for standard treatment of T1DM in the future.

Peer review

In this manuscript Sakata *et al* investigated whether islet transplantation in association with bone marrow transplantation improves diabetes related complications in streptozotocin induced diabetic mice. Although bone marrow cells yield similar results through vascular endothelial growth factor expression and this study is in press, their findings with NGF are still interesting and validates publication.

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Management of ruptured hepatocellular carcinoma: Implications for therapy

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Abstract

AIM: To assess the treatment and tumor-related variables associated with outcome after treatment of spontaneously ruptured hepatocellular carcinoma (HCC).

METHODS: Patients with ruptured HCC were identified. The complications, mortality and survival were assessed. The relationship between tumor size and the severity of hemoperitoneum and between tumor size and grade were examined.

RESULTS: From January 1993 to January 2008, 556 patients with HCC with or without cirrhosis were evaluated; of which, 16 (2.87%) presented with spontaneous rupture. All but 1 patient had cirrhosis. Twelve patients underwent surgical resection while 4 underwent trans-cutaneous arterial catheter embolization (TAE) (trans-cutaneous arterial embolization). Early mortality (< 30 d) was 25% (4 of 16) and was inversely related

to Child-Pugh score; 3 of the 4 early deaths occurred in patients treated with TAE with 1 of 12 occurring in the resected group. There was no correlation between tumor size and grade or between size and severity of hemoperitoneum.

CONCLUSION: Tumor size did not correlate with severity of the hemoperitoneum. There was an inverse relationship between G1-G3 (grade of cellular differentiation) HCC and dimensions.

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Key words: Bleeding hepatocellular carcinoma; Management of ruptured hepatocellular carcinoma; Ruptured hepatocellular carcinoma

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INTRODUCTION

Hepatocellular carcinoma (HCC) is a hypervascular tumor that almost always occurs in the setting of liver cirrhosis. It is the fifth most common cancer in the world and is gradually increasing^[1]. Spontaneous rupture is a complication seen in 3%-15% of cases, with an apparent lower incidence reported in Western compared with Asian centers^[2]. The exact mechanism leading to rupture is not clearly defined although it appears to be more

frequent when the tumor protrudes from the glissonian capsule^[3]. Spontaneous rupture is the third leading cause of HCC-related death, after tumor progression and liver failure, and is more common than rupture of oesophageal varices. Previously considered a problem of only large tumors, this is not always the case, and smaller tumors, particularly those with an aggressive behavior, are at risk. The symptoms are related to the position of the tumor. In fact, rupture of deep tumors may be asymptomatic or produce pain while a peripheral lesion will give rise to hemoperitoneum, often with peritonitis and hemodynamic instability.

Appropriate management of this problem remains the subject of some debate. In patients with advanced liver disease or multifocal HCC, trans-cutaneous arterial catheter embolization (TAE) is clearly the most appropriate approach. However, in patients with preserved liver function and resectable tumors, resection may be an option. Although burdened by high mortality in the acute phase and not always feasible, surgical resection offers the indisputable advantage of definitively treating the cancer, in addition to obtaining hemostasis, and may offer a better prognosis^[3]. This study analyzes the management of ruptured HCC in a single, tertiary referral center with experience of this disorder, specifically assessing peri-operative outcome with operative and non-operative therapy.

MATERIALS AND METHODS

Patients with HCC treated at the authors' institution were identified from a prospectively maintained hepatobiliary database and registry of Hepato-Biliary surgery. Patients who presented with ruptured tumors were taken from the database for in-depth analysis. Symptoms and clinical status on presentation were recorded and analyzed. Initial treatment was directed at patient resuscitation and hemodynamic stabilization according to the advanced trauma life support protocol. Blood products were administered as necessary. Cross-sectional imaging was used to determine the disease extent, and standard laboratory studies were used to assess hepatic function.

Definitive therapy was selected based on liver functional reserve according to Child-Pugh score, tumor extent and overall performance status. In patients with advanced cirrhosis (Child-Pugh C) or multifocal HCC, TAE was used as definitive therapy. In cases of surgical treatment, a right 'hockey-stick' incision was used as the favoured access; median laparotomy was used in one case. Procedures were classified based on Couinaud's classification^[4]. Non-anatomic resection (or wedge resection) denotes a non-segmental resection of the liver surrounding the cancer. The hepatic parenchyma was divided using the crush-clamping method and each vessel encountered was ligated or clipped. Hepatic inflow occlusion was carried out using the Pringle manoeuvre. Argon beam coagulator was used on the cut surface of the liver.

Table 1 Patient treatment after ruptured HCC

Patient	Age (yr)	Sex	Cirrhosis	HCC size	Grading	Treatment
1	54	F	C	80	1	Resection
2	42	M	-	80	1	Resection
3	59	F	Alc	40	1	Resection
4	76	M	Alc	30	3	Resection
5	65	M	Alc	40	1	Resection
6	84	M	Alc	20	3	Resection
7	74	M	C	19	3	Resection
8	80	M	Alc	70	1	Resection
9	73	F	C	30	3	Resection
10	78	M	C	28	3	Resection
11	60	M	Alc+C	50	1	Resection
12	49	F	Alc	30	3	Resection
13	67	F	C	70	-	TAE
14	74	F	Alc	90	-	TAE
15	71	M	Alc	40	-	TAE
16	67	M	C	45	-	TAE

Patient No. 2 cirrhosis was not present; patient No. 3 underwent R2 resection. HCC: Hepatocellular carcinoma; TAE: Trans-cutaneous arterial catheter embolization.

All patients, regardless of treatment, were monitored in the intensive care unit over the immediate post-treatment period. Macroscopic and microscopic histopathology tests were carried out on the resected specimens. The grade of cell differentiation was classified according to the Edmondson and Steiner system^[5]. Maximal diameter of the specimen was taken as the tumor size in patients submitted to resection, while radiological images were used to measure HCC in patients treated with TAE. Postoperative complications were documented and classified as minor or major. Peri-operative mortality was defined as death during the same stay in hospital or within 30 d of the date of operation.

Statistical analysis

Data were processed using Excel. The G1 and G3 groups were compared with the Student's *t* test for continuous data; the level of significance was assumed as $P < 0.05$.

RESULTS

From January 1993 to January 2008, 556 patients with HCC and cirrhosis were seen in our institution. One-hundred and four patients were treated with surgical resection, and 145 underwent percutaneous ethanol injection or radiofrequency ablation. Sixty were treated with TAE with or without chemotherapy. Sixteen patients presented with ruptured HCC, representing 2.87% of the entire population.

Twelve patients underwent surgical resection and 4 were treated with TAE (Table 1). There were 10 males and 6 females with a mean age of 67 years (range: 42-84 years). On admission, hemoperitoneum and abdominal pain were the first presentation in 14/16 cases. Hypovolemic shock was present in 10 patients. Abdominal ultrasonography (US) and/or computed tomography (CT)

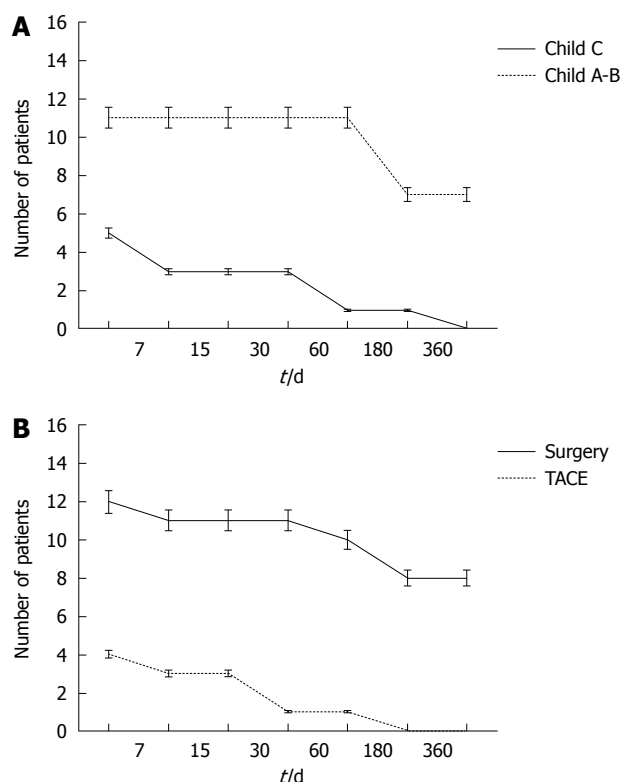


Figure 1 Summarizes early mortality in relation to liver functional reserve (A) and early survival in relation to treatment (B). A: Early overall mortality related to Child status; B: Early survival related to type of treatment.

showed hemoperitoneum in all 14 patients and delineated the liver disease in all but 1 case. Underlying cirrhosis was present in 15 patients (alcoholic in 8, hepatitis C infection in 6 and both in 1). Anemia with a haemoglobin level less than 10 g/dL was present in 7 patients. Four patients had alanine transaminase and aspartate transaminase levels more than twice the upper limits of normal, platelet count less than 100 000 mm³, pro-thrombin time less than 70% and albumin level less than 3.5 g/dL. These four patients were excluded from surgery and treated with TAE. One patient was referred to our unit after urgent laparotomy and packing performed in another hospital.

Resection was performed in 12 patients as a single stage operation, ranging from 1 to 6 d from the time of presentation. Seven of these patients had persistent anemia and hemodynamic instability despite aggressive resuscitation and underwent emergency surgery. There were 3 anatomic resections of 2 segments and 4 non-anatomic resections. Four patients stabilized after the initial resuscitative manoeuvres, and surgery was performed from 1-3 d later; the procedures performed in this group were 1 left hepatectomy and 3 wedge resections. One patient required emergency laparotomy for hemorrhagic shock before the investigations were completed and the diagnosis was known; he was found to have multifocal HCC, and a palliative resection of segment 2 and segment 3 was performed to stop the bleeding. This was the only patient with multifocal HCC and/or Child-Pugh C cirrhosis who underwent surgical resection in Child

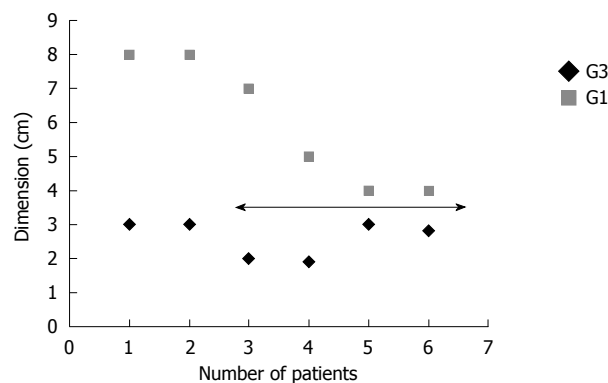


Figure 2 Relation between diameter and grading in ruptured HCC, average diameter of G1 is 60 mm and 26 mm for G3. HCC: Hepatocellular carcinoma.

C status and with multifocal and bilobar HCC; he died shortly after the operation from coagulopathy and liver failure. One patient required reoperation for bleeding on day 2 after the initial operation.

For all patients, the 30 d mortality rate was 25% ($n = 4$ patients, Figure 1A) and was directly related to underlying liver function. All four patients with Child-Pugh C cirrhosis died in the early post-treatment period – one after resection and three after TAE. The causes of death were coagulopathy ($n = 2$), recurrent bleeding ($n = 1$) and liver failure ($n = 1$). Three additional patients with Child-Pugh A or B cirrhosis and treated with surgical resection died within 6 mo due to liver failure (2 mo, $n = 1$), myocardial infarction (3 mo, $n = 1$), liver failure and progression of neoplastic disease (4 mo, $n = 1$, Figure 1B). Two of these patients had G3 HCC with vascular invasion and none of these patients underwent emergency surgery. They were transfused with 17 units of RPC.

Five patients were treated by emergency surgical resection and 2 patients who were treated with delayed surgical resection, survived more than 24 mo (long-term survival 46%).

Seven patients (43%) treated with resection, (emergency $n = 5$ and delayed $n = 2$) survived more than 24 mo. In all of these patients, the tumor grade was G1. Six of these patients were transfused with 27 units of RPC (range: 3-8). Postoperative ascites occurred in 5 patients, while 1 patient developed sub-phrenic abscess with collection drained percutaneously.

Histological study of the specimens confirmed HCC in all cases. Underlying cirrhosis was present in 11 cases. The average diameter of the HCC was 61 mm (range: 60-90 mm) for the patients who underwent TAE (measured with US or CT) and 43 mm for patients with resected HCC (range: 19-80 mm). The study on the histological specimen showed an average diameter of 60 mm for HCC in G1 status and 26 mm for G3 (Figure 2, $P = 0.001$).

DISCUSSION

HCC is the fifth most common tumor in the world and usually occurs in cirrhosis of alcoholic or viral (hepatitis

B or C) etiology^[1]. HCC is a hypervascular tumor with a high propensity for vascular invasion and the capability to produce growth factors which have a direct effect on endothelial cells to induce neo-angiogenesis. The rupture of HCC is a complication that can occur in 3%-15% of patients, although this has decreased now that screening programs for cirrhotics have been implemented^[1,2]. There is a relevant geographical difference between western and oriental countries where a major incidence has been reported in oriental countries^[2,6].

The mortality in the acute phase is very high and stands at around 25%-75% of cases^[6]. This complication is the third most common cause of death for HCC after neoplastic progression and liver failure; the mortality is higher than that due to bleeding resulting from rupture of esophageal varices^[6,7]. Although the incidence of viral cirrhosis is generally predominant, in the northeast area of Italy, as is documented both in the present study and in a recent publication, 50% of HCC occurs in cirrhosis due to alcohol abuse, while the remaining cases can be attributed to hepatitis virus C infection; the incidence of infection related to hepatitis virus B is occasional (Table 1)^[8].

The average age reported in the literature varies between 44 and 68 years, with a high prevalence in males (at least 3 times more frequent), confirming the overall impact of HCC^[6,9]. In this series, there was a high prevalence in men with respect to women (10 men and 6 women) with an average age of 67 years (range: 42-84 years).

According to the literature, the risk factors for rupture are not well known although rapid growth with necrosis, erosion of vessels, thrombosis of veins in HCC by tumor thrombi or direct invasion individually or combined could be responsible^[10,11].

The most common condition for bleeding into the peritoneum is when the HCC is located in one of the free surfaces of the liver. In this case there is no hepatic parenchyma surrounding the tumor and this is free to flood the peritoneum with blood and neoplastic cells. Many intra-parenchymal HCC may spontaneously break, but a deeper position in the center of the liver confines the bleeding around the cancer and the clinical manifestation is only pain without hemoperitoneum. Abnormal clotting and thrombocytopenia typical of cirrhosis may also worsen the hemorrhagic event.

The typical symptoms of spontaneous rupture are epigastric pain associated with clinical signs of shock and peritoneal irritation. This is especially true when the lesion is more peripheral on the liver. Peritoneal irritation due to bleeding is not evident in cases of rupture of a deeper lesion which does not interrupt the liver capsule. In addition to pain and hemorrhagic shock, the risk of peritoneal seeding may worsen the prognosis of these patients. In all of the cases in our study, rupture occurred in a peripheral tumor. A study by Kanematsu *et al*^[12] in 1992, reported that protrusion of the tumor is a relevant prognostic factor of rupture. The peri-

operative mortality in cases who undergo urgent surgery is considerably higher than those reported for planned liver resections in patients with cirrhosis and is related to Child status before the rupture^[9]. Of course, bleeding and shock are factors which strongly influence the prognosis of these patients, and bleeding "*per se*" worsens the prognosis, however, decompensated cirrhosis may be fatal in cases of poor liver function. Therefore, the first step of treatment is resuscitation and stabilization of the patient, but achieving hemostasis is the primary goal. When facing a rupture of HCC with underlying cirrhosis it is mandatory to obtain a correct diagnosis in order to achieve the best treatment for each patient. The therapeutic approach may be crucial for prognosis. This choice must take into account the hemodynamic conditions, functional status of the liver and stage of cancer.

Although associated with complications such as re-bleeding and a mortality rate of around 30%, TAE is the best method to achieve hemostasis without surgery^[6,13]. Moreover, when feasible, super-selective TAE is able to preserve liver function and has the dual outcome of being a definitive treatment or a bridge to resectable HCC. As in other studies, the four patients in our study treated with TAE should not be compared with those who were treated with surgical resection. In fact, all four patients had severe cirrhosis or bilobar HCC while the resected patients had favourable location of HCC and good functional reserve. For this reason a comparative study and analysis of the results reported in the literature are very difficult. Our experience confirms that TAE is indicated in cases of poor liver function (Child C) or in cases of multifocal or bilobar HCC. Alternatively, TAE can be used as a bridge to surgical resection^[14]. According to previously published reports and analysis of the data from the present study in patients with Child C status who underwent TAE, it is clear that the post-procedure mortality was very high, confirming that poor liver functional reserve determines survival regardless of treatment type^[6]. Moreover, it is worth remembering the demerits of TAE which include rebleeding, liver abscess and implanted peritoneal metastases^[15].

Histological examination revealed that the average size of the lesions in this study was 43 mm. Substantial differences were seen between the dimensions in relation to grading. In fact, G1 HCC had an average size of 60 mm, while G3 HCC had an average size of 26 mm ($P = 0.001$). However, all lesions were on the liver surface. We did not find a relationship between severity of hemoperitoneum and size or grading of HCC.

The experienced liver team should evaluate the next treatment steps according to liver functional reserve and extension of HCC. Preoperative assessment of the patient with blood tests is mandatory as is cancer staging using US and radiological imaging.

It was reported by Tanaka *et al*^[6] that rupture of HCC should be differentiated when it occurs in the early phase of cirrhosis rather than in the late phase. The prognosis is strongly determined by the functional status of the

liver immediately after bleeding. In fact, 3 patients in the present series were excluded from surgery due to poor liver function and not surprisingly had the worst outcome. In this cohort, functional liver reserve and G3 status influenced outcome, while emergency or delayed surgery and HCC dimensions did not seem to modify the prognosis. Several reports in the literature demonstrate acceptable results even in the case of one stage hepatectomy for ruptured HCC and limited resection is recommended^[2,16,17].

Moreover, peritoneal washing with saline solution can help to reduce the chance of spreading peritoneal cancer which may be a complication of TAE^[18,19].

In conclusion, in cases of ruptured HCC, any lesion should be considered for surgery if a low-risk curative resection is possible in a Child A-B patient. If necessary and possible, the intervention may be delayed. TAE is palliative procedure indicated when the liver function is compromised or in the case of multifocal-bilobar HCC. The introduction of screening programs should reduce the number of large HCCs which have broken, while we may have some concern about the rupture of smaller poorly differentiated tumors.

COMMENTS

Background

Hepatocellular carcinoma (HCC) is a hypervascular tumor that sometimes due to unknown reasons breaks spontaneously regardless of size, grading and state of cirrhosis. Location on the free surface of the liver seems to be a major factor in rupture of HCC. The choice of therapy is closely linked to liver functional reserve, grade of tumor and performance status of the patient.

Research frontiers

Larger prospective studies are necessary to assess the relationship between HCC dimensions and grading and the possibility of rupture.

Innovations and breakthroughs

There is an inverse relationship between G1-G3 HCC and dimensions. In the case of ruptured HCC, resection should not be excluded "a priori" but carefully considered since it has the dual purpose of both achieving hemostasis and being a definitive treatment.

Applications

In the case of advanced liver disease or bilobar multifocal HCC, a non-operative approach such as trans-cutaneous arterial catheter embolization must be attempted. In contrast, preserved liver function and resectable tumor should be considered as clear indications for surgical resection.

Peer review

This study aimed to assess the treatment and tumor-related variables associated with outcome after treatment of spontaneously ruptured HCC. The paper is interesting.

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Microalbuminuria in hepatitis C-genotype 4: Effect of pegylated interferon and ribavirin

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Abstract

AIM: To study the relation between hepatitis C virus (HCV) genotype 4 and microalbuminuria and renal impairment in relation to hepatic histology, and viremia in the absence of cryoglobulinemia, and to examine the effect of treatment on microalbuminuria.

METHODS: Three hundred subjects, including 233 HCV genotype-4 infected patients, were tested for cryoglobulinemia, microalbuminuria, albumin creatinine ratio (ACR), urea, creatinine, and estimated glomerular filtration rate (eGFR). The parameters were measured again in the HCV patients after 48 wk of treatment with pegylated interferon and ribavirin.

RESULTS: Significantly higher levels of microalbuminuria were detected in HCV-positive patients compared to HCV-negative controls (median 9.5 vs 5.9, respectively, Kruskal-Wallis $P = 0.017$). Log microalbuminuria was significantly correlated with hepatic inflammation ($r = 0.13$, $P = 0.036$) and fibrosis ($r = 0.12$, $P = 0.061$), but not with viral load ($r = -0.03$, $P = 0.610$), or alanine transaminase ($r = -0.03$, $P = 0.617$). Diabetes mellitus neither significantly moderated ($\chi^2 = 0.13$, $P = 0.720$), nor mediated (Sobel test $P = 0.49$) the HCV effect. HCV status was significantly associated with log microalbuminuria ($\chi^2 = 4.97$, $P = 0.026$), adjusting for age, gender, diabetes, cryoglobulinemia, urea and creatinine. A positive HCV status was not significantly associated with low eGFR (< 60 mL/min every 1.73 m²) [odds ratio (OR): 0.5, 95% confidence interval (CI): 0.2-1.4], nor with high ACR (OR: 1.7, 95% CI: 0.7-4.1). End-of-treatment response (ETR) was achieved in 51.9% of patients. Individuals with ETR had significantly lower microalbuminuria post-treatment ($\chi^2 = 8.19$, $P = 0.004$).

CONCLUSION: HCV affected the development of microalbuminuria independent of diabetes or cryoglobulinemia. Combination therapy of pegylated interferon-ribavirin had a positive effect in reducing microalbuminuria.

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Key words: Hepatitis C virus; Genotype; Kidney diseases; Albuminuria; Proteinuria; Peginterferon α -2a; Ribavirin

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INTRODUCTION

Hepatitis C virus genotype 4 (HCV-G4) is prevalent in the Middle East and Africa and has spread to several regions in Europe^[1]. HCV infection is associated with several renal diseases including mixed essential cryoglobulinemia, membranoproliferative glomerulonephritis and, less frequently, membranous nephropathy and crescentic glomerulonephritis^[2]. HCV is a significant cause of glomerulopathy in countries with a high prevalence of HCV infection^[3]. Several studies have postulated a causal link between HCV infection and renal diseases through the induction of cryoglobulinemia^[4]. The affinity for the kidney mesangium appears to be a major factor responsible for the precipitation of type-II mixed cryoglobulins in glomerular structures and the ensuing damage^[5]. The principal clinical manifestations of glomerular disease in HCV-infected patients are the presence of proteinuria and microscopic hematuria with or without impaired kidney function^[6]. Diabetes mellitus-mediated glomerulonephropathy in HCV patients may be considered, particularly with epidemiological studies showing a high occurrence of type 2 diabetes in patients with chronic HCV-G4^[7]. Various approaches have been tried for the treatment of HCV-associated glomerulonephritis, including immunosuppressive therapy (corticosteroids and cytotoxic agents), plasma exchange and antiviral agents. Limited data exist regarding antiviral treatment of HCV-associated glomerulonephritis.

We present a novel study reporting the prevalence of microalbuminuria, with other measures of renal insufficiency [albumin creatinine ratio (ACR), estimated glomerular filtration rate (GFR)] in HCV-G4 patients compared to HCV-negative controls, and the effect of viral load on liver histology, microalbuminuria, and renal insufficiency. Also, we investigated the effect of antiviral therapy with pegylated interferon and ribavirin on microalbuminuria, and other measures of renal insufficiency.

MATERIALS AND METHODS

In this prospective study, 300 consecutive eligible subjects who attended the gastroenterology clinic in Hamad Hospital were recruited in the period between January 2006 and 2009. Of these, 233 were chronic HCV-G4 patients who fulfilled the inclusion criteria, and 77 were control subjects who were either non hepatic patients followed in the clinic during the same period or healthy volunteers who were referred to Hamad hospital when they were ill. Controls were eligible if they did not have liver disease, evidenced by: persistently normal transaminases, negative serology for hepatitis serology, negative screening for auto-immune disease and no history of alcohol consumption, normal platelet count and α fetoprotein, and normal ultrasound scans.

All study participants were seen 2-3 times to provide repeated blood samples at baseline. Chronic HCV patients were also seen periodically to assess the response to treatment. All patients provided written informed

consent as stated in the Declaration of Helsinki of 1979, and the ethics research committee of the Hamad Medical Corporation provided ethical approval.

Chronic HCV infection was evidenced by persistently increased alanine aminotransferase (ALT) levels, positive serology for anti-HCV, active virus replication by detection of HCV RNA in serum, and histological findings of chronic active hepatitis according to the Scheur score. The patients were excluded if they had: (1) concurrent hepatitis B or human immunodeficiency virus infections, autoimmune hepatitis, hemochromatosis, or Wilson's disease; (2) systemic hypertension or if they reported current use of antihypertensive medication; (3) active alcohol consumption; (4) antiviral or corticosteroid treatment; and (5) chronic renal disease or history of dialysis.

The HCV patients who were candidates for treatment were given 180 μ g of peginterferon α -2a (Pegasys®; Hoffmann-LaRoche, Basel, Switzerland) subcutaneously once weekly and ribavirin (Copegus®; Hoffmann-La Roche, Basel, Switzerland) at a daily oral dose of 1000 mg (body weight < 75 kg) or 1200 mg (body weight > 75 kg) for 48 wk.

Laboratory measurement

Testing for anti-HCV was carried out using a commercial ELISA kit (AxSYM HCV version 3.0; Abbott Laboratories, Chicago, IL, USA). All patients were HCV-G4 as detected by the Inno-LiPA HCV II assay (Innogenetics Inc., Alphenet, GA, USA). Monitoring serum HCV RNA levels was by Amplicor (version 2.0, Hoffmann-La Roche) with a minimum detection limit of 50 IU/mL.

Microalbuminuria was measured on a spot of second morning urine after an overnight fast, taking the mean of at least 2 samples collected for each subject. Urine albumin measurements were obtained by an automated immunoturbidometric assay (Roche Hitachi 902, Roche Diagnostics, Indianapolis, IN 46250 USA).

For quantitative determination of creatinine in serum or urine, creatinine blanked kinetic Jaffé (Roche Diagnostics, Hitachi 917, Modulator P analyzer Roche Diagnostics) was used. We estimated GFR (eGFR) using the abbreviated modification of diet in renal disease equation.

After an overnight fast, samples for cryoglobulin measurement were collected at a temperature of 37°C, centrifuged at 37°C for 10 min, and the serum separated; 5 mL of the serum was allowed to stand in a cryocrit tube at 4°C for 2-7 d, with formation of precipitate confirmed visually. If the test was positive we proceeded to electrophoresis and immunofixation for typing. All subjects were tested for cryoglobulin.

Measures of renal insufficiency

Three measures of renal insufficiency were examined, namely eGFR, microalbuminuria, and serum creatinine. Low eGFR was defined as eGFR < 60 mL/min every 1.73 m². The presence of microalbuminuria was tested using (1) albuminuria level; or categories of microalbuminuria, defined as individuals with an albuminuria level higher than the upper tertile of the albuminuria level among controls

(2) ACR: to adjust for the variation in urine concentration, microalbuminuria was assessed by ACR. Gender-specific values for the ACR were 2.2 mg/mmol for males and 2.8 mg/mmol for females. High levels of serum creatinine were defined as > 1.2 mg/dL for males and > 1.1 mg/dL for females.

Outcomes and covariates definitions

Subjects were considered diabetic if they had a fasting blood glucose level ≥ 5.6 mmol/L, or self-reported as being diabetic or on anti-diabetic medication. End-of-treatment response (ETR) was defined as loss of detectable serum HCV RNA at the end of treatment. Normal ALT was defined as ALT ≤ 31 U/L for women and ≤ 40 U/L for men.

Statistical analysis

Unadjusted association between renal insufficiency and HCV infection was evaluated. For categorical variables, the unadjusted association was tested using the Chi-square test (Fisher exact test if there was limited sample size thus violating the Chi-square test assumptions), while for continuous variables, analysis of variance or the Kruskal-Wallis test (when the assumption of normality was violated) were employed. Multivariate logistic regression (for categorical variables) and multivariate linear regression (for continuous variables) were used to test for the null hypothesis of no significant association between renal insufficiency and HCV infection controlling for possible confounders and covariates. Microalbuminuria, ACR and eGFR data were log transformed to adjust for skewedness.

We tested for the presence of an interaction between HCV and diabetes, by adding the cross product term of HCV \times diabetes in addition to HCV and diabetes in the regression model predicting microalbuminuria.

In addition, we employed mediation analysis to test whether the effect of HCV on the risk of developing microalbuminuria was mediated by diabetes. Baron and Kenny's criteria was used to assess the presence of mediation^[8]. Mediation was expected if diabetes accounted for part or all of the relationship between HCV and microalbuminuria, as manifested by a decrease in the magnitude of the previously significant association between HCV and microalbuminuria upon controlling for diabetes^[8]. The significance of the mediation pathway was tested using the Sobel test as described elsewhere^[9].

To test for treatment effect, we examined differences between pre- and post-treatment using the Wilcoxon Signed Rank test which is a non-parametric equivalent of the paired *t*-test. In the multivariate analysis we tested for significant predictors of microalbuminuria post-treatment compared to pre-treatment, using the generalized estimating equation model.

All analyses were conducted using SAS 9.1 software.

RESULTS

The study population consisted of 300 subjects, of

Table 1 Study subjects characteristics by HCV status

Characteristics	HCV status	
	HCV-negative	HCV-positive
Age (yr)	46.0 (57.0)	46.0 (51.0)
Gender ^a <i>n</i> (%)		
Male	44 (18.4)	195 (81.6)
Female	23 (38.3)	37 (61.7)
Diabetes <i>n</i> (%)		
No	31 (19.6)	127 (80.4)
Yes	32 (23.2)	106 (67.8)
Fibrosis ^a <i>n</i> (%)		
No/mild	67 (54.0)	57 (46)
Moderate/severe	0 (0.0)	137 (100)
Inflammation ^a <i>n</i> (%)		
No/mild	67 (100)	0 (0.0)
Moderate/severe	0 (0.0)	194 (100)
Body mass index (kg/m ²)	27.9 (26.7)	29.2 (25.3)
Urea (mmol/L)	4.6 (41.4)	4.7 (128.9)
Creatinine (mg/dL)	75.0 (248.0)	77.0 (230.0)
ACR (mg/mmol)	1 (36.8)	1 (193.9)
eGFR (mL/min every 1.73 m ²)	99.5 (317.0)	98.0 (155.0)
Microalbuminuria ^{a,b} (μg/mg)	5.9 (299.0)	9.5 (399.0)
ALT ^{a,b} (U/L)	25.0 (110.0)	66.0 (583.0)
Hemoglobin ^{a,b} (g/dL)	14.0 (7.0)	14.9 (9.5)

^a*P* < 0.05; ^bKruskal-Wallis test. HCV: Hepatitis C virus; ACR: Albumin creatinine ratio; eGFR: Estimated glomerular filtration rate; ALT: Alanine aminotransferase. Quantities given as median (range).

whom 233 (77.7%) were HCV-positive. At enrolment 138 (46.6%) were diabetics. The majority of the study participants [239 (79.9%)] were male and 195 (81.6%) of the males were HCV-positive compared with 37 (61.7%) of females. Median age of the study participants was 46 years (inter-quartile range, 41-53 years). The histopathological changes of the liver in HCV patients were classified according to the Scheuer score, and fibrotic changes of Stages I, II, III and IV were seen in 58 (25.1%), 89 (38.3%), 60 (25.7%) and 26 (10.9%) patients, respectively. Necro-inflammatory changes were seen in 23 (9.9%), 109 (46.8%), 58 (25.1%) and 4 (1.7%) patients, respectively. A median microalbuminuria of 8.2 (inter-quartile range, 4.4-16.9) μg/mg was observed, while the median eGFR was 98 (inter-quartile range, 85-112) mL/min every 1.73 m². One hundred and eighteen individuals had microalbuminuria (39.7%), including 44 (37.6%) HCV-positive non-diabetic, 58 (49.6%) HCV-positive diabetic, 7 (6.0%) control non-diabetic, and 8 (6.8%) control diabetic subjects. Forty five individuals had high ACR (15.8%), including 13 (29.6%) HCV-positive non-diabetic, 25 (56.8%) HCV-positive diabetic, 2 (4.6%) control non-diabetic, and 4 (9.1%) control diabetic subjects. Only 15 (5.3%) had low eGFR, of whom there were 3 (20%) HCV-positive non-diabetic, 7 (46.7%) HCV-positive diabetic, 3 (20%) control non-diabetic, and 2 (13.3%) control diabetic subjects. Of 85 HCV patients who were on treatment, 44 (51.8%) showed an ETR.

Table 1 demonstrates the study participants' characteristics by HCV status. HCV-positive individuals had significantly higher ALT levels and higher microalbuminuria. No difference was detected in age, body mass index, urea, creatinine or gender (Table 1).

Microalbuminuria

Levels of microalbuminuria were significantly higher among HCV-positive individuals than HCV-negative patients (median 9.5 *vs* 5.9, respectively, Kruskal-Wallis $P = 0.017$). A significantly higher prevalence of microalbuminuria (defined as albuminuria > upper tertile of the controls) was observed among HCV-positive individuals (53.7%) compared to HCV-negative individuals (31.8%), ($\chi^2 = 9.8$, $P = 0.002$).

Log microalbuminuria was significantly correlated with grade ($r = 0.13$, $P = 0.036$), borderline correlated with older age ($r = 0.11$, $P = 0.069$) and more fibrosis ($r = 0.12$, $P = 0.061$), but not significantly associated with viral load ($r = -0.03$, $P = 0.610$), or ALT levels ($r = -0.03$, $P = 0.617$).

There was no significant interaction between HCV status and diabetes such that the odds ratio (OR) of microalbuminuria among diabetics compared to non-diabetics was not significantly higher in HCV-positive individuals compared to HCV-negative controls (P for interaction = 0.720) (Table 2).

For sensitivity analysis we restricted the analysis to non-diabetics. Among non-diabetics, the prevalence of microalbuminuria was significantly higher in HCV-positive individuals (50%) compared to HCV-negative controls (25.8%) ($\chi^2 = 5.9$, $P = 0.015$). Restricting the analysis to individuals with no cryoglobulinemia revealed that microalbuminuria was significantly higher in HCV-positive individuals (53.3%) compared to HCV-negative controls (31.8%) ($\chi^2 = 9.4$, $P = 0.002$).

To adjust for potential confounders and important covariates we employed multivariate regression to test for the significance of HCV as a predictor for microalbuminuria. Log microalbuminuria was used in the linear regression analysis because of the skewedness of the microalbuminuria data. Similar to the unadjusted analysis, we did not detect a significant interaction between diabetes and HCV ($\chi^2 = 1.2$, $P = 0.272$), thus no interaction term was included in the final model.

After adjusting for age, gender, diabetes, cryoglobulinemia, urea and creatinine, there was a significant association between HCV status and log microalbuminuria ($\chi^2 = 4.97$, $P = 0.026$). Microalbuminuria was significantly associated with urea ($\chi^2 = 8.2$, $P = 0.004$), creatinine ($\chi^2 = 27.0$, $P < 0.0001$), diabetes ($\chi^2 = 8.2$, $P = 0.004$), but not with age ($\chi^2 = 0.0$, $P = 1.0$) or gender ($\chi^2 = 0.4$, $P = 0.530$) or cryoglobulinemia ($\chi^2 = 0.2$, $P = 0.703$).

We tested whether diabetes mediated the effect of HCV on microalbuminuria risk. Diabetes was not a significant mediator of the effect of HCV on microalbuminuria risk, as manifested by the non fulfillment of the Baron and Kenny criteria for mediation, such that HCV did not significantly predict diabetes ($\chi^2 = 0.56$, $P = 0.45$), and therefore the Sobel test for mediation was not significant ($P = 0.49$).

Albumin creatinine ratio (ACR)

Median ACR was 1.0 (range, 36.8) mg/mmol in HCV-negative subjects *vs* 1.0 (range, 193.9) mg/mmol in HCV-

Table 2 Renal insufficiency in diabetics compared with non-diabetics among HCV-positive and HCV-negative controls

Renal function	Diabetes	
	OR (95% CI)	<i>P</i> -value ¹
Microalbuminuria		0.720
HCV-positive	1.4 (0.8-2.3)	
HCV-negative	1.7 (0.6-5.1)	
eGFR		0.175
HCV-positive	3.0 (0.8-11.9)	
HCV-negative	0.6 (0.1-4.0)	
Albumin creatinine ratio		0.752
HCV-positive	2.9 (1.4-6.1)	
HCV-negative	2.1 (0.4-12.7)	
Creatinine		0.627
HCV-positive	0.9 (0.3-2.8)	
HCV-negative	0.5 (0.1-2.5)	

¹ P for interaction is the P -value of the cross product term of HCV \times diabetes in the logistic regression models predicting renal insufficiency. OR: Odds ratio.

positive subjects. Positive HCV status was not associated with a significantly high ACR [OR: 1.7, 95% confidence interval (CI): 0.7-4.1], which also did not change by stratifying according to diabetic status (P for interaction = 0.752) (Table 2).

In multivariable regression analysis, and adjusting for age and gender, there was no significant association between HCV status and high ACR ($\chi^2 = 0.86$, $P = 0.35$).

eGFR

The prevalence of low eGFR < 60 mL/min every 1.73 m² was lower in HCV-positive individuals than HCV-negative individuals (4.4% *vs* 8.9%). Positive HCV status was not associated with significantly low eGFR < 60 mL/min every 1.73 m² (OR: 0.5, 95% CI: 0.2-1.4). Median eGFR was 99.5 (range, 317.0) mL/min every 1.73 m² in HCV-negative subjects and was 98.0 (range, 155.0) mL/min every 1.73 m² in HCV positive subjects. We did not detect a significant interaction between HCV status and diabetic status (P for interaction = 0.175) (Table 2).

Multivariate regression analysis revealed that, upon adjusting for age, and gender there was no significant association between HCV status and low eGFR ($\chi^2 = 1.12$, $P = 0.29$).

Serum creatinine

Median creatinine was 0.75 (range, 248) mg/dL in HCV negative subjects, *vs* 0.77 (range, 230) mg/dL in HCV positive subjects. Negative HCV status was borderline associated with high serum creatinine (OR: 2.6, 95% CI: 1.02-6.68, $P = 0.05$). There was no statistically significant difference in serum creatinine comparing diabetics to non-diabetics among HCV positive and HCV negative controls (Table 2).

In the multivariable regression analysis, and adjusting for age, and gender, there was significant association between HCV negative status and high serum creatinine (OR: 3.3, 95% CI: 1.2-9.2, $P = 0.02$).

Microalbuminuria and treatment effect

Log microalbuminuria pre-treatment median level was 2.26 (inter-quartile range, 1.5-3.1), while median post-treatment level was 2.04 (inter-quartile range 1.5-2.6), (Wilcoxon signed rank test $P = 0.09$). Adjusting for age, sex, fibrosis, grade, log ACR, ALT, diabetes and viral load the decline was more pronounced in individuals with ETR compared to individuals without ETR ($\chi^2 = 8.19$, $P = 0.004$).

The pre- to post-treatment log microalbuminuria difference was significantly correlated with pre-treatment older age ($r = 0.37$, $P < 0.001$), fibrosis ($r = 0.26$, $P = 0.017$), grade ($r = 0.23$, $P = 0.042$) and log ACR ($r = 0.38$, $P < 0.001$), but not correlated with male gender ($r = 0.14$, $P = 0.222$), diabetes ($r = 0.12$, $P = 0.265$), urea ($r = 0.015$, $P = 0.896$), creatinine ($r = -0.05$, $P = 0.658$) or ALT ($r = -0.07$, $P = 0.508$).

In multivariate regression, after adjusting for gender, age, pre-treatment ALT, log ACR, diabetes, fibrosis and grade, only log ACR, ETR, and fibrosis were moderately associated with a greater decline in log microalbuminuria post-treatment ($\chi^2 = 8.98$, $P = 0.003$; $\chi^2 = 8.19$, $P = 0.004$; $\chi^2 = 9.35$, $P = 0.053$, respectively), while age, gender, ALT, diabetes, and grade were not associated with log microalbuminuria decline ($\chi^2 = 0.70$, $P = 0.401$; $\chi^2 = 0.13$, $P = 0.718$; $\chi^2 = 1.31$, $P = 0.253$; $\chi^2 = 0.0$, $P = 0.969$; $\chi^2 = 1.33$, $P = 0.722$, respectively).

DISCUSSION

Hepatitis C infection is known to have a higher prevalence of some components of metabolic syndrome and to be associated with chronic renal disease. Renal involvement in the course of HCV infection is attributed to a high incidence of intrinsic diabetic renal disease or cryoglobulinemia. Studying microalbuminuria in HCV-G4 patients and its relationship to response to treatment is a novel report, especially after recent evidence for diabetes-inducing effects of HCV-G4^[10]. In the current study, using the same definition of microalbuminuria as Liangpunsakul *et al*^[11], the prevalence of microalbuminuria in HCV-G4 was 20%, similar to that reported by the Third National Health and Nutrition Examination Survey (12.4%). In contrast to the limitations of the NHANES III study, we were able to study the mean of multiple microalbuminuria readings, adjusting for stage of hepatic fibrosis, grade of inflammation, viral load and cryoglobulinemia.

In our study, not only was the prevalence of microalbuminuria higher among HCV-positive individuals but significantly higher levels were noted compared to non-HCV subjects. Although the prevalence of microalbuminuria was higher among diabetic HCV patients, testing for the effect of diabetes did not reveal a significant interaction with HCV infection nor a significant mediation of the HCV effect. In contrast to a previous suggestion of a link between HCV infection and diabetes^[12], our results revealed that HCV infection was not associated

with type 2 diabetes mellitus. Our results are in accordance with other reports that revealed no interaction between HCV infection and diabetes, and the association between HCV infection and albuminuria was not altered upon adjustment for diabetes^[13,14]. In addition HCV infection was not associated with cryoglobulinemia. Therefore, our results suggested that HCV-G4 was associated with microalbuminuria independently of cryoglobulinemia or diabetes status.

HCV-induced glomerulonephropathy in the absence of cryoglobulinemia was explained in previous reports by direct or indirect pathways: deposition in the glomerulus of a monoclonal IgM rheumatoid factor with particular affinity for the glomerular matrix, which is produced by permanent clones of B lymphocytes infected by the virus or immune complexes composed of HCV antigens and anti-HCV IgG antibodies can deposit directly in the glomerular structures in the absence of a concomitant type II mixed cryoglobulin. HCV RNA genomic sequences and HCV core protein detected in kidney glomerular and tubular structures point to distinct pathways of HCV-related damage in glomeruli and tubules^[15]. Kidney disorders constitute patho- and morphogenesis of systemic infection in HCV^[16].

In spite of HCV-induced microalbuminuria, which was in agreement with Liangpunsakul *et al*^[11], Moe *et al*^[17] and Tsui *et al*^[18] but in contrast to Dalrymple *et al*^[13] and Tsui *et al*^[14], we did not find an increased risk for renal disease in HCV-G4 as reflected by undetectable changes in creatinine, ACR or eGFR. This discrepancy might be explained by long-term infection in some studies, the definition of renal insufficiency, or the viral genotype. These findings suggested that the principal clinical manifestation of glomerular disease in HCV-G4-infected patients is the presence of microalbuminuria without impaired kidney function^[19].

The lack of increased risk of renal disease persisted even after restricting the analysis to diabetics which is consistent with a previous report^[20].

We found a significant correlation between microalbuminuria and necroinflammatory changes, but not fibrotic changes; this represents a relation with viral activity rather than progression of liver disease. In the absence of a significant correlation between microalbuminuria and viral load, hepatic fibrotic changes or platelet count, we suggest that HCV infection *per se* in HCV-G4 and not the stage of liver disease is the cause of microalbuminuria.

There was a moderate reduction in microalbuminuria after pegylated interferon therapy which was more pronounced in patients with an ETR, those with higher pre-treatment fibrosis and higher pre-treatment log ACR, regardless of the grade of inflammation, diabetes or liver function. This post-treatment reduction in proteinuria in HCV-G4 indirectly suggested an improvement in renal pathology^[21], and reinforced the hypothesis that the development of microalbuminuria in HCV infection is possibly by mechanisms other than diabetes or cryoglobulinemia.

In conclusion, renal disorders in the form of microalbuminuria increased in HCV-G4 infection, especially in older patients, regardless of the stage of liver disease or viral load. In our study, diabetes did not mediate or moderate the effect of HCV infection, suggesting that HCV independently affected the development of microalbuminuria. Combination therapy of pegylated interferon-ribavirin had a positive effect in reducing microalbuminuria.

COMMENTS

Background

Hepatitis C virus (HCV) is a significant cause of glomerulopathy in countries with a high prevalence of HCV. The principal clinical manifestations of glomerular disease in HCV-infected patients are the presence of proteinuria and microscopic hematuria with or without impaired kidney function.

Research frontiers

Microalbuminuria is observed in HCV-positive individuals. However, the prevalence of microalbuminuria in HCV-genotype 4 (HCV-G4) patients compared to HCV-negative controls and its association with liver histology, viral load, and response to treatment has not been indisputably addressed. In this study, the authors demonstrate that antiviral therapy with pegylated interferon and ribavirin could have a beneficial effect on microalbuminuria.

Innovations and breakthroughs

Latest reports have provided evidence of a significant increase in renal disease in HCV infection, in particular HCV-induced microalbuminuria. This is the first study to report the increase in microalbuminuria in HCV-G4 patients. Furthermore, the study demonstrated that antiviral therapy with pegylated interferon and ribavirin could have a beneficial effect on the microalbuminuria.

Applications

Understanding how *microalbuminuria* is related to HCV infection, grade, fibrosis and response to treatment, may provide an insight for future strategy for post-treatment follow-up and for predicting response to treatment.

Terminology

Microalbuminuria, estimated glomerular filtration rate, and serum creatinine are measures of renal insufficiency. End-of-treatment response is the loss of detectable serum HCV RNA at the end of treatment.

Peer review

In the current study, the authors demonstrated the independent effect of HCV-G4 infection on the development of microalbuminuria through prospective comparison to HCV-negative control groups and the positive effects of antiviral therapy. This study included some interesting points.

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Overlapping gastroesophageal reflux disease and irritable bowel syndrome: Increased dysfunctional symptoms

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GERD, whereas 34.7% of the non-IBS patients had GERD [odds ratio (OR) = 3.2, 95% confidence interval (CI): 2.9-3.7, $P < 0.0001$]. Among patients with GERD, 33.9% of subjects met Rome criteria compared to 13.5% of non-GERD patients (OR = 3.6, 95% CI: 3.1-4.3, $P < 0.0001$). Prevalence of all functional symptoms was higher in overlapping GERD and IBS subjects, when compared with their prevalence in the IBS subjects without GERD or GERD only subjects ($P < 0.05$).

CONCLUSION: This finding shows that in overlapping GERD and IBS, other functional abnormalities of the GI tract are also highly prevalent, suggesting a common underlying dysfunction.

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Key words: Gastro-esophageal reflux disease; Irritable bowel syndrome; *Helicobacter pylori*; Gastro-intestinal dysfunction; Endoscopy

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Abstract

AIM: To investigate the association of gastroesophageal reflux disease (GERD) and irritable bowel syndrome (IBS) in Iranian patients and examine the prevalence of functional symptoms of the gastrointestinal tract in patients presenting with either IBS, GERD or both.

METHODS: Six thousand four hundred and seventy six patients presented to the Gastro-intestinal (GI) clinic with symptoms of functional dysfunction of GI tract, 1419 patients (62.0% women, 38.0% men; mean age: 37.4 ± 11.5 years) met Rome II or Rome III criteria (depending on the year of diagnosis) for IBS. 2658 patients were diagnosed with GERD based on clinical presentation and endoscopic findings. We assessed other functional symptoms (epigastric pain, nausea, vomiting, belching, constipation and diarrhea) in patients suffering from GERD, IBS or both.

RESULTS: Among IBS subjects, 63.6% (69.0% women, 31.0% men; mean age: 36.4 ± 10.3 years) also had

INTRODUCTION

Irritable bowel syndrome (IBS), affecting approximately 10%-15% of the population is defined by Rome criteria as "abdominal pain or discomfort associated with

altered bowel habits and disordered defecation”^[1-5]; gastroesophageal reflux disease (GERD) is characterized by the symptoms of heart burn and regurgitation caused by reflux of stomach content and is experienced once daily by 7% and once monthly by 44%^[6,7] of patients. Originally, GERD and IBS were considered to be distinct issues, but common clinical features between IBS and GERD^[8,9] suggest that an overlap between these conditions might exist. Recently, a considerable overlap between GERD and IBS in both population based and clinical based studies have been reported^[10-13]. In addition, some evidence suggested that GERD, like IBS, should be considered as a gastrointestinal motility disorder, which suggests that similar pathophysiological characteristics might contribute to GERD and IBS^[9,14].

In the current study, we used a large clinic-based data-base to estimate the prevalence of overlap between GERD and IBS. We also examined the prevalence of a number of clinical symptoms of Gastro-intestinal (GI) functional motility disorders like belching, nausea and vomiting, constipation and diarrhea in patients presenting with GERD, IBS or both. We predicted that if GERD and IBS are different presentations of the same pathologic process in function and motility of GI tract, the more severe the disorder, the more prevalent the functional symptoms should be; therefore we expected a higher prevalence of dysfunctional symptoms in patients with overlapping GERD and IBS.

MATERIALS AND METHODS

All patients referred to a subspecialty practice medical group from March 1997 to March 2007 were enrolled. Five hundred and four patients were excluded because of not having follow up sessions, defined as having only one visit or lacking final diagnosis in the records, or having peptic ulcer found on endoscopy, and the remaining 6476 patients (mean age = 37.9 ± 13.4 years) were registered. Patients filled in a questionnaire about presence and severity of functional symptoms at every visit. All of the final diagnoses available in the records had been made by gastroenterologists who were participating in the gastroenterology department related to Tehran Medical University as academic staff.

GERD was defined as having clinical presentations such as heart burn, regurgitation and dysphagia. 91% of patients with clinically assumed GERD had undergone standard upper gastrointestinal endoscopy (UGIE). Patients who showed endoscopic evidence of GERD, like esophagitis, were not tested further. Those who did not show evidence of GERD were confirmed by pH monitoring. Patients with peptic ulcer found on endoscopy were excluded from the study. Subjects were diagnosed with IBS by fulfilling Rome II or Rome III criteria.

The records were then reviewed for the following items: (1) demographic data (including patient's record number, age and sex); (2) symptoms of GERD, IBS and other symptoms related to the dysfunction of upper and

lower GI tract such as: epigastric pain, nausea, vomiting and belching, constipation and diarrhea; (3) non-gastrointestinal complaints which included back pain and headache; (4) being a *Helicobacter pylori* (*H. pylori*) carrier or not. A number of patients diagnosed with GERD or IBS were evaluated for *H. pylori* during the clinical evaluation for the diagnosis and were found to have *H. pylori* infection by means of either C-urea breath test, urease test in obtained biopsies during UGIE or serum checking; (5) whether GERD in clinically suspicious patients is confirmed by UGIE or not; and (6) final diagnosis of GERD and IBS. Patients were classified according to their final diagnosis, as suffering from: (1) IBS (IBS group); (2) GERD (GERD group); and (3) both GERD and IBS (GERD + IBS group).

Statistical analysis

Extracted data was analyzed using SPSS version 14.0 (SPSS Inc., Chicago, IL, USA). χ^2 test was used to compare qualitative variables, whereas *t*-test was used to compare discontinuous quantitative variables. A *P* value less than 0.05 was considered to indicate a statistically significant difference.

RESULTS

Six thousand four hundred and seventy six patients including 3185 (49.2%) men and 3291 (50.8%) women were enrolled in the study. No statistically significant difference was found in mean age of men and women. One thousand four hundred and nineteen (21.9%) of patients were diagnosed with IBS, using Rome II or Rome III criteria for IBS. The IBS group was made up of 539 (38.0%) male and 880 (62.0%) female, with a mean age of 37.4 ± 11.5 years. There were no differences in mean age between men and women. Two thousand six hundred and fifty eight (41%) were diagnosed with GERD, 95.8% were experiencing heart burn, 3.0% presented with regurgitation and 1.2% presented with dysphagia (Figure 1). The diagnosis of GERD had been confirmed in 69.2% of GERD patients with UGIE and in 21.8% with pH monitoring. There was a slight difference in sex distribution of GERD, men making up 44.7% and women making up 55.3% of the patients; the mean age of GERD subjects was 37.9 ± 13.4 years without any considerable difference for men and women.

Among 1419 patients diagnosed with IBS, 902 (63.6%) also had GERD whereas 34.7% of non-IBS patients had GERD [odds ratio (OR) = 3.2, 95% confidence interval (CI) = 2.9-3.7, *P* < 0.0001]. On the other hand 33.9% of GERD subjects met Rome criteria for IBS compared to 13.5% of non-GERD sufferers (OR = 3.6, 95% CI: 3.1-4.3, *P* < 0.0001). 34.4% of the endoscopically diagnosed GERD patients had overlapping IBS; reciprocally 62.4% of the IBS patients had GERD diagnosed by endoscopy or pH monitoring.

69.0% of patients with both GERD and IBS were women and 31.0% were men; while women make up

Table 1 Comparison of frequency of functional symptoms in patients with IBS, GERD and GERD + IBS

Frequency	GERD only patients (n = 1522)	IBS only patients (n = 738)	GERD + IBS (n = 433)	P value		
				IBS <i>vs</i> GERD	GERD + IBS <i>vs</i> IBS	GERD + IBS <i>vs</i> GERD
Nausea	10.8%	6.2%	15.9%	0.02	< 0.0001	< 0.0001
Vomiting	11.6%	3.3%	18.8%	< 0.0001	< 0.0001	< 0.0001
Epigastric pain	18.9%	14.9%	22.9%	0.037	< 0.0001	< 0.0001
Belching	4.1%	6.4%	10.2%	0.03	0.015	< 0.0001
Headache	2.8%	4.4%	6.8%	0.058	0.07	< 0.0001
Back pain	3.6%	3.3%	6.0%	0.74	0.025	0.004

Pair-wise comparison of frequency of nausea, vomiting, epigastric pain, belching, headache and low back pain in patients with IBS alone, GERD alone and GERD + IBS showed that the frequency of all of these functional symptoms was significantly higher in patients in GERD + IBS group. IBS: Irritable bowel syndrome; GERD: Gastroesophageal reflux disease.

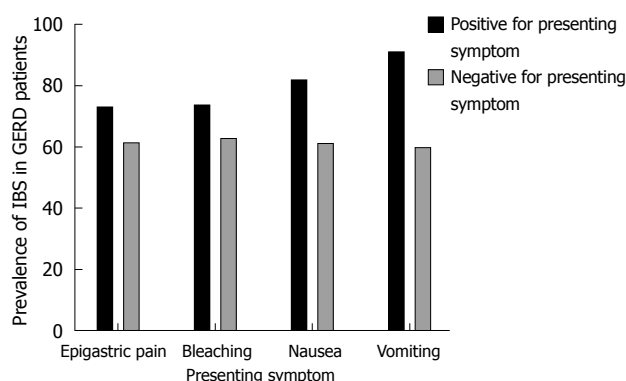


Figure 1 Prevalence of irritable bowel syndrome (IBS) in patients with gastroesophageal reflux disease (GERD) based on their presenting symptoms. The probability of having simultaneous GERD and IBS was significantly higher in patients presenting with either of these functional symptoms; however, nausea and vomiting were more strongly related to the overlap of two diseases.

50.0% of GERD only patients ($P < 0.0001$ *vs* GERD + IBS group) and 61.1% of IBS only patients ($P < 0.0001$ *vs* GERD + IBS group). The mean age of patients with GERD and IBS was 36.4 ± 10.3 , statistically similar to IBS patients without GERD and GERD subjects without IBS ($P > 0.5$).

Table 1 presents the prevalence of other gastrointestinal functional symptoms in the three mentioned groups. Remarkably, these symptoms were significantly more frequent in patients with both GERD and IBS compared to non-GERD non-IBS patients. Statistical analysis showed the following significant differences: epigastric pain (22.9% *vs* 9.6%, $P < 0.0001$), nausea (15.9% *vs* 4.8%, $P < 0.0001$), vomiting (16.0% *vs* 5.3%, $P < 0.0001$), constipation (12.2% *vs* 4.2%, $P < 0.0001$), diarrhea (18.7% *vs* 7.3%, $P < 0.0001$), headache (6.8% *vs* 2.4%, $P < 0.0001$), back pain (6.0% *vs* 3.7%, $P = 0.03$).

In comparison with men, women in the IBS group were more likely to experience epigastric pain (23.0% *vs* 14.1%, $P < 0.0001$), nausea (14.2% *vs* 8.7%, $P = 0.003$) and vomiting (15.6% *vs* 8.5%, $P < 0.0001$). 14.7% and 16.0% of women with GERD suffered nausea and vomiting, respectively, whereas 9.6% and 11.4% of male GERD patients had nausea and vomiting ($P < 0.0001$, $P = 0.001$, respectively). Women complaining of both

GERD and IBS had a trend toward experiencing nausea and vomiting more than men; 17.8% of women and 11.4% of men complained of nausea ($P = 0.015$) and 21.2% of women and 13.6% of men suffered from vomiting ($P = 0.007$, Figure 2).

We determined the differential prevalence of IBS in patients with GERD based on presenting and accompanying functional symptoms (Figure 1). 72.9% of patients with GERD who presented with epigastric pain also had IBS compared to 61.2% of GERD patients without epigastric pain ($P < 0.0001$). 73.6% of GERD patients reporting belching were also diagnosed to have IBS whereas 62.6% of GERD subjects without belching were suggested to have IBS ($P = 0.015$). IBS was present in 81.7% of GERD patients with a complaint of nausea while 61.0% of patients with GERD, not accompanied by nausea also suffered from IBS simultaneously ($P < 0.0001$). 90.9% of patients with GERD and vomiting were considered to have IBS compared to 59.4% of GERD subjects without vomiting who were assumed to have IBS ($P < 0.0001$). These findings suggest a considerable overlap between functional dyspepsia, GERD and IBS. Among the total upper GI symptoms assessed, 40.5% of the IBS group, 39.2% of the GERD group and 47.8% of both GERD + IBS group reported one or more functional symptoms; two or more symptoms were present in 10.9% of IBS group, 10.3% of GERD group and 15.9% of GERD + IBS; 2.7% of IBS patients, 1.7% of GERD patients and 3.9% of GERD + IBS patients complained of three or more symptoms while 0.2% of the IBS group, 0.1% of the GERD group and 0.3% of GERD + IBS group were known to have four symptoms. There was no significant difference when the association between number of overlapping symptoms and GERD and IBS was evaluated.

About 373 of IBS patients, 1323 of the GERD group and 287 of GERD + IBS group were evaluated for *H. pylori* infection. 70.9% of GERD patients were found to have *H. pylori* infection significantly more than non-GERD patients (29.1%, $P < 0.0001$). IBS group patients and GERD + IBS patients were not significantly affected by *H. pylori* infection in proportion to non-IBS and non-GERD + IBS patients ($P > 0.5$). This finding is interesting because it shows that in GERD +

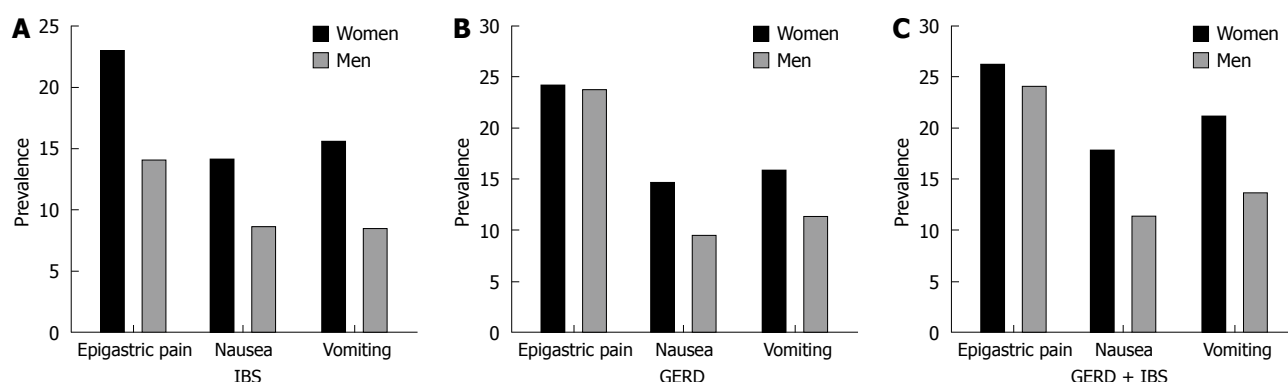


Figure 2 Sex distribution of functional symptoms in patients with IBS (A), GERD (B), GERD + IBS (C). In all of the groups the prevalence of nausea and vomiting was significantly higher in women than in men. Considering that nausea and vomiting were the most frequent symptoms in overlapping diseases, it is explainable by a high prevalence of overlap in women.

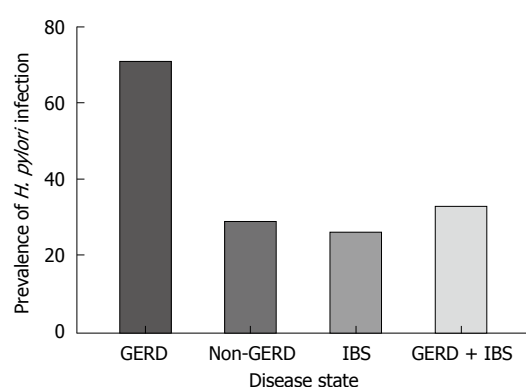


Figure 3 Frequency of *Helicobacter pylori* (*H. pylori*) infection in patients with IBS, GERD, GERD + IBS. The frequency of *H. pylori* infection was significantly higher in patients with GERD alone compared to patients with GERD + IBS.

IBS patients whose symptoms might have a common functional abnormality, the prevalence of *Helicobacter* infection is significantly lower than GERD only patients (Figure 3).

DISCUSSION

IBS and its comorbidities have recently been the focus of extensive research. In spite of the fact that other surveys have indicated the increased prevalence of GERD in IBS and *vice versa*^[8-13] to our knowledge, no similar survey to this study has evaluated the overlap between GERD, IBS and upper gastrointestinal symptoms suggestive of functional dyspepsia in such a large sample consisting of 6476 patients.

21.9% and 41.0% of patients enrolled in the present study had been diagnosed as having IBS and GERD, respectively. The high prevalence of IBS in this survey can be explained by the fact that the place of study had been a referral gastroenterology centre.

63.6% of IBS patients had GERD, and 33.9% of GERD patients also had IBS. Kennedy *et al*^[15] reported 46.5% of GERD patients to have IBS and 47% of IBS patients to suffer from GERD. Hungin *et al*^[2] stated that

IBS sufferers were twice as likely to suffer from GERD (22% *vs* 10%). Guillemot *et al*^[16] in a large clinical based data base reported prevalence equal to 27% for IBS in patients with GERD while Fass *et al*^[17] using a database consisting of about 6000 patients reported a very high prevalence of IBS in GERD patients (60%). Nastaskin *et al*^[18] in a systematic review, reported the prevalence to be about 49%. Lee *et al*^[12] reported a 24% overlap in a Korean population with a sample size of 1400, and a cohort study reported the relative risk of developing IBS in GERD patients to be 3.5^[13].

Although clinical judgment of GERD has a sensitivity of 78% and specificity of 60%^[6,19], in order to strengthen the method of study we also assessed the overlap of IBS with GERD patients whose diagnosis was confirmed by upper gastrointestinal endoscopy. The considerable overlap still existed and 62.4% of IBS patients also had endoscopically confirmed GERD and 34.4% of endoscopically confirmed GERD patients also had IBS.

By evaluating the frequency of these conditions in relation to the demographic aspects of the affected individuals, we found out that women are more likely to experience GERD and IBS at the same time compared to men, despite the similar sex distribution in GERD only patients. Other studies have demonstrated a higher prevalence of IBS in women^[2,20,21], but this study shows a higher proportion of women in GERD + IBS group compared to the prevalence of women in IBS only patients; probably because the same factors which contribute to IBS are more frequent in women than men. One of these factors is sex hormones which was assumed to play a role in IBS, based on the exacerbation of IBS symptoms with the onset of menses^[22]. Another possibility might be a lower pain perception threshold in women compared to men; although visceral hyperalgesia is a leading hypothesis for IBS^[23-27] there is no available data on the differences in pain perception between men and women.

Functional dyspepsia is consistent with gastrointestinal symptoms such as nausea, vomiting, bloating, and postprandial fullness in the absence of organic dis-

ease^[5,20,28,29]. It is stated in a review article that 23%-87% of patients with IBS had also functional dyspepsia and 13%-87% of dyspeptic patients complained of IBS^[30]. In the current study we examined epigastric pain, nausea, vomiting and belching which were shown to have a higher prevalence in patients presenting with both GERD and IBS in comparison to IBS only patients. This finding suggests a more advanced pathology in patients with both GERD and IBS. Other studies have estimated the prevalence of epigastric pain to be 20%-45%, nausea to be 24%-73% and vomiting to be 7%-27% of IBS patients^[31-35]. No similar finding in patients suffering from both GERD and IBS has been reported. 47.8% of patients with both GERD and IBS and 40.5% of IBS patients were associated with one or more functional symptoms. Stanghellini *et al*^[35] observed a high frequency (66%) of functional dyspepsia in IBS patients.

In the present study, we found nausea and vomiting to be the only symptoms differing between men and women. This higher prevalence in women with IBS, GERD and those with both GERD and IBS simultaneously might suggest that the higher occurrence of nausea and vomiting might be interrelated to sex hormones or some biological and psychological differences in men and women. On the other hand the mean age of IBS, GERD and GERD + IBS patients associated with either nausea or vomiting was lower than patients with other symptoms assessed in our survey.

Assessing the role of *H. pylori* infection in GERD and IBS patients could be a target of future research, as in the present study the prevalence of *H. pylori* infection in GERD patients was found to be greater than in non-GERD patients; however, we did not find any significant difference in the prevalence of *H. pylori* infection in patients with IBS or both IBS and GERD.

IBS is also associated with extra intestinal symptoms like frequency and urgency, sexual dysfunction, pains in muscles and joints, dyspareunia, lower back pain, and headaches^[36,37]. Whether this increase in frequency of extra intestinal symptoms reflects a systemic underlying disease with different manifestations in different organs or shows psychosomatic disorder is not clear. Here we investigated the frequency of two extra-intestinal symptoms, headache and low back pain in patients with IBS, GERD and GERD + IBS. Results showed that the frequency of these symptoms was significantly higher in patients with both GERD and IBS compared to patients with GERD or IBS alone. This finding might suggest that these diseases may have similar underlying pathophysiology, but they differ in severity. Thus, patients with a severe disorder are more likely to suffer from both IBS and GERD and also extra-intestinal symptoms. There are different hypotheses about the relationship between GERD and IBS. Some suggest that although there might be similar pathophysiology, these are two different diseases and therefore require two different treatments^[32,33]. Other groups postulate that visceral hyperalgesia in the GI tract in both GERD and IBS is the

main reason for the observed overlap^[38]. When patients are hypersensitive or hyperalgesic in the GI tract, there is a higher chance of reporting symptoms of both GERD and IBS, or other functional symptoms as reported by the current study. However, in our population of study, most of the patients presented endoscopic evidence of GERD, therefore hypersensitivity does not explain their GERD symptoms. Others suggest that the ineffective esophageal motility which contributes to GERD is part of a general disorder of smooth muscle movement in the GI tract which contributes to IBS as well^[39,40]. There is another hypothesis that suggests GERD might be a systemic disease and IBS like symptoms might be a systemic presentation of GERD^[16]. However, in our study there were many patients whose major complaint was IBS and GERD was found during the clinical evaluation.

In conclusion, we suggest that a considerable overlap between GERD and IBS exists with a remarkable predominance in women; we also point to the association of upper gastrointestinal symptoms with IBS in patients suffering from both GERD and IBS, particularly nausea and vomiting, with a significant trend toward affecting women. Because this is a clinic based study, the limitations of this study include the possibility of over-reporting of symptoms by patients and referral bias. Another limitation is that atypical GERD usually includes wheezing, chronic cough, hoarseness or sleep deprivation, however it might present as abdominal discomfort and alterations in bowel movement which may have led to overestimation of IBS prevalence in GERD patients. There is evidence in the literature that prevalence of GERD and IBS in the Iranian population is similar to those reported from western countries^[41]. Further studies to clarify the reason for the increased prevalence of these complaints in women are warranted.

COMMENTS

Background

Gastroesophageal reflux disease (GERD) and irritable bowel syndrome (IBS) are two very common disorders affecting the gastrointestinal tract. About 10% of the population experience daily discomfort from reflux of gastric contents into the esophagus. More than 10% of the population experience abdominal pain and disturbed bowel movements because of IBS.

Research frontiers

Until recently, the dominant view in clinical practice was that GERD and IBS are two different diseases with no link. This view has been challenged by a body of increasing evidence showing that prevalence of IBS like symptoms is very high in patients with GERD and vice versa. This finding inspired research and hypotheses regarding the link between these two diseases. Whether general hyperalgesia in the gastrointestinal tract or a general dysfunction in smooth muscle is responsible for this overlap is not clear.

Innovations and breakthroughs

In the current study, the authors used a large data base from a specialty clinic to investigate the prevalence of overlap between GERD and IBS. They also studied the prevalence of other functional symptoms not related to GERD and IBS in patients in addition to some symptoms outside of the gastrointestinal tract such as headache. They also investigated the correlation between *Helicobacter pylori* (*H. pylori*) infection and severity of symptoms. They found out that in addition to significant overlap between GERD and IBS, prevalence of other functional symptoms is much higher in patients with both conditions.

H. pylori did not play a significant role in this regard. This finding suggests that GERD and IBS should be considered as parts of the same spectrum of diseases.

Applications

Changes in the way that clinicians and scientists think about GERD and IBS could provide opportunities to change the way we treat these conditions. The response rate of treatment for both of these conditions is not very satisfactory at the moment, hinting that our understanding of the main underlying dysfunction might not be complete. This finding might provide further insight about the correct approach in treatment of these diseases.

Terminology

GERD is characterized by the symptoms of heart burn and regurgitation caused by reflux of stomach contents. IBS is defined as abdominal pain or discomfort associated with altered bowel habits and disordered defecation.

Peer review

In this manuscript, the authors suggest a considerable overlap between GERD and IBS. The study was uniquely performed and very interesting.

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Management of bleeding from pseudoaneurysms following pancreaticoduodenectomy

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angiography. Angiography was attempted in 23 cases. Eighteen (78.2%) cases succeeded to hemostasis; the five failed cases were explored. After embolization of the hepatic artery, five cases developed liver abscesses or infarction and a single case of hepatic failure expired. Gastroduodenal artery embolization with common hepatic artery stent insertion was performed to enhance hepatic artery flow in a single case and was successfully controlled.

CONCLUSION: Bleeding pseudoaneurysms are among the most serious and fatal complications following pancreaticoduodenectomy. Diagnostic angiography has been preferred over endoscopy and is rapidly becoming the standard therapeutic treatment for bleeding pseudoaneurysms.

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Key words: Pseudoaneurysm; Pseudoaneurysm; Angiography; Embolization; Stent graft

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Abstract

AIM: To review the clinical course and the management of pseudoaneurysms post-pancreaticoduodenectomy.

METHODS: Medical records of 907 patients who underwent pancreaticoduodenectomies from January 1995 to May 2007 were evaluated retrospectively. The clinical course, management strategy, and outcome of ruptured pseudoaneurysms cases were analyzed.

RESULTS: Twenty-seven (3.0%) of 907 cases had post-operative hemorrhage from ruptured pseudoaneurysms. Pancreatic fistula was evident in 12 (44%) cases. Sentinel bleeding appeared in 21 (77.8%) cases. Of the 27 cases, 11 (41%) cases demonstrated bleeding pseudoaneurysm of the ligated gastroduodenal artery, 8 (30%) of the right, proper, common hepatic artery, 2 (7%) of the right gastric artery, and 4 (15%) of the peripancreatic arteries. The remaining two patients died due to sudden-onset massive hemorrhage and pseudoaneurysm rupture was suspected. Emergent operation was performed on 2 cases directly without

INTRODUCTION

Major pancreatic surgery has advanced greatly in the last two decades with a dramatic decrease in its mortality rate. In the case of pancreaticoduodenectomy, several recent studies have reported mortality rates of less than

5%, with a few reporting mortality rates of less than 1%^[1,2]. Despite the reduction in mortality, the morbidity remains high at 40%. Some of the complications following major pancreatic surgery include pancreatic fistula, hemorrhage, delayed gastric emptying, and intra-abdominal abscess. Hemorrhage is one of the main complications following pancreaticoduodenectomy. Its incidence is anywhere between 1.5% and 15%^[1,3,4]. Hemorrhage on postoperative day #1 is defined as early, while hemorrhage after postoperative day #1 is termed late or delayed^[3,5,6]. Early-onset hemorrhage is usually due to poor hemostasis at the operation site. Delayed hemorrhage occurs with pancreatico-enterostomy leakage and pseudoaneurysm formation, intra-abdominal sepsis or abscess, or marginal ulcer^[1,3]. Without prior knowledge and experience, delayed hemorrhage is hard to diagnose and manage. Hence, it is crucial to understand the clinical features and etiology in anticipation and treatment of such delayed bleeding. Delayed hemorrhage, especially from a ruptured pseudoaneurysm, is a rare but rapidly progressing and potentially life threatening complication following pancreaticoduodenectomies. It has been widely studied, yet each study has reported different findings on its incidence, location, and clinical presentation. Recently, positive results have been achieved with angiographic intervention of ruptured pseudoaneurysms and a shift in management strategy from laparotomy to this less invasive alternative has occurred. The purpose of this study was to analyze data on delayed hemorrhage after pancreaticoduodenectomy due to pseudoaneurysm rupture. The clinical features, management strategy, and outcome of delayed hemorrhage from ruptured pseudoaneurysms were evaluated.

MATERIALS AND METHODS

Medical records of 907 patients who received pancreaticoduodenectomies between January 1995 and May 2007 were analyzed retrospectively. Based on the definition of postpancreatectomy hemorrhage from the International Study Group of Pancreatic Surgery (ISGPS), we suggest a definition that includes two times of onset: early hemorrhage, occurring in the first 24 h postoperatively, meaning 24 h after the end of the index operation, and late or delayed hemorrhage, occurring more than 24 h postoperatively. The severity of bleeding can be differentiated into two categories based on the amount of blood loss or transfusion requirements: (a) mild (no clinical impairment and transfusion requirements), or (b) severe (more than 4 or 6 units of packed cells transfused within 24 h; a decrease in hemoglobin of more than 4 g/dL or need for relaparotomy or interventional angiographic therapy due to severe blood loss). Evidence of bleeding in the surgical drains or GI tract 6 to 24 h prior to delayed massive hemorrhage was termed 'sentinel bleeding'. Pancreatic fistula or leakage was identified by the character of the drained fluid in the surgical drain. A definition of pancreatic fistula is a drain output of any measurable volume of fluid on or after postoperative day

Table 1 Clinical features of 27 patients with ruptured pseudoaneurysms

Initial sign or symptom	n (%)
Intraabdominal drain bleeding	11 (41)
Gastrointestinal (GI) bleeding	8 (30)
Intraabdominal drain bleeding with GI bleeding	2 (7)
Hypotension	2 (7)
Abdominal distension	2 (7)
Syncope	1 (4)
Arrest	1 (4)

3 with an amylase content greater than 3 times the serum amylase activity. Determination of the bleeding focus and management strategy were based on endoscopy, computed tomography (CT), angiography, and the details of the initial surgery. Medical records were analyzed to determine the effectiveness of the treatment provided and to review prognosis. Preceding complications were also evaluated.

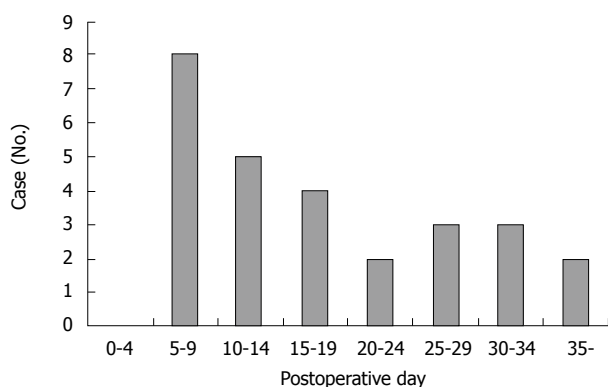
RESULTS

Nine hundred and five patients received pancreaticoduodenectomies [pancreatic duct to jejunal mucosa anastomosis (445 patients), invaginating pancreaticojejunostomy (454 patients), pancreaticogastrostomy (8 patients)]. Total frequency of pancreatic fistula was 43% (389 patients). Thirty-one (3.4%) of 907 patients who received pancreaticoduodenectomies were diagnosed with delayed massive hemorrhage. Twenty-seven [pancreatic cancer (11 patients), bile duct cancer (9 patients), ampulla of Vater cancer (7 patients)] of 31 patients were diagnosed with hemorrhage from a ruptured pseudoaneurysm. Twenty-three of the 27 patients were diagnosed by angiography and 2 were diagnosed by re-operation. The remaining 2 patients died due to sudden-onset massive hemorrhage and considering their clinical features, rupture of pseudoaneurysm is suspected in both cases. In total, taking into account both confirmed and suspected ruptured pseudoaneurysms, there were 27 patients with an incidence of 3.0%. Eleven patients died (mortality rate of 1.2%) following pancreaticoduodenectomy. Only 6 of the 11 patient deaths were related to ruptured pseudoaneurysm. Sentinel bleeding was evident in 21 of the 27 cases (prevalence of 77.8%). Reviewing the clinical features of the first episode of bleeding from ruptured pseudoaneurysms, sentinel bleeding appeared in the surgical drains in 11 cases (41%), GI bleeding appeared in 8 cases (30%), and sentinel bleeding and GI bleeding were observed in 2 cases (7%). The clinical features of the ruptured pseudoaneurysms are shown in Table 1.

Twenty-seven cases of first and recurrent hemorrhage were investigated to determine the exact onset of bleeding. Cases were evenly distributed after surgery, with most of the bleeding occurring between day 5 and 9 post surgery. The onset of hemorrhage from ruptured pseudoaneurysms is outlined in Figure 1. Table 2 is a list showing the number of cases for each preceding complication

Table 2 List of complications that present prior to the rupture of pseudoaneurysms

Complication	n (%)
Pancreatico-enterostomy leakage	12 (44)
Wound dehiscence or infection	3 (11)
Hepaticojejunostomy leakage	1 (4)
Abdominal abscess	1 (4)
No evidence of complication	10 (37)

**Figure 1** Number of cases based on the onset of pseudoaneurysm rupture.

of ruptured pseudoaneurysm. Pancreaticojejunostomy anastomotic leakage was the most common complication to occur before hemorrhage [12 cases (44%): pancreatic cancer (7 cases), bile duct cancer (4 cases), ampulla of Vater cancer (1 case)]. Other complications include hepaticojejunostomy anastomotic leakage, wound dehiscence and infection, and intra-abdominal abscess formation. Most of the bleeding occurred from vessels around the pancreaticojejunostomy. Eleven patients (40.7%) experienced bleeding from the gastroduodenal artery, 4 patients (14.8%) from the right hepatic artery, 2 patients (7.4%) each from the hepatic artery proper, common hepatic artery and the right gastric artery, and 4 patients (14.8%) from the peripancreatic artery. The bleeding sites of the ruptured pseudoaneurysms are presented in Table 3.

Reviewing the treatment strategy of the 27 patients with ruptured pseudoaneurysms, 23 patients were managed by angiography, 2 patients had surgical intervention, and 2 patients with rapidly escalating hemorrhage could not be intervened and died. Of 23 cases of angiography, hemostasis was achieved in 18 patients. In the other 5 patients, hemostasis could not be achieved and they required surgery; 2 for failed attempts at coil embolization of the bleeding vessel and 3 for clinically continuous bleeding and hemodynamic instability despite successful coiling. Of these latter 3 patients, 2 died. Continuing hemorrhage due to disseminated intravascular coagulation (DIC) after total pancreatectomy was responsible for one death and the other death was due to the development of pulmonary complications namely adult respiratory distress syndrome (ARDS) and pneumonia after becoming hemodynamically stable. Of 18 cases with hemostasis, 14 maintained their hemostatic state. However, 1 of 14 cases died of hepatic failure after common hepatic artery embolization. Four

Table 3 List of different sites of pseudoaneurysm rupture and hemorrhage

Site	n (%)
Gastroduodenal artery	11 (42)
Right hepatic artery	4 (15)
Proper hepatic artery	2 (7)
Common hepatic artery	2 (7)
Right gastric artery	2 (7)
Left hepatic artery	1 (4)
Superior mesenteric artery	1 (4)
Pancreatic branch of splenic artery	1 (4)
Inferior pancreaticoduodenal artery	1 (4)

cases presented with recurrent hemorrhage from ruptures of new pseudoaneurysms. They were successfully treated by another embolization. Evaluating the outcome of 23 angiography cases, 18 cases displayed functional/therapeutic success (success rate of 78.2%), that is, achievement of a hemostatic state by embolization. The outcome of the above mentioned patients is summarized in Figure 2. Figure 3A-C are angiographs of the ruptured pseudoaneurysms of different vessels.

Of the 2 patients who underwent emergent surgery without angiography, 1 recovered without further complications but the other patient died due to rebleeding from an unknown focus.

Eight patients underwent embolization of the right proper common hepatic artery. All 8 patients had normal portal blood flow and this procedure was only considered after thorough review by the radiologist and surgeon. There was a single case of hepatic failure, 2 cases where liver abscesses were detected on CT scans, and 3 cases of liver infarction without hepatic failure. In the remaining 2 cases, no radiological abnormalities were detected. Figure 4A and B are abdominal CT scans showing liver abscess and liver infarction, respectively, which developed after embolization of the common hepatic artery. The patient who died of hepatic failure was a 63-year-old male who underwent a pancreaticoduodenectomy with left lateral sectionectomy for pancreatic head cancer with liver metastases. Following surgery, the patient showed signs of pancreaticojejunostomy anastomotic leakage. Thirty days post operation, common hepatic artery embolization was performed to manage bleeding of a ruptured pseudoaneurysm. However, the patient passed away on day 41 post surgery.

Stent grafts were placed for pseudoaneurysms to maintain liver or visceral blood flow. Stent grafting was successfully attempted in 2 of our cases for hemorrhagic pseudoaneurysm of the gastroduodenal and superior mesenteric artery. For gastroduodenal artery pseudoaneurysm, embolization of the gastroduodenal artery with a short stump was performed with stent grafting (8 mm × 30 mm size) of the common hepatic artery (Figure 5A). In the other case, with the first episode of early bleeding, hemostasis was achieved by laparotomy without any intervention by angiography. This patient presented with recurrent hemorrhage from the rupture of a new pseudoaneurysm of the superior mesenteric artery. Embolization of the

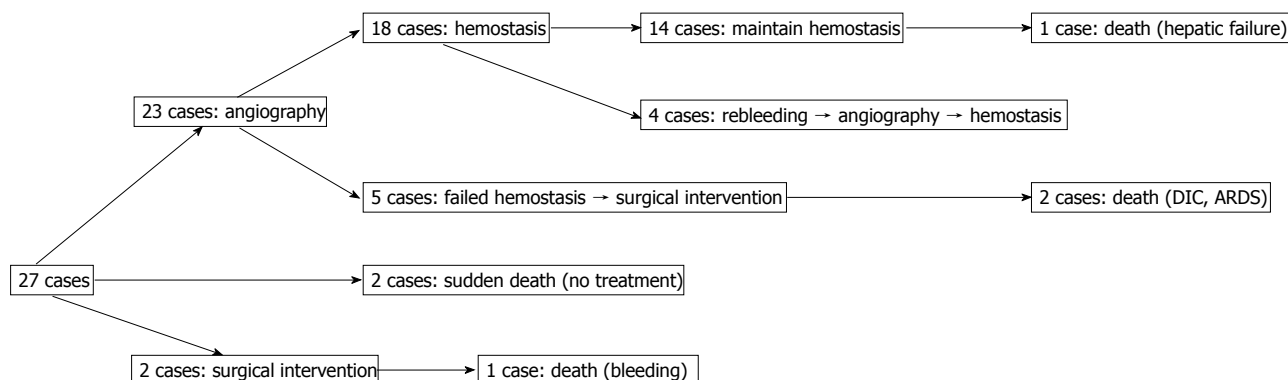


Figure 2 Flow chart summarizing the outcome of patients with hemorrhage from ruptured pseudoaneurysms. DIC: Disseminated intravascular coagulation; ARDS: Acute respiratory distress syndrome.

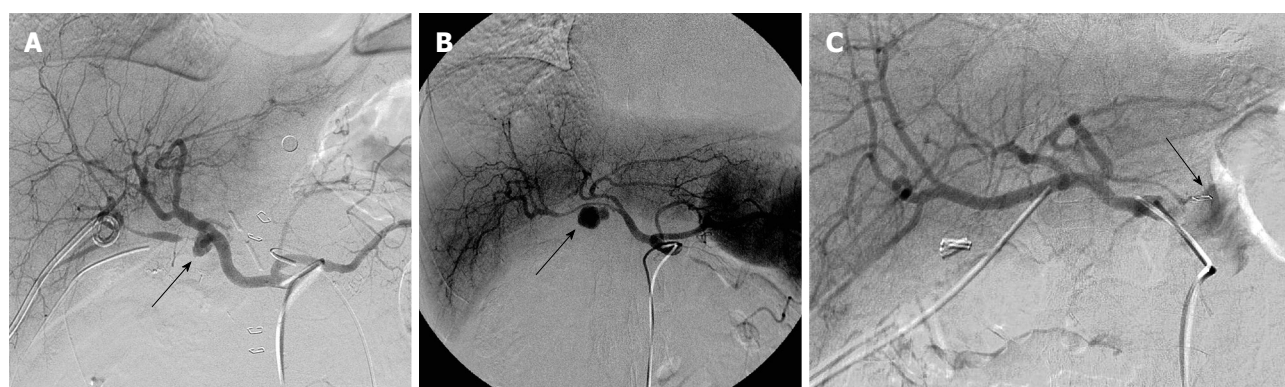


Figure 3 Angiograph. A: A pseudoaneurysm (arrow) of the gastroduodenal artery; B: A pseudoaneurysm (arrow) of the right hepatic artery; C: A ruptured pseudoaneurysm (arrow) of the right gastric artery.

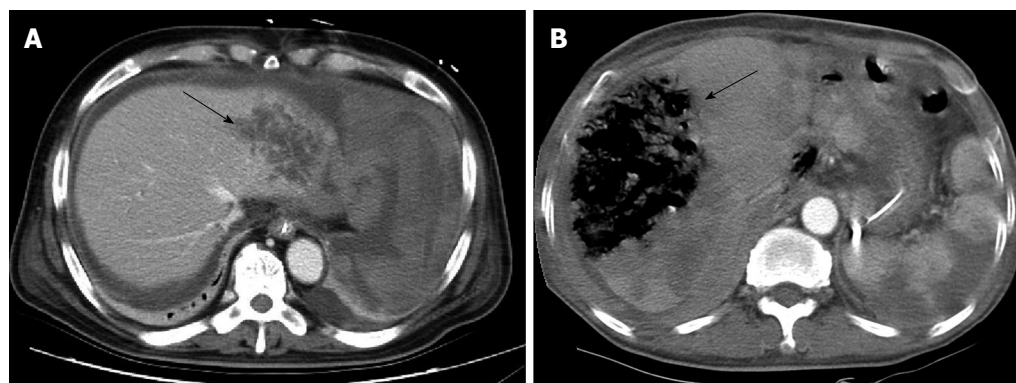


Figure 4 Abdominal CT scan. A: Liver abscess that may have developed after common hepatic artery embolization (arrow); B: An area of liver infarction following common hepatic artery embolization (arrow).

superior mesenteric artery results in severe ischemia of the end-organ and therefore needs careful consideration. Stent grafting (10 mm × 50 mm size) was also performed for recurrent bleeding (Figure 5B). Considering the risk of bleeding, anti-coagulant therapy was not administered and occlusion of the vessel by thrombus was shown on CT angiography taken 3 d later. This patient currently requires continuous total parenteral nutrition for GI malabsorption.

The mortality rate for hemorrhage of ruptured pseudoaneurysms was high at 22% (6/27 patients). As mentioned previously, 2 patients died due to rapidly developing hemorrhage of sudden onset. In these cases, no surgical or other intervening treatment could be applied in time. One patient died of continuing hemorrhage due to

disseminated intravascular coagulation and another due to hepatic failure after common hepatic artery embolization. One patient died of acute respiratory distress syndrome and pneumonia after becoming hemodynamically stable. Another death was due to recurrent hemorrhage from an unknown focus.

DISCUSSION

Hemorrhage after pancreaticoduodenectomy is one of the major life-threatening complications of this procedure and is responsible for 0.4%-5% of its mortality. According to numerous studies, mortality rates can range from 14%-58%^[1,4]. A ruptured pseudoaneurysm is among the

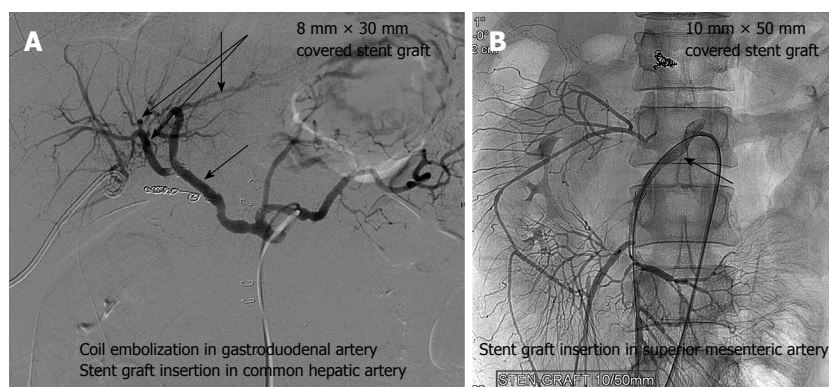


Figure 5 Angiograph. A: A stent graft of the common hepatic artery placed to manage the gastroduodenal artery pseudoaneurysm (arrow); B: A stent graft insertion for a pseudoaneurysm in the superior mesenteric artery (arrow).

most serious and life threatening causes of post pancreaticoduodenectomy hemorrhage. Pseudoaneurysmal bleeding refers to disrupted vascular wall integrity which becomes continuous with pulsatile hematoma^[7]. Many pseudoaneurysms develop secondary to pancreatitis in association with pseudocyst formation. The etiology of pseudoaneurysm formation after pancreaticoduodenectomies is unclear. Erosion of the arterial wall by trypsin and elastase, in the case of pancreaticojejunostomy site leakage, has been suggested as one of the causes. Furthermore, infections of post-operative hematomas remaining in the abdominal cavity and radiotherapy prior to surgery have both been associated with the development of pseudoaneurysms. According to other authors, preceding sepsis due to anastomotic leakage is evident in the majority of patients who present with hemorrhage from pseudoaneurysms (8%-25% of cases result in anastomotic leak after pancreaticoduodenectomies)^[7,8]. In our study, pancreaticojejunostomy anastomotic leak (44%) was the most common cause of subsequent complications. CT was performed on postoperative day 7 in all patients. Patients with pancreaticojejunostomy leaks are closely monitored and CT scans are taken immediately if there is sentinel bleeding and they are hemodynamically stable.

Sentinel bleeding was first described in 1989 by Shan-kar and Russell^[9]. They defined it as gastrointestinal (intraluminal) or intra-abdominal (placed drain) hemorrhage which occurs 6 h to 10 d prior to massive hemorrhage. Currently, different authors use different definitions for this term^[1,3,10,11]. Sentinel bleeding is evident in most cases of massive hemorrhage and it starts off as an intermittent or small-volume hemorrhage. Koukoutsis *et al.*^[3] reported that sentinel bleeding was evident in 57.1% of cases of post-operative hemorrhage 7 d after surgery, but they reached the conclusion that sentinel bleeding has no statistically significant association with post-operative mortality. In the study by Sato *et al.*^[8], sentinel bleeding was noted in 10 patients with massive arterial hemorrhage out of 81 pancreaticoduodenectomy patients and only one case of sentinel bleeding was not followed by massive hemorrhage. In our study, sentinel bleeding was evident in 21 of 27 cases (prevalence of 77.8%). It is important to adequately manage sentinel bleeding as soon as possible to prevent massive hemorrhage because sentinel bleeding is an important preceding sign of massive hemorrhage.

Current studies have suggested that angiography should be the treatment of choice for delayed massive hemorrhage after pancreaticoduodenectomies^[5,8,12]. They also argue that the therapeutic value of endoscopy is limited and should only be attempted if the precise location of the bleeding ulcer is obvious. Nevertheless, it is inappropriate to conclude that angiography is always more sophisticated than therapeutic endoscopy as their suitability varies on a case-by-case basis. Not all delayed massive hemorrhages are due to ruptured pseudoaneurysms. Furthermore, hemorrhage from ruptured pseudoaneurysms cannot be completely ruled out based on negative findings on angiography^[10]. Initial intermittent bleeding of ruptured pseudoaneurysms is hard to detect by angiography, which is only sensitive to bleeding greater than 2 mL per minute^[6,10]. In contrast, bleeding foci are hard to find using endoscopy in massive intraluminal hemorrhages^[5,8]. Location of the bleeding focus by endoscopy does not completely exclude bleeding from ruptured pseudoaneurysms elsewhere. Choi *et al.*^[5] stated that diagnostic endoscopy delays treatment in hemodynamically unstable patients and more often than not, intra-luminal hemorrhage is due to erosion of pseudoaneurysms. de Castro *et al.*^[1] recommended CT scanning for patients with sentinel bleeding who are hemodynamically stable. In the case where the bleeding focus cannot be identified by this method, or if the patient is hemodynamically unstable, angiography is suggested. Therefore, diagnosis and treatment strategies should be decided on a case-by-case basis. Angiography should be considered, with the understanding that most cases of hemorrhage from ruptured pseudoaneurysms present with preceding sepsis due to anastomotic leakage.

Transcatheter arterial embolization has been proven by many authors to be an efficacious and minimally invasive alternative to open surgery^[1,7,8]. It has a success rate of 67%-100%, morbidity rate of 14%-25%, and mortality rate of 0%-14%. Thirty-seven percent of patients in these studies required re-embolization either for rebleeding or recanalization of the vessels^[1,7,8]. When the bleeding focus cannot be identified by conventional investigation and hemostasis cannot be achieved by therapeutic angiography or endoscopy, emergency laparotomy is necessary for delayed massive hemorrhage^[1,7]. Thorough examination of the ligated vessel stumps should be carried out. In more difficult cases, inspection of the

anastomoses for suture line bleeding and bleeding of ruptured vessel stumps into the GI lumen is required^[6]. In other words, emergency laparotomy is rarely required for hemorrhagic pseudoaneurysms and therapeutic angiography has become the standard treatment strategy.

Stent grafting was successfully performed in 2 of our cases for hemorrhagic pseudoaneurysms. This procedure can be used as an alternative or in addition to embolization^[13]. This technique has the advantage of providing continued perfusion to the end-organ and, therefore, obviates the risk of occlusion and ischemia often seen with embolization. On the other hand, potential complications do occur and include stent occlusion, stent deformation or kinking, and exclusion of branch vessels. In our study, a stent graft was used for a pseudoaneurysm of the common hepatic artery and superior mesenteric artery to maintain the visceral and liver blood flow. Embolization of the superior mesenteric artery results in severe ischemia of the end-organ and therefore needs careful consideration. Stent grafting has the advantage of maintaining the peripheral blood flow and hence could be considered an alternative to embolization. However, it is a technically difficult procedure and requires adaptation to different-sized vessels. The limitation and the role of the interventions used to manage ruptured pseudoaneurysms need to be studied further.

Various surgeons have developed different surgical techniques in an attempt to prevent pseudoaneurysm formation. Turrini *et al.*^[11] suggested leaving 1 cm of the gastroduodenal artery stump to minimize direct contact of pancreatic juice with adjacent vessels. Koukoutsis *et al.*^[3] suggested spreading an omental flap behind the pancreaticojejunostomy site. We leave behind a long segment (5-10 mm) of the gastroduodenal artery stump to minimize gastroduodenal artery intimal injury with Hemolok[®] clipping.

Bleeding pseudoaneurysms are a rare cause of delayed hemorrhage but these delayed hemorrhages are among the most serious and fatal complications following pancreaticoduodenectomy. A decade ago, diagnostic or therapeutic endoscopy followed by laparotomy was the treatment strategy for delayed massive hemorrhage. However, in recent years, diagnostic angiography has been preferred over endoscopy and is rapidly becoming the standard therapeutic treatment for bleeding pseudoaneurysms. Fundamentally, however, the patient's clinical history and presentation should be taken into consideration when planning an appropriate management strategy that is tailored to that patient.

COMMENTS

Background

Delayed hemorrhage, especially from a ruptured pseudoaneurysm, is a rare but rapidly progressing and potentially life threatening complication following pancreaticoduodenectomies.

Research frontiers

To review the clinical course and the outcome of pseudoaneurysms post-pancreaticoduodenectomy.

Innovations and breakthroughs

It has been widely studied, yet each study has reported different findings on its incidence, location, and clinical presentation. The purpose of this study was to analyze data on delayed hemorrhage after pancreaticoduodenectomy due to pseudoaneurysm rupture.

Applications

Delayed massive hemorrhage following pancreaticoduodenectomies should be ruled out regardless of its association with rupture of pseudoaneurysms. In this study, arterial embolization was shown to be an effective and safe way to manage and control bleeding pseudoaneurysms.

Peer review

Well written article by very experienced authors in the field of pancreatic surgery.

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Specific endoscopic features of ulcerative colitis complicated by cytomegalovirus infection

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Abstract

AIM: To identify specific colonoscopic findings in patients with ulcerative colitis (UC) complicated by cytomegalovirus (CMV) infection.

METHODS: Among UC patients who were hospitalized due to exacerbation of symptoms, colonoscopic findings were compared between 15 CMV-positive patients and 58 CMV-negative patients. CMV infection was determined by blood test for CMV antigenemia. Five aspects of mucosal changes were analyzed (loss of vascular pattern, erythema, mucosal edema, easy bleeding, and mucinous exudates) as well as five aspects of ulcerative change (wide mucosal defect, punched-out ulceration, longitudinal ulceration, irregular ulceration, and cobblestone-like appearance). Sensitivity, specificity, positive predictive value, and negative predictive value of each finding for CMV positivity were determined.

RESULTS: The sensitivity of irregular ulceration for positive CMV was 100%. The specificity of wide mucosal defect was 95%. Punched-out ulceration and longitudinal ulceration exhibited relatively high sensitivity and specificity (more than 70% for each).

CONCLUSION: Specific colonoscopic findings in patients with UC complicated by CMV infection were identified. These findings may facilitate the early diagnosis of CMV infection in UC patients.

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Key words: Cytomegalovirus; Endoscopic findings; Ulcerative colitis

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INTRODUCTION

Ulcerative colitis (UC) is a worldwide, chronic, idiopathic, inflammatory disease of the rectal and colonic mucosa. Patients with UC suffer from abdominal pain, diarrhea with blood, tenesmus, and urgency to defecate. In Japan, the number of patients with UC was reported to be only 4400 in 1980. However, the number has increased sharply in recent years, and was estimated to be approximately 100 000 persons in 2008. This number is thought to

increase by about 5000 persons each year.

Cytomegalovirus (CMV) belongs to the herpes virus group and is prevalent in adults. Primary CMV infection in immunocompetent people is usually asymptomatic, but the virus remains in the white blood cells throughout the life of the host. Clinically significant diseases occur in patients with suppressed cellular immunity, especially in transplant patients, acquired immunodeficiency syndrome (AIDS) patients, and patients undergoing chemotherapy. The disease is usually the result of reactivation of the latent virus rather than reinfection with the virus. Significant CMV disease may occur in various organs such as the retina, lung, and gastrointestinal tract, and the target organ may be related to the etiology of immunosuppression. In the gastrointestinal tract, CMV disease can occur in all locations from the mouth to the rectum, and usually forms ulcers in the mucosa, often accompanied by hemorrhage^[1].

CMV infection has been described as a cause of relapse of inflammatory bowel disease^[2-5]. In particular, CMV infection was observed in UC patients, especially those receiving high-dose corticosteroid therapy^[4-7]. Moreover, CMV infection can exacerbate the disease^[2-5]. Retrospective studies of UC patients have found CMV infection in 5%-21% of surgically resected specimens^[2-4,8]. Steroid resistance is also reported to be one of the characteristics of CMV infection in UC^[6,8]. In recent prospective studies, 25%-81% of patients with steroid-refractory UC were found to harbor the virus^[5-7]. Thus, CMV infection in UC patients can have a severe clinical course and may cause death if appropriate treatment is not given. However, with the development of ganciclovir (GCV) therapy, outcomes have greatly improved^[9].

Therefore, it is necessary to make an early diagnosis of CMV infection in UC patients. Although several different methods have been developed to examine CMV infection, such as histology including immunohistochemistry^[10,11], serology^[10-12], CMV culture^[13,14], polymerase chain reaction (PCR) for CMV genome^[14-16], and CMV antigenemia^[14-16], it is necessary, with regard to clinical aspects, to suspect that UC patients also have CMV infection. In general, the symptoms of UC alone are not sufficient to distinguish exacerbation of UC due to CMV infection from exacerbation of UC unrelated to CMV infection. Specific colonoscopic findings for CMV infection, if any, may facilitate the early diagnosis of CMV infection.

Several reports have examined colonoscopic findings related to CMV infection. However, most of these reports focused on colonoscopic findings in patients with AIDS or in transplant patients^[17,18]. The spectrum of colonoscopic findings in those patients was variable and ranged from patchy erythema, exudates, and microerosions to diffusely edematous mucosa, multiple mucosal erosions, deep ulcers and pseudotumors^[1,13,17-22]. In contrast, colonoscopic findings of UC complicated by CMV infection have rarely been reported. Only a few studies have described findings without confirmation by statistical analysis^[20].

Accordingly, the aim of this study was to identify specific colonoscopic findings in patients with UC complicated by CMV infection. To achieve this, we compared

colonoscopic findings in CMV-positive and -negative patients, and determined the sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) of each specific finding for CMV positivity.

MATERIALS AND METHODS

Patients

This study was a retrospective analysis of medical charts and endoscopic images of UC patients. From January 1999 to August 2007, a total of 200 UC patients were hospitalized in Okayama University Hospital with exacerbation of UC symptoms. We began to routinely examine CMV antigenemia in these patients from 2003. Therefore, of these 200 patients, 111 did not receive a blood test for CMV antigenemia at admission, and 13 did not receive an endoscopic examination. Thus, a total of 76 patients who received both a blood test for CMV antigenemia and an endoscopic examination at admission were analyzed in this study. This study was approved by the institutional review board of Okayama University Graduate School of Medicine, Dentistry, and Pharmaceutical Sciences.

The diseases in these patients were pancolitis or left-sided colitis with moderate or severe activity, based on clinical, endoscopic, and histological findings according to established criteria^[23-25]. Medical charts provided clinical parameters including demographic data such as age, gender, and duration of the disease, as well as disease status such as severity of disease, extent of disease, and medications.

Diagnosis of CMV infection

Diagnosis of CMV infection was determined by CMV antigenemia, which was examined by detecting CMV pp65 antigen in peripheral blood leukocytes using the direct immunoperoxidase technique with horseradish peroxidase-conjugated Fab' fragment of human monoclonal antibody (C7-HRP) and immunofluorescence staining with monoclonal antibodies C10/11. The examinations were performed in the laboratory of SRL Inc. (Tokyo, Japan). We defined a patient as CMV-positive when one or more positive cells of either C7-HRP or C10/11 were detected in more than 10 000 leukocytes (the minimum number of positive cells).

Endoscopic findings

Endoscopic findings were evaluated by examining recorded colonoscopic images and reports. Two aspects of colonic changes were assessed: mucosal change and ulcerative change. Mucosal change was evaluated according to the following five features: easy bleeding, loss of vascular pattern, mucosal edema, erythema, and mucinous exudates. Each item was expressed as "severe" or "not severe" according to the severity of each change. Easy bleeding was defined as bleeding from the fragile membrane due to slight contact of the endoscope. Loss of vascular pattern was loss of visible vessels under the

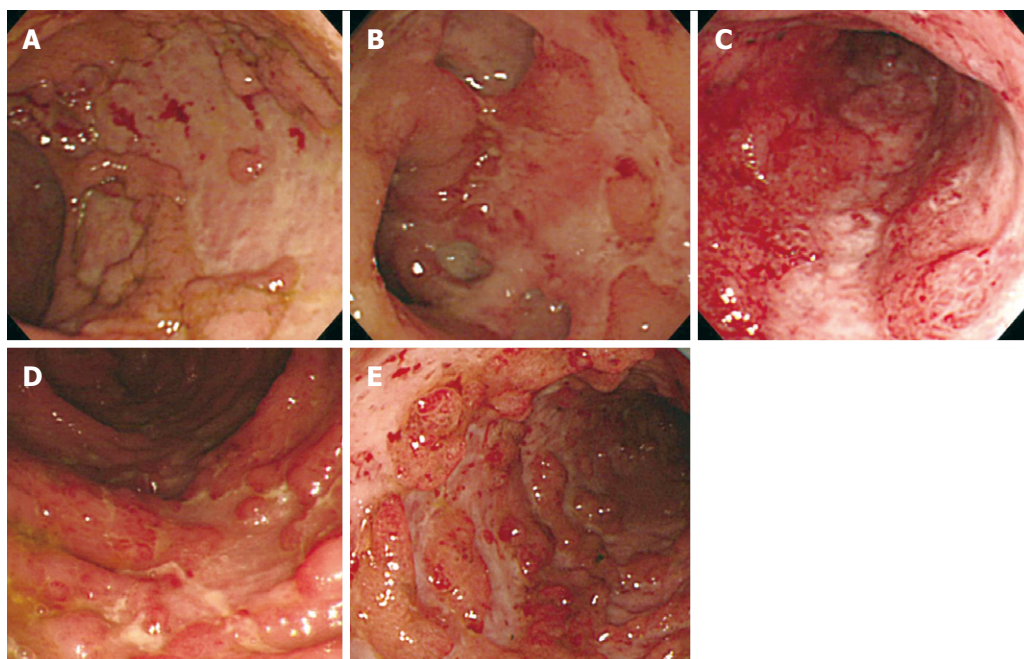


Figure 1 Typical endoscopic findings of ulcerative changes. A: Wide mucosal defect; B: Punched-out ulceration; C: Longitudinal ulceration; D: Irregular ulceration; E: Cobblestone-like appearance.

membrane, generally seen during colonoscopy. Mucosal edema was defined as thick and swollen membrane which pooled fluid. Erythema was red coloration of the membrane composed of dilated veins. Mucinous exudates were exudates composed of blood and pus.

For ulcerative changes, we determined whether or not the following forms of ulceration were observed: wide mucosal defect, punched-out ulceration, longitudinal ulceration, irregular ulceration, and cobblestone-like appearance. A wide mucosal defect was a wide area of this defect with a longitudinal and/or transverse spread, indicating that more than one-fourth of the mucosa in the endoscopic field was defective (Figure 1A). Punched-out ulceration formed an almost round shape of ulceration with clear demarcation (Figure 1B). Longitudinal ulceration had a longitudinal spread along the lumen of the colon (Figure 1C). Irregular ulceration was defined as ulceration with an irregular pattern and a branched shape (Figure 1D). Cobblestone-like appearance was an aggregate of elevated lesions as a result of the changes in the membrane, which looked like half-spheres of subpedunculated polyps with multiple ulceration as seen in Crohn's disease (Figure 1E). Each endoscopic image was evaluated by two (Suzuki H and Kato J) of the authors. If there were any disagreements in these evaluations, a third reviewer (Hiraoka S) made the final evaluation. At the time of interpretation of the images, the CMV antigenemia results were blinded to interpreters. The rate of disagreements between the two authors was less than 5%.

Statistical analysis

Of the clinical characteristics, the χ^2 test and Fisher's exact test were used to compare two discrete variables. The significance of differences for continuous variables was assessed by the Mann-Whitney *U*-test. Of the endoscopic findings, we calculated sensitivity, specificity, PPV, and

NPV for CMV positivity. In addition, odds ratios (OR) with 95% CI were determined by a univariate logistic regression model. The results were considered statistically significant when *P* values were less than 0.05. Statistical analysis of the data was carried out with SAS software version 9.2 (SAS Institute, Cary, NC, USA).

RESULTS

Patients

A total of 76 patients received a blood test for CMV antigenemia and a colonoscopy at admission. Of these, 18 patients were positive for CMV antigenemia, while the remaining 58 patients were negative. Of the 18 CMV-positive patients, symptoms in 3 patients were relatively mild, and those patients entered remission without antiviral therapy. The remaining 15 patients required GCV therapy for a period of two weeks, together with dose reduction of corticosteroids. As a consequence of these treatments, all patients entered remission. Therefore, a comparative analysis was performed on 15 CMV-positive patients *vs* 58 CMV-negative patients.

The clinical characteristics of these 73 patients are shown in Table 1. There were no significant differences in clinical characteristics such as gender, age, duration of disease, disease activity, extent of disease, use of corticosteroids, and use of immunomodulators (azathioprine or mercaptopurine) between CMV-positive and -negative patients. It is noteworthy that all the CMV-positive patients received corticosteroid therapy.

Specific endoscopic findings of UC with CMV infection

To identify specific endoscopic findings of UC complicated by CMV infection, five aspects of mucosal changes (loss of vascular pattern, erythema, mucosal edema, easy bleeding, and mucinous exudates) as well as five aspects of ulcerative change (wide mucosal defect, punched-out

Table 1 Characteristics of enrolled patients

	CMV positive (n = 15)	CMV negative (n = 58)	P value
Gender			
Male	8 (53)	26 (45)	0.55
Female	7 (47)	32 (55)	
Age (yr), median (range)	42 (17-65)	34 (14-81)	0.72
Duration of disease (d), median (range)	528 (15-9358)	1503 (5-10791)	0.40
Disease activity at admission			
Moderate	9 (60)	41 (71)	0.43
Severe	6 (40)	17 (29)	
Extent of disease			
Pancolitis	12 (80)	42 (72)	0.74
Left-sided	3 (20)	16 (28)	
Current use of corticosteroids			
Yes	15 (100)	47 (81)	0.11
No	0 (0)	11 (19)	
Current use of immunomodulators (AZA/6-MP)			
Yes	1 (7)	7 (12)	> 0.99
No	14 (93)	51 (88)	

Data are numbers with percentages in parentheses or medians with ranges in parentheses. CMV: Cytomegalovirus.

ulceration, longitudinal ulceration, irregular ulceration, and cobblestone-like appearance) were analyzed. The sensitivity, specificity, PPV and NPV of each finding for CMV positivity were determined. In addition, univariate logistic regression analyses were performed to determine the OR and 95% CI of each finding (Table 2).

Of the 5 mucosal changes, loss of vascular pattern, edema, and erythema exhibited relatively high sensitivity for CMV positivity (87%, 80%, and 87%, respectively), whereas easy bleeding and mucinous exudates exhibited high specificity (88% and 81%, respectively). Of the 5 ulcerative changes, irregular ulceration showed 100% sensitivity, but relatively low specificity (41%). Wide mucosal defect and cobblestone-like appearance showed relatively high specificity (95% and 83%, respectively), but low sensitivity. The sensitivity and specificity of punched-out ulceration and longitudinal ulceration were both relatively high (punched-out ulceration, sensitivity 80%, specificity 74%, longitudinal ulceration sensitivity 73%, specificity 78%, respectively).

Univariate logistic regression analysis revealed that severe easy bleeding was seen more frequently in CMV-positive patients than in CMV-negative patients (OR = 2.20, 95% CI: 1.14-4.28). Wide mucosal defect (OR = 4.58, 95% CI: 2.21-10.73), punched-out ulceration (OR = 3.39, 95% CI: 1.78-7.46), longitudinal ulceration (OR = 3.09, 95% CI: 1.66-6.26), and cobblestone-like appearance (OR = 2.05, 95% CI: 1.11-3.82) were more frequently observed in CMV-positive patients than in CMV-negative patients.

DISCUSSION

Colonoscopy is generally performed in UC patients who undergo flare-ups. Direct observation of the colon provides detailed information on the disease status and is useful for comprehending the severity of disease, and thus it is an important tool for formulating

treatments. However, although CMV infection can cause poor prognosis in UC patients (i.e. a high chance of colectomy), reports on the specific colonoscopic findings of UC with CMV infection are scarce. Therefore, in this study we analyzed the colonoscopic findings of UC patients with and without CMV infection, and showed that there were specific findings that predicted CMV infection in UC patients.

There have been reports on the endoscopic findings of CMV colitis in AIDS or transplant patients. These findings include patchy erythema, exudates, microerosions, diffusely edematous mucosa, multiple mucosal erosions, or deep ulcers and pseudotumors^[1,13,17-22]. In UC patients, however, the findings would be more complicated, because inflammatory changes due to UC itself in colonic mucosa exists prior to CMV infection. Therefore, in previous studies, no specific endoscopic findings were observed in UC patients with concomitant CMV infection^[6,26]. In contrast, in our study, specific colonoscopic findings of UC with CMV infection were observed; the sensitivity of irregular ulceration was 100%, the specificity of wide mucosal defect was 95%, and the sensitivity and specificity of punched-out ulceration and longitudinal ulceration were both greater than 70%. Therefore, in cases where these colonoscopic findings are seen in UC patients, examination to confirm CMV infection should be performed as soon as possible.

It is known that UC patients with CMV infection, especially those who are compromised by corticosteroid therapy, experience severe symptoms. These patients are likely to undergo colectomy, and CMV infection has been confirmed in 4.6%-25% of patients who have undergone colectomy^[2,3,7,8]. Recently the efficacy of GCV in CMV infection has become widely recognized. Pfau *et al*^[9] considers GCV a beneficial treatment that significantly decreases the mortality rate and the need for surgery. Wada *et al*^[6] found that GCV was effective in 8 (67%) of 12 patients with CMV infection. They

Table 2 The correlations between CMV positivity and endoscopic findings

	CMV positive (n = 15)	CMV negative (n = 58)	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Odds ratio	95% CI	P value
Easy bleeding									
Severe	6 (40)	7 (12)	40	88	46	85	2.20	1.14-4.28	0.02
Not severe	9 (60)	51 (88)							
Loss of the vascular pattern									
Severe	13 (87)	37 (64)	87	36	26	91	1.92	0.95-5.01	0.11
Not severe	2 (13)	21 (36)							
Edema									
Severe	12 (80)	33 (57)	80	43	27	89	1.74	0.92-3.79	0.11
Not severe	3 (20)	25 (43)							
Erythema									
Severe	13 (87)	39 (67)	87	33	25	90	1.78	0.88-4.64	0.15
Not severe	2 (13)	19 (33)							
Mucinous exudates									
Severe	4 (27)	11 (19)	27	81	27	81	1.25	0.61-2.36	0.51
Not severe	11 (73)	47 (81)							
Wide mucosal defect									
≥ 1/4 field	8 (53)	3 (5)	53	95	72	89	4.58	2.21-10.73	0.0001
< 1/4 field	7 (47)	55 (95)							
Punched-out ulceration									
Yes	12 (80)	15 (26)	80	74	44	93	3.39	1.78-7.46	0.0006
No	3 (20)	43 (74)							
Longitudinal ulceration									
Yes	11 (73)	13 (22)	73	78	46	92	3.09	1.66-6.26	0.0007
No	4 (27)	45 (78)							
Irregular ulceration									
Yes	15 (100)	34 (59)	100	41	31	100	ND	ND	ND
No	0 (0)	24 (41)							
Cobblestone-like appearance									
Yes	7 (47)	10 (17)	47	83	41	86	2.05	1.11-3.82	0.02
No	8 (53)	48 (83)							

PPV: Positive predictive value; NPV: Negative predictive value; 95% CI: 95% confidence interval; ND: Not determined.

also reported that steroid withdrawal improved steroid-resistant UC. Matsuoka *et al.*^[27] reported that most CMV reactivation-positive patients responded to conventional immunosuppressive therapies, however, high values of CMV antigenemia may be an indicator for antiviral treatment regardless of histology. Thus, treatment for UC patients with CMV infection should be different from that for UC patients without CMV infection. Therefore, our findings that predict CMV infection should be useful in therapeutic decision-making for UC patients.

In addition, there are several studies on the prophylactic/pre-emptive therapy of CMV diseases in patients with solid organ transplants, hematopoietic stem cell transplants, and human immunodeficiency virus disease^[27-29]. These reports suggested the importance of early detection of CMV reactivation and early administration of anti-viral drugs before the development of CMV disease. Although no concept of prophylactic therapy has been established for UC patients, early detection and early antiviral therapy may be considered because of the high rate of colectomy in these patients. In this context, early prediction of CMV infection in UC patients by colonoscopy would seem helpful in clinical settings.

There are several methods of detecting CMV infection: histology including immunohistochemistry^[10,11], serology^[10-12], CMV culture^[13,14], PCR for CMV ge-

nome^[14-16], and CMV antigenemia^[14-16]. Each method has advantages and disadvantages for the precise diagnosis of CMV infection. For example, histological examination is a relatively easy method, but the sensitivity is not so high (10%-87%)^[10-12]. Meanwhile, PCR for the CMV genome is very sensitive, but the false-positive rate is relatively high. In our analysis, CMV antigenemia was adopted to detect CMV infection, because examination of CMV antigenemia is relatively sensitive (60%-100%) and easy to measure within a short period^[10,14,16,30]. In addition, this method has been used for monitoring CMV infection in heart transplant recipients, and for the early diagnosis of CMV infection in renal transplant recipients^[29]. Moreover, it has been reported that positive or negative results of CMV antigenemia are a good indication for antiviral therapy^[28,31]. In fact, in our study, 15 (83%) of 18 patients who were positive for CMV antigenemia required antiviral therapy with GCV, and all of these patients entered remission without colectomy. In general, immunohistochemistry is considered the gold standard in the diagnosis of CMV infection. In our cohort, however, among 6 patients with positive CMV antigenemia whose biopsy specimens were immunostained with antibody for CMV, only 3 exhibited positive immunostaining. In addition, although we performed immunostaining in 16 of 58 patients with negative CMV

antigenemia, none of them exhibited positive staining. Therefore, the use of CMV antigenemia for CMV detection in this study was appropriate.

Our study has several limitations. First, CMV infection was determined by CMV antigenemia alone. As described above, there are several methods to detect CMV infection. A combination of these methods may have increased the CMV detection rate. Second, of the 18 CMV antigenemia-positive patients, 15 patients who were given GCV were analyzed in our study. However, it is not certain that all 15 patients really required GCV therapy. Some of these patients may have improved without GCV similar to the remaining 3 patients. Third, this is a retrospective study that relied on the examination of recorded colonoscopic images. Therefore, bias due to patient collection and missed recording of colonoscopic findings may inevitably exist. Finally, this is a relatively small scale study in Japan, and it is possible that the results might differ in patients of other ethnicities from other parts of the world.

In the case of a severe flare-up of UC, the possibility of a concurrent CMV infection causing or worsening colitis should be considered, especially when patients are on immunosuppressive medications that may make them more susceptible to this viral infection. The prognosis of UC cases with CMV infection is considered poor. Thus, CMV infection should be closely investigated in such cases, and anti-CMV treatment (GCV) should be given immediately to reduce the rate of colectomy. The specific colonoscopic findings of patients with UC complicated by CMV infection observed in this study may facilitate early diagnosis and treatment of CMV infection.

COMMENTS

Background

In general, the symptoms of ulcerative colitis (UC) alone are not sufficient to distinguish exacerbation of UC due to CMV infection from exacerbation of UC unrelated to cytomegalovirus (CMV) infection.

Research frontiers

Few studies have reported the colonoscopic findings of UC complicated by CMV infection.

Innovations and breakthroughs

Via retrospective research, authors found specific colonoscopic findings in UC patients with CMV antigenemia.

Applications

Specific colonoscopic findings in patients with UC complicated by CMV infection found in this study may facilitate early diagnosis and treatment of CMV infection.

Peer review

This is a potentially important study that is generally well done, well written, and makes a contribution to the field.

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Decreased levels of plasma adiponectin associated with increased risk of colorectal cancer

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Abstract

AIM: To investigate the association between adiponectin levels and risk of colorectal adenoma and cancer (early and advanced).

METHODS: A cross-sectional study in a cohort of hospital-based patients was conducted between January 2004 and March 2006 at Yamagata University Hospital. Male subjects, who had colorectal tumors detected by endoscopic examination, were enrolled according to inclusion and exclusion criteria. Based on the T factor of the TNM system, intraepithelial carcinoma and submucosally invasive carcinoma were defined as early cancer, and invasion into the muscularis propria or deeper was defined as advanced cancer. The plasma levels of glucose, insulin, total cholesterol, triglyceride, high sensitivity C-reactive protein, insulin like growth factor (IGF)-1, IGF binding protein-3, adiponectin, leptin, and resistin were measured. Each factor level was designated low or high, and the risk of adenoma

or cancer was estimated by univariate and multivariate logistic regression analysis.

RESULTS: We enrolled 124 male subjects (47 with adenoma, 34 with early cancer, 17 with advanced cancer, and 26 without tumors as controls). In patients with adenoma, high triglyceride and low adiponectin were associated with a significant increase in the odds ratio (OR) by univariate analysis. Only a low adiponectin level was related to increased adenoma risk, with an adjusted OR for low level ($< 11 \mu\text{g/mL}$) to high ($\geq 11 \mu\text{g/mL}$) of 5.762 (95% confidence interval (CI): 1.683-19.739, $P = 0.005$). In the patients with early cancer, high body mass index, high triglyceride, and low adiponectin were associated with a significant increase in OR in univariate analysis. In multivariate analysis, only low adiponectin was significantly associated with early cancer, with an adjusted OR of 4.495 (95% CI: 1.090-18.528, $P = 0.038$). However, in patients with advanced cancer, low adiponectin was not recognized as a significant risk factor for advanced cancer.

CONCLUSION: A decreased level of adiponectin is strongly associated with an increased risk of colorectal adenoma and early cancer. These data call for further investigation, including a controlled prospective study.

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Key words: Adenoma; Early colorectal cancer; Metabolic syndrome; Adipokines; Colonoscopy; Resistin; Leptin; Body mass index

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INTRODUCTION

Colorectal cancer is the world's third most common type of malignancy. Etiological studies have shown that body/abdominal fat or obesity increases the risk of colorectal cancer^[1]. Accumulation of body/abdominal fat is associated with abnormal secretion of adipokines, resulting in either insulin resistance or metabolic syndrome. Metabolic syndrome is clinically defined as an increase in waist circumference and in blood pressure and fasting glucose levels, as well as dyslipidemia^[2]. Many other factors, such as insulin, insulin-like growth factor-1 (IGF-1), IGF binding protein-3 (IGFBP-3), high sensitivity C-reactive protein (hsCRP), and inflammatory cytokines are also involved in the pathophysiology of insulin resistance or metabolic syndrome.

Adiponectin is a unique adipokine secreted from abdominal fat tissue and has anti-diabetic, anti-atherosclerotic, and anti-inflammatory effects^[3]. Previously, we demonstrated that an increase in visceral fat area and a decrease in plasma adiponectin level were strongly associated with an increased risk of colorectal adenoma^[4]. The present study investigated adiponectin levels in patients with colorectal adenoma and cancer. Several studies have previously described the adiponectin levels in patients with colorectal cancer^[5,6]. However, most focused on advanced cancer. Although plasma levels of adiponectin were described, evaluations of risk of cancer by multivariate analysis were not conducted. Therefore, the present study attempted to evaluate the risk of colorectal cancer in patients with decreased adiponectin levels, and elucidated the relationship between adiponectin levels and the risk of both early and advanced cancer, in addition to adenoma.

MATERIALS AND METHODS

A cross-sectional study in a cohort of hospital-based patients was conducted between January 2004 and March 2006 at Yamagata University Hospital.

Ethics

This study was approved by the Ethics Committee of Yamagata University Faculty of Medicine, and written informed consent to participate in the study was obtained from every patient and control subject.

Patients

Patients who visited our hospital for colonoscopic examination in that period were screened for this study. The subjects were enrolled according to the following inclusion criteria: (1) male aged at least 31 years; (2) colonoscopic observations of the cecum were performed successfully; (3a) histopathological confirmation by hospital patholo-

gists (in the case of patients with adenoma or cancer) and (3b) confirmation of lack of obvious polyps upon endoscopic examination of the entire colon and rectum (in the case of controls); and the following exclusion criteria: (a) previous gastrointestinal tract surgery; (b) inflammatory bowel disease; (c) patients with cancers in another organs and currently receiving therapy; (d) diabetes mellitus with insulin injection therapy, and (e) lack of consent to participate in the study. In the present study, only male patients were investigated. It has been shown that, in general, the levels of adiponectin, leptin, and resistin in males are significantly lower than in females^[7,8]. Also, the criteria for metabolic syndrome differ between males and females^[2]. Therefore, for this study, it was considered appropriate to evaluate only males.

Colonoscopy was performed by experienced gastrointestinal physicians using video-endoscopy (CF-240ZI, PCF-240, PCF-P240AI, or PCF-240Z; Olympus Medical Systems, Tokyo, Japan). The lesions of adenoma or cancer were treated appropriately by endoscopic polypectomy, endoscopic mucosal resection, laparoscopic colectomy, or open surgical colectomy. The degree of cancer invasion was evaluated according to the pathologic T factor of the TNM staging system^[9]. Based on the T factor classifications, cancer patients were divided into 2 subgroups: early and advanced cancer. Intraepithelial carcinoma (T_{im}) and submucosally invasive carcinoma (pT₁) were defined as the early cancer group. Those with a pathological depth of pT₂ or greater (muscularis propria invasion or deeper) were defined as having advanced cancer.

Observed factors and evaluations

All patients were interviewed about their smoking habits, alcohol intake, medications, and medical histories. Persons who had stopped smoking more than 1 year previously were counted as ex-smokers. Any person who was drinking an average of more than 15 g of alcohol daily was defined as a regular alcohol drinker. Body weight, height, and blood pressure were also recorded, and body mass index (BMI) was calculated. And blood samples were collected after overnight fasting. Glucose, insulin, total cholesterol (T-cho), triglyceride (TG), hsCRP, IGF-1, IGFBP-3, adiponectin, leptin, and resistin levels were measured in plasma samples. The homeostatic model assessment of insulin resistance (HOMA-IR) was calculated as: $HOMA-IR = \text{fasting glucose} \times \text{fasting insulin} / 405$. The IGF-1/IGFBP-3 ratio was also calculated. The plasma level of each factor was measured as follows: hsCRP by nephelometry (N Latex CRP II CardioPhase hsCRP, Siemens Healthcare Diagnostics Inc., Tokyo, Japan); IGF-1 by a radioimmunoassay (RIA; IGF-I IRMA Daiichi, TFB Inc., Tokyo, Japan); IGFBP-3 by an RIA2 antibody method (IGFBP-3 Cosmic, Bioclone Australia Pty Ltd., Sydney, Australia); leptin by an RIA2 antibody method (Human Leptin RIA Kit, LONCO Research Inc., St. Charles, USA); resistin by an enzyme-linked immunosorbent assay (ELISA; Human Resistin ELISA, BioVender Inc., Modrice, Czech Republic), and adiponectin by enzyme-linked immunosorbent assay (human adiponectin ELISA kit,

Otsuka Pharmaceuticals Inc., Tokyo, Japan).

The measured values of each factor in control patients were divided into 2 categories (Low and High), and the odds ratio (OR) (High *vs* Low) for adenoma or cancer was evaluated by univariate and multivariate logistic regression analysis. Criteria determining Low and High categorization were appropriately determined; the actual categorizations for each factor are described as follows. BMI was categorized as Low ($< 22.5 \text{ kg/m}^2$) and High ($\geq 22.5 \text{ kg/m}^2$), systolic blood pressure (BP-S) as $< 120 \text{ mmHg}$ and $\geq 120 \text{ mmHg}$, diastolic BP (BP-D) as $< 80 \text{ mmHg}$ and $\geq 80 \text{ mmHg}$, glucose as $< 100 \text{ mg/dL}$ and $\geq 100 \text{ mg/dL}$, insulin as $< 4.5 \text{ } \mu\text{U/mL}$ and $\geq 4.5 \text{ } \mu\text{U/mL}$, HOMA-IR as < 2 and ≥ 2 , T-cho as $< 180 \text{ mg/dL}$ and $\geq 180 \text{ mg/dL}$, TG as $< 120 \text{ mg/dL}$ and $\geq 120 \text{ mg/dL}$, hsCRP as $< 500 \text{ ng/mL}$ and $\geq 500 \text{ ng/mL}$, IGF-1/IGFBP-3 ratio as < 60 and ≥ 60 , adiponectin as $\geq 11 \text{ } \mu\text{g/mL}$ and $< 11 \text{ } \mu\text{g/mL}$, leptin as $< 3 \text{ ng/mL}$ and $\geq 3 \text{ ng/mL}$, and resistin as $< 42 \text{ ng/mL}$ and $\geq 42 \text{ ng/mL}$. Only adiponectin was inversely categorized (High and Low).

Statistical analysis

The SPSS statistical software package for Windows version 11.0J (SPSS Inc. Tokyo, Japan) was used for all statistical analyses. Distributions were evaluated by the χ^2 test. Comparisons of mean values among groups were tested by analysis of variance, and Dunnett's test was applied as an additional multiple comparison after the equality of variance had been confirmed. Risks were tested by univariate and multivariate logistic regression. Differences at $P < 0.05$ were considered to be significant. The exact P values are indicated when statistically significant.

RESULTS

Clinical background of patients

The study included 124 male subjects (47 with adenoma, 34 with early cancer, 17 with advanced cancer, and 26 without tumors as controls). The early cancer group included 14 T1m and 20 pT1 cases. The advanced cancer group included 6 pT2, 10 pT3, and one pT4 cases.

The clinical backgrounds of the controls and patients are shown in Table 1. There were no significant differences in mean age or blood pressure among the 4 groups (control, adenoma, early cancer, and advanced cancer). There were also no significant differences in the distribution of the numbers of diabetic patients, current smokers, ex-smokers, habitual alcohol drinkers, NSAID users, and other medicine users. Angiotensin II receptor antagonists, angiotensin converting enzyme inhibitors, and statin type anti-hyperlipidemia medicines were designated as other medicines. The BMI in the control group was $21.8 \pm 3.1 \text{ kg/m}^2$. Those in the adenoma, early cancer, and advanced cancer groups were $23.2 \pm 3.2 \text{ kg/m}^2$, $24.4 \pm 3.8 \text{ kg/m}^2$, and $22.7 \pm 3.8 \text{ kg/m}^2$, respectively. The BMI was highest in the early cancer group, and the difference was significant (Dunnett's test, $P = 0.015$). The BMI in patients with advanced cancer was lower than that in patients with early cancer.

Table 1 Background of the male patients examined by colonoscopy n (%)

	Control ($n = 26$)	Adenoma ($n = 47$)	Early cancer ($n = 34$)	Advanced cancer ($n = 17$)	P -value
Age (yr)	67.9 ± 12.4	65.1 ± 11.7	67.0 ± 8.8	66.5 ± 7.8	0.810
Diabetes	1 (3.8)	6 (12.8)	2 (5.9)	2 (11.8)	0.523
Smokers					
Current-	9 (34.6)	18 (38.2)	13 (38.2)	7 (41.8)	0.977
Ex-	6 (8.5)	14 (21.7)	10 (15.8)	2 (11.0)	0.480
Alcohol	15 (57.7)	30 (63.8)	27 (79.4)	14 (82.4)	0.151
NSAIDs	2 (7.7)	2 (4.3)	1 (3.0)	0 (0)	0.631
Other meds	6 (23.1)	11 (23.4)	8 (23.5)	3 (17.6)	0.965
BMI (kg/m^2)	21.8 ± 3.1	23.2 ± 3.2	24.4 ± 3.8^a	22.7 ± 3.8	0.025
BP-S (mmHg)	123 ± 14	129 ± 14	129 ± 13	133 ± 20	0.193
BP-D (mmHg)	76 ± 12	76 ± 10	78 ± 10	81 ± 11	0.331

NSAIDs: Non-steroid anti-inflammatory drug; Other meds: Other medicines such as angiotensin receptor antagonists, angiotensin converting enzyme inhibitors, or HMG-CoA reductase inhibitors; BMI: Body mass index; BP-S: Systolic blood pressure; BP-D: Diastolic blood pressure. Age, BMI, BP-S and BP-D are expressed as the mean \pm SD and P -value was evaluated by analysis of variance. BMI was additionally evaluated by Dunnett's test ($^aP = 0.015$). Diabetes (without insulin injections), current smoker, ex-smoker, alcohol habit (ethanol intake more than 15 g/d on average) and NSAID users and Other Meds. users are expressed as the number of patients, and P values were evaluated by χ^2 test.

Evaluation of risk for adenoma, early cancer and advanced cancer

In the patients with adenoma, TG [OR: 3.990, 95% confidence interval (CI): 1.173-13.574, $P = 0.027$] and adiponectin (OR: 6.667, 95% CI: 2.190-20.295, $P = 0.001$) levels revealed significant associations in univariate analysis (Table 2). Multivariate analysis (adjusted for age, BMI, BP-S, TG, and adiponectin) showed that only adiponectin was a significant risk factor for adenoma (OR: 5.762, 95% CI: 1.683-19.739, $P = 0.005$). The other factors including glucose, insulin, HOMA-IR, T-cho, hsCRP, IGF-1/IGFBP-3 ratio, leptin and resistin were not significant risk factors for adenoma.

In the patients with early cancer, BMI (OR: 4.200, 95% CI: 1.344-13.128, $P = 0.014$), TG (OR: 3.958, 95% CI: 1.103-14.201, $P = 0.035$), and adiponectin (OR: 6.300, 95% CI: 1.847-21.485, $P = 0.003$) showed significant associations in univariate analysis (Table 3). Multivariate analysis (adjusted for age, BMI, BP-S, TG, and adiponectin) showed that only adiponectin was a significant risk factor for early cancer (OR: 4.495, 95% CI: 1.090-18.524, $P = 0.038$). As was the case for adenoma, none of the other factors, including glucose, insulin, HOMA-IR, T-cho, hsCRP, IGF-1/IGFBP-3 ratio, leptin and resistin, were significant risk factors for early cancer.

In the patients with advanced cancer, only BP-D (OR: 4.950, 95% CI: 1.289-19.014, $P = 0.020$) revealed a significant OR in univariate analysis (Table 4). Unlike adenoma and early cancer, changes in adiponectin, BMI, and TG were not associated with a significantly increased risk. Multivariate analysis (adjusted for age, BP-D, hsCRP, and adiponectin) still recognized BP-D as a risk factor for advanced cancer (OR: 5.015, 95% CI: 1.159-21.696, $P = 0.031$).

Table 2 Evaluation of risks for adenoma

Criteria		(Case/Cont)		Univariate analysis OR (95% CI)	P value	Multivariate analysis OR (95% CI)	P value
		Low	High				
BMI (kg/m ²)	< 22.5, ≥ 22.5	18/14	29/10	2.256 (0.828-6.145)	0.112	1.432 (0.434-4.729)	0.556
BP-S (mmHg)	< 120, ≥ 120	14/14	33/12	1.030 (0.998-1.074)	0.073	1.576 (0.463-5.370)	0.467
BP-D (mmHg)	< 80, ≥ 80	32/18	15/8	1.055 (0.375-2.967)	0.920		
Glucose (mg/dL)	< 100, ≥ 100	27/14	19/10	0.992 (0.976-1.008)	0.328		
Insulin (ng/mL)	< 4.5, ≥ 4.5	12/12	35/14	1.033 (0.989-1.091)	0.249		
HOMA-IR	< 2, ≥ 2	27/15	19/9	1.173 (0.426-3.232)	0.758		
T-cho (mg/dL)	< 180, ≥ 180	19/13	27/10	1.005 (0.992-1.019)	0.449		
TG (mg/dL)	< 120, ≥ 120	25/19	21/4	3.990 (1.173-13.570)	0.027 ^a	2.713 (0.707-10.410)	0.146
hsCRP (ng/mL)	< 500, ≥ 500	20/16	27/10	2.160 (0.811-5.750)	0.123		
IGF-1/IGFBP-3	< 60, ≥ 60	29/15	18/11	0.846 (0.319-2.245)	0.074		
Adiponectin (μg/mL)	< 11, ≥ 11	40/12	7/14	6.667 (2.190-20.300)	0.001 ^b	5.762 (1.683-19.739)	0.005 ^b
Leptin (ng/mL)	< 3, ≥ 3	17/13	30/13	1.765 (0.668-4.665)	0.252		
Resistin (ng/mL)	< 42, ≥ 42	17/13	30/13	1.184 (0.524-3.642)	0.514		

OR: Odds ratio; CI: Confidence interval; TG: Triglyceride; T-cho: Total cholesterol; hs-CRP: High sensitivity C-reactive protein; HOMA-IR: Homeostatic model assessment of insulin resistance; IGF-1/IGFBP-3: Ratio of insulin-like growth factor-1 and IGF binding protein-3; Cont: Numbers of control persons; Case: Numbers of patients. The OR (High *vs* Low) was evaluated by univariate and multivariate logistic regression analysis. Only the OR of adiponectin was inversely evaluated (Low *vs* High). Multivariate analysis included age, BMI, BP-S, TG, and adiponectin. ^a*P* < 0.05, ^b*P* < 0.01.

Table 3 Evaluation of risks for early cancer

Criteria		(Case/Cont)		Univariate analysis OR (95% CI)	P value	Multivariate analysis OR (95% CI)	P value
		Low	High				
BMI (kg/m ²)	< 22.5, ≥ 22.5	8/14	24/10	4.200 (1.344-13.128)	0.014 ^a	2.814 (0.746-10.620)	0.127
BP-S (mmHg)	< 120, ≥ 120	11/14	22/12	2.333 (0.810-6.718)	0.116	1.702 (0.461-6.281)	0.425
BP-D (mmHg)	< 80, ≥ 80	21/18	12/8	1.286 (0.431-3.839)	0.653		
Glucose (mg/dL)	< 100, ≥ 100	15/14	18/10	1.680 (0.581-4.859)	0.338		
Insulin (ng/mL)	< 4.5, ≥ 4.5	12/13	21/14	1.500 (0.526-4.276)	0.448		
HOMA-IR	< 2, ≥ 2	13/15	17/9	1.771 (0.606-5.173)	0.296		
T-cho (mg/dL)	< 180, ≥ 180	13/15	18/8	1.560 (0.534-4.557)	0.416		
TG (mg/dL)	< 120, ≥ 120	18/19	15/4	3.958 (1.103-14.201)	0.035 ^a	2.705 (0.596-12.283)	0.197
hsCRP (ng/mL)	< 500, ≥ 500	16/17	17/10	1.700 (0.598-4.830)	0.319		
IGF-1/IGFBP-3	< 60, ≥ 60	18/15	15/11	1.136 (0.403-3.205)	0.809		
Adiponectin (μg/mL)	< 11, ≥ 11	27/12	5/14	6.300 (1.847-21.485)	0.003 ^b	4.495 (1.090-18.528)	0.038 ^a
Leptin (ng/mL)	< 3, ≥ 3	8/14	24/13	3.000 (0.989-7.100)	0.052		
Resistin (ng/mL)	< 42, ≥ 42	14/12	16/14	0.980 (0.324-2.808)	0.969		

The OR (High *vs* Low) was evaluated by univariate and multivariate logistic regression analysis. Only the OR of adiponectin was inversely evaluated (Low *vs* High). Multivariate analysis included age, BMI, BP-S, TG, and adiponectin. ^a*P* < 0.05, ^b*P* < 0.01.

DISCUSSION

The present data for adenoma were similar to those of our previous study^[4], and it was also revealed that a decreased level of adiponectin was a strong risk factor for early colorectal cancer. Furthermore, we were able to demonstrate that a low adiponectin level was a stronger risk factor than high TG or BMI in patients with adenoma and early cancer. However, other adipokines, including leptin and resistin, were not direct risk factors for colorectal tumors in this study. In contrast to adenoma and early cancer, a low adiponectin level was not recognized as a significant risk factor for advanced cancer.

In the present study, the mean BMI was significantly higher in the early cancer group than in the control group. However, the mean BMI in the advanced cancer group was lower than that in the early cancer group and nearly equal to that in the controls. One reason may be a reduction of BMI as a result of nutritional deficiencies in some

patients with advanced cancer. Generally, the adiponectin level is known to increase with a reduction in body weight^[10]. Thus the reduction of BMI may have been related to the fact that a low adiponectin level was not a significant risk factor for advanced cancer. As the number of patients with advanced cancer in the present study was relatively small, the low adiponectin level observed in this group must be evaluated carefully in further investigations.

Recently, 2 reports addressing adiponectin levels in patients with colorectal cancer were published. Kumor *et al*^[5] and Erarslan *et al*^[6] revealed that serum adiponectin levels were significantly low in patients with cancer. In fact, our present study revealed results consistent with those reports. Our results enhanced the strength of their data. However, we were able to add some information. Our study evaluated the risk of colorectal tumors, focusing on patients with early-stage colorectal cancer. Besides these 2 reports, Levy *et al*^[11] also reported adiponectin levels in patients with colorectal cancer, and demonstrated that

Table 4 Evaluation of risks for advanced cancer

	Criteria	(Case/Cont)		Univariate analysis OR (95% CI)	P value	Multivariate analysis OR (95% CI)	P value
		Low	High				
BMI (kg/m ²)	< 22.5, ≥ 22.5	8/14	8/10	1.400 (0.392-4.997)	0.604		
BP-S (mmHg)	< 120, ≥ 120	6/14	10/12	1.944 (0.545-0.940)	0.306		
BP-D (mmHg)	< 80, ≥ 80	5/19	11/8	4.950 (1.289-19.014)	0.020 ^a	5.015 (1.159-21.696)	0.031 ^a
Glucose (mg/dL)	<100, ≥ 100	6/15	10/10	2.333 (0.638-8.538)	0.201		
Insulin (μU/mL)	< 4.5, ≥ 4.5	5/12	11/14	1.885 (0.510-6.978)	0.342		
HOMA-IR	< 2, ≥ 2	10/15	6/9	1.000 (0.271-3.694)	1.000		
T-cho (mg/dL)	< 180, ≥ 180	7/13	9/10	1.671 (0.462-6.051)	0.434		
TG (mg/dL)	< 120, ≥ 120	10/19	6/4	2.850 (0.650-12.505)	0.165		
hsCRP (ng/mL)	< 500, ≥ 500	5/16	11/10	3.520 (0.941-13.174)	0.062	4.184 (0.896-19.529)	0.069
IGF-1/IGFBP-3	< 60, ≥ 60	7/14	9/11	1.753 (0.499-6.165)	0.382		
Adiponectin (μg/mL)	< 11, ≥ 11	11/12	5/14	2.567 (0.644-9.498)	0.158	3.355 (0.729-15.451)	0.120
Leptin (ng/mL)	< 3, ≥ 3	11/13	5/13	0.455 (0.123-1.680)	0.237		
Resistin (ng/mL)	< 42, ≥ 42	9/12	7/14	0.667 (0.190-2.334)	0.526		

The OR (High *vs* Low) was evaluated by univariate and multivariate logistic regression analysis. Only the OR of adiponectin was inversely evaluated (Low *vs* High). Multivariate analysis included age, BP-D, hsCRP, and adiponectin. ^a*P* < 0.05.

adiponectin levels were not influenced by cancer stage, including the early stage. However, their study did not consider the adiponectin levels in normal controls or adenoma patients.

While some reports have indicated a positive relationship between adiponectin and cancer, Lukanova *et al.*^[12] reported that the serum adiponectin level was not associated with the risk of colorectal cancer in a case control study in males. Also, Fukumoto *et al.*^[13] demonstrated only a weak correlation between large adenoma and adiponectin level in males working in the Japanese Self-Defense Forces. However, Wei *et al.*^[14] demonstrated that low levels of adiponectin were associated with a significantly increased risk of colorectal cancer in a prospective cohort study in males, with a mean follow-up period of 4.5 years. In addition to these epidemiologic studies, genetic polymorphisms of the adiponectin gene in patients with colorectal cancer have been investigated, and these showed that single nucleotide polymorphism of the adiponectin gene was associated with an increased risk of colorectal cancer^[15,16].

So far, several interesting reports on the molecular mechanism connecting adiponectin and colorectal tumors have been published. Fujisawa *et al.*^[17] reported that adiponectin normalized the expression of adenosine monophosphate-activated protein kinase (AMPK) and suppressed colonic epithelial cell proliferation *via* inhibition of the mammalian target of the rapamycin (mTOR) pathway. Moreover, a direct inhibitory effect of adiponectin on colorectal cancer cells *via* the AMPK-mTOR pathway has been demonstrated^[18]. Thus, the relationship between a decreased plasma adiponectin level and colorectal tumorigenesis is a major issue that needs to be investigated further.

Many studies have noted that a high TG level is associated with colorectal adenoma^[19,20]. Otani *et al.*^[21] have also reported that the OR (highest *vs* lowest quintile) of TG was significantly increased to 1.5 in Japanese patients. The present study revealed a significantly increased OR of TG in patients with adenoma and early cancer by univariate

analysis. The systemic inflammatory marker, hsCRP, is a factor associated with metabolic syndrome^[22]. The hsCRP levels were relatively increased in patients with advanced cancer (OR: 3.520, *P* = 0.062), but the difference was not statistically significant. CRP levels are increased by inflammatory cytokines. Kim *et al.*^[23] found that circulating levels of interleukin-6 and TNF- α were associated with colorectal adenoma risk. Association of inflammatory cytokines with colorectal cancer is also an interesting issue. The circulating IGF-1 level has been discussed in relation to colorectal adenoma and cancer^[24,25]. However, the relationship between IGF-1 and colorectal tumors is still obscure. In the present study, the IGF-1/IGFBP-3 ratio did not show a significant OR.

In our previous study, HOMA-IR was a significant risk factor for adenoma^[4]. However, HOMA-IR was not recognized as a risk factor for adenoma or cancer in the present study. This factor seemed not to be consistent. However, cohorts of both studies were different, and mean age and gender in the both cohorts were also different. To investigate this issue, further studies involving patients with wide age ranges must be performed.

In conclusion, our study has investigated risk factors for adenoma, early cancer, and advanced cancer. A decreased level of adiponectin was recognized as a strong risk factor for both colorectal adenoma and early cancer. The present results were similar to our previous data for adenoma, and we also demonstrated that a decreased adiponectin level was associated with an increased risk of early cancer. On the basis of these data, we consider that further investigations to clarify the relationship between low adiponectin and colorectal tumors, including controlled prospective studies, are warranted.

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COMMENTS

Background

Many biological factors vary according to accumulation of body/abdominal fat. Etiological studies have shown that body/abdominal fat or obesity increases the risk of colorectal cancer. What factors are strongly associated with colorectal tumors?

Research frontiers

Adiponectin is secreted from abdominal fat tissue, and has anti-diabetic, anti-atherosclerotic, and anti-inflammatory effects. A decrease in plasma adiponectin level may be strongly associated with an increased risk of colorectal adenoma or cancer.

Innovations and breakthroughs

The present study investigated the adiponectin levels in patients with colorectal adenoma and cancer (early and advanced). A low adiponectin level was a stronger risk factor than triglyceride or body mass index in patients with early cancer. However, a low adiponectin level was not recognized as a significant risk factor for advanced cancer.

Applications

The study enhanced the strength of previous data. Further investigations to clarify the relationship between low adiponectin levels and colorectal tumors, including controlled prospective studies, are warranted.

Peer review

The author investigate the association of adiponectin levels with risk of colorectal adenoma and cancer (early and advanced). It is a good paper and should be accepted with revision.

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T300A polymorphism of *ATG16L1* and susceptibility to inflammatory bowel diseases: A meta-analysis

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for cases of UC (OR = 0.98, 95% CI: 0.81-1.19, $P = 0.84$) relative to controls.

CONCLUSION: The *ATG16L1* T300A polymorphism contributes to susceptibility to CD and UC in adults, but different in children, which implicates a role for autophagy in the pathogenesis of IBD.

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Key words: *ATG16L1*; Inflammatory bowel diseases; Crohn's disease; Ulcerative colitis; Meta-analysis

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Abstract

AIM: To evaluate the association of the autophagy-related 16-like 1 (*ATG16L1*) T300A polymorphism (rs2241880) with predisposition to inflammatory bowel diseases (IBD) by means of meta-analysis.

METHODS: Publications addressing the relationship between rs2241880/T300A polymorphism of *ATG16L1* and Crohn's disease (CD) and ulcerative colitis (UC) were selected from the MEDLINE and EMBASE databases. To make direct comparisons between the data collected in these studies, the individual authors were contacted when necessary to generate a standardized set of data from these studies. From these data, odds ratio (OR) with 95% confidence interval (CI) were calculated.

RESULTS: Twenty-five studies of CD were analyzed, 14 of which involved cases of UC. The variant G allele of *ATG16L1* was positively associated with CD (OR = 1.32, 95% CI: 1.26-1.39, $P < 0.00001$) and UC (OR = 1.06, 95% CI: 1.01-1.10, $P = 0.02$). For child-onset IBD, a higher G allele frequency was found for cases of CD (OR = 1.35, 95% CI: 1.16-1.57, $P = 0.0001$) than

INTRODUCTION

Inflammatory bowel diseases (IBD), including Crohn's disease (CD) and ulcerative colitis (UC), are chronic inflammatory disorders of the gastrointestinal tract that have an increasing incidence and prevalence in developing countries^[1]. CD can affect any part of the alimentary tract, although it most commonly involves the terminal ileum, and is characterized by transmural discontinuous lesions. UC is a continuous disease that commences in the rectum and extends for a varying distance proximally in the colon, and its continuous inflammation is limited to mucosal and submucosal layers. Currently, the etiology and pathogenesis of IBD are not completely understood. IBDs are complex diseases with a number of contributing factors such as genetic predisposition, environmental factors, intestinal

microbial flora, and aberrant immune responses^[2].

Advances in genetic research, especially the genome-wide association (GWA) studies, have identified a number of IBD susceptibility loci. One of these loci, the autophagy-related 16-like 1 (*ATG16L1*) gene, has been shown to have a role in IBD. The *ATG16L1* gene is located on chromosome 2, at 2q37.1, and encodes a protein that is involved in the formation of autophagosomes during autophagy. Autophagy is a non-selective degradation system that has roles in starvation adaptation, intracellular protein and organelle clearance, development, anti-aging processes, elimination of microorganisms, cell death, tumor suppression, and antigen presentation^[3]. Autophagy has also been implicated in the innate and adaptive immune responses^[4].

Hampe *et al.*^[5] first identified *ATG16L1* as a susceptibility gene for CD in a GWA study of 19 779 non-synonymous single nucleotide polymorphisms (SNPs) present in 735 individuals with CD and 368 controls. Further analysis showed that the marker, rs2241880, was an SNP that encodes a threonine to alanine substitution (T300A) at amino acid position 300, which was correlated with the incidence of CD in two German and one British studies of CD. The rs2241880 SNP directly correlated with the majority of risk associated with this locus. In contrast, no correlation between rs2241880 and UC was detected. Many other studies^[6-29] have assessed the association of rs2241880 with predisposition to CD and UC, however, the results of these studies are inconsistent. In addition, a number of studies included child-onset IBD cases, the results of which were also confusing.

A meta-analysis was recently published that focused on the relationship between the T300A polymorphism and the incidence of CD, but not on the relationship between the T300A polymorphism and risk of UC, or the role of this polymorphism in child-onset IBD^[30]. With the publication of additional studies related to the T300A polymorphism, sufficient data were available to perform a comprehensive meta-analysis of these studies in order to identify consistencies in the data to determine disease susceptibility, as well as to identify areas that need to be further addressed.

MATERIALS AND METHODS

Study selection

As of June 1, 2009, published studies in MEDLINE and EMBASE containing the following key words were included in this study: “inflammatory bowel disease”, “IBD”, “Crohn’s disease”, “CD”, “Ulcerative colitis”, “UC”, “autophagy related 16 like 1”, and “ATG16L1”. The references of the eligible publications were searched manually to identify additional relevant studies. Relevant publications were also identified using the “Related Articles” option in PubMed. Studies reported by the same authors, yet published in different journals, were checked for possible overlapping participant groups. No language restrictions were made. When pertinent data

were not included, or data that were presented were unclear, the authors were directly contacted.

Inclusion criteria

To be eligible for inclusion in this meta-analysis, the following criteria were established: (1) the study must include a case-control study that addressed IBD (i.e. CD or UC) patients and unrelated controls; (2) the study must have evaluated the *ATG16L1* T300A polymorphism and the risk of CD or UC; and (3) the study must have included sufficient data for extraction.

Exclusion criteria

Studies were excluded from consideration if: (1) the study was based on family data; (2) the study did not have the outcomes of comparison reported or it was not possible to determine them; or (3) the study contained a smaller sample size and overlapped others meanwhile.

Data extraction

Using a standardized form, data from published studies were extracted independently by two investigators to populate the needed information. Data collected included first author, year of publication, inclusion and exclusion criteria, study population characteristics, and sample size. Any discrepancies between the two sets of data collected were resolved.

Statistical analysis

Odds ratio (OR) and 95% confidence interval (CI) were calculated for each study using Review Manager version 4.2 software. Between-study heterogeneity was estimated using the χ^2 -based Q statistic. Heterogeneity was considered statistically significant when $P < 0.05$. I^2 was also tested. If heterogeneity existed, data were analyzed using a random effects model. In the absence of heterogeneity, a fixed effects model was used.

A χ^2 test was performed to examine Hardy-Weinberg equilibrium when genotype data were available. $P < 0.05$ was considered statistically significant. If Hardy-Weinberg disequilibrium existed, or it was impossible to evaluate this equilibrium, sensitivity analysis was performed.

RESULTS

Patients and controls

Of the 41 papers identified in a literature search of MEDLINE and EMBASE databases relevant to IBD, CD and UC, 25^[5-29] were included in this meta-analysis, while 16 were excluded^[31-46] (Figure 1). The reasons for exclusion are listed in Table 1. Three of the 41 papers^[5,10,15] evaluated more than one study. Two of these studies contained relevant data and were combined^[5,10], while the third was excluded^[15]. Each of the 25 studies (Table 2) included cases of CD, with various subsets of papers considering different ethnic populations. For example, there were three studies of Asian popula-

Table 1 Reasons for study exclusion

Studies	Reasons
Glas <i>et al</i> ^[31] , Török <i>et al</i> ^[32]	Data overlapped those of another article ^[17]
Cooney <i>et al</i> ^[33]	Data overlapped those of another article ^[8]
Weersma <i>et al</i> ^[34] , Weersma <i>et al</i> ^[35]	Data overlapped those of another article ^[26]
Franke <i>et al</i> ^[36] , Franke <i>et al</i> ^[37]	Data overlapped those of another article ^[5]
Fisher <i>et al</i> ^[38] , Wtccc <i>et al</i> ^[40] , Parkes <i>et al</i> ^[43] , Yamazaki <i>et al</i> ^[45]	Other SNPs rather than rs2241880 SNP of <i>ATG16L1</i> were analyzed
Beckly <i>et al</i> ^[39] , Barrett <i>et al</i> ^[41] , Libioulle <i>et al</i> ^[42] , Raelson <i>et al</i> ^[44] , Kugathasan <i>et al</i> ^[46]	Data could not be extracted

Table 2 Studies included in the meta-analysis

No.	First author	Year	Population	Number of participants used in analysis		
				CD	UC	Controls
1	Hampe <i>et al</i> ^[51]	2007	Germany, UK	2122	1227	2056, 1032 for CD, UC respectively
2	Baldassano <i>et al</i> ^[6]	2007	USA	142	NA	281
3	Büning <i>et al</i> ^[7]	2007	Germany, Hungary, Netherlands	614	296	707
4	Cummings <i>et al</i> ^[8]	2007	UK	645	676	1190
5	Prescott <i>et al</i> ^[9]	2007	UK	727	877	579
6	Rioux <i>et al</i> ^[10]	2007	North-America	1571	353	1184, 207 for CD, UC respectively
7	Roberts <i>et al</i> ^[11]	2007	New Zealand	496	466	549
8	Yamazaki <i>et al</i> ^[12]	2007	Japan	481	NA	437
9	Amre <i>et al</i> ^[13]	2009	Canada	286	NA	290
10	Baptista <i>et al</i> ^[14]	2008	Brazil	180	NA	189
11	Fowler <i>et al</i> ^[15]	2008	Australia	154	NA	420
12	Gaj <i>et al</i> ^[16]	2008	Poland	59	NA	140
13	Glas <i>et al</i> ^[17]	2008	Germany	768	507	1615
14	Lakatos <i>et al</i> ^[18]	2008	Hungary	266	149	149
15	Lappalainen <i>et al</i> ^[19]	2008	Finland	240	459	190
16	Latiano <i>et al</i> ^[20]	2008	Italy	667	668	749
17	Okazaki <i>et al</i> ^[21]	2008	Canada	208	113	314
18	Perricone <i>et al</i> ^[22]	2008	Italy	163	NA	160
19	Peterson <i>et al</i> ^[23]	2008	USA	555	NA	486
20	Van Limbergen <i>et al</i> ^[24]	2008	Scotland	629	580	345
21	Zhi <i>et al</i> ^[25]	2008	China	40	40	50
22	Weersma <i>et al</i> ^[26]	2009	Holland	1684	1120	1350
23	Hancock <i>et al</i> ^[27]	2008	UK	586	NA	1156
24	Yang <i>et al</i> ^[28]	2009	Korea	377	NA	372
25	Newman <i>et al</i> ^[29]	2009	Canada	435	NE	895
Total				14095	7531	15849, 13852 for CD, UC respectively

¹Data were extracted from the combination of panel A, panel B, panel C, and UC cohort; ²Only GWA study and replication cohort 2 study were included; ³Data from the cohort 1 study were not included in the analysis based on the use of data for controls from 251 families with IBD; ⁴Data of the UC group were obtained by communication with the authors of this publication. CD: Crohn's disease; UC: Ulcerative colitis; NA: Not applicable (no such group); NE: Non-extractable or unavailable data.

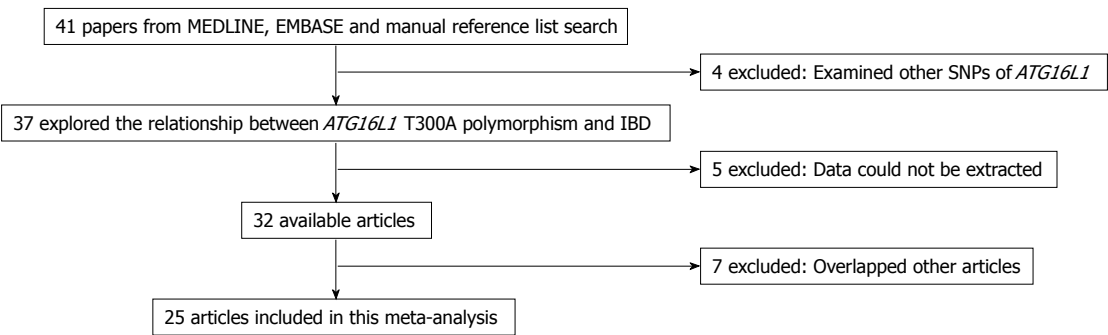


Figure 1 A flowchart illustrating the selection of published studies included in this meta-analysis.

tions^[12,25,28], two of Oceanic populations^[11,15], seven studies based in the United States^[6,10,13,14,21,23,29], and 13 studies of European populations^[5,7-9,16-20,22,24,26,27]. Of the 25 studies, 14 included both CD and UC cases and

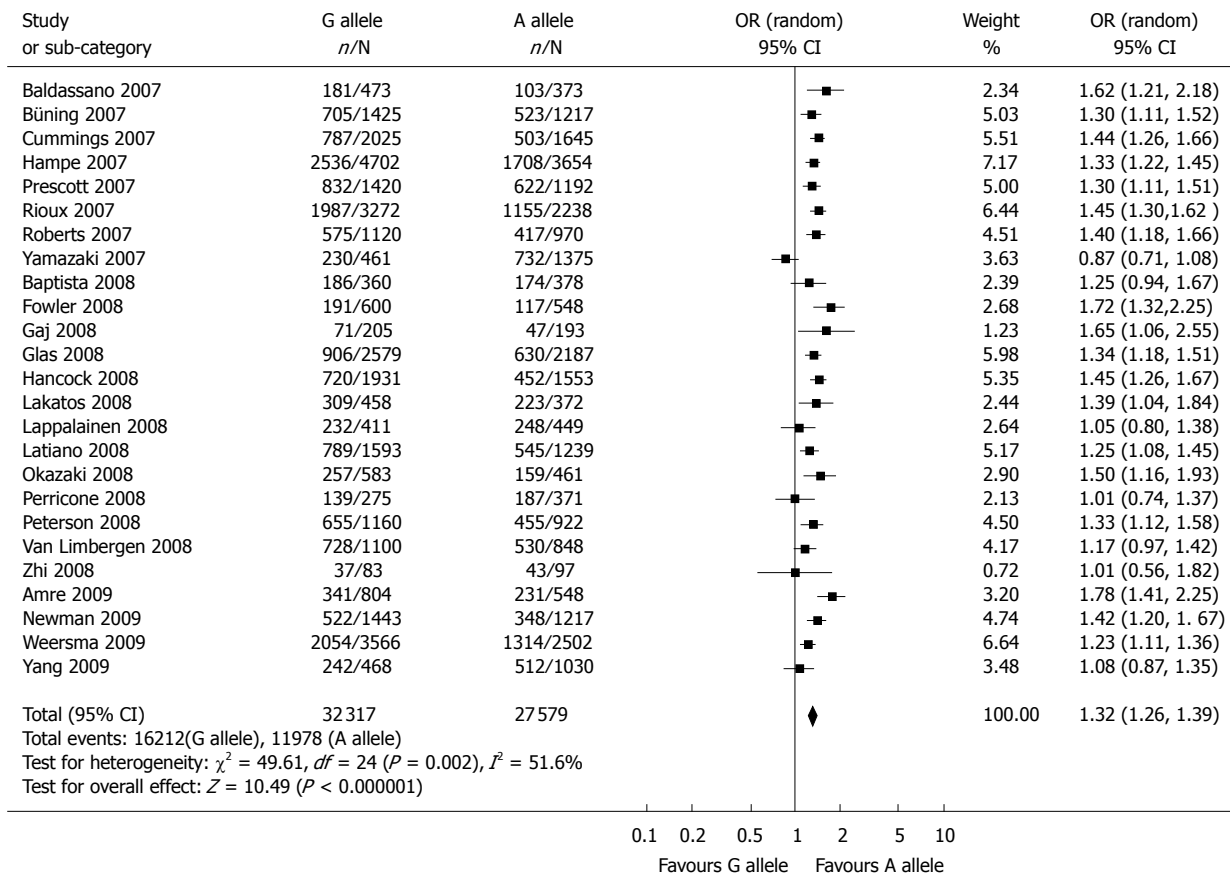


Figure 2 Meta-analysis of the association between the rs2241880 polymorphism of the *ATG16L1* gene and CD for the G allele vs the A allele.

were based on data from an Asian population^[25], an Oceanic population^[11], two studies from the United States^[10,21], and 10 European populations^[5,7-9,17-20,23,24]. Seven of the included studies explored the relationship between the incidence of the T300A polymorphism of *ATG16L1* and child-onset CD^[6,11,13,17,20,23,24], while only two reported cases of child-onset UC^[20,24]. For six of the total studies, genotype data were not available even after authors were contacted for further inquiry. Therefore, a Hardy-Weinberg equilibrium test was performed on the remaining 19 studies, and three of them showed disequilibrium^[15,25,29] ($P = 0.04$, $P = 0.00$, and $P = 0.03$, respectively). Among the six untested studies due to unavailable data, five^[5,8,10,17,26] reported Hardy-Weinberg equilibrium data in the original paper.

Association between *ATG16L1* T300A polymorphism and IBD

Meta-analysis of the 25 studies that fulfilled the inclusion criteria identified a significant association between the G allele of *ATG16L1* and susceptibility to CD (OR = 1.32, 95% CI: 1.26-1.39, $P < 0.00001$) (Table 3 and Figure 2). Sensitivity analysis was performed with the omission of three studies^[15,25,29] and Hardy-Weinberg disequilibrium showed similar results (data not shown). No notable change in the results of the statistic analyses was obtained when the Peterson *et al.*^[23] study was eliminated. As shown in Table 3 and Figure 3, a significant association between

Table 3 Summary of the association of the rs2241880 polymorphism and IBD determined in the meta-analysis

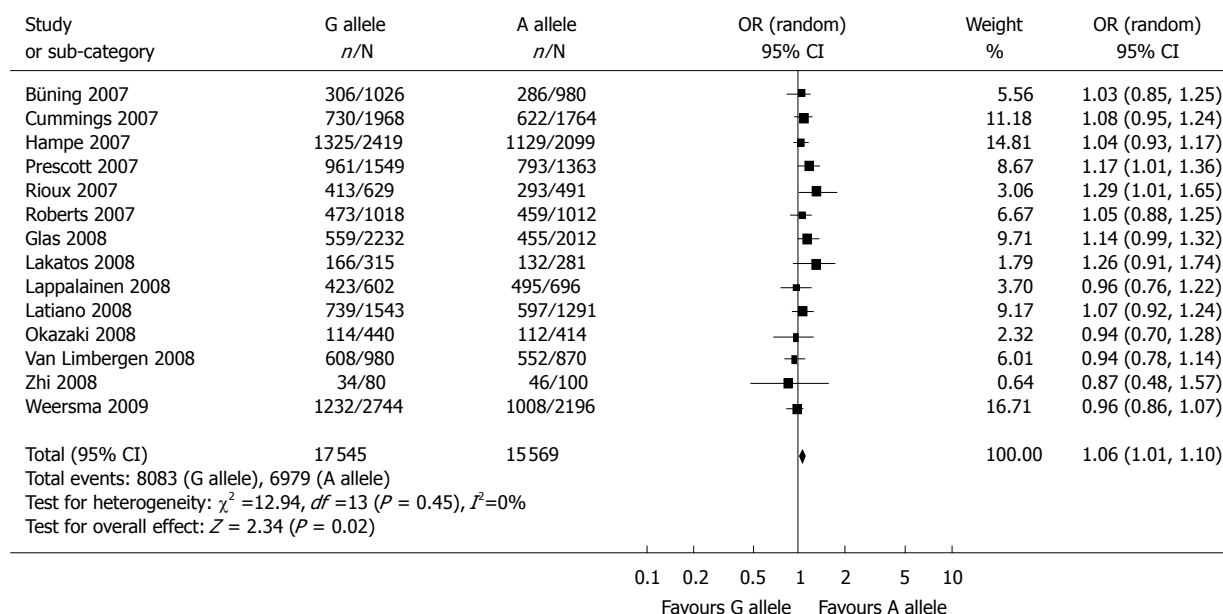
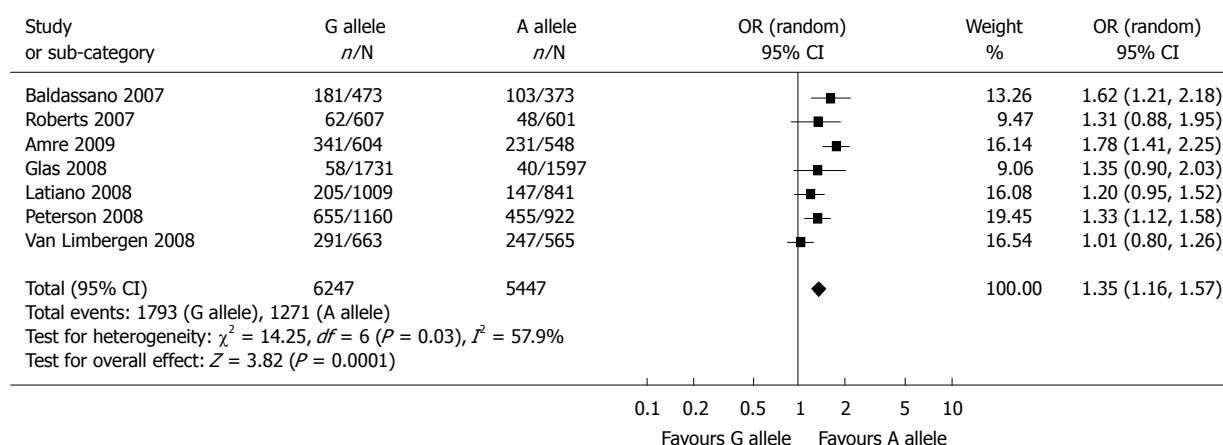
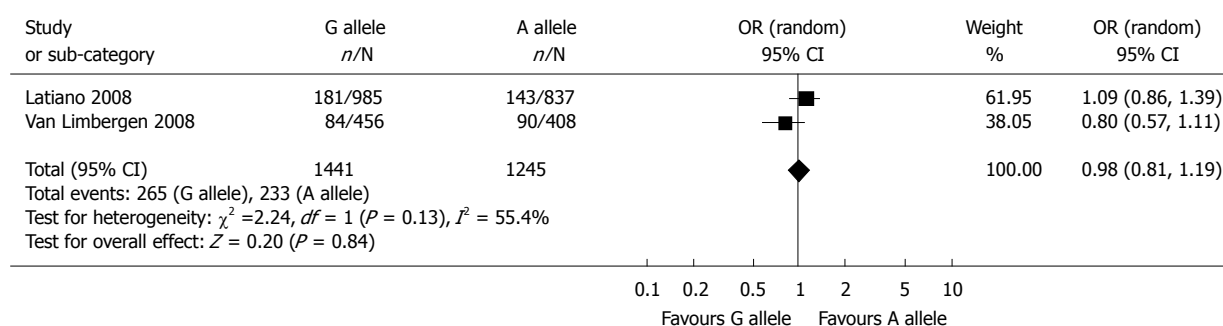
Comparisons	No. of studies	Effects model selection	OR (95% CI)	P-value
CD vs control	25	R	1.32 (1.26-1.39)	< 0.00001
UC vs control	14	F	1.06 (1.01-1.10)	0.02
Child-onset	7	R	1.35 (1.16-1.57)	0.0001
CD vs control				
Child-onset	2	F	0.98 (0.81-1.19)	0.84
UC vs control				

R: Random effects model; F: Fixed effects model.

the G allele of *ATG16L1* and the risk of UC was also detected (OR = 1.06, 95% CI: 1.01-1.10, $P = 0.02$). When the data of Zhi *et al.*^[25] were excluded, similar results were obtained: OR = 1.05, 95% CI: 1.01-1.10, $P = 0.02$.

Association between *ATG16L1* T300A polymorphism and child-onset CD and UC

The pooled analysis for child-onset cases of CD identified the T300A polymorphism of *ATG16L1* as a factor for predisposition to child-onset CD in a total of seven studies^[6,11,13,17,20,23,24] (OR = 1.35, 95% CI: 1.16-1.57, $P = 0.0001$) (Table 3 and Figure 4). When the study of Peterson *et al.*^[23] was excluded, similar data were acquired: OR = 1.35, 95% CI: 1.11-1.64, $P = 0.003$. Two studies^[20,24] discussed the association of the T300A polymorphism of *ATG16L1* and

Figure 3 Meta-analysis of the association between the rs2241880 polymorphism of the *ATG16L1* gene and UC for the G allele vs the A allele.Figure 4 Meta-analysis of the association between the rs2241880 polymorphism of the *ATG16L1* gene and child-onset CD for the G allele vs the A allele.Figure 5 Meta-analysis of the association between the rs2241880 polymorphism of *ATG16L1* gene and child-onset UC for the G allele vs the A allele.

child-onset UC, yet the results of the combined analysis indicated there was no correlation (Table 3 and Figure 5).

DISCUSSION

Over the past few years, knowledge of the genetics of

IBD has advanced tremendously since the first IBD GWA study was published in 2006^[47]. Hence, several novel susceptibility loci have been identified; one of them being the *ATG16L1* gene. Hampe *et al*^[5] first identified the association of the rs2241880 SNP in *ATG16L1* with cases of CD, but not with UC. Several subsequent studies

have tried to verify these results, however, the results of these latter studies were inconsistent, especially regarding the relationship between *ATG16L1* and UC, further perplexing our understanding of the role of *ATG16L1* in IBD. Thus, we conducted a meta-analysis of the currently published reports of CD and UC to attempt to clarify the correlation between *ATG16L1* and IBD. In order to obtain as much relevant data as possible, we retained some studies that presented a Hardy-Weinberg disequilibrium, however, there were no notable change in the results of our analyses whether these studies were included or omitted.

Our meta-analysis confirmed a positive association between the rs2241880 polymorphism of *ATG16L1* and susceptibility to CD. Meanwhile, a modest but significant association of the rs2241880 polymorphism with predisposition to UC was also observed. Taken together, these outcomes demonstrate that the *ATG16L1* variant containing the T300A substitution confers risk for both CD and UC.

Many aspects of child-onset IBD differ from adult-onset IBD, especially in regard to the type, location, and behavior of the disease, as well as sex preponderance and genetically attributable risk^[48]. Considering the relatively short time of exposure to environmental factors (e.g. smoking) in children compared to adults with IBD, genetic background is hypothesized to be a more important factor in early-onset IBD. Therefore, we further analyzed the association between *ATG16L1* and child-onset IBD. Our results indicated that *ATG16L1* was associated with the risk of child-onset CD, but not with child-onset UC. Regarding the negative finding in early-onset UC, small sample sizes must be recognized as a contributing factor. Only two studies were included in the final analysis, therefore, this may have precluded the detection of a significant association. Additional studies are needed to determine whether an association exists in this cohort or not.

There were some limitations in the present meta-analysis. For example, because of publication limitations, some relevant studies could not be included in our analysis. Secondly, heterogeneity existed among studies of CD and child-onset CD, which had the potential to influence the results of our meta-analysis. The lack of information provided by some published studies, especially with regard to genotype data, was another limitation. Although we contacted authors of publications that did not provide a comprehensive set of data, genotype data remained unavailable for six of the total number of studies. Finally, there were only two studies that examined the relationship between *ATG16L1* and child-onset UC. As a result, the small sample size available was not ideal for detecting small genetic effects.

Although the precise impact of the *ATG16L1* variant on the pathogenesis of IBD remains unknown, accumulating evidence suggests that microbes play an important role in the initiation and etiopathogenesis of IBD. IBD lesions have been shown to develop preferentially in regions with the highest concentrations of

bacteria^[49]. In addition, enteric flora has been found to be more commonly associated with IBD patients than with control groups^[50,51]. IBD animal model studies also have demonstrated that gut inflammation does not develop in a germ-free environment^[52]. Autophagy is a fundamental intracellular degradation system that protects cells against various bacterial pathogens and the cytotoxic effect of bacterial toxins. Autophagy also has been identified to play a role in both innate and adaptive immune responses^[53,54]. The specific role of *ATG16L1* in these processes remains unclear. *ATG16L1* is expressed not only in intestinal epithelial cells, but also in lymphocytes and macrophages. It interacts with two other autophagy proteins, *ATG5* and *ATG12*, to form a complex essential for the process of autophagy. Several studies have characterized the influence of *ATG16L1* deficiency. Rioux *et al*^[10] have shown that knockdown of *ATG16L1* mRNA in HeLa cells with siRNA reduced targeting of *Salmonella typhimurium* to autophagic vacuoles, which implicates a role for *ATG16L1* in the clearance of intracellular bacteria *via* autophagy. Saitoh *et al*^[55] have reported that *ATG16L1* deficiency disrupts the recruitment of the *ATG12-ATG5* conjugate to the isolation membrane, which results in loss of microtubule-associated protein 1 light chain 3 binding to phosphatidylethanolamine. Consequently, autophagosome formation and degradation of proteins with a long half-life are severely impaired in *ATG16L1*-deficient cells. *ATG16L1*-deficient macrophages also have been shown to produce large amounts of the inflammatory cytokines, IL-1 and IL-18, and mice that lack *ATG16L1* expression in hematopoietic cells are highly predisposed to dextran sulfate sodium-induced acute colitis. Cadwell *et al*^[56] have generated hypomorphic *ATG16L1* (*ATG16L1^{HM}*) mice that express *ATG16L1* at 30% of its normal level. In this model, notable abnormalities have been observed in Paneth cells. These ileal epithelial cells are hypothesized to play a role in the control of intestinal microbiota by secreting granules that contained antimicrobial peptides and lysozymes. Aberrant Paneth cells exhibit deficiencies in their granule exocytosis pathway, which results in a decreased capacity to eliminate microbes. The mammalian *ATG16L1* protein consists of three distinct domains: the N-terminal region mediates interactions with other autophagy proteins; a coiled-coil domain provides the capacity for *ATG16L1* oligomerization and *ATG5-ATG12* association; and there is a region of 7 WD repeats^[57]. The T300A mutation is located within the WD repeats, which are associated with protein interactions. Therefore, the T300A mutation is hypothesized to affect protein interactions necessary for the formation of autophagosomes, which results in dysfunction of the autophagy pathway and a subsequent decrease in pathogen clearance^[9]. It has been shown that the *ATG16L1* coding variant is defective in mediating efficient antibacterial autophagy in cultured HeLa and Caco2 cells, and the *ATG16L1**300A protein is unstable under conditions of high microbial load^[58]. These observations suggest the effect of this mutation has a direct impact on autophagic effectiveness. In

summary, the T300A mutation of *ATG16L1* impairs specific autophagic, innate resistance mechanisms to gut commensals, which facilitate inflammation after invasion^[59].

In addition to decreased ability to remove intestinal microbes directly, there may be other mechanisms that underlie the association between the *ATG16L1* variant and increased risk for IBD. For example, since autophagy contributes to immune tolerance against self tissues, it seems likely that decreased autophagy may lead to a failure of immune tolerance by autoantigen presentation on major histocompatibility complex class II molecules, which causes immune inflammation^[59]. Another possible mechanism involves autophagy and apoptosis. Accumulating studies have detected an acceleration in the rate of epithelial cell apoptosis and inhibition of inflammatory cell apoptosis in CD and UC^[60-63]. Autophagy and apoptosis share many common triggers and cross-inhibitory interactions^[64], thus, it is hypothesized that defective autophagy might alter the process of intestinal cell apoptosis, ultimately contributing to IBD pathogenesis.

In conclusion, our meta-analysis of published cases of CD and UC suggests that the *ATG16L1* T300A polymorphism is associated with susceptibility to CD and UC. However, the effect of this polymorphism differs between child-onset CD and UC. These findings implicate the role of autophagy and intestinal microbes in the pathogenesis of IBD, and demonstrate the need for further studies.

COMMENTS

Background

Inflammatory bowel diseases (IBD), Crohn's disease (CD) and ulcerative colitis (UC), are polygenic disorders. An A to G transition of the autophagy-related 16-like 1 gene (*ATG16L1*) has been implicated as a risk factor for IBD, but individual studies have been inconclusive or controversial.

Research frontiers

Much attention has been paid to the potential role of autophagy in the pathogenesis of IBD, since Hampe *et al* identified *ATG16L1* as a CD susceptibility gene in 2007. Some of them have been trying to confirm the definite relationship between *ATG16L1* polymorphism and IBD, and meanwhile, others attempting to uncover underlying mechanisms.

Innovations and breakthroughs

The current study demonstrated that *ATG16L1* is a susceptibility gene for CD and UC in adults, but different in children.

Applications

It is seen from this study that the *ATG16L1* T300A polymorphism contributes to susceptibility to CD and UC in adults, which provides an insight into the role of autophagy in the pathogenesis of IBD. Studies for further exploring the mechanism by which altered ability of autophagy predisposes to IBD are needed.

Terminology

Autophagy is a cytoplasmic homeostasis process that cleanses the interior of all eukaryotic cells. It has an important role in cell and tissue homeostasis, and has been implicated in a range of human diseases.

Peer review

This study is an interesting meta-analysis about T300A polymorphism of *ATG16L1* and susceptibility to inflammatory bowel diseases. The authors carefully reviewed the available literature and concluded that *ATG16L1* T300A polymorphism contributes to susceptibility to both CD and UC in adults, but not in children, implicating a role for autophagy in the pathogenesis of IBD.

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Establishment of a new quantitative detection approach to adefovir-resistant HBV and its clinical application

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RESULTS: Both the sensitivity and specificity of this new detection approach to ADV-resistant HBV quasispecies were 100%, which were much higher than those of direct HBV DNA sequencing. The approach was able to detect 0.1% of mutated strains in a total plasmid population. Among the 32 clinical patients, single rtA181 and rtN236T mutation and double rtA181T and rtN236T mutations were detected in 20 and 8, respectively, while ADV-resistant mutations in 6 (including, rtA181V/T mutation alone in 5 patients) and no associated mutations in 26.

CONCLUSION: This new approach is more feasible and efficient to detect ADV-resistant mutants of HBV and ADV-resistant mutations before and during ADV treatment with a specificity of 100% and a sensitivity of 100%.

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Key words: Chronic hepatitis B; Adefovir; Drug resistance; Quantitative detection; Real-time fluorescent quantitative polymerase chain reaction

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Abstract

AIM: To establish the more feasible and sensitive assessment approach to the detection of adefovir (ADV) resistance-associated hepatitis B virus (HBV) quasispecies.

METHODS: Based on the characteristics of rtA181V/T and rtN236T mutations, a new approach based on real-time fluorescent quantitative polymerase chain reaction (RT-PCR) was established for the detection of ADV-resistant HBV quasispecies, total HBV DNA, rtA181 and rtN236 mutations in blood samples from 32 chronic hepatitis B (CHB) patients with unsatisfactory curative effect on ADV and compared with routine HBV DNA sequencing.

INTRODUCTION

Chronic hepatitis B virus (HBV) infection affects approximately 400 million people worldwide and is still an important cause of morbidity and mortality, as well as a

source of potential new infections^[1]. Chronic HBV carriers may develop chronic hepatitis, cirrhosis, and hepatocellular carcinoma (HCC) with HBV as the most frequent cause^[2]. Since chronic hepatitis B (CHB) patients require a prolonged if not a lifelong therapy, data about its long-term safety and effect are indispensable for the assessment of its risk and benefit. Treatment of CHB has been improved due to available nucleoside/tide analogues, such as lamivudine (LAM), adefovir dipivoxil (ADV), entecavir (ETV), and telbivudine (LdT), which act as inhibitors of HBV reverse transcriptase and decrease viral load in most cases^[3]. ADV has become a treatment option for HBV infection due to its effect on lamivudine-resistant mutations occurring upon prolonged treatment. However, viral resistance to ADV develops and increases over time. ADV-resistance is mainly associated with rtN236T and rtA181V/T mutations within the D and B functional domains of HBV polymerase^[4]. Many methods have been developed to detect ADV-resistant mutations, such as direct polymerase chain reaction (PCR) sequencing, INNO-LiPA, restriction fragment length polymorphism (PCR-RFLP), matrix-assisted laser desorption/ionization-time of flight-mass spectrometry (MALDI TOF MS)^[4-9], with their respective advantages and disadvantages. Sequencing remains the best approach to the identification of new mutations. However, it cannot detect rtN236T and rtA181V/T mutations in less than 25% of a total viral population and is not appropriate for large-scale use in large cohort studies or clinical laboratories because of its labor-intensive and time-consuming manipulations^[5]. INNO-LiPA and MALDI TOF MS are capable of detecting variants, but more strict experiment conditions and equipments are required. PCR-RFLP^[8] could only detect mutations in a high proportion and is also labor-intensive and time-consuming. This study described a feasible and sensitive approach to quantitative detection of ADV-resistant HBV quasispecies by real-time fluorescent quantitative PCR.

MATERIALS AND METHODS

Materials

Thirty-two patients (29 males and 3 females, mean age 35 years, range 20-63 years) who were diagnosed as CHB from March 2003 to October 2007 in our hospital following the guidelines of prevention and treatment of chronic hepatitis B (2005)^[10] were enrolled in this study. ADV was administered with oral dipivoxil (10 mg) for 58 wk (range: 42-82 wk), during which viral breakthrough, viral rebound, partial or inadequate virus response occurred^[11]. Exclusion criteria included a coexisting severe illness, organ or bone marrow transplantation, recent treatment with systemic corticosteroids, immunosuppressants or chemotherapeutic agents, liver disease not due to hepatitis B, and seropositivity for human immunodeficiency virus (HIV) or hepatitis C or D virus. Informed consent was obtained from each patient in accordance with the Declaration of Helsinki. The study was approved by the institutional review board of So-

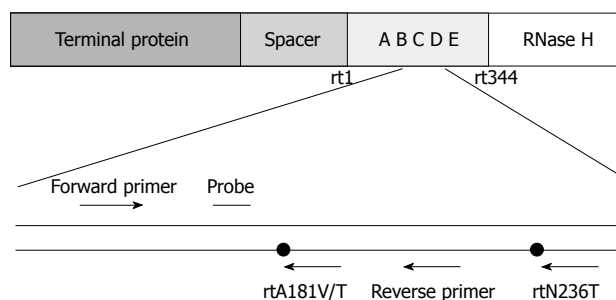


Figure 1 Targeted sequences of primers and probes designed for real-time polymerase chain reaction (PCR) to detect and quantify rtA181V/T and rtN236T adefovir (ADV)-resistance mutations.

ochow University. The average load of HBV DNA at baseline before ADV therapy was $6.30 \pm 0.23 \log_{10}$ IU/mL, and dropped to $5.61 \pm 0.17 \log_{10}$ IU/mL after treatment with ADV for 82 wk.

Serum specimens, quantitative reference substances for correlated mutations

HBV DNA was extracted from 100 μ L of sera as previously described^[6]. Extracted DNA pellets were resuspended in 30 μ L of sterile distilled water and stored at -20°C until use. HBV DNA fragments containing the RT domain were amplified by PCR using special primers (RTS: 5'AAAATCCTCACAATACCAC3', RTSA: 5'GACATACTTTCCAATCAATAG3') as previously described^[6]. The PCR products were cloned into the pUC-18 vector (TAKARA) following its manufacturer's instructions. Following transformation into DH5 α competent cells (Tiangen, Shanghai), each wild strain plasmid, rtA181V/T and rtN236T variant plasmid of types B and C were picked out respectively according to the sequencing results, and standardized with a quantitative test kit for HBV DNA. After serial dilutions, quantitative reference substances for related rt181 and rt236 variant mutations were prepared.

Quantification of HBV DNA and related mutations

Based on the characteristics of rtA181V/T and rtN236T mutations, specific primers and Taqman probes were designed (Figure 1). Mutations of rtA181 and rtN236 and total HBV DNA isolated from the same serum specimens were detected by real-time fluorescent quantitative PCR as previously described^[7].

Direct sequencing of HBV DNA

Primers for PCR were designed to target highly conservative sequences of HBV gene in oligonucleotides: P1, 5'CCTGCTGGTGGCTCCAGTTCAGGAACAG3' (nt56-83), P2, 5'AAGCCCCAACAGTGGGGGTTGCGTCA3' (nt1188-1214). The amplified fragments contained the polymerase RT domain and overlapped the S gene. Viral DNA was amplified with a HBV sequencing kit (Shenyong, Shanghai, China). Positive PCR products identified by agarose gel electrophoresis were directly sequenced with an ABI PRISM 3730 genetic analyzer.

Table 1 Sensitivity and specificity of the new method for the detection of ADV-associated HBV quasispecies

Plasmid	HBV DNA reagent	rtA181 reagent	rtN236 reagent
Wild strain plasmid of Type B	7.27E+06	-	-
Wild strain plasmid of Type C	7.23E+06	-	-
rtA181V variant plasmid	8.78E+06	1.88E+06	-
rtA181T variant plasmid	1.14E+07	2.26E+06	-
rtN236T variant plasmid of Type B	1.14E+07	-	1.25E+06
rtN236T variant plasmid of Type C	1.12E+07	-	1.31E+06
Mixed variants plasmid	5.92E+06	2.00E+06	1.18E+06

ADV: Adefovir; HBV: Hepatitis B virus.

Table 2 Sensitivity of the new method for the detection of mixed variants

Variant: wild strain	HBV DNA reagent	rtA181 reagent	rtN236 reagent	a/b	a/c
0:1	6.06E+06	-	-		
1:1	2.94E+06	4.80E+06	5.10E+06	0.61	0.58
1:10	3.98E+06	486 276.30	337 566.80	8.18	11.79
1:50	4.28E+06	83 242.16	85 867.71	51.42	49.84
1:200	5.63E+06	23 130.80	22 000.00	243.40	255.91
1:1000	5.76E+06	7880.00	6223.36	730.96	925.55
1:10000	6.62E+06	-	-		

a/b: The total DNA to rtA181 mutation-associated DNA; a/c: The total DNA to rtN236 mutation-associated DNA.

Assessment criteria for HBV DNA mutation

Mutations of rtA181 or rtN236 were assayed with the ratio of total HBV DNA to rtA181 or rtN236 mutation-associated DNA. The assessment criteria used were as follows: (1) If the ratio (a/b or a/c) of total DNA to rtA181 or rtN236 mutation-associated DNA was between 1 and 10, it was defined as complete variants or the proportion of mutations was more than 10% in the pool of quasispecies; (2) If the ratio (a/b or a/c) of total DNA to rtA181 or rtN236 mutation-associated DNA was between 10 and 200, it was defined as the proportion of variants was lower than 10% in the pool of quasispecies; and (3) If the ratio (a/b or a/c) of total DNA to rtA181 or rtN236 mutation-associated DNA was more than 200, it was defined as complete wild strains or the proportion of variants was below the limit of identification.

Statistical analysis

All data were analyzed using the SAS V8.1 statistical package version. Analysis of one way variance (ANOVA) and *t*-test were used to compare the average value. $P < 0.05$ was considered statistically significant

RESULTS**Sensitivity and specificity of real-time fluorescent quantitative PCR for detecting ADV-associated HBV quasispecies**

Each plasmid listed in Table 1 with a concentration of

Table 3 PCR of HBV DNA, mutations of rtA181 and rtN236, a/b and a/c in patients who did not receive ADV treatment

Serum samples	HBV DNA reagent	rtA181 reagent	rtN236 reagent	a/b	a/c
Sample 1	2.45E+07	541.19	-	45 270.61	
Sample 2	6.79E+06	-	-		
Sample 3	1.09E+07	935.07	-	11 656.88	
Sample 4	7.48E+06	-	-		
Sample 5	3.24E+07	4285.91	185.27	7559.65	174 879.91
Sample 6	3.02E+07	1652.39	-	18 276.56	
Sample 7	4.22E+06	-	-		
Sample 8	1.64E+07	2871.36	-	5711.58	

PCR: Polymerase chain reaction.

6×10^6 IU/mL was picked out for real-time fluorescent quantitative PCR. The rtN236T and rtA181 were found to be wild type plasmids at positions 181 and 236, respectively (Table 1), indicating that the new approach has a sensitivity of 100% and a specificity of 100%.

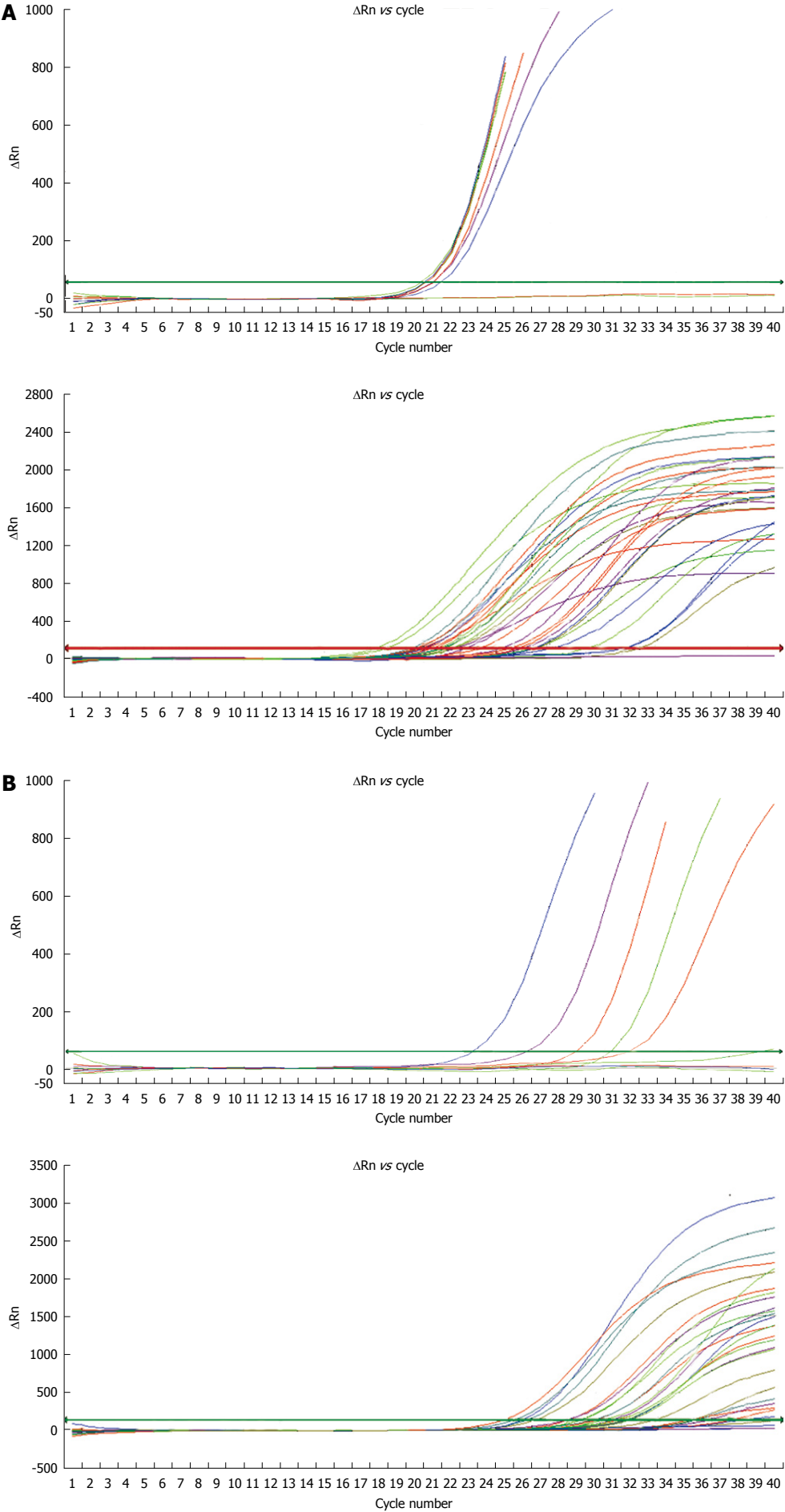
Sensitivity to mixed variants

Variant and wild type plasmids with a concentration of 6×10^6 IU/mL were picked out and diluted at 0/1, 1/1, 1/10, 1/50, 1/200, 1/1000, and 1/10000, respectively. The results of fluorescent quantitative PCR are shown in Figure 2A and Table 2. The Ct value for the wild type plasmids was different.

Total HBV DNA, and rtA181 or rtN236 mutations in 8 serum samples from the patients who did not receive ADV treatment and had no associated mutations detected by sequencing were detected by fluorescent quantitative PCR. The ratio (a/b or a/c) of total HBV DNA to rtA181 or rtN236 mutation-associated DNA was lower than 1000 when the variant plasmids were mixed with wild type plasmids at a dilution of 1:1000 and higher than 5000 in the patients who did not receive ADV therapy (Table 3).

Detection of ADV-resistant mutations

The ratio of total HBV DNA to rtA181 and rtN236 mutation-associated DNA (a/b or a/c) in mutations of variant and wild-type plasmids was less than 1000 at a dilution of 1:1000, while the ratio of total HBV DNA to ADV-resistant mutations was higher than 5000 in the naïve patients. Among the 32 patients, mutations of rtA181 and rtN236T were detected in 20 and 8, respectively. Based on the current assessment criteria (the ratio of a/b cut-off was 1, 10 and 200, respectively), the 32 patients were divided into 3 groups with the a/b ratio = 1 to 10 in 6, = 10 to 200 in 7 and ≥ 200 in 12 patients, respectively, and the a/c ratio = 1 to 10 in 1, = 10 to 200 in 7 and ≥ 200 in 24 patients, respectively. The rtA181 mutation level in HBV DNA was $6.22 \pm 0.55 \log_{10}$ IU/mL, $4.78 \pm 0.21 \log_{10}$ IU/mL, and $3.14 \pm 0.33 \log_{10}$ IU/mL, respectively, in these three groups ($P < 0.0001$). The rtN236 mutation level in HBV DNA was $5.31 \pm 0.25 \log_{10}$ IU/mL and $2.49 \pm 0.19 \log_{10}$ IU/mL, respectively, in patients with their a/c ratio = 1 to 200 or ≥ 200 ($P < 0.0001$) (Figure 2B and Table 4).



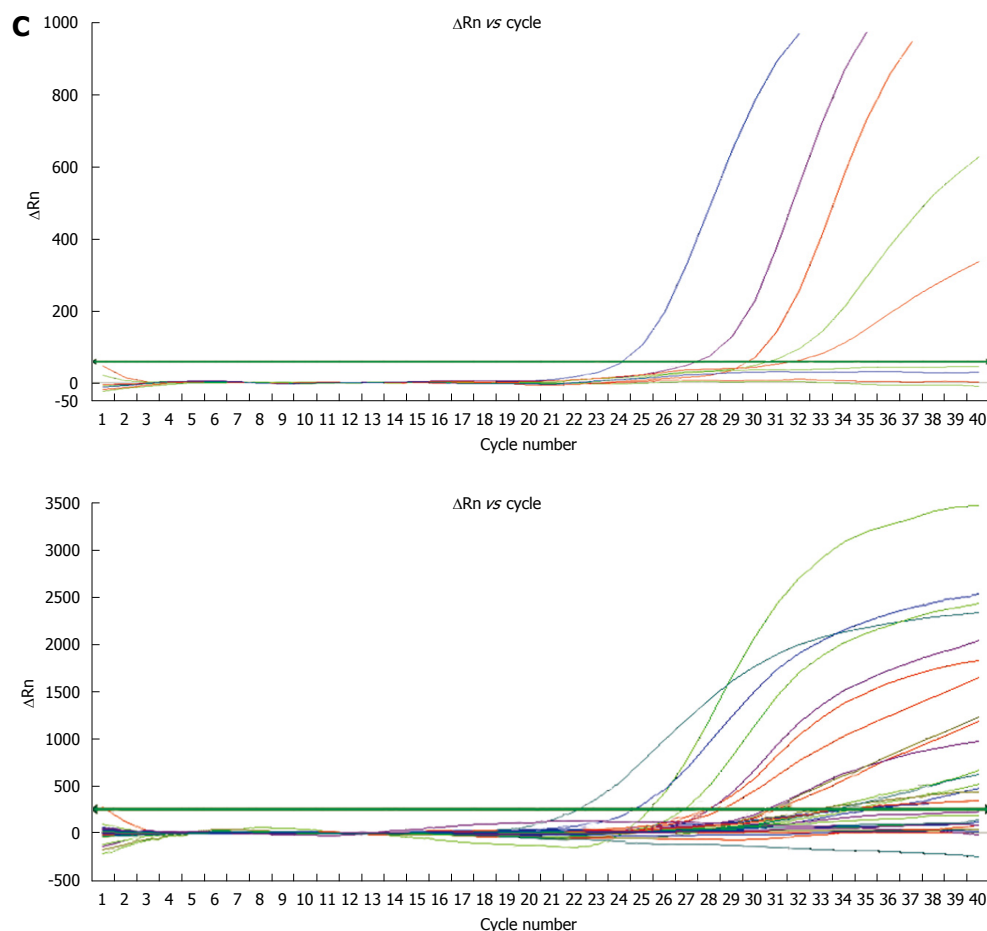


Figure 2 Real-time fluorescent quantitative PCR amplification curves for the sensitivity of mixed variants (the upper part of each diagram) and patients (the lower part of each diagram) to the new method. A: Total HBV DNA; B: rtA181 mutation HBV DNA; C: rtN236T mutation HBV DNA.

Table 4 Quantification of rtA181 and rtN236 mutations

a/b	n	rtA181 mutation (\log_{10} IU/mL)	P ¹	a/c	n	rtN236 mutation (\log_{10} IU/mL)	P ²
1-10	6	6.22 ± 0.55	< 0.0001	1-200	8	5.31 ± 0.25	< 0.0001
10-200	14	4.78 ± 0.21					
≥ 200	12	3.14 ± 0.33		≥ 200	24	2.49 ± 0.19	

¹Analysis of one way variance (ANOVA) result; ²t-test result. n: Number of samples.

Table 5 ADV-resistant mutations detected by sequence analysis and their quantification

Patients	rtA181	rtN236	a/b	a/c
#1	rtA181T		8.32	
#2	rtA181V		1.66	
#3	rtA181T		0.93 ¹	
#4	rtA181T		1.76	
#5	rtA181T	rtN236T	1.03	3.11
#6	rtA181T		7.13	

¹The ratio below 1 due to PCR error.

Comparison between sequencing chromatograms and quantitative results

Of the 6 patients with ADV-resistant mutations, rtA181T and rtN236T mutations and single rtA181V/T mutation

were observed in patients 1 and 5, respectively. Among the 26 patients with no associated mutations, rtA181 variants and wild type strains were found in patients 1, 5 and 6 (Figure 3) while only rtA181 variants were detected in patients 2, 3 and 4 (Table 5).

DISCUSSION

Dipivoxil, a nucleotide analogue, has become a treatment option for HBV infection due to its efficacy on lamivudine-resistant mutations occurring upon prolonged treatment. However, resistance to ADV develops and increases over time. ADV therapy for naive patients is associated with delayed and less frequent drug-resistant mutations. The reported cumulative incidence of mutations in hepatitis B e antigen (HBeAg)-negative and positive chronic

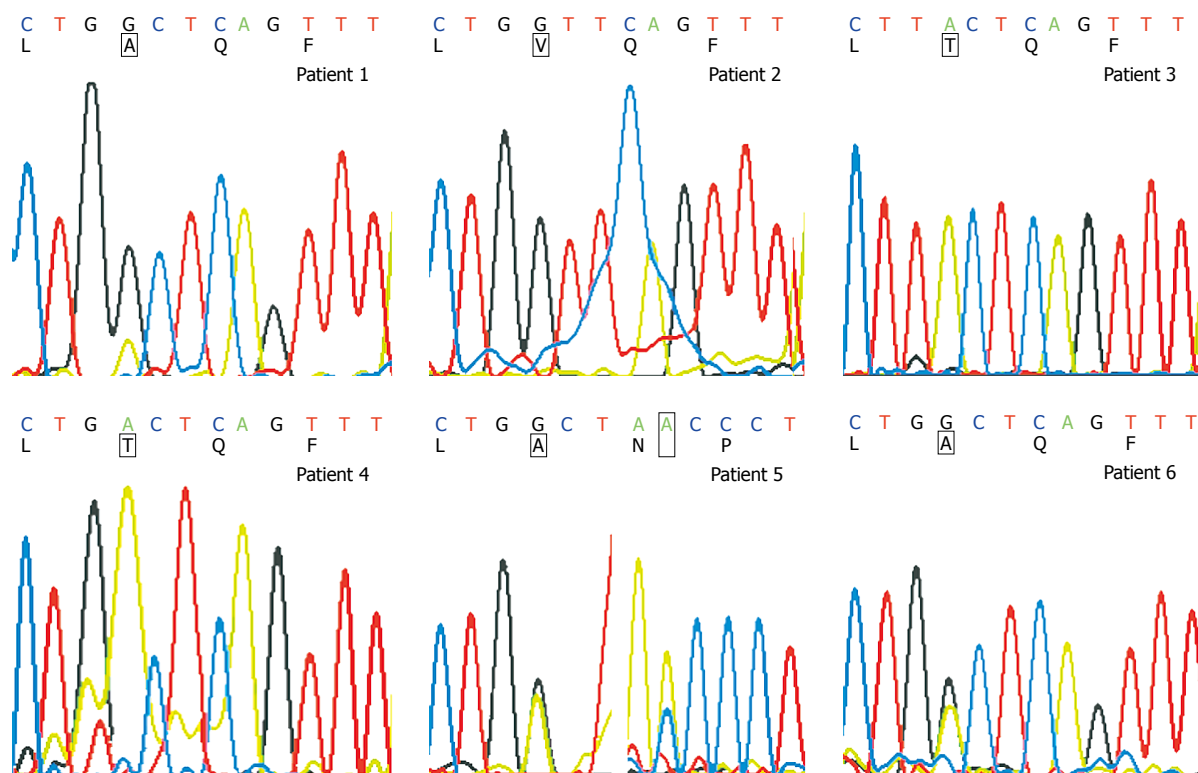


Figure 3 Sequence analysis of ADV-resistant mutations. rtA181 wild strains (amino acid codon: GCT) with few rtA181T mutations (amino acid codon: ACT) in patient 1, rtA181V mutant (amino acid codon: GTT) in patient 2, rtA181T mutant (amino acid codon: ACT) in patients 3 and 4, rtA181 wild strains (amino acid codon: GCT) mixed with half of rtA181T mutant (amino acid codon: ACT) and rtN236 wild strains (amino acid codon: AAC) mixed with partial rtN236T mutants (amino acid codon: ACC) in patient 5, and rtA181 wild strains (amino acid codon: GCT) mixed with partial of rtA181T mutants (amino acid codon: ACT) in patient 6.

hepatitis B patients is 29% and 20%, respectively after 5 years of ADV treatment^[12,14]. LAM-resistant patients treated with ADV monotherapy are at a higher risk of developing ADV-resistant mutations than nucleoside-naïve patients^[7,13]. In accordance with the current guidelines, ADV should be used in combination with other drugs in treatment of LAM-resistant patients instead of ADV monotherapy. It has been shown that ADV-resistant mutants occur infrequently in patients on combined therapy^[15]. An open-label study in HBeAg-negative LAM-resistant patients demonstrated that combined therapy does not result in development of resistance to ADV over a period of 3 years^[16]. It has been recently reported that the rate of ADV resistance is 1% and 4% 1 and 3 years after ADV + LAM therapy^[17]. Two mutations in the HBV polymerase gene, alanine to valine or threonine (rtA181V/T) and asparagine to threonine substitution (rtN236T) have been described to confer resistance to ADV^[4], suggesting that genotypic resistance monitoring is important to anticipate and confirm the observed phenotypic resistance and new treatment modalities can be selected. The incidence of genotypic resistance may vary with the sensitivity of different methods used to detect resistant mutations. Thus, methodological options for detecting HBV mutations are an increasingly important issue in clinical management of CHB. Several other methods have been used to detect ADV-resistant HBV. However, the disadvantages of each method restrict its wide-range use. So

developing a more sensitive, specific and easy approach is always the major concern for researchers. Malmström *et al.*^[18] developed a method based on real-time polymerase chain reaction with TaqMan chemistry which can target wild type and mutant viral strains at rtL180 and rtM204, later, Lupo *et al.*^[19] described a selective real-time PCR (sPCR) for the quantitative detection of rtM204I/V and rtN236T mutations associated with resistance to LAM and ADV, with a lowest limit of 0.1% of strains carrying rtM204V/I or rtN236T mutations for a total of 105 copies of plasmid. In this study, primers and probes for quantitative amplification of HBV DNA, rtA181 and rtN236 mutations were designed, and the results showed that they could detect more than 100 IU/mL of variants. Mutants could be obtained by calculating the ratio of total HBV DNA to the variants. To assess the feasibility of this approach, HBV DNA, rtA181 and rtN236 mutations were detected in 32 patients with unsatisfactory response to adefovir dipivoxil by direct sequencing. The results showed that this new approach could detect rtA181 and rtN236 mutations as well as some ADV mutations not identified by direct sequencing, thus supporting the new approach with a high concordance and a higher sensitivity. ADV-resistant HBV mutations can be pre-monitored, which can provide valuable reference for shifting the treatment in order to prevent hepatitis deterioration and decompensation. Furthermore, real-time fluorescent quantitative PCR can be widely used.

Meanwhile, there is a large room for the improvement of this new approach. The major improvement in RT-PCR is to design the optimal primers for each mutant. Moreover, due to the variability of genotypes, a number of primers may be required for the detection of a single nucleotide polymorphism. In this study, the primers were restricted to the most frequent codons associated with rtA181 and rtN236T mutations. However, further study is needed to verify the other mutations.

In conclusion, the new approach based on real-time fluorescent quantitative PCR is more feasible than direct DNA sequencing. It is more sensitive and specific for the detection of wild-type and variant viruses as well as ADV-resistant mutations. We believe that this easy-to-use approach will play an important role in detection of ADV-resistant mutations and in optimization of ADV therapy.

COMMENTS

Background

With the availability of more potent nucleotide/nucleoside analogues, early detection of drug-resistant mutants of hepatitis B virus (HBV) is important for the treatment of chronic hepatitis B (CHB). The incidence of genotypic resistance may vary with the sensitivity of methods used to detect resistant mutations. Thus, methodological options for detecting HBV mutations are an increasingly important issue in clinical management of CHB.

Innovations and breakthroughs

The study described a practical and accurate method for quantitative detection of adefovir (ADV)-resistant HBV quasiespecies using real-time fluorescent quantitative polymerase chain reaction (PCR). The results demonstrated that this method could detect and monitor ADV resistance mutations in patients chronically infected with HBV and is more sensitive than sequencing.

Applications

ADV-resistant HBV quasiespecies can be detected by real-time fluorescent quantitative PCR, which should be extensively popularized in clinical practice.

Peer review

The method described in this paper is specific, sensitive and accurate for the detection of ADV-resistant mutants of HBV in ADV-treated patients, thus adding a new tool for monitoring ADV-resistant mutations before and during ADV therapy.

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Clinical significance of Fas and FasL protein expression in gastric carcinoma and local lymph node tissues

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expression level was significantly higher in invasive lymph nodes than in non-invasive lymph nodes (82.9% vs 56.5%, $P < 0.003$) and in well-differentiated gastric carcinoma tissue samples than in poorly-differentiated gastric carcinoma tissue samples (50.0% vs 18.0%, $P = 0.015$). The FasL expression level was significantly lower in well-differentiated gastric carcinoma tissue samples than in poorly-differentiated gastric carcinoma tissue samples (42.9% vs 84.0%, $P = 0.021$). The Fas and FasL expression levels (25.0% and 81.3%) were significantly different in gastric carcinoma tissue samples ($P < 0.001$), but had a non-linear correlation ($P = 0.575$).

CONCLUSION: Abnormal Fas and FasL expressions in gastric carcinoma and lymph node tissues are involved in carcinogenesis and metastasis of gastric cancer.

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Key words: Gastric carcinoma; Lymph nodes; Fas; Fas ligand FasL

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Li Q, Peng J, Li XH, Liu T, Liang QC, Zhang GY. Clinical significance of Fas and FasL protein expression in gastric carcinoma and local lymph node tissues. *World J Gastroenterol* 2010; 16(10): 1274-1278 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v16/i10/1274.htm> DOI: <http://dx.doi.org/10.3748/wjg.v16.i10.1274>

Abstract

AIM: To investigate the relation of Fas and Fas ligand (FasL) protein expression with carcinogenesis and metastasis of gastric carcinoma.

METHODS: Immunohistochemistry was used to detect Fas and FasL protein expression in 64 gastric carcinoma tissue samples and 20 normal gastric tissue samples. Relation between FasL and Fas expression, age and gender of gastric cancer patients, and pathological subtype and lymph node metastasis of gastric cancer was analyzed.

RESULTS: The Fas expression level was significantly higher in normal gastric tissue samples than in gastric carcinoma tissue samples (85.0% vs 25.0%, $P < 0.001$), while the FasL expression level was significantly lower in normal gastric tissue samples than in gastric carcinoma tissue samples (30.0% vs 81.3%, $P < 0.001$). The Fas

INTRODUCTION

Gastric carcinoma is one of the most common malignant tumors in the world. Currently, no effective treatment modalities are available for its metastasis and recurrence. Fas, a cell-surface receptor, activates the apoptosis signal

pathway by binding to its ligand, FasL, resulting in cancer cell apoptosis^[1,2]. It has been shown that Fas protein is abnormally expressed in esophageal, colon, and gallbladder carcinomas^[3-5]. FasL is over-expressed in transformation and metaplasia of esophageal carcinoma^[6].

By investigating the role of Fas and FasL expression in the carcinogenesis and metastasis of gastric carcinoma, several investigators have found that Fas expression in gastric carcinoma is decreased^[7]. Bennett *et al*^[8] showed that the high FasL expression level in gastric carcinoma can induce apoptosis of T lymphocytes infiltrating cancerous regions, suggesting that Fas and FasL induce apoptosis of activated lymphocytes, allowing cancer cells to escape immune attack.

However, no research is available on the clinical significance of Fas and FasL in local lymph nodes. To explore the relation between gastric carcinoma and regional lymph node metastasis, Fas and FasL protein expressions were detected using immunohistochemistry method in this study.

MATERIALS AND METHODS

Materials

Specimens were obtained from 64 cases of gastric carcinoma admitted to the Department of Pathology, Second Affiliated Xiangya Hospital, Central South University, in May 2007-May 2008. Of these specimens, 38 were from male patients and 26 from female patients, (including 3 patients at the age of 20-29 years, 6 patients at the age of 30-39 years, 20 patients at the age of 40-49 years, 19 patients at the age of 50-59 years, and 16 patients at the age of over 60 years), 14 were from well-differentiated adenocarcinoma patients, and 50 were from poorly-differentiated adenocarcinoma patients. Lymph node metastasis was detected in 41 cases. The patients did not receive radiotherapy or chemotherapy before operation. Twenty normal gastric tissue samples were also obtained from the same hospital.

Immunohistochemistry

Specimens, fixed with 10% neutral formaldehyde solution and embedded in paraffin, were cut into 3- μ m thick sections. Streptavidin-biotin complex (SABC) immunohistochemistry kit, primary antibodies: rabbit anti-human Fas and FasL, second antibody: biotin goat anti-rabbit IgG, and 3,3'-diaminobenzidine chromogenic kit were purchased from Wuhan Boster Company (China). PBS was used instead of primary antibody as a negative control following its manufacturer's instructions.

Evaluation of score

Intensity of staining was scored as 0: negative, 1: light yellow, 2: brown-yellow and 3: brown. Eight random high-power fields were observed under optical microscope and 4 high-power fields were recorded as the percentage of positive cells. Extent of staining was scored as 0: < 5%, 1: 5%-25%, 2: 26%-50%, 3: 51%-75% and 4: > 75%.

Table 1 Expression of Fas and FasL in gastric carcinoma and normal gastric tissues

	Fas ^a		FasL ^b	
	Normal gastric tissues	Gastric carcinoma	Normal gastric tissues	Gastric carcinoma
-	3	48	14	12
+	5	6	2	10
++	4	4	3	18
+++	8	6	1	24

^aZ = -4.780, *P* < 0.001; ^bZ = -4.115, *P* < 0.001.

Table 2 Expression of Fas and FasL in invasive and non-invasive lymph nodes

	Fas ^a		FasL ^b	
	Invasive	Non-invasive	Invasive	Non-invasive
-	7	10	18	7
+	7	6	6	6
++	9	4	9	6
+++	18	3	8	4

^aZ = -2.954, *P* = 0.003; ^bZ = -0.534, *P* = 0.593.

The final score was determined by multiplying the scores of intensity and extent of staining, ranging 0-12. Scores 9-12 were defined as strong staining (+++), scores 4-8 as moderate staining (++), scores 1-3 as weak staining (+), and 0 as negative staining (-)^[7].

Statistical analysis

Statistical analysis was performed using SPSS 15.0. The data were analyzed by rank-sum test and χ^2 test. *P* < 0.05 was considered statistically significant.

RESULTS

Expression of Fas and FasL in gastric carcinoma and normal gastric tissue samples

The expression level of Fas protein was higher in normal gastric tissue samples than in gastric carcinoma tissue samples (85.0% *vs* 25.0%, *Z* = -4.780, *P* < 0.001). The FasL protein expression level was lower in normal gastric tissue samples than in gastric carcinoma tissue samples (30.0% *vs* 81.3%, *Z* = -4.115, *P* < 0.001) (Table 1 and Figure 1).

Expression of Fas and FasL in invasive and non-invasive lymph nodes

The expression level of Fas protein was higher in invasive lymph nodes than in non-invasive lymph nodes (82.9% *vs* 56.5%, *Z* = -2.954, *P* = 0.003). The expression level of FasL protein was lower in invasive lymph nodes than in non-invasive lymph nodes (56.1% *vs* 69.6%, *Z* = -0.593, *P* = 0.593) (Table 2 and Figure 2).

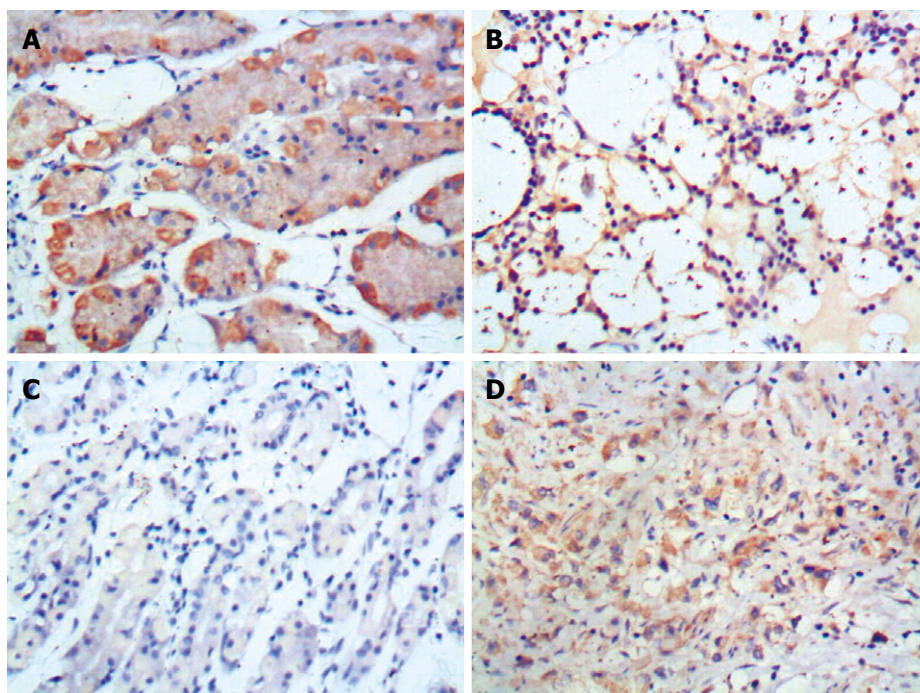


Figure 1 Expression of Fas and FasL in normal gastric tissues (A, C) and gastric carcinoma tissue (B, D) (SABC $\times 200$).

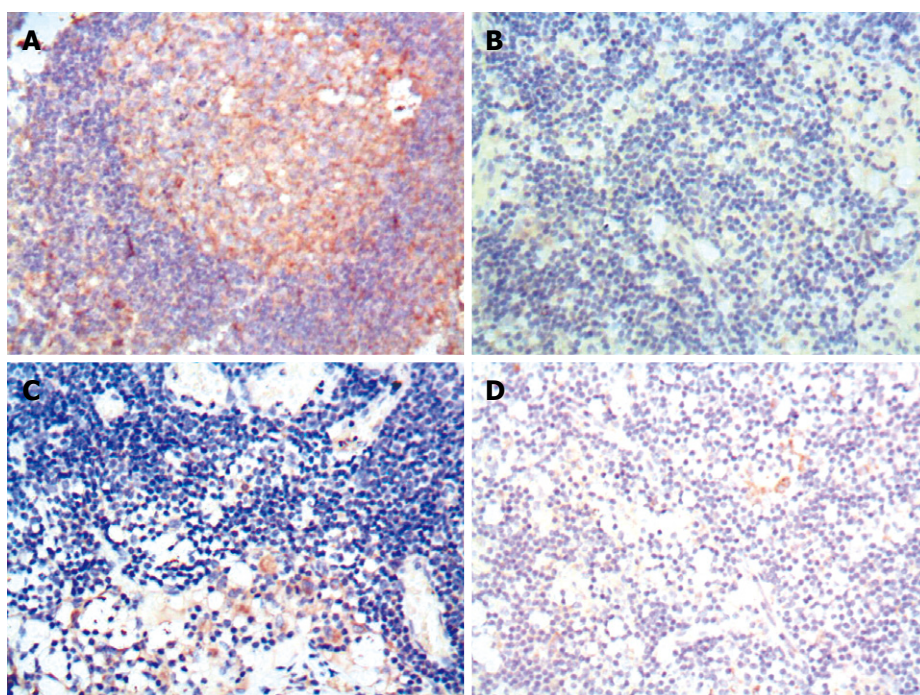


Figure 2 Expression of Fas and FasL in invasive lymph nodes (A, C) and non-invasive lymph nodes (B, D) (SABC $\times 200$).

Expression of Fas and FasL in various pathological subtypes of gastric carcinoma

The Fas expression level was higher in well-differentiated gastric carcinoma tissue samples than in poorly-differentiated gastric carcinoma tissue samples (50.0% *vs* 18.0%, $Z = -2.421$, $P = 0.015$). The FasL expression level was lower in well-differentiated gastric carcinoma tissue samples than in poorly-differentiated gastric carcinoma tissue samples (42.9% *vs* 84.0%, $Z = -2.307$, $P = 0.021$) (Table 3).

The expression level of Fas and FasL protein in gastric carcinoma tissue samples was not significantly different in patients at different ages with or without lymph node metastasis (Table 4).

Expression of Fas and FasL in gastric carcinoma tissue samples

The Fas and FasL expression rate was 25.0% and 81.3%, respectively, in gastric carcinoma tissue samples ($Z = -4.780$, $P < 0.001$). Rank correlation test showed that the Fas and FasL expression in gastric carcinoma tissue samples had a non-linear correlation (correlation coefficient: -0.425 , $P = 0.575$).

DISCUSSION

The binding of Fas to its natural ligand, FasL, induces a signal transduction pathway leading to apoptosis^[9]. Fas is

Table 3 Expression of Fas and FasL in well- and poorly-differentiated gastric carcinoma tissues

Differentiated	Fas ^a		FasL ^b	
	Well	Poorly	Well	Poorly
-	7	41	8	8
+	3	4	2	21
++	3	3	3	9
+++	1	2	1	12

^aZ = -2.421, P = 0.015; ^bZ = -2.307, P = 0.021.

a type I transmembrane protein belonging to the tumor necrosis factor (TNF) superfamily. Combination of Fas and FasL or a corresponding membrane-antibody, activates caspases, and is a major route to apoptosis^[10].

Dysfunction of Fas or FasL will lead to inactivation of the Fas system and inhibit normal apoptosis, resulting in abnormal survival and proliferation of tumor cells, and may promote malignancies. It has been shown that inhibition of apoptosis plays a significant role in various stages of tumor formation and abnormal expressions of Fas and FasL protein can inhibit apoptosis^[1,2].

In this study, the Fas expression level was lower in gastric carcinoma tissue samples than in normal gastric mucosa tissue samples, and the positive FasL expression in gastric carcinoma tissue samples had a trend to increase, which is considered to be the general mechanism by which tumor cells escape the Fas-mediated host immune system by reducing or inhibiting Fas expression, suggesting that Fas-mediated cytotoxic T cells (CTL) are unable to identify carcinoma cells, and carcinoma cells can thus avoid attack by T-lymphocytes^[11].

Fas and FasL can be simultaneously expressed in tumor tissues. It has been reported that the susceptibility to Fas-mediated cell death is not positively correlated with the expression of tumor cell surface ligand FasL^[12]. The molecular mechanism may be related to the expression of both FasL and decoy receptor 3 (DcR3) in gastric cancer cells. DcR3, a newly identified member of the tumor necrosis factor receptor (TNFR) family, binds to some specific protein ligands and may competitively inhibit Fas binding to FasL, thus blocking FasL and inhibiting apoptosis^[13].

Although activated T lymphocytes and their ligand FasL can combine with Fas receptors in carcinoma cells, they do not induce apoptosis. However, membrane FasL in carcinoma cells can combine with Fas receptors of activated T lymphocytes, resulting in T lymphocyte apoptosis^[14]. In this study, some invasive lymph nodes could over-express Fas, indicating that FasL can combine with over-expressed Fas receptors on T cells, resulting in T cell apoptosis and tumor proliferation. Since a high Fas expression level in lymphoid tissue signifies a higher susceptibility to tumor cells, the Fas/FasL system is involved in gastric carcinoma tumors evading the immune system. On the one hand, since Fas expression is reduced or lost in gastric carcinoma, anti-tumor immune

Table 4 Expression of Fas and FasL in patients with or without lymph node metastasis of gastric carcinoma

Item (cases)	Fas		P value	FasL		P value
	Positive	Negative		Positive	Negative	
Sex						
Male (38)	11	27	$\chi^2 = 0.777$ 0.378	29	9	$\chi^2 = 0.086$ 0.769
Female (26)	5	21		19	7	
Age (yr)						
< 40 (9)	3	6	$\chi^2 = 2.105$ 0.551	6	3	$\chi^2 = 5.891$ 0.117
40-49 (20)	5	15		18	2	
50-59 (19)	6	13		15	4	
> 60 (16)	2	14		9	7	
Lymph node metastasis						
Yes (41)	10	31	$\chi^2 = 0.023$ 0.880	29	12	$\chi^2 = 1.109$ 0.292
No (23)	6	17		19	4	

cells expressing FasL are less able to clear them. On the other hand, gastric carcinoma cells expressing high FasL levels are able to attack tumor-infiltrating immune cells expressing high Fas levels by promoting apoptosis of immune cells, thus allowing gastric cancer cells to escape immune attack^[15]. Kume *et al.*^[16] reported that a large number of lymphocytes in gastric carcinoma can express FasL. In our study, the Fas expression level was significantly higher in invasive lymph nodes than in non-invasive lymph nodes, suggesting that lymph nodes with a high Fas expression level may be more susceptible to self-apoptosis, thereby promoting lymph node metastasis.

In this study, the expression of Fas and FasL in gastric carcinoma tissue samples was not correlated with the sex and age of patients with or without lymph node metastasis, suggesting that the characteristics of Fas and FasL expression may have already formed in malignant transformation of gastric tumor cells^[17].

In this study, the Fas and FasL expression rates were significantly different between well- and poorly-differentiated gastric carcinomas, which is consistent with the reported findings^[7]. A possible mechanism is that a high FasL expression level in well-differentiated gastric carcinoma combines with Fas in an autocrine or paracrine manner to promote gastric carcinoma cell apoptosis and reduces tumor growth, while poorly-differentiated gastric carcinoma can produce soluble Fas (sFas), which competes with tumor cell membrane Fas receptor for FasL, leading to the inhibition of cancer cell apoptosis^[18]. Meanwhile, sFas released from cancer cells plays an important role in the immunosuppressive effect. An elevated sFas level in hepatocellular carcinoma patients is negatively correlated with humoral (IgA, IgG) and cellular immune function parameters (e.g. lymphocyte transformation rate, natural and lymphokine-activated killer cell killing rate). Tumor cells produce sFas and combine with FasL expressed by T lymphocytes, a mechanism to evade immune attack^[19], indicating that sFas may play an important role in carcinogenesis and cancer progression. However, how sFas generates and regulates such a mechanism is unclear, and needs to be further investigated.

In conclusion, abnormal expression of Fas and FasL is involved in carcinogenesis and metastasis of gastric carcinoma by escaping T lymphocyte attack and inducing lymphocyte apoptosis.

COMMENTS

Background

Gastric carcinoma is one of the most common malignant tumors in the world. Fas, a cell-surface receptor, activates the apoptosis signal pathway by binding to its ligand FasL, resulting in cancer cell apoptosis. Abnormal expressions of Fas and FasL are associated with carcinogenesis.

Research frontiers

A high FasL expression level in gastric carcinoma can induce apoptosis of T lymphocytes infiltrating the tumor. However, no current research is available on the clinical significance of Fas and FasL protein expression in local lymph nodes. In this study, the authors showed that a high Fas expression level in lymphoid tissue might indicate a greater susceptibility to tumor cell invasion.

Innovations and breakthroughs

Binding of Fas to its natural ligand, FasL, induces the signal transduction pathway leading to apoptosis. This is the first study to report that Fas-mediated cell death susceptibility and expression of the tumor cell surface ligand FasL are not positively correlated. Furthermore, in the study, lymph nodes with a high Fas expression may be more susceptible to self-apoptosis, thus promoting lymph node metastasis.

Applications

By exploring the relation between the expressions of Fas and FasL in gastric carcinoma and lymph node tissues with regional lymph node metastasis, this study may convey a strategy for therapeutic intervention in gastric carcinoma patients.

Peer review

It is a well written paper. The authors investigated the expression of Fas and FasL in gastric cancer, which helps to understand the pathogenesis of gastric cancer. The experimental procedure is quite well performed.

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Influence of efflux pump inhibitors on the multidrug resistance of *Helicobacter pylori*

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Abstract

AIM: To evaluate the effect of efflux pump inhibitors (EPIs) on multidrug resistance of *Helicobacter pylori* (*H. pylori*).

METHODS: *H. pylori* strains were isolated and cultured on Brucella agar plates with 10% sheep's blood. The multidrug resistant (MDR) *H. pylori* were obtained with the inducer chloramphenicol by repeated doubling of the concentration until no colony was seen, then the susceptibilities of the MDR strains and their parents to 9 antibiotics were assessed with agar dilution tests. The present study included periods before and after the advent of the EPIs, carbonyl cyanide m-chlorophenyl hydrazone (CCCP), reserpine and pantoprazole, and the minimum inhibitory concentrations (MICs) were determined accordingly. In the same way, the effects of 5 proton pump inhibitors (PPIs), used in treatment

of *H. pylori* infection, on MICs of antibiotics were evaluated.

RESULTS: Four strains of MDR *H. pylori* were induced successfully, and the antibiotic susceptibilities of MDR strains were partly restored by CCCP and pantoprazole, but there was little effect of reserpine. Rabeprazole was the most effective of the 5 PPIs which could decrease the MICs of antibiotics for MDR *H. pylori* significantly.

CONCLUSION: *In vitro*, some EPIs can strengthen the activities of different antibiotics which are the putative substrates of the efflux pump system in *H. pylori*.

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Key words: Multidrug efflux pump; *Helicobacter pylori*; Multidrug resistance; Proton pump inhibitor; Real-time polymerase chain reaction

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INTRODUCTION

Helicobacter pylori (*H. pylori*) is an important human pathogen that infects 50% of the world's population^[1,2]. *H. pylori* infection in humans is associated with the development of numerous gastric pathologies, such as peptic ulcer, chronic gastritis and gastric cancer. Once *H. pylori* is established in the gastric submucosa, infected

individuals usually carry it for life unless treated^[1,2]. In recent years, the rate of resistance of *H. pylori* to standard therapies has increased as a result of the widespread use of antibiotics. *H. pylori* resistance to metronidazole and clarithromycin has increased worldwide, and multidrug resistant (MDR) strains that are simultaneously resistant to amoxicillin, metronidazole and clarithromycin have been reported^[3,4]. Boyanova *et al*^[3] reported that 26.4% of the strains were resistant to metronidazole and clarithromycin among antibiotic-treated patients; Wueppenhorst *et al*^[4] found that 15% of the *H. pylori* isolated from patients showed resistance to 2 or 3 types of antibiotic.

Efflux pump systems in bacteria which can eject the drugs and toxic compounds, including antibiotics, have a critical role in the development of multidrug resistance^[5]. We have observed previously that the efflux pump gene *hcfA* of *H. pylori* has valuable applications in multidrug resistance^[6]. Until now, efflux pump inhibitors (EPIs) as promising therapeutic agents, have been widely investigated in other bacteria, where they could decrease the intrinsic bacterial resistance to antibiotics, and reverse the acquired resistance associated with efflux pump overexpression. Different classes of EPIs have been exploited and studied, including analogues of antibiotic substrates and new molecules^[7,8]. However, whether the EPIs can also reverse the multidrug resistance of *H. pylori* has not been fully researched. Carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP), as an energy blocker, can induce a decrease in the transmembrane electrochemical gradient, and inhibit the efflux pumps driven by hydrogen ion gradients. Previous research showed that it can inhibit the multidrug and toxic compound extrusion (MATE) family efflux transporter pumps, such as NorM in *Bacteroides thetaiotaomicron*, and the resistance/nodulation/division superfamily (RND) family multidrug efflux transporter (MexAB-OprM system in *P. aeruginosa*), the small multidrug resistance subfamily of the DMT (drug/metabolite transporters) superfamily, such as QacE in *Escherichia coli*, and thus restore the antibiotic sensitivity of the bacteria^[9-11]. Reserpine belongs to the alkaloid family, and could inhibit the major facilitator superfamily (MFS) and the adenosine triphosphate (ATP)-binding cassette (ABC multidrug efflux) superfamilies^[12,13]. CCCP and reserpine are employed in the present study as different types of EPI, and a chloramphenicol-induced multidrug resistance model was developed. The aim of this investigation was to elucidate whether EPIs can influence the antibiotic susceptibilities of *H. pylori* MDR strains.

MATERIALS AND METHODS

Reagents

Chemical reagents were purchased from TaKaRa Biotechnology Co. Ltd (Dalian, China). Chloramphenicol, tetracycline, and CCCP were purchased from Sigma Co. Ltd (Shanghai, China). Quant SYBR Green PCR Kits, polymerases and other molecular biology reagents were purchased from Biosail Biotechnology Co. Ltd (Beijing,

China), and used according to their manufacturers' instructions. Clarithromycin, amoxicillin, penicillin G, cefotaxime, polymyxin B, piperacillin ciprofloxacin clindamycin ceftriaxone ampicillin, metronidazole, erythromycin and other drugs were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China).

Bacterial strains and culture conditions

H. pylori isolates were obtained from routine cultures of clinical gastric biopsies from patients with peptic ulcer or chronic active gastritis at the Second Affiliated Hospital of Zhengzhou University (Zhengzhou, China). *H. pylori* NCTC11637 obtained from Henan Key Laboratory of Molecular Medicine was used as a reference. Isolates were cultured on Brucella agar medium plates containing 7% lysed sheep blood at 37°C under microaerobic conditions (50 mL/L O₂, 100 mL/L CO₂, 850 mL/L N₂) for 48-72 h. Identification of *H. pylori* isolates was based on the results of Gram staining, cell morphology, and positive reactions for catalase, oxidase and urease activities.

Induction of multiple antibiotic resistances of clinical isolates

Susceptible strains were isolated from gastric biopsy samples, with an established protocol^[6], and the MDR strains were developed. Induction of chloramphenicol resistance in susceptible isolates was performed by selecting resistant colonies that arose in the agar plates containing 1/2 × minimum inhibitory concentration (MIC) chloramphenicol. The resistant colonies were incubated for 48-72 h under microaerobic conditions with repeated doubling of the chloramphenicol concentration until no colony was seen. The induced strains were further incubated on fresh plates with no chloramphenicol for 4 generations, to insure that the induced strains were non-adaptive resistance strains, and then transferred onto plates containing 4 × MIC chloramphenicol. Induced colonies were maintained on plates containing 4 × MIC of tetracycline, ampicillin, penicillin G, cefotaxime, piperacillin, ciprofloxacin, clindamycin, ceftriaxone, and erythromycin. The colonies were incubated for 48-72 h under microaerobic conditions.

Real-time reverse transcription polymerase chain reaction (RT-PCR)

RNA was isolated using the total RNA kit (SBS Genetech Co., Ltd Beijing, China) and reverse transcribed into cDNA. *hcfA*, versus *gyrB* (a housekeeping gene encoding for gyrase B), was utilized to study the relative expression of the *hcfA* gene in 4 MDR strains and their parent strains. cDNA of *hcfA* and *gyrB* was amplified using a real-time PCR 5700 sequence detector (Perkin Elmer Company) in the presence of Real Master Mix (SYBR Green). The gene-specific primers used were designed based on the sequence alignments of the genes from *H. pylori* 11637 in GenBank. The sequences of *hcfA* (accession No: AF059041) are F: (5'-CTCGCTCGCATGATCGC-3')

Table 1 Induced multidrug resistant MIC profiles of *H. pylori* ($\mu\text{g/mL}$)

Strains	Exptl condition	ERY	CIP	TET	CTX	CRO	PIP	CLI	PEN	CAM
4151	Before induction	0.25	0.25	0.5	0.25	0.25	0.25	0.5	0.125	0.5
	After induction	2	0.5	4	1	2	0.5	1	0.25	32
8022	Before induction	0.125	1	1	0.5	0.5	0.5	0.5	0.063	0.25
	After induction	2	1	4	4	4	0.5	0.5	0.25	16
11021	Before induction	0.5	1	1	0.5	0.125	0.25	0.25	0.063	0.5
	After induction	2	2	2	4	4	1	1	0.5	32
11637	Before induction	0.063	0.5	0.25	0.25	0.125	0.25	1	0.25	0.5
	After induction	2	1	4	2	2	0.25	2	1	16
Geometric mean	Before induction	0.177	0.595	0.595	0.354	0.210	0.297	0.500	0.105	0.420
	After induction	2.000	1.000	3.364	2.378	2.828	0.500	1.000	0.420	22.627

ERY: Erythromycin; CIP: Ciprofloxacin; TET: Tetracycline; CTX: Cefotaxime; CRO: Ceftriaxone; PIP: Piperacillin; CLI: Clindamycin; PEN: Penicillin G; CAM: Chloramphenicol; Exptl: Experimental; MIC: Minimum inhibitory concentration.

and R: (5'-CGTATTTCGCTCAAAATCCCT-3'). The PCR primers were predicted to amplify a 162-bp amplicon. Expression of the housekeeping gene *gyrB* (accession No: AB084049) was assessed in parallel with the primer pair *gyrB*: F: (5'-TTACTACGACTTATCCTGGGGCTAGCGCTG-3') and R: (5'-CCCCATCAATTTCCACA TTCTCCGC-3'). The PCR primers were predicted to amplify a 267-bp amplicon. According to the methods reported^[6,14,15], *hefA* gene expressions in MDR and parent strains were determined.

Assessment of susceptibilities to antimicrobials

H. pylori strains grown for 48 h on sheep blood agar plates were resuspended in phosphate-buffered saline. Suspensions of *H. pylori* were adjusted to an optical density of 0.1 at 625 nm, and 1 μL of these suspensions containing approximately 10^5 colony forming units/mL, was spread on horse blood agar plates containing approximately 5×10^4 bacteria within 5 mm. (1) MICs of tetracycline, ampicillin, penicillin G, cefotaxime, piperacillin ciprofloxacin clindamycin ceftriaxone, erythromycin in MDR strains and their parent isolates were determined with the conventional 2-fold agar dilution tests; (2) After addition of CCCP (100 $\mu\text{mol/L}$), reserpine (20 $\mu\text{g/mL}$) and pantoprazole (10 $\mu\text{g/mL}$) to the agar, MICs of the antibiotics were determined again; and (3) To determine whether proton pump inhibitors (PPIs) can affect the *H. pylori* eradication effect of the antibiotics, 5 PPIs (omeprazole, rabeprazole, pantoprazole, lansoprazole, esomeprazole, 10 $\mu\text{g/mL}$) usually used in treatment of *H. pylori* infection were added to the incubation media as above, and MICs of metronidazole, amoxicillin, furazolidone and clarithromycin were tested. A 4-fold increase in MIC of antibiotics for the MDR strains was considered significant^[16].

RESULTS

Induction of multidrug resistance in *H. pylori* strains

From 65 *H. pylori* isolates, 9 strains with no antibiotic resistance phenotype were selected. Following consecutive doubling of the concentration of chloramphenicol, MICs of chloramphenicol-induced strains to 9 antibiotics were

Table 2 Relative expression of *hefA* in PT and MDR strains

Strains	PT (<i>hefA/gyrB</i>)	MDR (<i>hefA/gyrB</i>)
4151	1.93	4.67
8022	1.85	5.84
11021	1.35	5.94
11637	2.45	6.88
mean \pm SD	1.90 \pm 0.45	5.83 \pm 0.91

PT: Parent isolates; MDR: Multidrug resistant strains.

determined. The MICs in 4 MDR strains (including 3 clinical isolates and *H. pylori* NCTC11637) were significantly increased (≥ 4 -fold) compared with their parent strains (Table 1).

Expression of *hefA* in MDR strains and their parent isolates

The expression of *hefA* in MDR strains and their parent isolates was assessed by real-time RT-PCR. Each relative expression value was the mean of 3 replications. The relative expression of *hefA* versus *gyrB* in 3 clinical isolates and *H. pylori* 11637 was significantly higher in MDR strains (5.83 ± 0.91) than in parent strains (1.90 ± 0.45). The difference in *hefA* expression was statistically significant ($P < 0.001$) (Table 2).

MICs of the antibiotics in *H. pylori* strains after treatment with CCCP

When treated with CCCP (100 $\mu\text{mol/L}$), MICs of the 9 antibiotics were tested. MICs of 5 antibiotics decreased at least 4-fold in MDR strains: 19-fold with chloramphenicol, 10-fold with tetracycline, 7-fold with erythromycin, 4-fold with cefotaxime and ceftriaxone (geometric mean), while there was little difference in the MIC of antibiotics in parent strains (sensitive strains) after challenge with CCCP (Figure 1).

Difference in MICs of the antibiotics in *H. pylori* strains after treatment with pantoprazole or reserpine

After treatment with pantoprazole (10 $\mu\text{g/mL}$), MICs of the 9 antibiotics were tested. MICs of 5 antibiotics

Table 3 MICs of the antibiotics after treatment with pantoprazole or reserpine (μg/mL)

Antibiotic	Treatment	PT					MDR				
		4151	8022	11021	11637	Geometric mean	4151	8022	11021	11637	Geometric mean
Erythromycin	+Res	0.250	0.125	0.250	0.063	0.149	2.000	2.000	2.000	2.000	2.000
	+Pan	0.063	0.016	0.125	0.016	0.037	0.250	0.250	0.130	1.250	0.318
Ciprofloxacin	+Res	0.250	1.000	1.000	0.250	0.500	0.250	0.500	1.000	1.000	0.595
	+Pan	0.250	0.500	0.500	0.500	0.420	0.500	1.000	2.000	0.500	0.841
Tetracycline	+Res	0.500	0.500	1.000	0.125	0.420	4.000	4.000	2.000	4.000	3.364
	+Pan	0.125	0.500	0.250	0.063	0.177	0.250	0.250	0.250	0.250	0.250
Cefotaxime	+Res	0.125	0.500	0.250	0.250	0.250	1.000	4.000	1.000	2.000	1.682
	+Pan	0.063	0.250	0.250	0.063	0.125	0.063	0.500	0.500	0.250	0.250
Ceftriaxone	+Res	0.250	0.500	0.063	0.125	0.177	2.000	2.000	4.000	2.000	2.378
	+Pan	0.063	0.125	0.031	0.031	0.053	0.250	0.250	0.500	0.250	0.297
Piperacillin	+Res	0.125	0.500	0.250	0.250	0.250	0.500	0.500	1.000	0.063	0.354
	+Pan	0.125	0.250	0.125	0.125	0.149	0.250	0.250	0.500	0.125	0.250
Clindamycin	+Res	0.500	0.500	0.125	1.000	0.420	0.500	0.500	1.000	2.000	0.841
	+Pan	0.500	0.500	0.125	0.250	0.297	0.250	0.125	0.250	0.500	0.250
Penicillin G	+Res	0.125	0.032	0.063	0.250	0.089	0.250	0.250	0.125	1.000	0.297
	+Pan	0.063	0.032	0.016	0.125	0.044	0.125	0.063	0.125	0.500	0.149
Chlor-amphenicol	+Res	0.500	0.250	0.250	0.500	0.354	16.000	8.000	16.000	8.000	11.314
	+Pan	0.500	0.125	0.125	0.500	0.250	8.000	8.000	8.000	4.000	6.727

PT: Parent strain; MDR: Multidrug resistant strain; Pan: Pantoprazole; Res: Reserpine.

Table 4 Influence of proton pump inhibitors on MICs of the antibiotics in MDR strains (μg/mL)

	Before treatment	Omeprazole	Pantoprazole	Lansoprazole	Rabeprazole	Esomeprazole
Metronidazole	4.757	3.364	1.414	2.378	1.189	2.828
Amoxicillin	3.364	2.000	1.189	1.682	1.000	1.682
Furazolidone	2.378	2.000	2.378	2.000	2.378	2.378
Clarithromycin	5.657	4.757	3.364	5.657	4.000	4.757

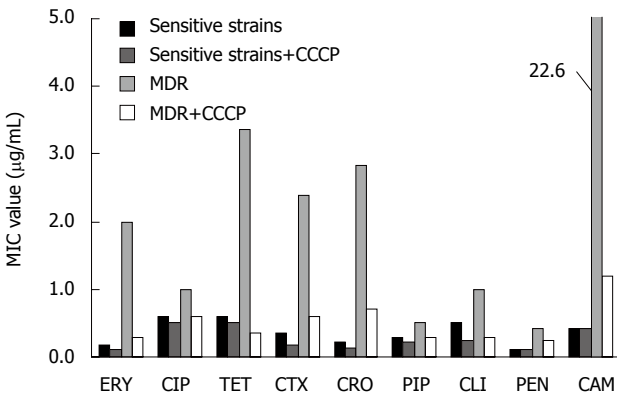


Figure 1 MICs of the antibiotics in *H. pylori* strains after treatment with CCCP (carbonyl cyanide m-chlorophenyl hydrazone). ERY: Erythromycin; CIP: Ciprofloxacin; TET: Tetracycline; CTX: Cefotaxime; CRO: Ceftriaxone; PIP: Piperacillin; CLI: Clindamycin; PEN: Penicillin; CAM: Chloramphenicol.

decreased at least 4-fold in MDR strains: 6-fold with erythromycin, 13-fold with tetracycline, 9-fold with cefotaxime and ceftriaxone, 4-fold with clindamycin (geometric mean), while MICs with erythromycin and ceftriaxone were also decreased 4-fold in their parent strains. MICs of antibiotics in MDR strains and parent strains had no obvious difference after reserpine (20 μg/mL) was added to the agar (Table 3).

Influence of PPIs on MICs of the antibiotics in MDR strains

To further clarify the effect of PPIs on MICs of antibiotics used in eradicating *H. pylori* in the clinic, and to determine which PPI was the most efficient in *H. pylori* treatment when combined with 2 antibiotics, MICs of metronidazole, amoxicillin, furazolidone and clarithromycin in MDR *H. pylori* strains were measured after treatment with one of the 5 PPIs (10 μg/mL). Our data indicated that MICs of metronidazole and amoxicillin were decreased 4-fold by treatment with rabeprazole, while there was a 3-fold decrease with pantoprazole. There was little effect of omeprazole, lansoprazole and esomeprazole on the MICs of antibiotics (Table 4).

DISCUSSION

With the extended use of antibiotics, the rate of resistance of antibiotics for *H. pylori* has increased worldwide, and appearance of an MDR strain that is simultaneously resistant to amoxicillin, metronidazole and clarithromycin, has been reported. In addition to chromosomally-encoded drug resistance, resistance to toxic compounds through increased export may be of importance in the multidrug resistance of *H. pylori*. Efflux systems have been identified in *H. pylori*^[17], but the mechanism of multidrug resistance

in *H. pylori* is far from well understood, and a tetracycline resistance-related gene (*HP1165*) was found in 2006^[18]. A non-specific MDR protein has also been reported, though we have little knowledge about the function of multidrug resistance^[19,20]. Preliminary studies suggested that overexpression of the efflux pump systems in *H. pylori* were associated with multidrug resistance to antibiotics^[6,21,22]. In the current investigation, the findings suggested that efflux pump systems were overexpressed under the pressure of increasing concentrations of antibiotics, which led to multidrug resistance to antibiotics in *H. pylori* strains, indicating that the long-term administration of antibiotics in the clinic may be responsible for *H. pylori* multidrug resistance.

Five families of multidrug efflux transporters have been described, which are primarily active transporters with energy from ATP hydrolysis or driven by ion gradients^[23,24]. MDR efflux pumps are recognized as an important component of resistance in *H. pylori*. To combat resistant pathogens, in recent years EPIs, as promising therapeutic agents, have been widely investigated in other bacteria^[25,26]. EPIs can be divided into several categories according to their mechanisms^[27]; to interfere with the structural synthesis of the efflux pumps, to block the energy supply of the bacterial membrane, or to act as a competitive substrate of efflux pumps. CCCP, as an energy blocker, can reduce the transmembrane electrochemical gradient, and inhibit the efflux pumps driven by hydrogen ion gradients. The results of this study showed that CCCP could decrease MICs of the 5 antibiotics at least 4-fold in MDR *H. pylori* strains, while it had little influence on MICs of antibiotics in parent strains, indicating that overexpression of efflux pump systems in *H. pylori*, as in other bacteria, can cause multidrug resistance. Only MICs of 5 out of 9 antibiotics were reduced after treatment with CCCP, suggesting that these types of antibiotic are the substrates of the *HefABC*-RND efflux pump in MDR *H. pylori*. Reserpine could inhibit MFS and the ATP-binding cassette (ABC multidrug efflux) superfamilies^[12,13]. MICs of antibiotics in MDR strains and parent strains showed little difference when treated with reserpine, confirming that MFS and ABC family efflux pumps may be not responsible for multidrug resistance in *H. pylori*.

PPIs including omeprazole, rabeprazole, pantoprazole, lansoprazole and esomeprazole combined with 2 or 3 kinds of antibiotics have been used widely in the treatment of *H. pylori* infection. As proton motive forced uncoupling agents, however, less is known about whether PPIs could restore the antibiotic sensitivity of the MDR *H. pylori* strains. Other studies reported that PPIs, as one class of EPI, could decrease the MICs of norfloxacin, ciprofloxacin and levofloxacin by 4-8 fold by inhibiting the NorA efflux pump in MDR *Staphylococcus aureus*^[28]. In this study, when treated with pantoprazole, MICs of the 5 antibiotics were decreased at least 4-fold in MDR strains, indicating that not only could PPIs act as antacids in the treatment of *H. pylori* infection, but also might inhibit the efflux pumps, and strengthen the effect of antibiotics by increasing their

intracellular accumulation. Previous studies have indicated that 5 PPIs used in treatment of *H. pylori* infection had little effect on the eradication rate of *H. pylori*^[29,30]. While 5 different PPIs were compared *in vitro* for their effects on the MICs of antibiotics in MDR *H. pylori* strains, our data showed that rabeprazole was the most efficient in reducing the multidrug resistance of these strains, with pantoprazole in second place. The underlying mechanisms require further study, but, based on the results in this study, it is suggested that rabeprazole could be used in the treatment of MDR *H. pylori* strains rather than other PPIs.

The antibiotic resistance rate of *H. pylori* has increased worldwide, and MDR strains have been reported, though limited information is available on the mechanisms of multidrug resistance and the role of EPIs in MDR *H. pylori*. To our knowledge, this study demonstrates for the first time that EPIs could partly reverse some types of antibiotic resistant phenotype in MDR *H. pylori* strains, and some PPIs contribute to enhance the antibacterial effect in *H. pylori* treatment.

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COMMENTS

Background

More than 50% of the world's population have *Helicobacter pylori* (*H. pylori*) infection, which is the major pathogen in human gastritis, peptic ulcers and gastric cancer. Nowadays, because of the extensive application of antibiotics, not only does the *H. pylori* resistance rate continue to rise, but also multidrug resistant (MDR) strains have emerged. The efflux pump systems of bacteria play an important role in the mechanism of multidrug resistance.

Research frontiers

To deal with the problem of multidrug resistance, in recent years, efflux pump inhibitors (EPIs) have been given extensive attention in many other bacteria, such as *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, etc. However, whether EPIs could influence the resistance of *H. pylori* has not yet been reported.

Innovations and breakthroughs

In the present study, some types of EPI were used to explore their effect on multidrug resistance of *H. pylori*, and for the first time it was demonstrated that EPIs could partly reverse an antibiotic-resistant phenotype in MDR *H. pylori* strains, and some proton pump inhibitors (PPIs) contributed to enhance the antibacterial effect in *H. pylori* treatment.

Applications

In vitro, the study found that EPIs could improve antibiotic sensitivity of MDR strains of *H. pylori*, and for clinical application, the EPI most efficient in decreasing the resistance should be investigated.

Peer review

The study by Zhang *et al.* explores the hypothesis that bacterial efflux pumps play an important role in the development of MDR *H. pylori*. Three chloramphenicol-induced MDR strains (out of 9 strains) were isolated from stomachs of patients and were shown to express higher levels of *hefA* (a efflux pump gene) mRNA.

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Heme oxygenase-1 protects donor livers from ischemia/reperfusion injury: The role of Kupffer cells

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Abstract

AIM: To examine whether heme oxygenase (HO)-1 overexpression would exert direct or indirect effects on Kupffer cells activation, which lead to aggravation of reperfusion injury.

METHODS: Donors were pretreated with cobalt protoporphyrin (CoPP) or zinc protoporphyrin (ZnPP), HO-1 inducer and antagonist, respectively. Livers were stored at 4°C for 24 h before transplantation. Kupffer cells were isolated and cultured for 6 h after liver reperfusion.

RESULTS: Postoperatively, serum transaminases were significantly lower and associated with less liver injury when donors were pretreated with CoPP, as compared

with the ZnPP group. Production of the cytokines tumor necrosis factor- α and interleukin-6 generated by Kupffer cells decreased in the CoPP group. The CD14 expression levels (RT-PCR/Western blots) of Kupffer cells from CoPP-pretreated liver grafts reduced.

CONCLUSION: The study suggests that the potential utility of HO-1 overexpression in preventing ischemia/reperfusion injury results from inhibition of Kupffer cells activation.

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Key words: Heme oxygenase-1; Kupffer cells; Ischemia/reperfusion injury; Liver transplantation

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Zeng Z, Huang HF, Chen MQ, Song F, Zhang YJ. Heme oxygenase-1 protects donor livers from ischemia/reperfusion injury: The role of Kupffer cells. *World J Gastroenterol* 2010; 16(10): 1285-1292 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v16/i10/1285.htm> DOI: <http://dx.doi.org/10.3748/wjg.v16.i10.1285>

INTRODUCTION

Orthotopic liver transplantation (OLT) is an effective treatment for end-stage liver diseases^[1]. However, ischemia/reperfusion (I/R) injury of the liver remains a major cause of graft injury, causing liver dysfunction and even failure posttransplantation^[2,3]. The destructive effects of I/R arise from the acute generation of reactive oxygen species subsequent to reoxygenation, which inflicts direct tissue damage and initiates a cascade of deleterious

cellular responses leading to inflammation, cell death, and organ failure^[4]. Endothelial cell damage results from free radicals produced from Kupffer cells and adherent polymorphonuclear leukocytes (PMNs)^[5]. Ultimately, this results in loss of microvascular integrity and reduction of blood flow ('no-reflow phenomenon')^[6]. Methods to protect liver grafts against I/R injury have considerable clinical consequences.

Kupffer cells, the resident macrophages of the liver, are involved in liver I/R injury through the release of cytokines such as tumor necrosis factor- α (TNF- α) and other biologically active mediators^[7-9]. Multiple lines of evidence have suggested that Kupffer cells are critical to the onset of liver injury and following secretion, cytokines aggravate hepatocyte damage. The use of *in vitro* and animal models has shown that inactivation of Kupffer cells prevents liver injury^[10,11]. Activation of Kupffer cells directly or indirectly by endotoxin (lipopolysaccharide, LPS) results in the release of an array of inflammatory mediators, growth factors, and reactive oxygen species.

The heme oxygenase (HO) system is the rate-limiting step in the oxidative degradation of heme into biliverdin, carbon monoxide (CO) and free iron^[12]. Three HO isoforms have been identified: inducible HO-1, also known as heat shock protein 32; constitutively expressed HO-2; and a related but less well-characterized HO-3. HO-1 is induced in a variety of organs during diverse stress-related conditions and is thought to provide cytoprotection^[13,14]. Upregulation of HO-1 is known to be a protective response from cellular stress, following I/R injury, radiation and inflammation^[15]. Overexpression of HO-1 exerts a cytoprotective function in a number of I/R injury and liver transplant models^[16-18]. Thus, HO-1 is an attractive target for anti-inflammatory therapies and potential candidate responsible for cell injury.

Immunochemical studies with specific monoclonal antibodies have revealed the distribution of HO-1 in the rat liver with distinct topographical patterns^[19]. HO-1 has been shown to be expressed principally in Kupffer cells^[16,20-22]. However, exact mechanisms by which HO-1 induction may lead to cytoprotection during I/R injury in organ transplantation have not been fully clarified. We designed a study to evaluate the role of HO-1-mediated cytoprotection in rat liver transplantation models. The aim was to demonstrate whether HO-1 plays a critical role in inhibiting Kupffer cells activation.

MATERIALS AND METHODS

Animals

Male Sprague-Dawley (S-D) Rats (Third Military Medical University Laboratory Animal Center, Chongqing, China) weighing 220-250 g were used. Animals were fed standard rodent chow and water *ad libitum* and cared for according to the local animal welfare guidelines. All procedures used in this study were approved by the ethics committee for the use of experimental animals at Kunming Medical College.

Synthetic metalloporphyrins

Metalloporphyrins (CoPP and ZnPP) were purchased from Sigma Chemical inc. USA. They were dissolved in 0.1 mol/L NaOH, subsequently adjusted to pH 7.4 with HCl, and diluted in 0.85% NaCl. The stock concentration was 0.5 mg/mL (CoPP) and 2 mg/mL (ZnPP).

Experimental design

S-D rats underwent ether anesthesia. The basic techniques of liver harvesting and orthotopic transplantation without hepatic arterial reconstruction were performed according to the method described previously by Kamada *et al.*^[23]. In the control group ($n = 8$), no drugs were applied. There were two treatment groups. In ZnPP group ($n = 8$), donors received ZnPP, an HO-1 inhibitor (20 mg/kg ip) 24 h prior to harvest. CoPP group ($n = 8$) rats received CoPP, an HO-1 inducer (5 mg/kg ip) 24 h prior to harvest. All liver grafts were harvested and stored with UW solution for 24 h at 4°C, and orthotopically transplanted into syngeneic S-D recipients. All transplant experiments in this study were performed by a single person. The anhepatic phase was 11.3 ± 0.7 min. Separate groups of rats were killed at 6 h after their vessels were unclamped, and liver samples were collected for further analysis.

Blood sample collection and serum liver enzymes

At 6 h following by liver reperfusion, blood was collected into ethylenediaminetetraacetic acid (EDTA) tubes. After centrifugation of whole blood (2000 g, 15 min), serum was stored at -70°C until analysis. Serum alanine aminotransferase (ALT) and aspartate transaminase (AST) levels were measured using an automated clinical analyzer (7060 automatic analyzer, Hitachi, Japan).

Histopathology

Liver graft tissues were fixed in 10% formalin, embedded in paraffin. Serial sections of 3 μ m thickness were stained with hematoxylin and eosin for routine pathological examination.

Isolation and culture of Kupffer cells

Kupffer cells were isolated from integral left liver tissue obtained from fresh specimens. The liver was perfused *in vitro* through the vena cava with 80 mL Hanks' balanced salt solution (HBSS) Ca²⁺/Mg²⁺-free (Hyclone, Germany) at 37°C, and transferred to a 100 mm culture dish and perfusion was continued with complete HBSS containing 0.05% collagenase IV (Sigma, USA) and 3 mmol/L Ca²⁺ at 37°C. Liver tissue was finely diced into 2 mm³ sized pieces and the suspension incubated under constant agitation at 37°C for 30 min. The liver homogenate was filtered through gauze mesh and the cells suspension was centrifuged at 50 g for 3 min at 4°C to remove hepatocytes. The non-parenchymal cells-enriched supernatant was centrifuged at 400 g for 6 min. The cell pellet was resuspended in 30% Percoll (Pharmacia, Sweden) with a density of 1.040 g/mL, and this was carefully layered onto 60% Percoll with a density

Table 1 Primers used for RT-PCR

Gene	Primer sequence	Size of amplified DNA (bp)	Annealing temperature (°C)
HO-1	F 5' TGAAGAGGAGATAGAGCGA 3'	451	53
HO-1	R 5' TGTGAGCAGGAAGGCGGTC 3'	451	
CD14	F 5' GGACCCGATCTCAACACCT 3'	377	55
CD14	R 5' CCAGCAGTATCCCGCAGT 3'	377	
β -actin	F 5' CGGAAATCGTGCGTGAC 3'	443	55
β -actin	R 5' TGAAGGTGGACAGCGAGG 3'	443	

RT-PCR: Reverse-transcriptase polymerase chain reaction; F: Forward; R: Reverse.

of 1.075 g/mL. The double layer discontinuous gradient formed was overlaid with 3 mL of HBSS and centrifuged at 400 *g* for 15 min at 4°C. The opaque interface was collected, resuspended in HBSS and centrifuged at 400 *g* for 5 min at 4°C. The cells were seeded onto tissue culture plates at a density of 2×10^6 /mL and cultured in RPMI 1640 medium (Gibco, USA) containing 10% heat-inactivated fetal bovine serum (FBS, Gibco, USA), 100 U/mL Penicillin/Streptomycin (Hyclone, Germany) and 10 mmol/L HEPES at 37°C with 5%CO₂. Nonadherent cells were removed after 2 h by gentle washing the plated cells and replacing the culture medium. Adherent cells were incubated for 48 h before performing uptake assays. More than 95% of adherent cells were ED2 (Serotec, UK) positive.

Reverse-transcriptase polymerase chain reaction (RT-PCR)

Total RNA was extracted from Kupffer cells at 48 h after culture or liver tissues with Trizol (Invitrogen, USA). Reverse transcription was performed on 1 μ g RNA using random primers. Reverse-transcription reaction product (PrimeScript™ RT-PCR Kit, TaKaRa) was used for PCR reaction, with initial heating at 94°C for 5 min, followed by 40 cycles of 94°C for 30 s, 53-55°C for 30 s, 72°C for 1 min, and finally 72°C for 7 min. The primers are listed in Table 1. The PCR products were subjected to electrophoresis on 1.5% agarose gel containing ethidium bromide and visualized by UV illumination. β -actin was used as an internal control for RNA integrity.

Western blotting analysis

Proteins were extracted from Kupffer cells with radioimmunoprecipitation (RIPA) containing phenylmethyl sulfonylfluoride (PMSF). Protein quantification of samples was performed using the BCA assay. Proteins (15 μ g/sample) in SDS-loading buffer were heated to 100°C for 5 min, subjected to 12% SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to nitrocellulose membrane. The membrane was blocked overnight in 5% nonfat dry milk in TBST buffer at 4°C. Blots were incubated at room temperature (RT) for 2 h with anti-CD14 (dilution, 1:200, Santa Cruz Biotechnology, Inc. USA) in TBST buffer with 5% nonfat dry milk. After washing in TBST buffer three times at RT for 10 min, blots were incubated for 2 h at RT

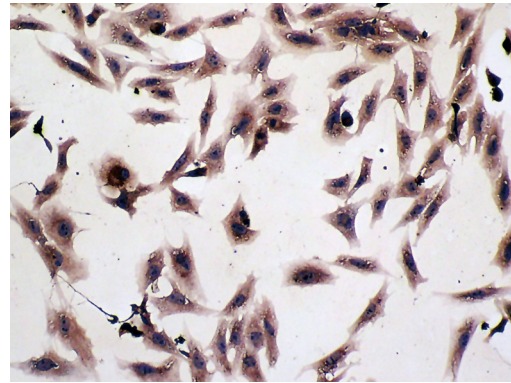


Figure 1 ED2 immunocytochemistry staining of cultured Kupffer cells. More than 95% are ED2 positive. Cells adhere to plastic and exhibit a spread out morphology and irregular shape ($\times 400$).

with horseradish peroxidase-conjugated goat anti-rabbit IgG antibodies (dilution, 1:50 000, Pierce Biotechnology) in TBST buffer with 5% nonfat dry milk. Finally, membranes were washed three times and developed with ECL (Amersham Pharmacia Biotech, Piscataway, New Jersey).

Measurement of cytokine release

The medium from Kupffer cells culture was collected and centrifuged at 1000 *g* for 5 min, and supernatant was kept -70°C until assayed. IL-6 and TNF- α levels in the supernatant were determined with rat-specific enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems, Inc. USA). All samples, including standard and control solution, were assayed in duplicate.

Statistical analysis

Statistical analysis was performed using SPSS Version 13.0 for Windows (SPSS, Inc, Chicago, IL). All data are expressed as mean \pm SE. Difference between experimental groups were analyzed using one-way analysis of variance or Student's *t* test. All differences were considered statistically significant at the *P* value of < 0.05 .

RESULTS

Primary cultures of Kupffer cells

The isolated Kupffer cells were seeded onto tissue culture plates and incubated for 48 h. Cells attached rapidly to the dish surface, and spread in an irregular outline after 48 h in culture (Figure 1). More than 95% were ED2 positive on immunocytochemistry staining.

Liver HO-1 expression levels before transplantation

To assess HO-1 expression in experimental livers that were either untreated or pretreated with an HO-1 inducer or inhibitor, we performed RT-PCR analyses. A wide variation in HO-1 gene expression was detected in liver tissues that were collected before transplantation (Figure 2A). Livers harvested from donors that were pretreated with CoPP significantly up-regulated HO-1 mRNA expression levels as compared with control or the ZnPP

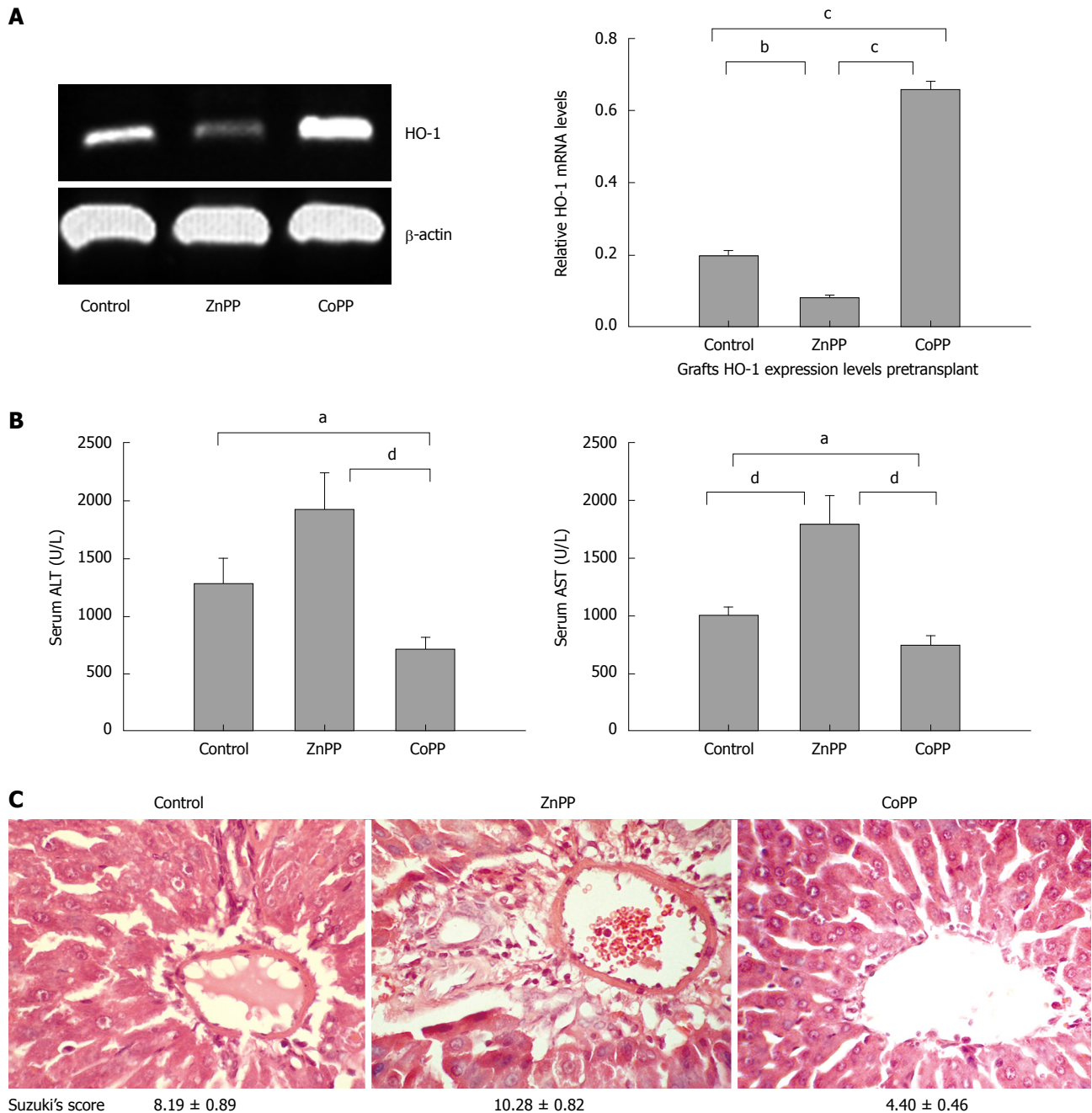


Figure 2 HO-1 overexpression ameliorates hepatic I/R injury. A: The expression of hepatic HO-1 was evaluated by RT-PCR before liver transplantation. β-actin was used as an internal control. Decreased expression of HO-1 mRNA was detected 24 h after ZnPP treatment. CoPP treatment increased HO-1 mRNA ($n = 4/\text{group}$). ^b $P < 0.001$; ^c $P < 0.0001$; B: Hepatocellular function. Serum ALT/AST levels (U/L) were higher in the ZnPP group as compared with controls. Significantly lower serum transaminase levels were seen in the CoPP group ($n = 8/\text{group}$). ^a $P < 0.05$; ^d $P < 0.01$; C: Representative photomicrographs of rat livers following 6 h reperfusion. Livers in ZnPP group show severe lobular distortion, sinusoidal congestion, ballooning, hepatocyte necrosis. Livers pretreated with CoPP exhibit good preservation of lobular architecture. Original magnification, $\times 400$; HE stain.

group (0.658 ± 0.022 , 0.198 ± 0.0165 , 0.083 ± 0.008 , respectively; $P < 0.0001$). ZnPP treatment decreased HO-1 mRNA as compared with control ($P < 0.001$).

HO-1 overexpression improves liver function

We analyzed the hepatocellular function in rats that underwent cold ischemia followed by 6 h of reperfusion in three animal groups. As shown in Figure 2B, pretreatment with CoPP significantly decreased serum ALT levels as

compared with the control or the ZnPP group (711.37 ± 111.13 , 1285.00 ± 219.46 , 1932.13 ± 313.41 , respectively, $P < 0.05$ *vs* control, and $P < 0.01$ *vs* ZnPP-treated control). Serum AST levels were also significantly reduced in the CoPP treatment group as compared with both the control and the ZnPP group (740.38 ± 84.71 , 998.63 ± 69.45 , 1795.50 ± 244.59 , respectively, $P < 0.05$ *vs* control, and $P < 0.01$ *vs* ZnPP-treated control). Both ALT and AST levels were higher in the ZnPP group.

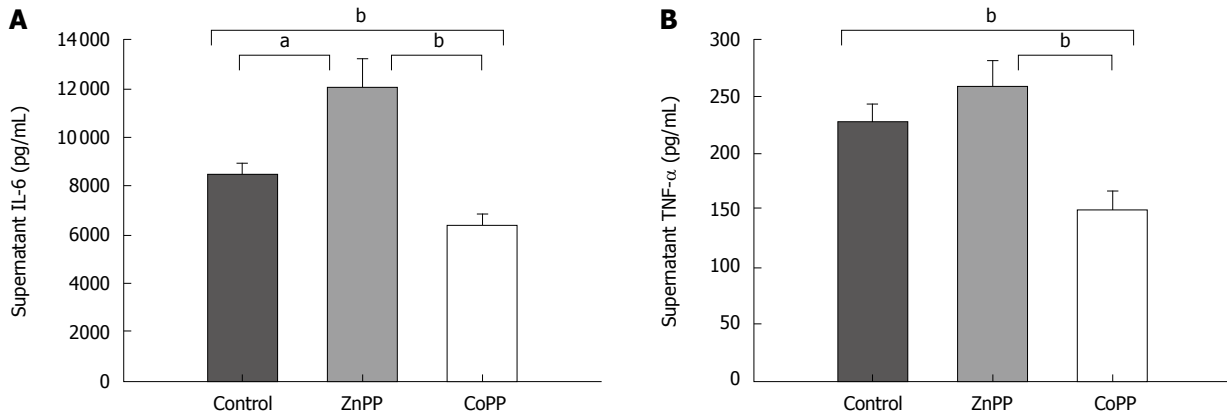


Figure 3 Concentration of IL-6 and TNF- α in cultured supernatant. Detection of cytokines at 48 h of Kupffer cell culture. ZnPP treatment increased cytokine levels of IL-6 (A) and TNF- α (B) were assayed. Significantly lower cytokine levels were detected in the CoPP group as compared with controls. The cytokine levels in cultured supernatant were measured by ELISA technique as described in Materials and methods ($n = 5/\text{group}$). ^a $P < 0.05$, ^b $P < 0.01$.

HO-1 overexpression ameliorates hepatic I/R injury

The hepatocellular damage was evaluated by liver histology. Livers in the ZnPP group showed a more severe hepatocyte necrosis, sinusoidal congestion and ballooning, as compared with controls. In contrast, livers in the CoPP group revealed almost complete preservation of lobular architecture without sinusoidal congestion, ballooning, or necrosis (Figure 2C). The hepatocellular damage was graded using a modified Suzuki's criteria^[24].

Cytokines release

The cytokine levels (IL-6/TNF- α) in supernatant from the release of cultured Kupffer cells revealed a substantial increase in the ZnPP pretreated group as compared with the CoPP group ($12019.25 \pm 1244.89/257.75 \pm 21.56$ pg/mL *vs* $6360.13 \pm 522.36/152.00 \pm 14.66$ pg/mL, respectively; $P < 0.01$). The IL-6/TNF- α levels of supernatant in CoPP pretreated group were significantly lower, when compared to the control group ($8459.86 \pm 453.51/228.38 \pm 15.04$, $P < 0.01$; Figure 3A and B).

HO-1 and CD14 expression of Kupffer cells (Effect of HO-1 on Kupffer cells activation)

HO-1 mRNA expression levels of Kupffer cells pretreated with CoPP were significantly up-regulated, and those pretreated with ZnPP were down-regulated (Figure 4A). A prominent increase of CD14 mRNA levels in Kupffer cells was detected in the ZnPP group as compared with the control group or the CoPP group (1.547 ± 0.227 , 0.772 ± 0.135 , 0.325 ± 0.084 pg/mL, respectively; $P < 0.05$ *vs* control, and $P < 0.01$ *vs* CoPP-pretreated group). CoPP treatment was significantly lower, when compared to the control group ($P < 0.05$). As shown in Figure 4B and consistent with mRNA data, Western blot-assessed CD14 protein levels of Kupffer cells were also significantly up-regulated in the ZnPP group as compared with the control group (1.996 ± 0.446 pg/mL *vs* 1.303 ± 0.081 pg/mL, $P < 0.05$). CoPP treatment reduced CD14 protein levels (0.771 ± 0.136 , $P < 0.05$ *vs* control).

DISCUSSION

I/R injury is one of the major obstacles in liver

transplantation. HO-1 overexpression exerts cytoprotection and ameliorates hepatocellular damage in a number of liver I/R injury models^[25-27], and prolongs cold ischemia followed by *ex vivo* perfusion or OLT^[18]. In our study, CoPP-induced HO-1 overexpression significantly decreased hepatocyte injury posttransplantation. Donor livers with enhanced HO-1 expression reduced serum ALT/AST levels of recipients, ameliorated hepatic injury and suppressed cytokine release. In the transplant model, cultured Kupffer cells from donors pretreated with CoPP down-regulated CD14 mRNA and protein expression levels and reduced cytokine release.

HO-1 has been suggested as a cytoprotective gene during liver transplantation. However, the mechanism of HO-1-cytoprotection against I/R injury remains elusive. Tsuchihashi *et al.*^[28] showed that basal HO-1 levels are more critical than the ability to up-regulate HO-1 in response to I/R injury. Generally, the cellular mechanisms of HO-1-derived protection included regulation of the inflammatory response, improvement of microvascular flow and the antiapoptotic effects. The beneficial effects of HO-1 may be the result of the ability of the end-products of heme degradation^[18]. Kaizu *et al.*^[29] reported that exogenous CO treatment suppressed early proinflammatory gene expression and neutrophil infiltration, and efficiently ameliorated hepatic I/R injury. CO has vasodilating effects, thereby maintaining microvascular hepatic blood flow^[18,30]. Furthermore, Tomiyama *et al.*^[31] recently demonstrated that CO ameliorated hepatic I/R injury and *in vitro* rat primary Kupffer cells culture also showed significant down-regulation of LPS-induced inflammatory responses. Biliverdin and the subsequently formed bilirubin possess potent antioxidant effects.

The mechanism of hepatic I/R injury is largely attributed to Kupffer cell activation and release of proinflammatory mediators such as reactive oxygen species and cytokines. During the initial stages of reperfusion Kupffer cells are activated. Activated Kupffer cells release a large amount of proinflammatory cytokines (TNF- α , IL-6, and IL-1)^[32-34], which lead to aggravation of I/R injury. Liver I/R injury can be attenuated by the suppression of Kupffer cells^[35]. Therefore modulation

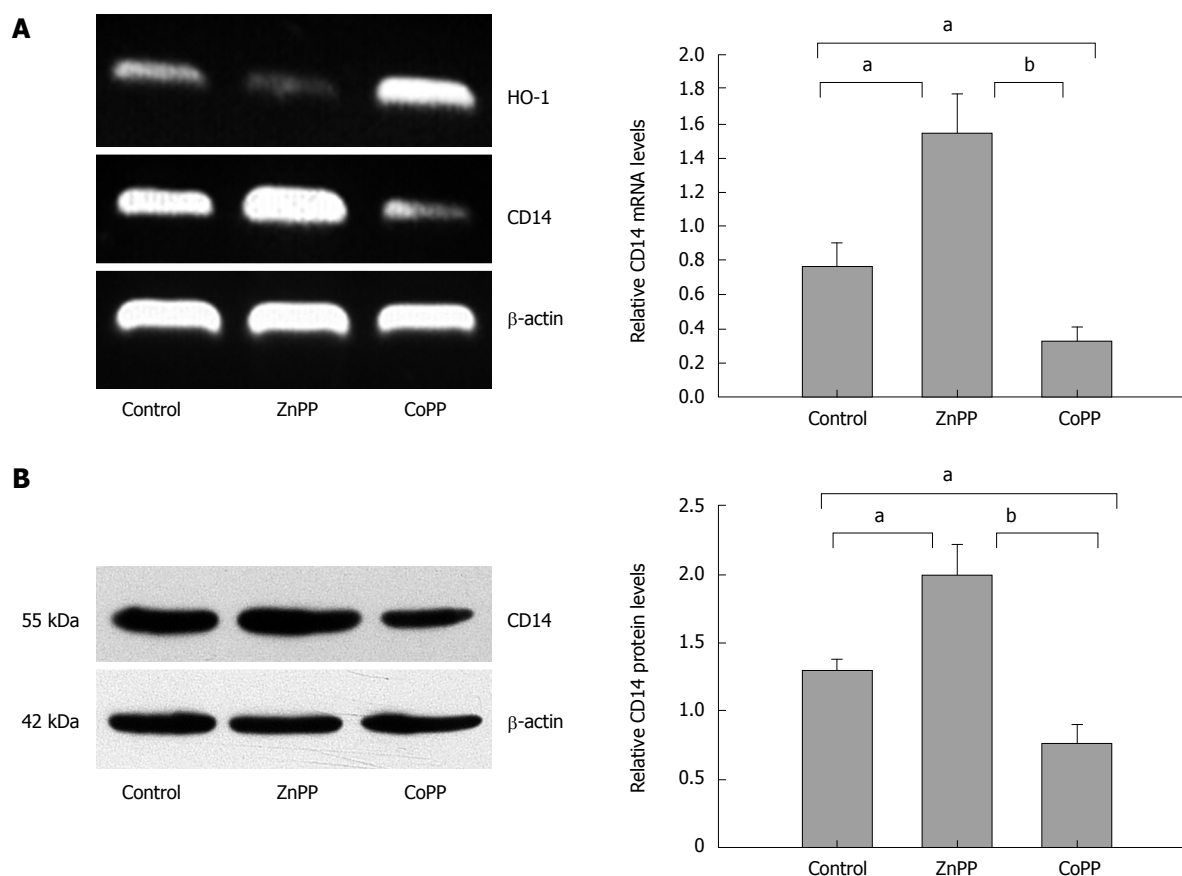


Figure 4 HO-1 and CD14 expression levels of cultured Kupffer cells. A: HO-1 mRNA expression levels of Kupffer cells were significantly downregulated in the ZnPP group and upregulated in the CoPP group. ZnPP treatment CD14 mRNA expression was expressed as relative increase compared with control. CoPP pretreated was lower ($n = 4/\text{group}$); B: Western blot analysis for CD14 protein of Kupffer cells. β -actin was used as internal control. CD14 protein expression levels increased in the ZnPP group and decreased in the CoPP group ($n = 4/\text{group}$). ^a $P < 0.05$, ^b $P < 0.01$.

of Kupffer cell activity can attenuate I/R injury in liver transplantation models.

In this study, we observed that donor pretreatment with CoPP significantly improved liver function, ameliorated hepatic injury and reduced plasma proinflammatory mediators. The elimination of oxidants from the cell is considered to be an important mechanism of HO-1-mediated protection against oxidative stress. Biliverdin (BV) and bilirubin (BR), which have been regarded as toxic metabolites of heme degradation, may serve as important mediators of nitrosative injury through a similar mechanism^[36]. BV and BR were shown to scavenge peroxynitrite. Superinduction of HO-1 leads to BR-mediated reductions in oxidative stress following I/R injury and provides cytoprotection in hepatocytes that are subjected to reperfusion injury^[14,18]. CO, another byproduct of HO-1, has been shown to modulate intrahepatic sinusoidal tone and improve microcirculation of the liver^[29]. Kupffer cells, liver resident macrophages, are the prime source of HO-1. We monitored the circulating levels of TNF- α and IL-6, both of which are proinflammatory cytokines mainly released by activated Kupffer cells. Kupffer cells overexpressing HO-1 exhibit low levels of cytokines in supernatant. In CoPP treatment, the activation of Kupffer cells was suppressed by lower TNF- α and IL-6 levels compared with ZnPP

treatment. In many models of liver injury elevated TNF- α levels are present and correlate with injury, and inhibition of TNF- α activity can decrease liver injury^[9]. As potent producers of inflammatory cytokines, Kupffer cells have been implicated in the pathway leading to liver injury^[37].

Interestingly, there was an association of Kupffer cell HO-1 levels with lower levels of CD14 mRNA and protein seen in the CoPP treatment group as compared with the ZnPP treatment group. Kupffer cells have relatively low baseline expression of CD14^[38]. Some studies showed that activation of Kupffer cells up-regulated expression of CD14 protein and its gene^[39-41]. Interpretations of studies in liver injury should take into account the relative contributions of Kupffer cells and hepatocyte CD14^[9]. Thus, increased baseline HO-1 levels as seen in CoPP treatment may represent a diminished ability to activate Kupffer cells. Kupffer cells are the major site of expression of hepatic HO-1. It is well known that liver I/R injury results in Kupffer cell activation and subsequent cytokines release, leading to localized hepatic damage. However, Devey *et al*^[42] reported that Kupffer cells depletion resulted in loss of HO-1 expression and increased susceptibility to hepatic I/R injury. Therefore, HO-1 expression levels of Kupffer cells are likely to play a crucial role in liver I/R injury.

In conclusion, CoPP-induced HO-1 overexpression

ameliorates liver I/R injury and suppresses cytokine release by inhibiting activation of Kupffer cells. Our data provides evidence for a novel mechanism of HO-1 dependent cytoprotection in liver I/R injury.

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COMMENTS

Background

Orthotopic liver transplantation (OLT) is an effective treatment for end-stage liver diseases. However, ischemia/reperfusion (I/R) injury of the liver remains a major cause of graft injury, causing liver dysfunction and even failure posttransplantation. Overexpression of heme oxygenase (HO)-1 exerts a cytoprotective function in a number of I/R injury and liver transplant models. Studies of the mechanism by which HO-1 protects donor livers against I/R injury contribute to the development of liver transplantation.

Research frontiers

The mechanism of HO-1-mediated cytoprotection is complicated. Kupffer cells are critical to the onset of liver injury and following secretion, cytokines aggravate hepatocyte damage. HO-1 is principally distributed in Kupffer cells. Recently, it has been reported that Kupffer cells depletion resulted in loss of HO-1 expression and increased susceptibility to hepatic I/R injury.

Innovations and breakthroughs

Since the mechanism of HO-1-mediated cytoprotection against I/R injury in organ transplantation remains unclear, upregulation of HO-1 is known to be involved in regulation of inflammatory response, improvement of microvascular flow and antiapoptotic effects. Liver I/R injury can be attenuated by the suppression of Kupffer cells. This study is an attempt to evaluate whether activation of Kupffer cells is related to HO-1 expression levels.

Applications

HO-1 can inhibit Kupffer cell activation and reduce release of proinflammatory mediators. This study showed that HO-1 is an attractive target against I/R injury in liver transplantation.

Terminology

Kupffer cells are the resident macrophages of the liver and involved in I/R injury through the release of cytokines and other biologically active mediators. Modulation of Kupffer cells activity can attenuate I/R injury in liver transplantation models.

Peer review

The paper deals with the role of heme oxygenase-1 in connection with ischemia/reperfusion injury in liver. It is important to find methods to prevent or reduce the injury that may follow ischemia/reperfusion, and the results presented may be useful for further research in the field. All the different parts of the paper are concise and clear.

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Malignant transformation of ectopic pancreatic cells in the duodenal wall

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Abstract

Ectopic pancreas (EP) is the relatively uncommon presence of pancreatic tissue outside the normal location of the pancreas. This condition is usually asymptomatic and rarely complicated by pancreatitis and malignant transformation. A few cases of neoplastic phenomena that developed from EP into the duodenal wall are described in the literature. Herein we report a case of gastric outlet obstruction due to adenocarcinoma arising from EP of the duodenal wall. The patient underwent a Whipple's procedure and had an uneventful post-operative recovery. Traditional imaging studies are often inconclusive in the definitive diagnosis, whilst endoscopic ultrasonography and guided biopsy may aid accurate pre-operative diagnosis. Diagnostic uncertainty warrants surgical exploration and if necessary extended resection is indicated.

INTRODUCTION

The presence of ectopic pancreas (EP) is relatively uncommon and generally asymptomatic. EP is defined as pancreatic tissue abnormally situated, without connection to the normal pancreas but provided with its own vascular and ductal systems^[1]. Other terms such as heterotopic and aberrant pancreas have also been described in the literature. EP has been found in both abdominal and extra abdominal locations, but is most frequently encountered in the duodenum (25%-35%)^[2], stomach (25%-60%)^[3], and rarely in the mesocolon^[4,5] and Meckel diverticulum^[6]. The origin of EP is unknown, it is possible that during rotation of the foregut and fusion of the ventral and dorsal parts of the pancreas in early foetal life, small pieces of tissue become detached from the forming organ leading to entrapment in different locations^[7]. The following is a case of upper gastrointestinal obstruction caused by malignant transformation of ectopic pancreatic tissue located into the duodenal wall.

CASE REPORT

A 56-year-old man was admitted to the authors' institution with a one month history of recurrent vomiting after meals. The patient's medical history consisted of type 1 diabetes and polydistrectual vasculopathy with no previous surgical history. The physical examination on admission did not reveal palpable abdominal masses. The vital signs were normal. Serum tumour markers such as CA 19-9, CA 125, AFP (α fetoprotein), CEA (carcinoembryonic antigen) and amylase were all within the normal range as was the basic metabolic screen.

The patient underwent a sequence of radiologic investigations. A double contrast barium meal of the upper gastrointestinal tract demonstrated reduced gastric emptying together with a degree of gastric distension. Contrast-enhanced CT demonstrated significant gastric distension and increased wall thickness in the first portion of the duodenum. The radiologic appearance of the pancreas was normal (Figure 1). We actually perform EGD (esophagogastroduodenoscopy) before CT in case of upper gastrointestinal obstruction.

EGD was used to localize an area of narrowed lumen in the first portion of the duodenum. EUS (endoscopic ultrasonography) showed heterogeneous hypoechoic areas in the submucosal tissue in relation to the narrowed first part of the duodenum, where we performed multiple biopsies. Cytology of biopsy specimens revealed the presence of an adenocarcinoma of uncertain origin. A multi-disciplinary discussion led to surgical resection. The patient subsequently underwent a pancreaticoduodenectomy. A classic Whipple's operation was performed in the distal part of the stomach (antrectomy), the distal portion of the common bile duct (choledochectomy), head of the pancreas, and the entire duodenum and proximal jejunum were resected en-block along with the regional lymph nodes. Gastrointestinal reconstruction was performed with pancreaticogastrostomy, Roux en Y choledochojejunostomy and gastrojejunostomy. Two abdominal drains were inserted and short term antibiotics as well as thrombo-prophylaxis were given according to the institutional protocol.

Figure 2 demonstrates the resected specimen consisting of gastric antrum, duodenum, common bile duct and proximal pancreas. The margins of the resection were negative for tumour and there was no nodal involvement. The conclusive histological report showed an infiltration of the duodenum by adenocarcinoma arising from ductal structures of heterotopic islets of pancreatic tissue into the duodenal wall, and pT4 grading according to WHO G2 (Figure 3A). The neoplasm involved the duodenal serosa with focal invasion of the orthotopic pancreas. The uninvolved EP had a microscopic (Figure 3B) appearance consistent with Heinrich's Class 1, where the EP was characterized by cystically dilated ducts, islets and acini^[8]. The post-operative recovery was uneventful. A nasogastric tube was maintained in aspiration until the sixth post-operative day and total parenteral nutrition was continued for 10 d. Progressive oral feeding was introduced from the sixth post-operative day. Upon discharge the patient

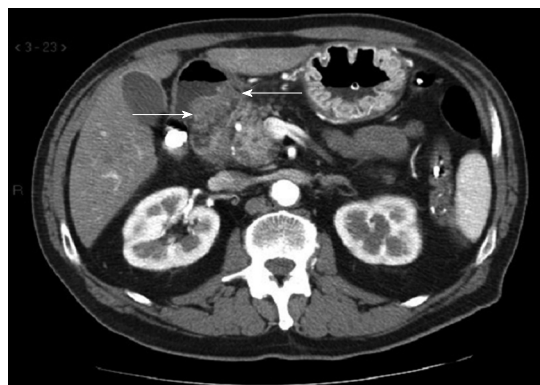


Figure 1 Contrast-enhanced CT of upper abdomen demonstrating a duodenal mass which narrowed the lumen (arrows).

was referred to the oncology department for adjuvant chemotherapy.

DISCUSSION

EP rarely gives rise to symptoms and its discovery is usually accidental^[3]. EP is found in about 1/500 laparotomies carried out for upper abdominal conditions and is reported in 0.6%-13% of post-mortem examinations^[7,9]. Macroscopically EP is often located in the submucosal layer, but may also be found in the muscularis mucosae, sub-serosa and serosa^[10]. According to Guillou *et al*^[11], to confirm the malignant transformation of EP, a series of criteria require to be fulfilled: (1) presence of a neoplasm located in/or in the proximity of the EP site; (2) concurrent presence and continuity of normal ectopic pancreatic tissue and adenocarcinoma; and (3) ectopic pancreatic tissue with normal histological structure. Our report met all the criteria. The simultaneous absence of primary neoplasm of orthotopic pancreas as well as the evidence of EP in the duodenal wall, along with other criteria aforementioned (Guillou *et al*^[11]) confirmed the malignant transformation of duodenal ectopic pancreas in the case described here.

The ectopic tissue may develop the same pathological changes that occur in a normal pancreas, such as cyst formation and acute pancreatitis. Malignant transformation has been rarely reported in the literature in cases of duodenal EP. Depending on anatomical location and pathology involved, EP may also give rise to symptoms such as abdominal pain, nausea, vomiting, gastric outlet obstruction, jaundice and gastrointestinal bleeding.

Pre-operative diagnosis of EP may be difficult with conventional imaging studies such as CT, radiographic contrast study and EGD. EUS is typically used to evaluate the submucosal lesions in the upper gastrointestinal tract^[12]. EUS can in fact determine the layer of origin of the intramural lesion, which is of high importance for the diagnosis^[13]. EP usually appears hypoechoic and heterogeneous with indistinct margins during EUS and most commonly originates from the third or fourth layer or a combination of these two layers of the gastrointestinal (GI) tract^[14]. EUS also allows the use of targeted fine needle aspiration biopsy (FNAB), particularly helpful for

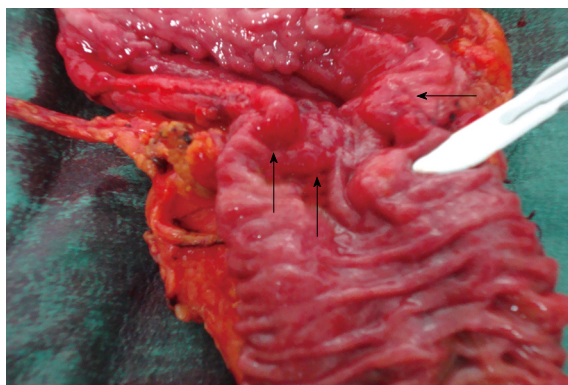


Figure 2 Duodenum. Arrows indicate the EP adenocarcinoma into the duodenal wall; the scalpel indicates the Ampulla of Vater.

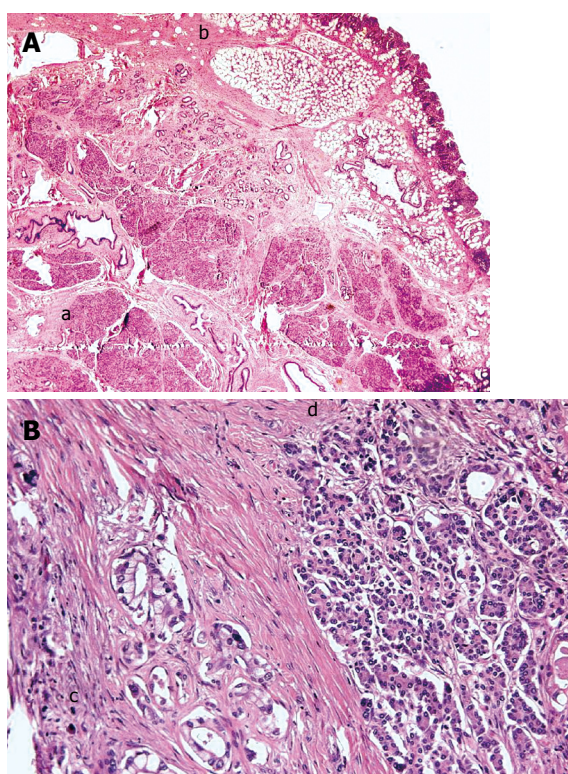


Figure 3 Histological findings. A: Duodenal wall (a) and ectopic pancreatic tissue (b) (HE, $\times 25$); B: Adenocarcinoma arising from ectopic pancreatic tissue (c) and ectopic pancreatic tissue (d) (HE, $\times 200$).

the final diagnosis. However, cytological examinations are inconclusive in about 50% of cases^[7].

Due to its relative rarity, there is no evidence in the scientific literature showing a greater tendency to develop neoplasms in EP as opposed to orthotopic pancreas. Most of the patients with EP are asymptomatic and require no treatment, but on discovery of EP as an accidental finding, its excision is recommended^[10]. In symptomatic EP or in the case of uncertain diagnosis, surgical resection may be both diagnostic and curative. Prognosis of adenocarcinoma arising from EP is not known. In the literature, only ten

cases have been reported with a survival time of between six months and ten years and all with a life expectancy longer than that in the case of orthotopic pancreas surgery^[9].

In summary, we report a rare case of malignant transformation of EP of the duodenal wall in a patient with gastric outlet obstruction. Routine imaging studies provided inconclusive information on the origin of the lesion, whilst EUS was an important adjunct in locating the lesion supplemented by fine needle aspiration cytology. Although rare, EP should be considered in the differential diagnosis of a submucosal mass of the duodenal wall.

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Concurrent amoebic and histoplasma colitis: A rare cause of massive lower gastrointestinal bleeding

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colitis with *Histoplasma capsilatum* was diagnosed. *In vitro*, amoebic parasites can increase virulence and pathogenicity of histoplasma which may account for the fulminant presentation in this patient. Although rare, this unusual dual infection should be considered in the differential diagnosis of infective colitis, as appropriate antimicrobial treatment may prevent progression to massive lower gastrointestinal bleeding, obviating the need for urgent surgical intervention.

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Key words: Gastrointestinal hemorrhage; Histoplasma; Amoebic colitis; Colectomy; Infective colitis

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Abstract

Infective colitis can be a cause of massive lower gastrointestinal bleeding requiring acute surgical intervention. Causative organisms include entamoeba and histoplasma species. However, concurrent colonic infection with both these organisms is very rare, and the *in vivo* consequences are not known. A 58-year-old male presented initially to the physicians with pyrexia of unknown origin and bloody diarrhea. Amoebic colitis was diagnosed based on biopsies, and he was treated with metronidazole. Five days later, the patient developed massive lower gastrointestinal bleeding with hemorrhagic shock. Emergency total colectomy with end-ileostomy was performed. However, he deteriorated and died on the second postoperative day. Histopathological examination revealed multiple deep ulcers at the hepatic flexure where fungal bodies of mycelial and yeast forms were noted. Isolated lymph nodes showed abscess formation with fungal bodies. Infective fungal

Koh PS, Roslani AC, Vimal KV, Shariman M, Umasangar R, Lewellyn R. Concurrent amoebic and histoplasma colitis: A rare cause of massive lower gastrointestinal bleeding. *World J Gastroenterol* 2010; 16(10): 1296-1298 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v16/i10/1296.htm> DOI: <http://dx.doi.org/10.3748/wjg.v16.i10.1296>

INTRODUCTION

Massive lower gastrointestinal bleeding can be fatal and emergency surgery may be required. There are various etiologies leading to such a presentation, with infective colitis being one of the causes. Causative organisms in infective colitis include entamoeba, a parasite, and histoplasma, a dimorphic fungus. Both these organisms can be virulent individually, where mortality is estimated to be 50% in fulminant amoebic colitis, and mortality rates of 20%-25% are estimated in gastrointestinal histoplasma

in those who are immunocompromised^[1,2]. However, concurrent infection with both these organisms is very rare. We present a case of amoebic and histoplasmic colitis causing fatal massive lower gastrointestinal bleeding.

CASE REPORT

A 58-year-old male presented initially to the physicians with pyrexia of unknown origin for a month prior to admission. There was associated bloody diarrhea with loss of appetite and weight. There was no history to suggest any respiratory or urinary tract infection and he denied any high risk behavior. There was also no alteration in bowel habit prior to these symptoms.

Blood investigations were unremarkable except for elevated white cell count, erythrocyte sedimentation rate and C-reactive protein levels. Cultures were negative and stools did not show evidence of ova and cysts.

Colonoscopy revealed mucosal inflammation along the rectum and sigmoid interspersed with normal colonic mucosa, with further inflammation and ulcers seen along the hepatic flexure (Figure 1). There was no active bleeding noted. Amoebic colitis was diagnosed based on biopsies from colonoscopy (Figure 2), and he was treated appropriately with metronidazole.

Five days later, the patient developed massive lower gastrointestinal bleeding with hemorrhagic shock. Resuscitation was commenced but he remained unstable and underwent emergency laparotomy. A total colectomy (Figure 3) with end-ileostomy was performed. Mesenteric lymphadenopathy was noted intra-operatively. His condition deteriorated postoperatively with subsequent disseminated intravascular coagulopathy. The patient died on the second postoperative day.

Histopathological examination revealed multiple deep ulcers into the muscularis propria at the hepatic flexure, where fungal bodies with mycelial and yeast formations were seen (Figure 4). Abscesses were also seen in the submucosal and serosal layers containing fungal bodies of yeast form. Isolated lymph nodes showed abscess formation with fungal bodies (yeast form). There was no evidence of malignancy or residual amoebic parasites. Infective fungal colitis with *Histoplasma capsulatum* was diagnosed.

DISCUSSION

Infective colitis can be severe and fatal. The spectrum of clinical presentation ranges from an asymptomatic state, a mild form with symptoms such as abdominal pain, fever or diarrhea, to a severe state which may result in gastrointestinal bleeding or colonic perforation^[3-5]. When presenting with massive gastrointestinal bleeding, surgery is warranted^[6].

Appropriate therapy with antimicrobials is vital in the treatment of infective colitis. Metronidazole had been shown to be effective in treatment of amoebic colitis^[4]. Despite a negative stool culture and examination as seen

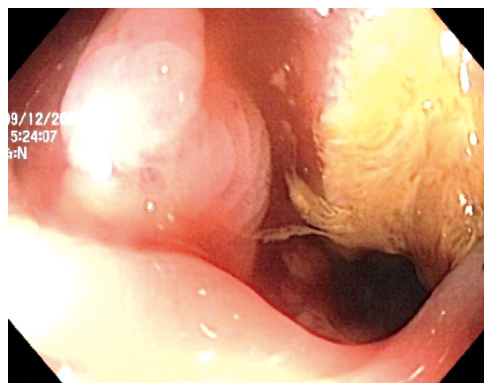


Figure 1 Colonoscopic appearance of an ulcer at the hepatic flexure.

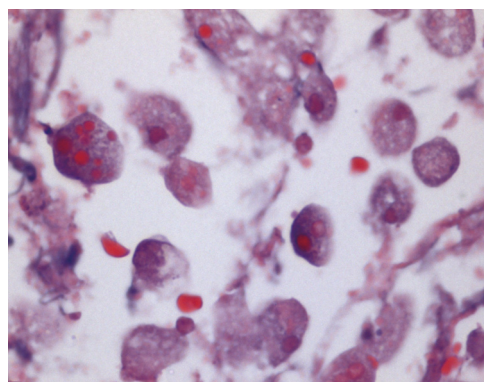


Figure 2 Colonoscopic biopsy showing amoeba (x 40).

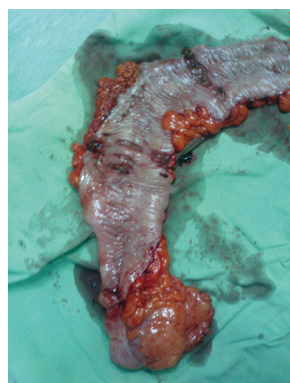


Figure 3 Gross appearance of ulcers in the colectomy specimen.

in our case, the endoscopic biopsy was positive, and this had been shown to be most effective in diagnosing amoebic infection^[7,8]. It is also interesting to note skip colonic ulcerative lesions were present on our endoscopic findings, which may suggest an inflammatory bowel disease. One tends to associate their appearance with Crohn's colitis but skip lesions in infective colitis does occur^[9,10].

Histoplasma infection is an endemic mycosis commonly found in North and Central America, and complete recovery has been well documented with the use of antifungal therapy such as amphotericin B or itraconazole^[11,12]. Isolated gastrointestinal histoplasmosis

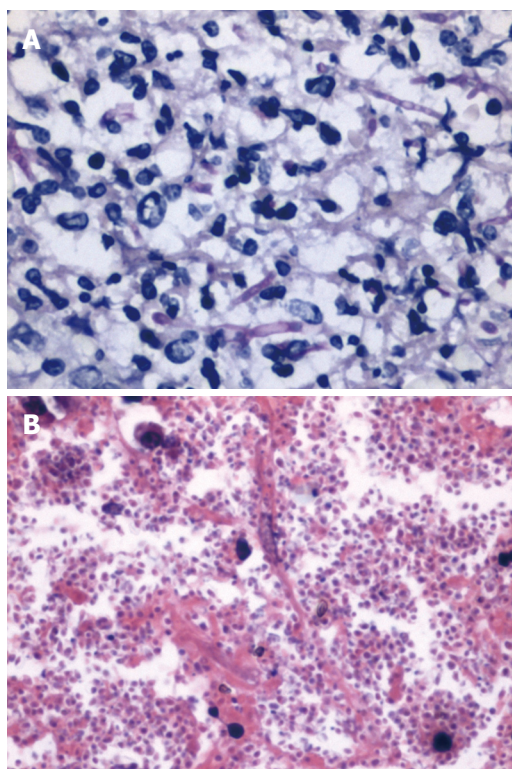


Figure 4 Histopathological examination. A: Mycelial formations (Periodic acid-Schiff, $\times 40$); B: Yeast formations (HE, $\times 40$).

is rare as it is usually asymptomatic with clinical manifestation occurring in less than 5% of the population, though it may be more common in those who are immunocompromised^[12-14].

In our case, concurrent histoplasmic infection was initially unsuspected, which may have caused progression to the severe end of the clinical spectrum. *In vitro* tests have shown that amoebic parasites may contribute to the virulence or pathogenic traits of histoplasma^[15].

Concurrent colonic infection with both amoeba and histoplasma has not been documented in the literature to our knowledge, although it has been reported in lung infections^[15]. The above case presents to us as a very rare form of colitis, which was eventually fatal.

In conclusion, massive lower gastrointestinal bleeding warrants urgent surgical intervention. Infective colitis,

especially with this unusual dual infection, as a cause of such a presentation is rare, but should be considered in the differential diagnosis.

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Negligence and Litigation in Medical
Practice

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Waikoloa, HI, United States
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January 26-27
Dubai, United Arab Emirates
2nd Middle East Gastroenterology
Conference

January 28-30
Hong Kong, China
The 1st International Congress on
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February 11-13
Fort Lauderdale, FL, United States
21th Annual International Colorectal
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February 26-28
Carolina, United States
First Symposium of GI Oncology at
The Caribbean

March 04-06
Bethesda, MD, United States
8th International Symposium on
Targeted Anticancer Therapies

March 05-07
Peshawar, Pakistan
26th Pakistan Society of
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Meeting

March 09-12
Brussels, Belgium
30th International Symposium on
Intensive Care and Emergency
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March 12-14
Bhubaneswar, India
18th Annual Meeting of Indian
National Association for Study of
the Liver

March 23-26
Cairo, Egypt
14th Pan Arab Conference on
Diabetes PACD14

March 25-28
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March 27-28
San Diego, California, United States
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2010

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April 14-18
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2010

April 28-May 01
Dubrovnik, Croatia
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Congress of Surgery

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Digestive Disease Week Annual
Meeting

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Munich, Germany
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May 15-19
Minneapolis, MN, United States
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June 04-06
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- 3 **Tian D**, Araki H, Stahl E, Bergelson J, Kreitman M. Signature of balancing selection in Arabidopsis. *Proc Natl Acad Sci USA* 2006; In press

Organization as author

- 4 **Diabetes Prevention Program Research Group**. Hypertension, insulin, and proinsulin in participants with impaired glucose tolerance. *Hypertension* 2002; **40**: 679-686 [PMID: 12411462 PMID:2516377 DOI:10.1161/01.HYP.0000035706.28494.09]

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- 5 **Vallancien G**, Emberton M, Harving N, van Moorselaar RJ; Alf-One Study Group. Sexual dysfunction in 1, 274 European men suffering from lower urinary tract symptoms. *J Urol* 2003; **169**: 2257-2261 [PMID: 12771764 DOI:10.1097/01.ju.0000067940.76090.73]

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

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- 9 Outreach: Bringing HIV-positive individuals into care. *HRSA Careaction* 2002; 1-6 [PMID: 12154804]

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- 11 **Lam SK**. Academic investigator's perspectives of medical treatment for peptic ulcer. In: Swabb EA, Azabo S. Ulcer disease: investigation and basis for therapy. New York: Marcel Dekker, 1991: 431-450

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- 12 **Breedlove GK**, Schorfheide AM. Adolescent pregnancy. 2nd ed. Wiczorek RR, editor. White Plains (NY): March of Dimes Education Services, 2001: 20-34

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- 13 **Harnden P**, Joffe JK, Jones WG, editors. Germ cell tumours V. Proceedings of the 5th Germ cell tumours Conference; 2001 Sep 13-15; Leeds, UK. New York: Springer, 2002: 30-56

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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/eid.htm>

Patent (list all authors)

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

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Write as mean \pm SD or mean \pm SE.

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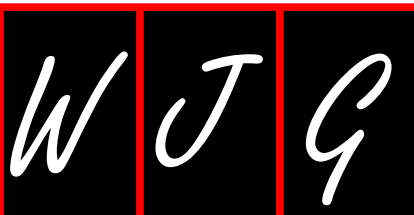
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Current role of hyperthermic intraperitoneal chemotherapy in the treatment of peritoneal carcinomatosis from colorectal cancer

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Abstract

Peritoneal carcinomatosis is one of the most common routes of dissemination of colorectal cancer (CRC). It is encountered in 7% of patients at primary surgery, while it develops in about 4% to 19% of patients after curative surgery and in up to 44% of patients with recurrent CRC. Peritoneal involvement from colorectal malignancies has been considered traditionally as a manifestation of terminal disease, due to limited response to conventional surgical and chemotherapeutic treatments. In the past few years the introduction of cytoreductive surgery combined with hyperthermic intraperitoneal chemoperfusion has shown promising results in selected patients. Currently, the surgical management of peritoneal surface malignancies of colonic origin with this combined locoregional therapy has resulted in a significant improvement in survival of these patients. However, further controlled studies will help to standardize indications and the technique of this locoregional therapy in order to achieve an improvement of morbidity and mortality rates.

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INTRODUCTION

Colorectal cancer (CRC) is the second most common cause of cancer-related deaths in western countries. Despite the improvement in its prognosis, recurrence following resection of CRC remains a substantial problem.

Peritoneal carcinomatosis (PC) is one of the most common routes of dissemination of CRC. It is encountered in 7% of patients at primary surgery, while it develops in about 4% to 19% of patients after curative surgery and in up to 44% of patients with recurrent CRC^[1,2].

In the past, PC from colorectal malignancies has been considered as a manifestation of terminal disease, due to limited response to conventional surgical and chemotherapeutic treatments^[3,4].

Sugarbaker^[5], one of the pioneers of treatment of this disease, has suggested that PC is a locoregional cancer spread as a result of a molecular crosstalk between cancer cells and host elements.

For this reason the only chance to achieve long term

survival in these patients is the eradication of microscopic residual disease.

CYTOREDUCTIVE SURGERY AND HYPERTHERMIC INTRAPERITONEAL CHEMOTHERAPY

In 1980 Spratt *et al.*^[6], after an experimental study with hyperthermic peritoneal perfusion in dogs, were first to clinically test cytoreductive surgery followed by hyperthermic intraperitoneal chemoperfusion (HIPEC) with thiotepea in a case of pseudomyxoma peritonei.

Later Sugarbaker *et al.*^[7,8] introduced intraperitoneal chemotherapy as a new innovative therapeutic option for selected patients with PC.

This treatment is based on surgical cytoreduction and HIPEC. The underlying rationale of this combined approach is that on one hand an aggressive surgical approach combining visceral resection and peritonectomy procedures addresses the macroscopic disease, whereas HIPEC is aimed at residual microscopic disease^[9].

Drugs selected for intraperitoneal administration usually are hydrophilic and have large molecular size, so that they pass slowly through a peritoneal-plasma barrier and are therefore more effectively sequestered in the peritoneal cavity. Adding hyperthermia to intraperitoneal chemotherapy may increase the tumor response to cancer chemotherapy drugs by several mechanisms. Firstly, heat alone has a direct anti-tumor effect. Hyperthermia above 41°C induces selective cytotoxicity of malignant cells. Secondly, the cytotoxic effects of some chemotherapeutic agents (doxorubicin, platinum complexes, mitomycin C, melphalan, docetaxel, irinotecan and gemcitabine) are augmented by applying mild hyperthermia^[10].

This locoregional therapy provides an elevated and persistent drug concentration for the tumor, with a limited systemic concentration.

HIPEC with mitomycin C, cisplatin and doxorubicin or oxaliplatin has been used for gastrointestinal PC. Oxaliplatin has been used intraperitoneally in Europe for colorectal PC, as pioneered by Elias *et al.*^[11]. This drug has a very low area under the curve ratio, which means that oxaliplatin is rapidly absorbed and does not require a long dwell time in the peritoneal cavity for maximal local-regional effect^[11].

However, the extent of intraperitoneal chemotherapy penetration into tumor nodules by passive diffusion is limited to a few cell layers. For this reason Elias *et al.*^[11] first suggested that intraperitoneal oxaliplatin should be combined with intravenous 5-FU administered just before HIPEC (bidirectional intraoperative chemotherapy). By combining intraoperative intravenous and intraoperative intraperitoneal cancer chemotherapy a bidirectional diffusion gradient is created through the intermediate tissue layer which contains the cancer nodules. This offers opportunities for optimizing cancer chemotherapy delivery to the target peritoneal tumor nodules.

Nevertheless, further pharmacologic studies are needed to clarify the most efficient method of administration (continuous *vs* bolus *vs* repeated bolus), doses and choice of cancer chemotherapy drugs for this bidirectional approach.

RESULTS TO DATE IN TREATING COLORECTAL PC

Since the introduction of HIPEC, promising results have been reported by several groups in the treatment of PC^[12,13].

In all published series, the most important prognostic factors in predicting survival are the involvement of disease encountered at laparotomy (measured by peritoneal cancer index, PCI) and the completeness of resection^[2].

A retrospective analysis of prognostic factors in 71 patients treated by cytoreductive surgery plus HIPEC for PC from CRC reported a median survival of 41 mo in patients with a PCI < 20, while in cases with PCI > 20 the survival was 16 mo^[14].

When there is extension of disease to six or seven regions of abdomen patients have a poor prognosis, with a median survival of 5.4 mo *vs* 29 mo in patients with a lower number of regions affected^[14-16].

Glehen *et al.*^[17], in a study of 523 patients with PC from CRC, have reported a survival of 32.4 mo in patients who had a complete cytoreduction, compared with 8.4 mo in patients in whom a radical cytoreductive surgery was not possible.

The morbidity rates reported after HIPEC combined with cytoreductive surgery range from 20% to 50% and mortality rates from 1% to 10%^[18,19].

The main morbidities associated with this advanced treatment are caused by complications of surgery and hematological toxic effects. The most frequent surgical complications include anastomotic leakage, intestinal perforation, pancreatitis, prolonged ileus, bile leak, intra-abdominal bleeding/sepsis, wound dehiscence, pulmonary embolism and renal failure.

Intra-abdominal sepsis and enteric fistulas often necessitate re-operation. Most of these complications can be attributed to the extensive surgery performed, especially when the patient has had multiple previous operations. This procedure on average may take up to 10-12 h with a median blood loss of 3-5 L, and necessitates blood transfusion, complex anesthetic management, intensive unit care, and re-operation. To add to the complexity, Sugarbaker stresses that the dosing of intraperitoneal chemotherapy often needs to be modified to keep the morbidity and mortality for these patients at 30% and 2%, respectively. He suggests using a one-third dose reduction in patients who have had prior extensive surgeries, extensive cytoreductive surgery, if multiple anastomoses needed to be performed, those who have had prior chemotherapy or radiation therapy, and those who are older than 65 years^[20].

Results of multivariate analyses have shown that the independent factors affecting morbidity are duration of

surgery, extent of carcinomatosis, the number of anastomoses performed, and sex^[21,22].

The advanced stage of neoplastic disease and immunodeficient status of patients previously subjected to chemotherapy were found to be important factors that probably contributed to the occurrence of septic complications after an extended surgical procedure.

Furthermore, there are several technical and procedural nuances, which mandate that only those who have considerable experience should perform these procedures. Indeed, both the cytoreductive surgery and the administration of HIPEC are technically demanding procedures for which learning curves exist.

In a study of 70 patients treated with HIPEC combined with cytoreductive surgery, Yan *et al*^[23] have reported a mortality of 4%, a moderate morbidity in 44% of patients and that 20% displayed severe morbidity; these workers suggested that there is a learning curve associated with this advanced treatment in order to achieve an acceptable morbidity rate. Smeenk *et al*^[24] observed that the peak of the learning curve, graded by the percentage of complete cytoreductions, was reached after approximately 130 procedures. Therefore, such a complex, aggressive modality can be a considerable drain on the resources of an institution in order to provide benefit for a small group of patients.

Recently, Elias *et al*^[25] reported a multi-institutional retrospective analysis of cytoreductive surgery and HIPEC for patients with PC from CRC. This investigation involved treatment of 523 patients over a 17-year period with almost 30% ($n = 152$) from a single institution whereas, importantly, 18 centers recorded fewer than 20 cases each. The purpose of their study was to evaluate the short and long-term efficacy of this combined approach and determine prognostic factors affecting outcome. The overall mortality was 3.3%, with 31% grade 3 to 4 morbidity, and 30.1 mo median survival. The risk of postoperative morbidity and mortality was significantly influenced by two factors: the peritoneal index (reflecting the peritoneal metastatic burden) and the center in which the treatment was performed (inexperienced centers were those with < 7 years of practice). The center experience was an independent predictor for achieving the important goal of complete cytoreductive surgery^[25].

Currently patients with CRC who develop peritoneal recurrence might benefit from cytoreductive surgery and HIPEC even in the presence of resectable liver metastasis^[15,19,26-29]. Because this treatment has been shown to be most effective in patients with absent or minimal residual disease, there might be a role for preventive HIPEC in patients at high risk of peritoneal tumor spread. In a study of 29 patients with CRC at high risk of peritoneal recurrence, PC not detected by preoperative imaging was present in 16 patients. In these cases, HIPEC resulted in a disease-free status in half of the patients after a median follow up of 27 mo^[30,31].

CONCLUSION

The clinical outcome after a diagnosis of PC has radical-

ly changed during the last two decades due to combined locoregional treatment. At the moment, HIPEC provides a promising therapeutic option for patients with PC from CRC. Patients who have minimal residual disease as a result of cytoreductive surgery are candidates for HIPEC. This approach has become an important part of CRC treatment and should become a standard modality for prevention and treatment of cancer that involves the peritoneal surface. The national health care systems of the Netherlands and France have approved this approach for colon carcinomatosis. In other countries in Europe, approval is given on a case-by-case basis.

In order to achieve maximum benefit, correct selection of the patients approved for submission to this procedure, a radical cytoreduction and an adequate learning curve are necessary.

However, further controlled studies will help to standardize indications and the technique of this locoregional therapy in order to achieve an improvement of morbidity and mortality rates.

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Alcohol and liver, 2010

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Abstract

Liver is known as an organ that is primarily affected by alcohol. Alcoholic liver disease (ALD) is the cause of an increased morbidity and mortality worldwide. Progression of ALD is driven by “second hits”. These second hits include the complex of nutritional, pharmacological, genetic and viral factors, which aggravate liver pathology. However, in addition to liver failure, ethanol causes damage to other organs and systems. These extrahepatic manifestations are regulated *via* the similar hepatitis mechanisms. In the Topic Highlight series, we provide an update of current knowledge in the field of ALD.

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Key words: Liver; Alcohol

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Our Topic Highlight: “Alcohol and liver” is annually published in *World Journal of Gastroenterology* and reviews the most important discoveries in the field of alcoholic liver disease (ALD). In the current third edition of “Alcohol

and liver”, we focus on the ethanol-induced cross-talk between the liver and other alcohol-affected organs, such as gut, brain and pancreas^[1-3]. Also, signal transduction aspects of ALD include the role of an adiponectin/interleukin-10/heme oxygenase-1 pathway^[4], the impact of alcohol on hepatitis C virus replication and interferon signaling^[5] and the role of Toll-like receptor signaling^[6]. In addition, certain reviews published in this issue shed light on molecular mechanisms of liver cell damage, as well as other aspects of ALD pathogenesis, such as the effects of ethanol on proteasome-interacting proteins^[7], alterations of hepatocyte cytoskeleton^[8], hepatoprotective effect of S-adenosyl-L-methionine against alcohol in CYP2E1-dependent liver injury^[9] and gender/hormonal differences in ALD development^[10]. We believe that this issue will be of strong interest not only for gastroenterologists, but also for those involved in alcohol, liver and brain research.

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Alcohol, inflammation, and gut-liver-brain interactions in tissue damage and disease development

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Abstract

Chronic inflammation is often associated with alcohol-related medical conditions. The key inducer of such inflammation, and also the best understood, is gut microflora-derived lipopolysaccharide (LPS). Alcohol can significantly increase the translocation of LPS from the gut. In healthy individuals, the adverse effects of LPS are kept in check by the actions and interactions of multiple organs. The liver plays a central role in detoxifying LPS and producing a balanced cytokine milieu. The central nervous system contributes to anti-inflammatory regulation through neuro-immunoendocrine actions. Chronic alcohol use impairs not only gut and liver functions, but also multi-organ interactions, leading to persistent systemic inflammation and ultimately, to organ damage. The study of these interactions may provide potential new targets for therapeutic intervention.

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Key words: Chronic alcohol use; Chronic inflammation; Lipopolysaccharides; Pro-inflammatory and anti-inflamma-

INTRODUCTION

Chronic inflammation is commonly associated with alcohol-related medical conditions. Accumulating evidence suggests that it acts as an etiological factor in the initiation and progression of many of these conditions. A significant number of illnesses of individuals with alcoholic liver diseases can be explained readily by a high level of circulating pro-inflammatory cytokines^[1]. Humans with variants of pro- and anti-inflammatory cytokine genes show increased susceptibility to alcoholic liver disease^[2]. Alcohol-mediated activation of inflammation signaling pathways can increase tumorigenesis in mice^[3]. An alcohol-induced increase of tumor necrosis factor (TNF) α , a pro-inflammatory cytokine, in the mouse brain is closely linked to neurodegeneration^[4]. In addition, mice in which the receptor gene for TNF α has been knocked out are resistant to alcohol-induced liver injury^[5].

There are two broad sources of alcohol related inflammation inducers: those derived from alcohol damaged cells and those derived from gut microflora, specifically, lipopolysaccharide (LPS). Alcohol metabolism

directly leads to the production of reactive oxygen species, known for their ability to stimulate activation of a key inflammation transcription factor nuclear factor- κ B (NF- κ B)^[6]. Hypoxia, resulting from alcohol metabolism, is also known to induce the inflammatory response^[7]. On the whole, information on such inflammation inducers and their contribution to alcohol related inflammatory conditions is still limited.

In comparison, LPS derived from gut microflora has been extensively studied as a key inducer of inflammation in alcohol-related conditions. Alcohol stimulates LPS translocation across the gut *via* a number of mechanisms, and alcoholics with liver diseases are known to have significantly elevated circulating LPS^[8]. LPS is a major cell wall component of all Gram-negative bacteria and can mimic bacterial infection in causing an acute inflammatory response. Humans have evolved complex and coordinated defenses against LPS and its adverse effect, in which the gut, the liver, the brain, as well as innate immune cells within these organs, play crucial roles. Effects of LPS in combination with alcohol on individual organs have been reviewed extensively in the past, but less attention has been given to the communication and cooperation among these organs and their actions against LPS. In this review, our goals are to capture the latest understanding of (1) the effect of alcohol on LPS translocation and dissemination; (2) the actions of the liver and brain to manage the adverse effect of LPS; and (3) the impairment by alcohol use to the integrated host control of inflammation. We hope that such an approach will lead to a better understanding of the pathogenesis of alcohol-related tissue damage and disease.

INFLAMMATORY RESPONSE

Inflammation, mediated largely by the innate immune system, is the physiological response triggered by “danger” signals derived from tissue injury or infection^[9,10]. In response to such signals, innate immune cells, such as macrophages, release pro-inflammatory cytokines and chemokines that stimulate neutrophil recruitment to the site of injury and increase vascular permeability to fluid and plasma proteins. Neutrophils, in turn, release reactive oxygen species and anti-microbial peptides and phagocytose dead and foreign material. The plasma proteins contribute proteases and opsonins to combat infection. The liver is an important innate immune organ that plays a major role in the inflammatory response by producing acute phase reactants, including C-reactive protein, mannose binding protein, complement factors, ferritin, serum amyloid A and P, and surfactant proteins^[11,12]. All acute inflammation facets work together to eliminate the insult, prevent further damage, and promote tissue repair.

An inflammatory response is triggered by the binding of a microbial product or an endogenous product of tissue damage to pattern recognition receptors (PRRs) including toll-like receptors (TLRs). Upon ligand binding, PRRs activate intracellular signaling that ultimately leads

to the expression of genes encoding pro-inflammatory cytokines and other effectors that promote, sustain and amplify the inflammatory response. However, inflammation is a double-edged sword, equipped to destroy invading pathogens, but also equally capable of damaging healthy tissue. Thus, the process also includes the synthesis of anti-inflammatory effectors to regulate the extent and duration of the inflammatory response. Neuroimmunoendocrine mechanisms coordinated in the central nervous system (CNS) also contribute to anti-inflammatory regulation. In contrast to acute inflammation, chronic inflammation in alcoholic liver disease is characterized by persistent expression of pro-inflammatory cytokines and persistent recruitment of monocytes and neutrophils^[13,14]. These cytokines and immune effector cells maintain the damaging oxidative state, leading to sustained damage to host tissue.

INTERACTIONS OF GUT, LIVER AND BRAIN IN INFLAMMATION, AND ALCOHOL'S ROLE IN ORGAN DAMAGE

Translocation of gut microbial products

Gut microflora and translocation of microbial products: The human gastrointestinal tract is home to approximately 10^{14} microorganisms, consisting mostly of commensals. Besides providing the host nutritional and energy needs and shaping host mucosal and systemic immunity, the gut microflora are increasingly recognized as an important pathogenic factor in chronic inflammation-related diseases^[15-18]. These physiological and pathological roles of gut microflora are mediated by two interactions: (1) inside the lumen of the gastrointestinal (GI) tract between microflora, or their products, and different types of host cells at the surface of the intestinal epithelium; and (2) outside the GI tract between microbial products that have translocated across the intestinal epithelial barrier and the host cells in the circulation or in different organs.

A causal role for a bacterial factor in human diseases in the absence of bacterial infection was first demonstrated in animal models of severe hemorrhagic shock-associated mortality and dietary hepatic injury^[19,20]. In both cases, there was no apparent bacterial infection and sterilization of gut microflora with oral administration of non-absorbable antibiotics significantly improved the clinical outcomes. In addition, germfree animals were resistant to diet induced liver injury^[21]. Search for a causative factor led to the findings that the diseased animals had increased circulating LPS or endotoxemia^[22]. Oral administration of LPS resensitized the antibiotic treated animals to diet-induced liver injury^[23]. Later studies showed that in healthy individuals LPS is readily detectable in the portal blood that flows from the GI tract to the liver^[24]. More recently, evidence has emerged for translocation of microbial products and their potential pathogenic role in a variety of chronic diseases including chronic heart failure, spondyloarthritis, chronic fatigue syndrome, HIV/AIDS and AIDS-associated dementia,

chronic hepatitis C, non-alcoholic steatohepatitis (as part of metabolic syndrome), and alcoholic liver disease^[25-33].

Translocation of LPS across the gut epithelial barrier has been frequently ascribed to simple diffusion across epithelial cell membranes or leaking through tight junctions between adjacent cells^[34]. Consistently, factors that reduce membrane permeability to various molecules and improve the function of tight junctions appear to reduce LPS translocation^[35,36]. Tomita *et al.*^[37] have shown that a majority of LPS transport across colonic epithelial cells uses a receptor-mediated endocytosis system involving LPS binding proteins, CD14 and TLR4. A recent study demonstrated that LPS can also be translocated across the gut epithelium by its association with chylomicrons formed on the surface of the epithelium as a vehicle for fatty acid transport^[38]. Whether these two mechanisms, receptor-mediated endocytosis and chylomicron association, represent most, if not all, of *in vivo* LPS transport across gut epithelial cell membranes is not yet known.

Enhancement of LPS translocation by alcohol: It has been well established in humans that heavy alcohol consumption is associated with an increase in gut permeability and LPS leakage, with or without liver disease. Abstinence for 2 wk or longer is necessary for increased gut permeability to return to a baseline level^[39]. Acute heavy alcohol consumption is associated with a transient appearance of LPS in the circulation in normal human subjects^[40]. Individuals with alcoholic fatty liver but not severe liver disease also show an elevated LPS level in plasma^[8]. Studies of animal models show that acute alcohol feeding in mice increases LPS in plasma approximately five-fold within 30-90 min^[41,42]. Daily binge feeding of alcohol in rats for 4 wk increases the plasma LPS level approximately 15-fold compared to control animals^[43].

Alcohol may increase LPS leakage from the gut by a variety of mechanisms. Recent studies have demonstrated that alcohol and/or acetaldehyde can directly alter gut permeability and leakiness by induction of inducible nitric oxide synthase (iNOS) and NF- κ B signaling, which, in turn, modulates a differential expression of tight junction proteins that involves the action of miRNA-122^[44-46]. In addition, chronic alcohol use can cause barrier structure defects^[42,47]. In addition, alcohol can also alter gut integrity and permeability indirectly. First, the Zn²⁺ deficiency that is common in alcoholics also adversely affects gut epithelial integrity^[48,49]. Second, in patients with alcoholic liver disease, increased circulating inflammatory cytokines and LPS may promote further increases in gut leakiness^[50,51]. Third, alcohol use also alters the mucosal immune system, which, in turn, could have an adverse effect on the structure or function of the gut barrier. For example, alcohol feeding in Rhesus Macaques alters the number of CD4⁺ and the ratio of CD4⁺/CD8⁺ cells in the mucosal immune compartment^[52]. Whether these changes affect gut barrier structure and function is yet to be explored. Finally, alcohol might also affect gut permeability *via* its stimulatory effect on neuroendocrine hor-

mones, known to regulate gut permeability. Locally released corticotropin-releasing hormone can stimulate the degranulation of mast cells in the GI tract thus releasing mediators for “enhanced” gut permeability^[53].

In addition to the adverse effect on gut barrier structure and permeability, chronic alcohol exposure also alters microflora content and composition. Alcoholics have an overgrowth of microflora compared to non-alcoholic individuals^[54,55]. Such overgrowth includes LPS-producing Gram-negative bacteria and is expected to increase microbial products in the gut and thus promote their translocation. Chronic alcohol consumption also alters the composition of the microflora^[54,56,57]. Consistent with these findings, probiotic or synbiotic treatments in alcoholic patients and animals with alcoholic liver disease restore gut permeability and reduce tissue injury^[56,58-60].

Routes of LPS dissemination and their distinct responses: After passing through the gut epithelium, LPS in the interstitial fluid can enter the systemic circulation by two routes: the portal vein and the GI tract lymphatic vessels^[61].

By the lymphatic route, lymph fluid from the GI tract moves through the ascending lymphatic vessel and the mesenteric lymph node and eventually releases LPS into the blood stream at the thoracic duct opening. By the portal vein route, LPS in the portal blood is delivered directly into the liver, where only a minor fraction that is not detoxified or excreted enters the systemic circulation *via* the hepatic central vein. In rats, under normal conditions, a vast majority of LPS absorbed from the GI tract is delivered to the liver *via* portal blood^[62], possibly due to a faster flow rate of portal blood compared to that of lymph.

A recent study revealed, surprisingly, that dietary fatty acids can drive LPS dissemination *via* the lymphatic route^[38]. Significant increases of lymphatic LPS dissemination have also been observed in experimental peritonitis and intestinal ischemic injury caused by hemorrhage or burn^[62-64]. Although experimental data are lacking, increased portal hypertension and intestinal interstitial fluid volume associated with liver disease would be expected to drive more LPS into the lymphatics. Whether alcohol affects the routes of dissemination remains to be explored.

The lymphatic LPS dissemination, rather than the portal vein, plays a dominant role in determining the level of LPS in systemic circulation^[61,62]. Upon entering the liver from the portal route most LPS is detoxified and only that escaping the process can enter circulation. However, the lymphatic route, with no significant detoxification organ in transit, will release most of the bioactive LPS into the circulation and thus make it available to different organs. Consequently, LPS released from the lymphatics is responsible for most of the LPS-induced inflammatory injury in many organs. Indeed, preference of the lymphatic LPS transport in the case of experimental peritonitis is associated with lung injury^[65]. Interestingly, acute alcohol feeding causes a rapid rise of circulating LPS in healthy animals^[41,42], implicating the involvement of the lymphatic route in alcohol use.

The most important host cell types that bind LPS and produce a large amount of inflammatory mediators are macrophages and monocytes. The liver contains the largest pool of tissue macrophages, Kupffer cells (KCs), whereas the spleen contains the largest reserve of monocytes^[66]. In response to LPS, KCs produce a significant amount of both pro- and anti-inflammatory cytokines, whereas monocytes produce predominantly pro-inflammatory cytokines (see below). Therefore, the route of LPS dissemination will also influence the types of inflammatory mediators released into circulation, which would, in turn, affect the inflammatory state in an individual.

Liver plays a key role in controlling the impact of microbial products and bears its burden during pathogenesis

Dampening the inflammatory response by detoxification and induction of anti-inflammatory cytokines:

Both clinical observation and experimental research suggest that the liver plays a critical role in LPS clearance and inactivation. Two of the best known mechanisms for dampening LPS-induced inflammation are: (1) the liver's ability to detoxify (by both Kupffer cells and hepatocytes) and excrete (by hepatocytes) LPS; and (2) the Kupffer cell's ability to generate a large amount of the anti-inflammatory cytokine, interleukin (IL)-10.

This role of liver in LPS detoxification (clearance and inactivation) has been best demonstrated by tracking the fate of labeled LPS introduced by i.v. injection. Most LPS is rapidly concentrated in the liver and either degraded in hepatocytes or excreted into the gut *via* the bile duct^[67-69]. In a healthy liver, LPS rapidly loses its biological activity^[22], and its uptake by the spleen increases when liver is diseased^[69]. Thus, the liver plays a primary role in protecting cells from exposure to microbial products.

IL-10 is a major anti-inflammatory cytokine. Like TNF α , it is produced mainly by KCs in response to LPS. IL-10 has a pleiotropic inhibitory effect on innate and adaptive immune responses. In KCs and macrophages, IL-10 strongly inhibits LPS-induced production of cytokines, including TNF α , IL-1, IL-6, and IL-10 itself^[70,71]. IL-10 also inhibits the differentiation and maturation of pro-inflammatory Th1 T cells of the adaptive immune system^[72,73].

Significantly, KCs are responsible for producing most infection- or LPS-induced IL-10 in circulation^[74,75]. Two recent studies reveal that cell-cell interactions in the liver play an important role in KC's ability to make IL-10. A liver endothelial cell-derived factor, hepatocyte growth factor (HGF), enhances the expression of the heme oxygenase-1 (HO-1) gene, which, in turn, stimulates IL-10 production in the KCs^[76]. A study in a hepatocyte-monocyte coculture model also showed that LPS-TLR4 signaling in hepatocytes stimulates IL-10 production in monocytes and likely in KCs^[77].

When liver function is compromised, more microbial products enter and/or stay in circulation^[78]. LPS can ac-

cumulate in other organs, especially the spleen^[79]. Consequently, the inflammatory response to the microbial products becomes amplified and prolonged. The impact of such circulating LPS on many organs might be significant, especially on the brain, and might exacerbate disease conditions in these organs.

Interconnections among microbial products, liver innate immune function, injury, and repair:

Gut microflora and its products also play an important role in shaping hepatic innate immunity. Livers from germ-free animals have a significantly lower number of KCs, which also show reduced activity in response to LPS^[80]. Translocation of microbial products including LPS from the gut is thus necessary for the host to maintain a sufficient number of KCs in the liver *via* proliferation or recruitment, and to stimulate and sustain (by priming) the maturation of these cells. Although additional definitive studies are needed, such primed KCs could play a number of important roles in the liver's innate immune defense. First, by phagocytosis and by producing immune mediators, primed KCs are effectors for immune attack against infection in the liver and for clearing microbial products, damaged tissue and debris. In addition, such primed KCs are responsible for initiating the acute phase response (APR), another important innate immune function of liver, in which hepatocytes synthesize many accessory factors involved in combating infection by phagocytosis, leukocytosis and leukocyte infiltration^[81]. Finally, primed KCs are likely to be competent in producing IL-10 to protect the host against an overzealous inflammatory response.

By playing a central role in detoxifying microbial products and supplying mediators for immune defense, the liver also uniquely subjects itself to non-specific inflammatory damage. The pro-inflammatory cytokines TNF α , IL-1, and IL-6, produced by KCs in response to LPS and endogenous inflammatory inducers (e.g. from apoptotic cells), are critical for inducing secondary cytokines and chemokines, and the acute phase proteins, which, in turn, play roles in other vascular events associated with inflammation, including infiltration of neutrophils. Neutrophils are cytotoxic due to the release of superoxides (*via* the respiratory burst) and proteolytic enzymes that can cause cell injury. Activation of the liver APR and chemokine production, for example, due to injury in the brain, can cause liver injury^[82]. The same cytokines also play important roles in liver tissue regeneration and repair^[83]. Therefore, it is important to identify and recognize those conditions under which this balance of injury and regeneration is disrupted and a pathological course ensues.

Alcohol's synergistic interaction with LPS in amplifying liver and systemic inflammatory conditions:

The liver is the first internal organ after the GI tract to be exposed to alcohol and the principal organ for its metabolism. Simultaneously, the liver is exposed to increased alcohol concentrations and LPS translocated

from the gut microflora. Alcohol and its metabolism can modulate the cellular response to LPS in the liver and independently generate endogenous inflammatory inducers. Thus, both alcohol and LPS act simultaneously to influence the inflammatory response in the liver and other organs.

There are four broad mechanisms by which alcohol can modulate inflammatory conditions in the liver and circulation. First, chronic alcohol exposure can amplify inflammation in the liver *via* a short term sensitizing/priming of KCs. For example, daily alcohol feeding for up to 8 wk sensitizes KCs in their response to LPS. KC sensitization depends on the increased LPS translocation by alcohol feeding^[84]. The sensitized KCs produce an increased amount of the pro-inflammatory cytokine TNF α that contributes to liver inflammation and injury^[5]. Consistent with these observations, depletion of KCs can prevent early liver injury associated with alcohol exposure^[85].

Second, long term alcohol exposure impairs the liver's ability to produce pro-inflammatory, and more significantly, anti-inflammatory cytokines. Circulating monocytes in alcoholics show reduced IL-10 production in response to LPS^[86,87]. Similarly, production of plasma IL-10 in mice is depressed after 7 wk of alcohol exposure^[88]. Although it remains to be confirmed in this setting, KC dysfunction may be responsible for the reduced plasma IL-10 because depletion of them abolishes circulating IL-10^[83]. The reduced IL-10 production in alcoholics coincides with an increased production of plasma TNF α by circulating monocytes^[89]. In supporting the anti-inflammatory function of IL-10, knockout of IL-10 and macrophage/neutrophil specific knockout of STAT3, a key IL-10 downstream target, drastically increased alcohol-induced liver inflammation in mice^[90] (Bin Gao, personal communication). In addition, reduced circulating IL-10, in combination with increased IL-6, might also play a direct role in the appearance of pro-inflammatory Th17 cells in circulation and in the liver^[91]. Thus, chronic alcohol exposure creates an imbalance in the cytokine milieu favoring systemic inflammatory conditions that can cause cell injury in various tissues.

Third, chronic alcohol consumption can independently stimulate inflammation *via* the formation of endogenous inflammation inducers: (1) Alcohol metabolism generates highly reactive byproducts, including reactive oxygen species and acetaldehyde^[92,93]. These reactive byproducts modify proteins and lipids to form adducts that are processed as foreign antigens by the host immune system. Antibodies and lymphocytes against these products are commonly found in patients with alcoholic liver diseases^[94,95]. A recent study has shown that alcoholics with liver disease have increased levels of the pro-inflammatory T-cell, Th17, both in circulation and in the liver^[91], which might be related to a cytokine imbalance caused by chronic alcohol, as in the case of IL-10 knockout mice^[96]; (2) Alcohol metabolism can promote necrosis or apoptosis of hepatocytes that can independently induce inflammation. Alcohol metabolism, especially

by pericentral hepatocytes, can lead to mitochondrial damage and hypoxia, events that cause cells to undergo necrosis and apoptosis^[97-99]. Intracellular components derived from these dead cells can also act as endogenous "danger signals" to activate the inflammatory response^[9]; and (3) Alcohol metabolism also leads to dysregulation of fatty acid metabolism, which invariably contributes to the accumulation of fatty acids in hepatocytes. Fat accumulation in cultured cells can induce the synthesis of IL-8, a pro-inflammatory chemokine important for neutrophil recruitment^[100]. Though the relative contribution to systemic inflammation remains unknown, the above alcohol-involved mechanisms are expected to significantly affect the inflammatory state within the liver.

Finally, chronic alcohol use impairs the liver's ability to detoxify LPS and other microbial products. Circulating LPS increases significantly even in those alcoholics with no clinical liver disease other than fatty liver^[8]. Thus, it is likely that fat accumulation in hepatocytes can significantly reduce their ability to detoxify LPS. Other alcohol-induced effects, such as reactive oxygen species formation, decrease in antioxidants, hypoxia, reduction of ATP production, and mitochondrial defects can cause cell injury or death. Such liver damage is expected to further diminish the liver's ability to detoxify LPS. Consequently, more LPS will escape from the liver and stay in the circulation, which in turn increases systemic inflammatory conditions and injury among different organs. This conclusion is supported by the observations that in alcoholic hepatitis patients there is a significant increase of multi-organ failure^[101]. Interestingly, the rise of circulating LPS and increased production of pro-inflammatory cytokines also raises the possibility that circulating LPS (monocyte response), not liver LPS (KC response), plays a more important role in liver and other organ injury^[87,101,102].

Brain participates in the regulation of peripheral inflammation and also bears its burden

Inflammation induces brain damage: Any event leading to an increased pro-inflammatory cytokine presence in the brain creates the potential for oxidative conditions, which can result in neuronal damage. In cases of CNS trauma or infection, further escalation of the cytotoxic conditions occurs when leukocytes infiltrate the brain. While necessary to stem the infection, leukocytes in the brain are also believed to contribute to neurodegeneration in CNS disorders such as Alzheimer's disease and multiple sclerosis. Surprisingly, even when the injury initiating event is CNS inflammation, chemokines originating in the liver are responsible for mobilizing neutrophils from the bone marrow into circulation, making them available for infiltration into the brain. Although the mechanism by which CNS inflammation is signaled to the liver has not been determined, there is evidence that liver KCs are the source of the chemokines that mobilize the neutrophils^[103,104].

Peripheral LPS injection, as a model of bacterial infection in rodents, leads to elevation of the pro-inflammatory

cytokine TNF α in the serum and the CNS, even though LPS does not cross the blood brain barrier. Remarkably, TNF α remains elevated in the brain for months after exposure to LPS, thus exerting a prolonged detrimental effect on the brain^[105].

The central pro-inflammatory cytokines induced by peripherally-injected LPS, in particular IL-6, inhibit neurogenesis in the hippocampus through blockade of the differentiation of neural progenitor cells into neurons^[106]. The TLR signaling pathway can also be activated by currently unidentified endogenous ligands and is believed to contribute to inflammation-mediated cell damage in neurodegenerative disorders such as Alzheimer's disease^[107-109].

Alcohol-related inflammation causes brain damage:

Even in the absence of specific neurological or hepatic complications, excessive drinking can lead to regional structural brain damage and cognitive dysfunction^[110], neuronal death and inhibition of neurogenesis^[111], resulting in a reduction in brain volume, including white matter^[112].

Similar to the CNS response to peripheral LPS, TNF α , and the pro-inflammatory chemokine MCP-1 are induced in the brains of mice treated intragastrically with alcohol^[4]. Separately, it has been proposed that alcohol causes neurodegeneration by reducing the activity of the pro-neuronal survival transcription factor CREB and by activating the inflammation-promoting transcription factor NF- κ B^[111]. Alcohol can also inhibit neuronal differentiation by disrupting activity-dependent neuroprotective protein signaling^[113].

Neuroimmune sensing and regulation of peripheral inflammation:

The brain plays a vital role in coordinating defense against systemic infection or injury and in restoring homeostasis to safeguard the integrity of the entire organism. Physiologically important CNS responses include fever, hyperalgesia, and other sickness behaviors, as well as activation of homeostatic stress responses [the hypothalamo-pituitary-adrenal (HPA) axis and the sympathetic nervous system (SNS)]. The presence of systemic infection or injury is rapidly signaled to the brain, both by neural routes and by circulating pro-inflammatory cytokines^[114].

The very early effectors of the immune response also communicate to the CNS that homeostasis has been threatened. The CNS, in turn, responds by activating the HPA axis and the SNS, the actions of which are largely anti-inflammatory, to keep inflammation in check. The anti-inflammatory signals of the HPA and SNS supplement intrinsic immune anti-inflammatory mechanisms to restrict the extent and duration of inflammation.

Signals reaching the paraventricular nucleus of the hypothalamus lead to the activation of the HPA axis, resulting in the synthesis of corticotropin releasing hormone by the hypothalamus, which in turn activates the pituitary gland to produce adrenocorticotrophic hormone, which subsequently induces synthesis and release of the

glucocorticoid cortisol from the adrenal cortex. Glucocorticoids inhibit the production of pro-inflammatory cytokines and promote the release of anti-inflammatory cytokines including IL-10 following binding to glucocorticoid receptors on leukocytes^[115-119].

In parallel, the SNS is also activated by peripheral inflammation. SNS activation leads to peripheral release of epinephrine and norepinephrine, which also have anti-inflammatory effects at the systemic level. These catecholamines act through specific receptors expressed on innate immune cells to suppress the pro-inflammatory response and promote anti-inflammatory cytokine synthesis^[120]. Thus, the glucocorticoids and neuroendocrine catecholamines produced by the HPA axis and SNS response work in concert with the anti-inflammatory cytokines produced by immune cells to contain inflammation. Since an unchecked pro-inflammatory response will damage healthy tissue due to the highly oxidative and cytotoxic milieu, the counter-regulatory mechanisms are essential to maintain tissue integrity.

Alcohol interferes with neuroimmune response and regulation:

Alcohol ingestion is recognized as a stressful event by the HPA axis; a single dose of alcohol resulting in a blood alcohol level of 100 mg percent activates the HPA axis, leading to elevated cortisol levels^[121]. Chronic exposure to alcohol increases the tolerance of the HPA axis to alcohol, such that serum cortisol after alcohol consumption is lower than in non-alcohol-dependent controls^[121]. In addition, chronic alcohol also leads to a blunting of the HPA axis in response to external stressors other than alcohol. Tolerance to alcohol by the neuroendocrine system seems to begin to develop prior to full blown alcohol dependence and may correlate with the amount of alcohol consumed^[122]. In addition, alcohol-induced complications can uncouple the HPA axis and the SNS responses to stress. Patients with alcoholic cirrhosis have increased basal sympathetic tone, as measured by neuropeptide Y production, compared to healthy controls while displaying reduced serum cortisol levels compared to controls^[123]. The absence of cortisol due to the blunting of the stress responses allows inflammation to persist beyond the point where it is beneficial. Furthermore, in the context of alcoholic cirrhosis, the uncoupling of the HPA axis and the SNS has harmful hemodynamic consequences^[123]. The potential role of neuroendocrine factors to mediate the effects of acute alcohol on inflammation has been investigated^[124]. However, whether the blunting of the HPA axis directly mediates the effect of chronic alcohol consumption on the inflammatory state remains to be determined.

Since the low-grade chronic inflammation associated with chronic alcohol consumption is present even in the absence of any obvious infection, it is possible that LPS leakage from the gut and poor clearance by the liver may be underlying factors. In view of the tolerance that humans and animals develop to the stimulation of the HPA axis by either alcohol^[125-127] or LPS^[128], LPS will not be able to sufficiently activate the HPA axis and subsequent

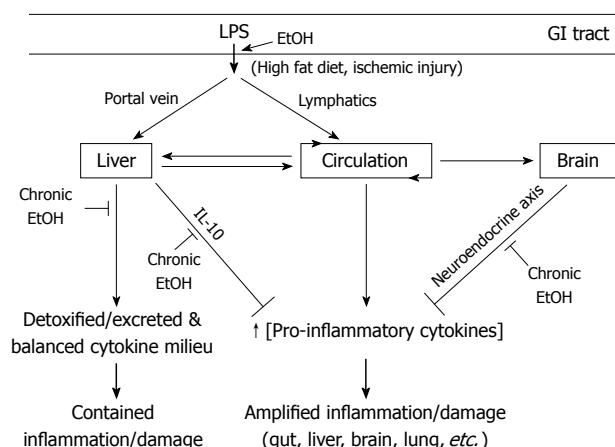


Figure 1 Gut microflora derived lipopolysaccharide (LPS)-dissemination, detoxification, and multi-organ host response and effects of alcohol use. A number of key events in LPS translocation and host response are depicted. First, upon translocation across the gut barrier, LPS enters circulation via two different routes: the portal vein or lymphatic vessels. LPS in the portal vein is directly delivered to the liver and most of it is detoxified and excreted, whereas most LPS in lymphatics are released into the circulation at the thoracic duct opening. Between the two routes, portal dissemination is dominant under normal conditions whereas a few known conditions including fatty diet and ischemic injury stimulate lymphatic dissemination. Second, a healthy liver plays a key role in the detoxification of LPS in the portal vein and systemic circulation but also generates a balanced cytokine milieu that lead to a contained inflammation and limited damage. Third, LPS in circulation is accessible to many organs and plays a major role in multi-organ damage, especially when LPS detoxification in liver is compromised. Lastly, LPS and its immune mediators (pro-inflammatory cytokines) can activate the neuroendocrine response in the central nervous system (CNS) that leads to the activation of the hypothalamo-pituitary-adrenal (HPA) axis and then the synthesis of cortisol, which down-regulates inflammatory responses in the periphery. A number of key sites under alcohol influence are depicted. First, alcohol enhances LPS translocation across the gut barrier by increasing microflora content and impairing gut barrier function. Second, alcohol stimulates LPS dissemination via lymphatics, evidenced by a rapid rise in circulating LPS in healthy animals fed with acute alcohol. The potential use of the portal route remains to be determined. Third, chronic alcohol use not only adversely affects the liver's ability to detoxify LPS but also affects the liver's ability to synthesize a key anti-inflammatory cytokine, interleukin (IL)-10. Lastly, chronic alcohol reduces the synthesis of neuroendocrine effectors that regulate systemic inflammation. Together, chronic alcohol impairs the balance of microflora in the gut, gut barrier function, the liver's ability to detoxify bacterial products and to generate a balanced cytokine milieu, and the brain's ability to regulate inflammation in the periphery. GI: Gastrointestinal.

glucocorticoid release. The net outcome is that the alcohol-dependent individual would have a reduced capacity to suppress LPS-induced inflammation.

CONCLUSION

Heavy alcohol consumption contributes to systemic inflammation by interfering with the body's natural defenses against the influx of gut microbiota and its products. Chronic alcohol use impairs the balance of microflora in the gut, the gut barrier function, the liver's ability to detoxify bacterial products and to generate a balanced cytokine milieu, and the brain's ability to regulate inflammation in the periphery. When these defenses are impaired, systemic inflammation ensues. The sustained inflammation has the potential to damage host tissues, beyond the local injury to the gut and the liver, including

the brain. It also has the potential to alter adaptive immunity that can lead to lymphocyte-mediated inflammation and tissue injury.

As detailed in this review and Figure 1, many potential sites for alteration of gut and liver function and the complex network of neuroimmunoendocrine interactions by alcohol remain to be explored. Both acute and chronic alcohol consumption can cause devastating morbidity and mortality in the drinking population. Research into the effects of alcohol on gut leakiness and LPS translocation, mucosal and liver immunity, and CNS-liver interactions are important to the understanding of alcohol's potential to disrupt host inflammation control mechanisms and consequent damage to all tissues in the body. Delineating the mechanism by which alcohol disrupts homeostatic control of inflammation may provide insights into effective preventative or therapeutic measures.

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Alcoholic pancreatitis: Lessons from the liver

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of the knowledge that we have gained regarding the effects of alcohol on the liver to the pancreas.

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Abstract

The association between alcohol consumption and pancreatitis has been recognized for over 100 years. Despite the fact that this association is well recognized, the mechanisms by which alcohol abuse leads to pancreatic tissue damage are not entirely clear. Alcohol abuse is the major factor associated with pancreatitis in the Western world. Interestingly, although most cases of chronic pancreatitis and many cases of acute pancreatitis are associated with alcohol abuse, only a small percentage of individuals who abuse alcohol develop this disease. This situation is reminiscent of the association between alcohol abuse and the incidence of alcoholic liver disease. The liver and the pancreas are developmentally very closely related. Even though these two organs are quite different, they exhibit a number of general structural and functional similarities. Furthermore, the diseases mediated by alcohol abuse in these organs exhibit some striking similarities. The diseases in both organs are characterized by parenchymal cell damage, activation of stellate cells, aberrant wound healing, and fibrosis. Because of the similarities between the liver and the pancreas, and the alcohol-associated diseases of these organs, we may be able to apply much

INTRODUCTION

Alcohol abuse is a major cause of morbidity and mortality in the United States; each year it is responsible for more than 100 000 deaths and directly or indirectly costs over 185 billion dollars^[1]. Alcohol affects every organ system in the body causing a variety of disorders. Although not the most common alcohol-associated disease, alcoholic pancreatitis is one of the more painful and serious consequences of alcohol abuse.

Alcoholic pancreatitis has been recognized for well over 100 years, yet it remains one of the least understood alcohol-associated diseases. The natural course of alcoholic pancreatitis is not well known. This is, in part, a result of the fact that traditionally pancreatitis has been classified by morphology rather than etiology, as well as the fact that confusing and imprecise criteria have been used to differentiate between different types of pancreatitis. Additionally, there is no good animal model of chronic pancreatitis, and progression of this disease in human beings has been difficult to elucidate.

The mechanism(s) by which alcohol abuse induces alcoholic pancreatitis is not well understood. Although it is evident that alcohol abuse can have an important role in the development of pancreatitis, it does not appear that alcohol abuse alone is responsible for the development of pancreatitis. Rather, it appears that ethanol sensitizes the pancreas to injury and other factors are required to actually develop alcoholic pancreatitis. A number of factors, including cigarette smoking, high-lipid diet, genetics, and infections have been suggested as possible cofactors^[2].

There are a number of similarities between alcoholic liver disease and alcoholic pancreatitis; there are also a number of differences. In this review we will attempt to highlight some of the similarities, as well as some of the important differences between alcoholic liver disease and alcoholic pancreatitis. It is hoped that this approach will provide further insight into the pathogenesis of alcoholic pancreatitis.

ALCOHOLIC PANCREATITIS: ACUTE VS CHRONIC

Pancreatitis is a necroinflammatory disease that is normally classified as either acute or chronic. In developing countries, alcohol abuse has been reported to be the second most common factor associated with acute pancreatitis. In these countries, alcohol abuse is associated with approximately 35% of the cases^[3]. Inappropriate intracellular activation of trypsinogen has long been considered an initiating event in acute pancreatitis, resulting in destruction of pancreas parenchymal cells and inflammation^[4]. In most individuals, acute pancreatitis is a mild self-limiting disorder, but in up to 20% of the cases there are severe clinical complications and mortality^[3].

Alcoholic chronic pancreatitis is a complex disease normally thought to have an early stage that is associated with recurrent attacks of acute pancreatitis, and a late stage that is characterized by steatorrhea, diabetes, fibrotic scarring, and pancreatic calcification^[5]. In the western world, chronic alcohol abuse is the major etiological factor of chronic pancreatitis and accounts for approximately 70% of the reported cases^[6]. Although pancreatitis may remain an acute disease, it appears that in many cases alcoholic acute pancreatitis progresses to alcoholic chronic pancreatitis. The progression of alcoholic acute pancreatitis to alcoholic chronic pancreatitis is generally associated with the frequency and severity of acute attacks^[7].

ETHANOL METABOLISM

It is generally thought that the liver is a target of the toxic effects of alcohol because of its ability to metabolize alcohol. Like the liver, the pancreas possesses the enzymes responsible for ethanol metabolism. Therefore, it has been proposed that the pancreas is also a target for the toxic effects of ethanol metabolism^[8-10].

Alcohol can be metabolized by oxidative, as well as nonoxidative, pathways. The oxidative metabolism of ethanol is primarily carried out by two enzymes, the cytosolic enzyme alcohol dehydrogenase and the microsomal enzyme cytochrome P450 2E1. Metabolism of ethanol by either of these enzymes results in the production of the reactive intermediate acetaldehyde, and the production of reactive oxygen species. Although the pancreas expresses both alcohol dehydrogenase and cytochrome P450 2E1, the capacity for oxidative metabolism by the pancreas is significantly less than that of the liver^[11,12].

Nonoxidative metabolism of ethanol is carried out by a number of enzymes, the most important being the fatty acid ethyl ester synthases. Metabolism of ethanol by these enzymes results in the formation of fatty acid ethyl esters (FAEEs). Although the capacity of oxidative metabolism in the pancreas is lower than in the liver, the capacity for nonoxidative metabolism in the pancreas is high because the pancreas has high fatty acid ester synthetic activity^[13]. The nonoxidative and oxidative pathways of ethanol metabolism are linked; when the oxidative pathway is low or impaired the nonoxidative pathway is enhanced^[9]. Because the oxidative metabolism of ethanol in the pancreas is relatively low, the nonoxidative metabolism of ethanol may be more important and the production of FAEEs and their toxic effects accentuated. It has been shown that infusion of FAEEs into rats causes edema, trypsin activation, and vacuolization of pancreas acinar cells^[14]. FAEEs have also been shown to activate the transcriptional activators nuclear factor (NF)- κ B and AP-1, which are involved in the activation of proinflammatory cytokines^[8]. Additionally, FAEEs have been shown to increase the fragility of lysosomes in pancreatic acinar cells^[15]. It has also been shown that FAEEs can inhibit the degradation of extracellular matrix proteins, and therefore may have an involvement in the fibrotic scarring characteristic of alcoholic chronic pancreatitis^[16].

Because the expression of alcohol dehydrogenase and cytochrome P450 2E1 are low in the pancreas, the oxidative metabolism of ethanol is also relatively low and is not considered to contribute a major role in many of the biochemical and pathologic changes associated with pancreas acinar cells. That being said, acetaldehyde, a reactive metabolite of the oxidative metabolism of ethanol, has been shown to mediate some detrimental effects in the pancreas. It has been shown that acetaldehyde is involved in the regulation of the transcriptional activators NF- κ B and AP-1 in pancreatic acinar cells^[8]. Additionally, Masamune *et al.*^[17-19] demonstrated that acetaldehyde, as well as the fatty acid ethyl ester, palmitic acid ethyl ester, activates the mitogen-activated protein kinases p38, ERK1/2, and JNK/SAPK in isolated pancreatic stellate cells. Furthermore, these authors reported that acetaldehyde activated the transcriptional activator AP-1, but not NF- κ B in these cells. In addition, activation of the AP-1 pathway was inhibited by the antioxidant N-acetyl-cysteine (NAC). Because the effects of acetaldehyde were inhibited by inclusion of NAC in the media, the authors concluded

that activation of these signal transduction pathways were mediated by the presence of reactive oxygen species.

Despite the fact that the contribution of the oxidative and nonoxidative pathways of ethanol metabolism differs between the pancreas and the liver, it appears that metabolism of ethanol plays an important role in the disease process in both organs.

CELL DEATH IN PANCREATITIS

Acinar cell death is a major complication of alcoholic pancreatitis and has been linked to the eventual fibrotic scarring associated with this disease. The two major pathways of cell death, apoptosis and necrosis, have both been shown to occur in experimental models of pancreatitis, and it appears that the severity of disease is linked to whether apoptosis or necrosis predominates^[20-22].

Cell death as a result of apoptosis is mediated by a group of cysteine proteases known as caspases. Apoptotic cell death generally preserves the integrity of the cell membrane, whereas necrotic cell death generally results in rupture of the cell membrane and release of the cellular contents. Release of the cellular contents can damage neighboring cells and initiate an inflammatory response^[23]. It has been shown that the severity of experimental pancreatitis directly correlates with the amount of necrosis, and inversely correlates with the extent of apoptosis^[20,22]. Additionally, one of the main indicators of the outcome of pancreatitis in human beings is the extent of necrosis^[24]. In experimental animals, it has been shown that alcohol treatment prior to induction of pancreatitis enhances the necrotic response by decreasing the expression of the initiator caspase, caspase-8. This reduces the apoptotic response, and results in more severe pancreatitis^[25,26]. Thus, modulating the pathway of cell death during pancreatitis may be a mechanism by which alcohol consumption predisposes the pancreas to more severe damage, or alters the severity of pancreatic injury. This modulation can potentially change a subclinical episode of pancreatitis into a clinical episode.

PANCREATIC STELLATE CELLS

The pancreas, like the liver, possesses stellate cells^[27]. Pancreatic stellate cells appear to be morphologically and functionally similar to hepatic stellate cells. In fact, transcriptome analysis of pancreatic and hepatic stellate cells revealed that only 29 of the 23000 genes analyzed differed in expression^[28]. These results indicate that the two populations of cells are very closely related. Stellate cells in the pancreas, as in the liver, are thought to have a major role in extracellular matrix remodeling^[29,30]. Thus, pancreatic stellate cells appear to have a major role in fibrotic disorders of the pancreas, such as chronic pancreatitis and pancreatic cancer^[31,32]. Whereas hepatic stellate cells normally reside in the space of Disse, pancreatic stellate cells are primarily located in the periacyinar space,

but are also present in the periductal and perivascular areas of the pancreas^[33,34]. Pancreatic stellate cells comprise approximately 4% of all pancreatic cells; in contrast, hepatic stellate cells comprise approximately 8% of all hepatic cells^[33]. In the normal pancreas, stellate cells are characterized by the presence of vitamin A-containing lipid droplets in their cytoplasm, and by expression of the cytoskeletal proteins desmin and glial fibrillary protein^[33,34]. In response to pancreas injury or acetaldehyde, these cells are activated into a highly proliferative state and are transformed to myofibroblast-like cells^[27]. Activated stellate cells are characterized by the absence of lipid droplets, expression of α -smooth muscle actin, and the production of type-1 collagen, as well as other extracellular matrix proteins^[30]. Activated pancreatic stellate cells have been shown to be closely associated with areas of pancreatic fibrosis. They are the principal source of type 1 collagen in pancreatic fibrosis, both in humans and experimental animal models^[35].

Numerous paracrine factors have been demonstrated to participate in the activation of pancreatic stellate cells. Included in these factors are cytokines such as interleukin (IL)-1, IL-6, IL-8, and tumor necrosis factor α , growth factors such as platelet-derived growth factor (PDGF) and transforming growth factor (TGF)- β 1, angiotensin II, acetaldehyde, and reactive oxygen species released by damaged parenchymal cells, or leukocytes recruited in response to tissue injury^[36-39]. In turn, the activated stellate cells produce autocrine factors such as PDGF, TGF- β 1, IL-1, and IL-6, which are thought to perpetuate the activation of the pancreatic stellate cells.

It has been demonstrated that both hepatic and pancreatic stellate cells express NADPH oxidase, and it has been proposed that this enzyme has a role in the fibrosis associated with chronic pancreatitis^[40,41]. NADPH oxidase is a multicomponent enzyme originally described in phagocytic cells as a source of reactive oxygen species. In phagocytic cells, the activated complex produces extracellular superoxide that has an important role in defense against microbial infection. The NADPH oxidase expressed by stellate cells and the enzyme complex expressed by phagocytic cells exhibit some important differences. One of the most important differences is the fact that in stellate cells NADPH oxidase is constitutively active, producing low levels of reactive oxygen species.

Originally, it was shown that angiotensin II mediated its profibrotic action in the liver through NADPH oxidase activity in hepatic stellate cells^[40]. *In vivo*, mice lacking NADPH oxidase activity demonstrated attenuated liver fibrosis after bile duct ligation. Investigation of the role of NADPH oxidase in pancreatic stellate cells revealed that PDGF- $\beta\beta$, IL-1 β , and angiotensin II induced production of reactive oxygen species in cultured pancreatic stellate cells. The increase in reactive oxygen species and subsequent activation of downstream signaling pathways were abolished by inhibition of NADPH oxidase activity with diphenylene iodonium (DPI)^[42].

Additionally, treatment with DPI attenuated the development of pancreatic fibrosis in Wistar Bonn/Kobori rats and in rats with dibutyltin dichloride-induced chronic pancreatitis^[42]. In a separate study, it was shown that ethanol enhanced PDGF-mediated activation of pancreatic stellate cells, indicating that NADPH oxidase may have a role in alcoholic pancreatitis^[41].

The cascade of pancreatic damage, activation of pancreatic stellate cells, and progression to fibrosis has been termed a necroinflammatory response. It has been suggested that alcohol abuse can result in pancreas damage by a nonnecroinflammatory response as well. Interestingly, pancreatic stellate cells express alcohol dehydrogenase and are able to produce acetaldehyde^[27]. Because acetaldehyde has been shown to activate pancreatic stellate cells, it has been suggested that this ability may itself result in the activation of pancreatic stellate cells. In turn, this activation may result in the initiation, as well as the perpetuation of, alcohol induced pancreatitis and pancreatic fibrosis^[27].

Tissue repair is a process that is regulated by an intricate balance between the synthesis and degradation of extracellular matrix components^[43]. As mentioned above, pancreatic stellate cells are critically important in the remodeling of the extracellular matrix. This remodeling involves not only synthesis of extracellular matrix components but their degradation as well. Pancreatic stellate cells secrete a number of matrix metalloproteinases (MMPs), enzymes that are involved in extracellular matrix degradation^[44]. The MMPs are a family of zinc-dependent enzymes secreted by a variety of cells that are able to degrade extracellular matrix components. Thus, pancreatic stellate cells are not only involved in pancreatic damage; they are also critically involved in recovery and repair of pancreatic damage^[45].

Specifically, pancreatic stellate cells have been shown to secrete MMP1, MMP2, MMP3, MMP9, and MMP13^[39,44,46]. MMPs have different substrate specificities; MMP2 and MMP9 primarily degrade basement membrane collagen (type-4), whereas MMP1 and MMP13 degrade type-1 collagen. The secretion of MMP2 is greatly increased by ethanol and acetaldehyde, and is secreted far in excess of MMP13^[44]. This finding led the authors to suggest changes in MMPs secretion as an explanation for the accumulation of fibrotic type 1 collagen in pancreatitis.

Pancreatic stellate cells have also been shown to secrete enzymes that inhibit the activity of MMPs, these enzymes are known as tissue inhibitors of metalloproteinases (TIMPs). TIMP2 is an important inhibitor of MMP2, the matrix metalloproteinase responsible for the degradation of basement membrane collagen. Ethanol and acetaldehyde have been shown to increase the secretion of TIMP2. However, this increase in TIMP2 was significantly less than the increase in MMP2. The overall result was an increase in MMP2 activity^[44]. Interestingly, similar findings have been reported in hepatic stellate cells, again indicating the close functional relationship between these two cell types^[47].

In a model of acute pancreatitis where the fibrotic damage was resolved, both MMP2 and MMP1 activity were shown to increase, but were regulated in a different temporal fashion. MMP2 activity increased soon after injury, while MMP1 activity increased later^[46]. These results indicate different roles for different MMPs in repair and regeneration of the pancreas. They also demonstrate that there is a delicate balance and interplay between different MMPs in the normal recovery of pancreatic injury.

Understanding the factors and mechanisms that maintain stellate cells in a quiescent state or return them to a quiescent state is as important as understanding the factors and mechanisms of stellate cell activation and fibrotic scarring. In both the liver and the pancreas, the nuclear hormone receptor peroxisome proliferator-activated receptor- γ (PPAR- γ) has been implicated as being involved in inhibition of stellate cell activation^[18,48,49]. PPAR- γ is a nuclear hormone receptor that is activated by specific ligands and has been shown to inhibit key characteristics of activation, including induction of collagen synthesis in both hepatic and pancreatic stellate cells^[18,48,49]. Acetaldehyde has been shown to activate hepatic stellate cells, at least in part, by abrogating PPAR- γ -mediated transcriptional activity in cultured hepatic stellate cells^[50]. Additionally, the profibrogenic actions of acetaldehyde were shown to be associated with increased phosphorylation of PPAR- γ at a MAP kinase phosphorylation site. These results demonstrate the involvement of MAP kinases in the inhibition of PPAR- γ -mediated transcriptional activity, and ultimately stellate cell activation^[50].

Vitamin A (retinol) also appears to be a major contributor to the maintenance of quiescence in pancreatic stellate cells; its absence in active pancreatic stellate cells may be related to ethanol metabolism^[51,52]. In support of this notion, McCarroll *et al.*^[52] demonstrated that retinol and its metabolites all trans retinoic acid and 9-cis retinoic acid induce quiescence in activated pancreatic stellate cells. Furthermore, quiescence of pancreatic stellate cells was associated with a decrease in the activity of all three classes of MAP kinases. In the presence of ethanol, retinol was not able to fully inhibit activation of pancreatic stellate cells. Because retinol and ethanol can both be metabolized by either alcohol dehydrogenase or by retinol dehydrogenase, the authors suggest that ethanol may act as a competitive inhibitor of normal retinol metabolism, and that this inhibition may, in part, explain the ability of ethanol to activate pancreatic stellate cells^[52].

REGENERATION AND REPAIR

The pancreas, like the liver, has a tremendous capacity to regenerate after injury^[53,54]. As with all fibrotic diseases, pancreatitis can be considered a result of an aberrant or inappropriate repair process. Thus, it is as important to have a detailed understanding of the mechanisms of pancreas repair and the effects of ethanol on this process, as it is to determine the mechanisms by which alcohol abuse leads to pancreatitis. In animal models of

pancreatitis, normal regeneration is characterized by a sequence of events that includes activation and proliferation of pancreatic stellate cells, deposition of extracellular matrix, and proliferation of acinar cells^[53,55]. It is now evident that this regenerative process is orchestrated by the temporal and sequential expression of growth factors that act in either an autocrine or paracrine manner to stimulate cells. This coordinated process ultimately results in full structural and functional restitution of the pancreas. Little is known regarding the effects of ethanol on this process.

While investigating the effects of ethanol consumption on regeneration after cerulein-induced pancreatitis, one group, using the Lieber-DeCarli diet, found that ethanol feeding of rats for 2-8 wk significantly decreased amylase content of the pancreas. This finding indicated impaired functional regeneration. This treatment did not affect protein, DNA, or RNA content. Although no histological evaluation was performed, these authors concluded that ethanol had no effect on pancreatic regeneration^[56,57]. In contrast, Pap *et al.*^[58] found that gastric intubation of ethanol over a 2 mo period inhibited the recovery of pancreatic weight and enzyme content in rats that had common bile ducts and main pancreatic ducts occluded to induce pancreatic injury. During this 2 mo period, the animals exposed to ethanol developed chronic calcifying pancreatitis. Cessation of the ethanol treatment resulted in recovery of the pancreas. These results led the authors to suggest that inhibition of pancreatic regeneration by ethanol was required to maintain this chronic pancreatitis. Investigating the role of cholecystokinin (CCK) in pancreatic regeneration, it has been shown that alcohol administration reduced CCK release and prevented pancreas regeneration^[59]. Additionally, using a model of virally induced pancreatitis, it has been shown that ethanol administration to mice delays pancreas recovery^[60].

The pancreas and the liver are developmentally very closely related. Therefore, it is tempting to speculate that the regenerative process, and the effects of ethanol on this process, may be similar in the pancreas and the liver^[61]. In the liver, it has been demonstrated that ethanol abuse impairs regeneration^[62,63]. Both the pancreas and the liver contain a population of progenitor cells that are multipotent and able to differentiate into multiple cell types^[64,65]. In the liver, these hepatic progenitor cells have been shown to be important in regeneration in alcoholic liver diseases and in other hepatic diseases where oxidative stress has occurred^[66,67]. Thus, it may be that repair initiated by pancreatic precursor cells may be impaired by ethanol as well. It is clear that more work is required to determine the effects of ethanol on regeneration and repair of pancreatic damage.

CLINICAL IMPLICATIONS AND FUTURE DIRECTIONS

It is obvious that alcoholic pancreatitis is an important

health concern and more focus on this disease is warranted. It appears that, as with alcoholic liver disease, the fibrotic scarring associated with chronic pancreatitis is primarily associated with activation of stellate cells. Because of this, it is likely that future research directed at the mechanisms by which ethanol and its metabolites mediate or enhance the activation of these cells will provide targets for therapeutic intervention. Currently, some promising therapeutic candidates include NADPH oxidase and receptors located on the surface of stellate cells.

Future work should strive to understand the mechanisms by which stellate cells remain quiescent. It is clear that balancing the need for activation of stellate cells for repair and wound healing with maintenance of the quiescent state will be challenging. Because similar mechanisms of fibrotic scarring occur in the liver, it will be prudent to monitor progress in this field and apply it to the pancreas.

CONCLUSION

The pancreas and the liver are developmentally closely related and, in a general sense, share certain structural and functional similarities. It is well known by the general public that alcohol abuse can result in liver damage and lead to cirrhosis. It is not as well known that alcohol abuse can also result in pancreatic damage and ultimately in alcoholic chronic pancreatitis. Although there are many differences between the liver and the pancreas, and the alcohol-associated diseases of these organs, there are also many similarities. Because of these similarities, lessons learned regarding the effects of ethanol on the liver may be useful in determining mechanisms by which ethanol damages the pancreas.

Both the liver and the pancreas possess stellate cells. Although some of the biochemical changes that occur as a result of ethanol metabolism in the liver and the pancreas differ, the end result in both organs is the activation of stellate cells. In both organs the activated stellate cells are the major source of fibrotic collagen. Because fibrosis is a characteristic of alcohol-associated damage in both organs, stellate cells are the focus of therapeutic intervention. Among the questions that require further investigation are the mechanisms that will inhibit hyperactivation or inappropriate activation of stellate cells. Paradoxically, activated stellate cells are also involved in the removal of fibrotic collagen. Therefore, determining the mechanisms of appropriate activation, and enhancing the ability of these cells to degrade fibrotic collagen are paramount. Additionally, mechanisms of repair and regeneration must be further delineated. Are progenitor cells involved in repair of alcohol-associated damage in the pancreas, as they are in the liver?

It is clear that although there are differences between the effects of alcohol on the liver and the pancreas, there are also striking similarities. Lessons learned from the liver may be useful in answering questions regarding the pancreas.

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Alcoholic liver disease and the gut-liver axis

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INTRODUCTION

The clinical spectrum of alcoholic liver disease (ALD) includes alcoholic fatty liver, alcoholic steatohepatitis, alcoholic cirrhosis (Laennec's cirrhosis), and increased risk of hepatocellular carcinoma^[1,2]. The pathomechanism of ALD involves complex interactions between the direct effects of alcohol and its toxic metabolites on various cell types in the liver, induction of reactive oxygen species (ROS), upregulation of the inflammatory cascade, and other cell-specific effects in the liver^[3,4]. Lipopolysaccharide (LPS), also known as endotoxin, has been identified as a major factor in the pathogenesis of ALD. Indeed, LPS can lead to liver steatosis, as it induces inflammation and contributes to cirrhosis, which are all features of ALD^[5,6]. These effects of LPS are manifested in the various cell types in the liver and the source of LPS appears to be the gut in ALD, resulting from alcohol-induced disturbance of gut permeability. At the cellular and molecular level, LPS is recognized by the Toll-like receptor 4 (TLR4) complex and induces specific intracellular activation pathways. This review will focus on the role of LPS in ALD and will summarize the current state of art on alcohol-related changes in the gut-liver axis.

GUT-LIVER AXIS

The gut is a habitat for billions of microorganisms and the gut mucosal epithelium serves as a barrier between microbiota and gut lumen^[7]. LPS (endotoxins) derived from Gram-negative bacteria in the intestinal microflora normally penetrate the mucosa only in trace amounts,

Abstract

Alcoholic liver disease (ALD) is one of the leading causes of liver diseases and liver-related death worldwide. Of the many factors that contribute to the pathogenesis of ALD, gut-derived lipopolysaccharide (LPS) plays a central role in induction of steatosis, inflammation, and fibrosis in the liver. In this review, we discuss the mechanisms by which alcohol contributes to increased gut permeability, the activation of Kupffer cells, and the inflammatory cascade by LPS. The role of the Toll-like receptor 4 (TLR4) complex in LPS recognition and the importance of the TLR4-induced signaling pathways are evaluated in ALD.

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Key words: Kupffer cell; Gut permeability; microRNA; Tumor necrosis factor- α ; Endotoxin

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enter the portal circulation, and become cleared in the liver to maintain the control of immune homeostasis. Resident macrophages (Kupffer cells) and hepatocytes both contribute to this process through different LPS recognition systems^[3,8-10]. There is a positive correlation between liver dysfunction and the occurrence of bacterial translocation^[11-14], and the clearance of LPS from the circulation is decreased in states of hepatic dysfunction, such as cirrhosis^[15]. Studies in animals suggest that the liver quickly removes about 40%-50% of an intravenous dose of LPS from the bloodstream^[16,17]. Hepatic uptake and detoxification is important for preventing systemic reactions to blood-borne LPS. It has been proposed that LPS initially is taken up by Kupffer cells and then by hepatocytes^[18]. LPS is removed *via* several mechanisms, including molecules that bind LPS and prevent it from activating TLR4, enzymes that degrade the lipid A moiety to decrease its activity, inactivation of LPS following uptake into the liver and spleen, and cellular adaptations that modify target cell responses^[19]. Another mechanism for LPS neutralization is by serum lipoproteins, HDL, LDL, VLDL, and chylomicrons, apolipoproteins apoE and apoA-I LPS^[20-22]. All of these mechanisms can chaperone endotoxin to hepatocytes, Kupffer cells, or sinusoidal endothelial cells, resulting in clearance of LPS without significant inflammatory cell activation.

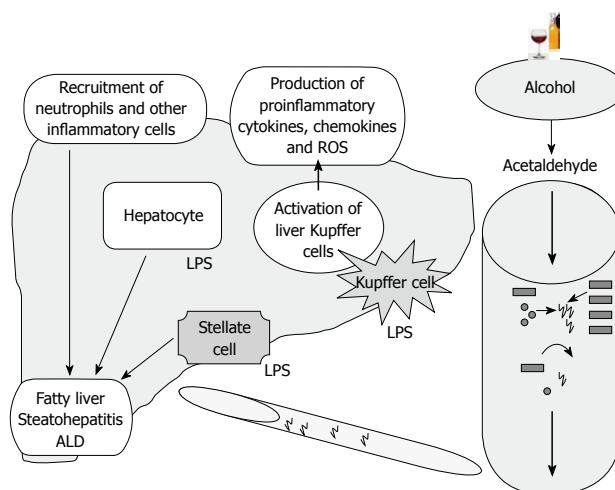


Figure 1 Mechanisms of alcohol induced liver damage. Alcohol consumption alone, or with its metabolites, disrupts the gut integrity by various mechanisms, including increased reactive oxygen species (ROS), inducible nitric oxide synthase (iNOS), alteration of microRNAs, proliferation of Gram-negative bacteria, and changes in bacterial species. These factors alone, or in combination, mediate increased gut permeability and subsequent bacterial or microbial translocation into intestinal lumen and thus an increase in lipopolysaccharide (LPS) in the portal circulation. The excess of LPS in the liver affects immune, parenchymal, and non-immune cells and in response there is release of various inflammatory cytokines, and recruitment of neutrophils and other inflammatory cells. Persistence of the above mentioned factors are hallmark of alcoholic liver disease (ALD).

ALD AND ENDOTOXIN

The role of LPS in alcoholic liver injury has been shown in several studies^[12,23,24]. The importance of gut-derived endotoxin in ALD was suggested by experiments where treating the animals either with antibiotics or with lactobacilli to remove or reduce the gut microflora provided protection from the features of ALD^[12,25,26]. In mice and rats, circulating endotoxin levels were increased after chronic alcohol feeding^[27,28] and plasma endotoxin levels were also increased in patients with ALD compared to normal subjects^[29]. The persistence of endotoxin not only activates the liver immune cells but also affects the function of liver parenchymal cells.

The progression of ALD is a complex phenomenon, as it not only results from the direct effects of alcohol and its metabolites, but other factors also play an important role in its pathogenesis, such as leaky gut, which results in endotoxemia^[30]. Both chronic ethanol-mediated microbial proliferation^[31,32] and acetaldehyde-mediated opening of intestinal tight junctions (TJs)^[33] enhance the passage or release of endotoxins into the intestinal lumen, which are later transported to liver. However, when excess amounts of endotoxin are not cleared efficiently by the liver and accumulate in blood circulation, innate immune cells, including Kupffer cells, are activated, leading to the release of various pro-inflammatory cytokines, chemokines, and other factors^[34,35].

Kupffer cell activation has been identified as one of the key elements in the pathogenesis of alcoholic steatohepatitis. Studies in mice and rats demonstrated that

inactivation of Kupffer cells with gadolinium chloride or clodronate injection can almost fully ameliorate alcohol-induced liver disease^[36,37]. These observations led to the currently accepted model of ALD, where Kupffer cell activation by gut-derived endotoxin, induction of chemokines such as MCP-1, and upregulation of the inflammatory cascade represent a central component of the pathomechanisms of ALD (Figure 1).

MECHANISMS OF GUT BARRIER DISRUPTION BY ALCOHOL

The mechanisms underlying the disruption of the intestinal barrier by alcohol appear to be at multiple levels, including disruption of the gut barrier and changes in microbial flora.

Disruption of gut integrity by alcohol and its metabolites

Tight junctions are scaffolds of various transmembrane proteins (e.g. claudins, occludin, JAMs, and tricellulin) and a complex network of adaptor proteins that cross-link junctional membrane proteins (i.e. ZO-1/2/3, PATJ, PAR-3, and PAR-6) to the actin cytoskeleton as well as to different intracellular signaling components. Both alcohol and its metabolites affect the integrity of TJs.

Several studies in the literature suggest the role of acetaldehyde (one of the metabolites of alcohol) in increasing intestinal permeability^[30,38,39]. Acetaldehyde causes the redistribution of tight junction proteins (occludin and ZO-1) and adherens junction (E-cadherin and

β -catenin) proteins from the intercellular junctions^[40,41]. Furthermore, acetaldehyde increases the tyrosine phosphorylation of ZO-1, E-cadherin, and β -catenin, without affecting tyrosine kinase activity^[40]. Acetaldehyde also disrupts the interactions between E-cadherin, β -catenin, and PTP1B, which are the vital components of adherens junctions and epithelial cell-cell adhesion^[42]. These studies indicate the central role of acetaldehyde in disruption of gut integrity, however, not much is known about the effects of other metabolic products of alcohol on gut permeability.

Increased expression of inducible nitric oxide synthase (iNOS) is another factor by which alcohol disrupts the intestinal barrier function. Increase in iNOS, NO, and superoxide correlates with an increase in nitration and oxidation of tubulin, causing increased levels of disassembled tubulin that subsequently damage the microtubule cytoskeleton and result in disruption of barrier function in alcohol treated CaCo2 cells^[43,44]. NF- κ B is involved in oxidation-induced upregulation of iNOS as well in nitration and oxidation of cytoskeleton^[45]. Interestingly epidermal growth factor has a protective role in intestinal barrier function *via* downregulation of iNOS activity, which results in the stabilization of cytoskeleton^[46-48].

Not only chronic alcohol intake results in the disruption of intestinal barrier, but acute alcohol consumption also damages intestinal mucosal membrane, as reported in a rat model^[49]. In a mouse model, a single dose of acute ethanol (6 g/kg) causes injury to the mucosal lining of the small intestine^[50].

Exploitation of mircoRNAs (miRs) by alcohol to target the tight/adherent junction proteins

Another mechanism by which alcohol increases intestinal permeability is by indirectly affecting tight junction proteins through miRs. In particular, a recent study showed the involvement of miRs in gut barrier disruption in alcohol treated cells. miR-212 targets the ZO-1 protein negatively, thus increasing intestinal permeability^[51]. Consistent with this *in vitro* observation, higher levels of miR-212 and lower amounts of ZO-1 protein were found in colon biopsy tissues from patients with ALD^[51]. However, more work needs to be done to explore the role of miRs in regulating tight and adherent junction proteins in ALD.

Change or increase in gut microflora by alcohol

Chronic alcohol abuse not only causes gut leakiness, but also affects the composition of colonic mucosa-associated bacterial microbiota in alcohol-fed rats^[52]; however, the latter finding needs to be validated in human subjects. While there is evidence of bacterial overgrowth (Gram negative) in the gut of alcoholics^[53], little is known about how alcohol consumption is related to increased intestinal bacterial growth. Interestingly, we do not know whether alcohol consumption affects Gram-positive bacteria, which are the source of peptidoglycan. Nevertheless,

increased peptidoglycan levels were found in mice after prolonged administration of alcohol in their drinking water. Interestingly, this mode of alcohol administration does not result in ALD^[54].

ENDOTOXIN RECEPTORS AND SIGNALING PATHWAYS

LPS is a major component of the outer membrane of Gram-negative bacteria and it comprises three distinct parts: a carbohydrate “O-antigen”, the oligosaccharide core region, and a lipid portion “lipid A”. Only the lipid A moiety is toxic and is responsible for the activation of the innate immune response in mammals^[55]. LPS and other bacterial cell wall constituents are released during bacterial multiplication or when bacteria die or lyse^[56]. As soon the immune system recognizes the presence of microorganisms (bacteremia) or LPS in the blood stream (endotoxemia), various proinflammatory cytokines, chemokines, ROS, and other mediators are released to activate monocytes, macrophages, and to recruit lymphocytes. The liver plays an important role in the body’s defense mechanism against bacteria and bacterial products.

LPS is recognized by various receptors in the cells. CR3 (CD11b/CD18) was the first described LPS receptor^[57] in human macrophages. Later on, cluster of differentiation 14 (CD14) and LPS binding protein (LBP) were recognized as receptors for LPS^[58]. Recently, myeloid differentiation factor-2 (MD-2) was found as another LPS binding molecule (direct binding)^[59]. However, CD14 and MD-2 lack a transmembrane domain and, therefore, a second receptor is required to activate the signaling cascade, which was recently described as TLR4 (indirect binding)^[60,61].

TLR4

Toll receptors were first discovered in *Drosophila*^[62] and later on their human homologs were identified^[63]. TLR4 recognizes LPS with the cooperation of its co-receptors, CD14 or MD-2^[64,65]. LPS recognition by TLR4 results in recruitment of the adaptor molecules MyD88 and TRIF, which each activate separate downstream signaling cascades (Figure 2). Formation of the TLR4-MyD88 complex activates the IRAK kinases, which turn on the IKK complex to activate NF- κ B, which results in increased production of pro-inflammatory cytokines, such as tumor necrosis factor (TNF)- α , interleukin (IL)-6 and IL-1 β ^[64]. Activation of TRIF pathway results in TBK/IKK ϵ phosphorylation and activation of the interferon regulatory factor-3 (IRF3), which leads to induction of Type- I interferons (IFNs)^[66]. Activation of both of the pro-inflammatory and Type- I IFN pathways by TLR4-LPS is unique, and evaluation of these specific pathways has recently received attention in ALD.

The TLR4-LPS signaling pathway plays a critical role in alcohol-induced liver injury. Both chronic and acute (or binge) alcohol use affect the various components of TLR4 signaling^[67-71]. The effect of alcohol use on TLR4

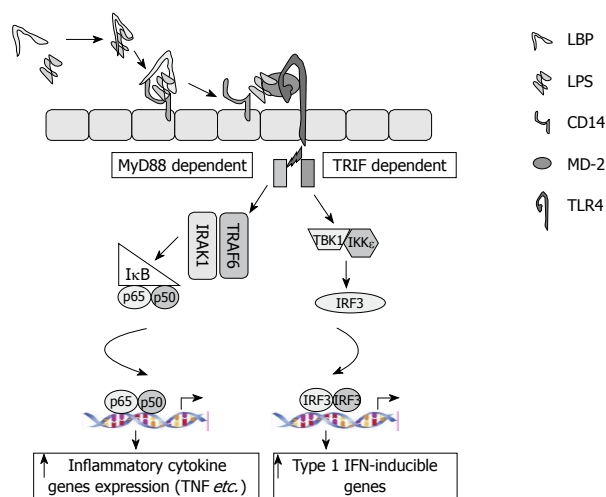


Figure 2 Role of the Toll-like receptor 4 (TLR4)-LPS signaling cascade in alcohol induced liver injury. LPS binds to LPS binding protein (LBP), transfers to cluster of differentiation 14 (CD14) and then to the TLR4 myeloid differentiation factor-2 (MD-2) complex. This signal is passed either through MyD88-dependent or TRIF-dependent intracellular pathways, which activate various transcription factors and induce various pro-inflammatory cytokine and Type I interferon genes.

signaling was recently reviewed in detail^[3]. There is increased expression of TLR4 and its co-receptors, as well as other TLRs, in ALD in mice. Early studies in TLR4 mutant mice demonstrated protection from early ALD^[72] and more recent reports using TLR4 deficient mice validated the important role of TLR4 in the pathogenesis of ALD^[73]. We also investigated the specific role of the MyD88 adapter in ALD and found that MyD88-deficient mice were not protected from alcoholic steatosis and inflammation. Consistent with the hypothesis that MyD88-independent, TLR4-mediated, pathways are involved in ALD, we found protection from ALD in TLR4-deficient, as well as in IRF3-deficient, mice^[73]. The role of IRF3 in ALD was also indicated by another study^[74].

MD-2

MD-2 is a type II acute phase protein and is expressed on the surface of myeloid and endothelial lineage cells^[75,76]. Although it lacks a transmembrane domain, it attaches to the cell surface through its interaction with TLR4^[77,78]. MD-2 also presents in a soluble form (sMD-2) and is secreted by various cells^[79,80]. Increased sMD-2 activity is found in plasma of sepsis patients^[81,82]. It is postulated that at high concentrations, sMD-2 might inhibit endotoxin induced cell activation in a similar way to LBP and soluble CD14^[78,83]. IL-1 β regulates the production of MD-2 in hepatocytes and myeloid cells^[83]. Chronic alcohol feeding results in an upregulation of MD-2 in the liver^[73].

CD14

CD14 is expressed in various cell types, including monocytes, macrophages, B cells, liver parenchymal cells, and some fibroblast cells^[84,85]. It is absent in early myeloid progenitor cells; however, with maturation, its expression increases. Human CD14 transgenic mice are hypersensitive to LPS^[86], whereas CD14 knockout mice are

resistant to endotoxin shock^[87], indicating its crucial role in LPS signaling. CD14 is also present in soluble forms, as sCD14 α and sCD14 β , and is secreted by macrophages^[88] and liver parenchyma cells^[89].

Alcohol consumption affects CD14 expression and plays an important role in LPS induced immune activation in alcoholics. Increased expression of CD14 is found in Kupffer cells or whole livers of chronic ethanol-fed animals^[25,90,91]. A correlation between CD14 expression and the severity of ALD has been reported in humans and it has been suggested that CD14 is one of risk factor in ethanol-induced pathology^[92,93]. Interestingly, acute alcohol treatment also induces CD14 expression in whole liver cells^[94] and CD14-deficient mice were protected from alcohol-induced liver steatosis^[92].

LBP

LBP is an acute phase protein and is induced by LPS, IL-6, and IL-1 β ^[95,96]. Although liver is a major source of LBP production, other organs, such as lungs, kidneys, and heart, also produce LBP^[97]. This protein is present in normal serum; however, its levels become elevated during acute phase responses^[98,99]. LBP catalyzes the transfer of LPS to CD14, and thus enhances the LPS-induced activation of monocytes, macrophages, and other immune cells^[100]. Anti-LBP antibodies, together with LPS, protected the mice from death^[101]. Neutralization of LBP protects the host from LPS-induced toxicity, suggesting its critical role in innate immunity^[102].

In addition to its pro-inflammatory role, it also acts as an antiinflammatory, where it transfers LPS (Gram negative) or LTA (Gram positive) to HDL and other lipoproteins, and also aids the neutralization of LPS^[103]. The antiinflammatory role of this protein is well described in various reports^[99,104-107]. It is postulated that low concentrations of LBP enhance the LPS-induced activation of mononuclear cells, whereas the acute-phase rise in LBP concentrations inhibit LPS-induced immune cell activation^[108].

Not much is known about the role of LBP in alcoholics, except one report where its role is described in early alcohol-induced liver injury where it enhances the production of cytokines, such as TNF- α . Ethanol fed LBP KO mice showed reduced TNF- α expression and reduced liver damage^[109]. There was no change in endotoxin levels of both wild-type and LBP knockout mice; however, decreased steatosis in LBP knockout ethanol-fed mice was observed^[109]. A potential antiinflammatory role of the above mentioned LBPs in the pathogenesis of ALD is yet to be explored.

In summary, it appears that LBPs and receptors modulate the LPS response bifunctionally, either by neutralizing or enhancing its response.

EFFECTS OF LPS ON THE LIVER

Activation of inflammatory cells in ALD

There is ample evidence for increased inflammatory

cascade activation in ALD^[3]. Alcoholic steatohepatitis is characterized by infiltration of various inflammatory cells into the liver, including neutrophils, leukocytes, monocytes, and macrophages and this occurs as a result of chemokine activation (e.g. IL-8, MCP-1, and MIPs)^[110-112]. In humans with alcoholic steatohepatitis, serum TNF- α , IL-6, and IL-8 levels are increased and there is also evidence for activation of circulating monocytes based on increased TNF- α production and increased NF- κ B activation^[113-115]. Serum levels and liver expression of these LPS-inducible pro-inflammatory cytokines are also increased in animal models of ALD^[73]. Isolated Kupffer cells from mice and rats show increased production of TNF- α after chronic alcohol feeding^[116] and this has been linked to increased TNF- α mRNA stability, as well as to upregulation of Erk, MAPK, and Egr-1 kinases^[117]. While LPS has been proposed to play a major role in Kupffer cell and macrophage activation in ALD, *in vitro* studies in human monocytes/macrophages suggest that chronic alcohol exposure itself can promote a pro-inflammatory phenotype and amplify LPS-induced pro-inflammatory responses^[71]. Our laboratory showed that increased LPS responsiveness after chronic alcohol exposure in monocytes is due to reduced expression of IRAK-M, which is a negative regulator of TLR4 activation^[71]. Thus, chronic alcohol exposure alone not only results in pro-inflammatory activation of macrophages, but also sensitizes cells to LPS-induced pro-inflammatory signals^[71].

Effects of endotoxin on the liver parenchymal and other non-immune cells in ALD

TLR4, the LPS receptor, is expressed in all cell types in the liver; thus, gut-derived endotoxin can modulate the function of all liver cells in ALD^[3]. In hepatocytes, LPS can promote apoptosis, particularly in combination with other hepatotoxins^[118,119]. TLR4 expression in hepatic stellate cells (HSC) has been shown to mediate inflammatory signaling by LPS and manifests in activation of Jnk kinase and NF- κ B^[120]. Oxidative stress induced by alcohol and its metabolites has also been shown to sensitize HSC to LPS-induced activation and subsequent induction of hepatic fibrosis^[121,122]. Thus, LPS affects hepatocytes as well as HSC, both directly and *via* inflammatory cell activation.

ROLE OF GUT-LIVER AXIS IN OTHER LIVER DISEASES

The balance of gut microbial flora, intestinal permeability, hepatocyte function, and Kupffer cell activation appears to be critical in the maintenance of normal homeostasis (Figure 1). Indeed, increasing evidence suggests an importance for a gut-liver connection in different liver diseases where gut-derived LPS delivered to the liver through the portal circulation might play a role. For example, increased intestinal permeability was detected

in patients with intrahepatic cholestasis of pregnancy^[123], and in hepatitis C virus (HCV)-induced liver injury in human immunodeficiency virus infected individuals^[124]. An increase in serum endotoxin levels was associated with pro-inflammatory activation of circulating monocytes in chronic HCV infection, even in the absence of cirrhosis^[125]. These observations underscore the importance of the gut-liver axis in the pathogenesis of ALD, as well as in other types of liver injuries.

CONCLUSION

The gut-liver axis, particularly gut-derived endotoxin, seems to play a crucial role in the pathogenesis of liver diseases caused by various insults, including alcohol. However, the mechanisms and source of endotoxin in liver diseases are not fully understood. The importance of alcohol-induced alterations in the gut and the role of the liver in elimination of gut-derived pathogen-derived compounds require further investigation. Furthermore, interactions between immune, non-immune, and parenchymal cells, which take place *in vivo*, contribute and determine the progression of ALD. Understanding the role of TLR signaling and the cell-specific effects of gut-derived microbial products will provide new insights, not only into the pathomechanisms of ALD, but might also reveal new targets for therapeutic interventions.

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Anti-inflammatory pathways and alcoholic liver disease: Role of an adiponectin/interleukin-10/heme oxygenase-1 pathway

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Abstract

The development of alcoholic liver disease (ALD) is a complex process involving both the parenchymal and non-parenchymal cells in the liver. Enhanced inflammation in the liver during ethanol exposure is an important contributor to injury. Kupffer cells, the resident macrophages in liver, are particularly critical to the onset of ethanol-induced liver injury. Chronic ethanol exposure sensitizes Kupffer cells to activation by lipopolysaccharide *via* Toll-like receptor 4. This sensitization enhances production of inflammatory mediators, such as tumor necrosis factor- α and reactive oxygen species, that contribute to hepatocyte dysfunction, necrosis, apoptosis, and fibrosis. Impaired resolution of the inflammatory process probably also contributes to ALD. The resolution of inflammation is an active, highly coordinated response that can potentially be manipulated *via* therapeutic interventions to treat chronic inflammatory diseases. Recent studies have identified an adiponectin/interleukin-10/heme oxygenase-1 (HO-1) pathway that is profoundly effective in dampening the enhanced activation of innate immune responses in primary cultures

of Kupffer cells, as well as in an *in vivo* mouse model of chronic ethanol feeding. Importantly, induction of HO-1 also reduces ethanol-induced hepatocellular apoptosis in this *in vivo* model. Based on these data, we hypothesize that the development of therapeutic agents to regulate HO-1 and its downstream targets could be useful in enhancing the resolution of inflammation during ALD and preventing progression of early stages of liver injury.

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Key words: Liver disease; Alcohol; Macrophages; Heme-oxygenase-1; Inflammation

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INTRODUCTION

Chronic abuse of ethanol in humans leads to liver disease. An ordered progression of increasingly more serious liver injury, culminating in liver failure in 15%-20% of all alcohol abusers, has been described^[1]. Fatty liver (steatosis) is the earliest stage of liver injury and is characterized by triglyceride accumulation in hepatocytes. If alcohol abuse continues, some patients with steatosis will develop inflammatory changes in their livers, a pathology described as steatohepatitis or simply hepatitis. Hepatitis is characterized by marked hepatomegaly and infiltration

of several different subtypes of leukocytes including neutrophils, monocytes/macrophages and both T and B lymphocytes. Of the patients who develop hepatitis, 50% will progress further and exhibit fibrotic changes in the liver. Of note, alcohol abuse is a leading cause of fibrosis in the USA^[1,2]. Hepatic stellate cell activation is a major contributor to fibrosis through overproduction of excessive extracellular matrix components such as type I and type III collagens and proinflammatory molecules^[3]. Alcohol-induced steatosis and fibrosis can resolve, provided that the underlying stimulus, i.e. alcohol, is removed. Unfortunately, when the liver progresses to cirrhosis, a stage of liver injury characterized by significant hardening of the liver, decreased hepatocyte regeneration and significant loss of liver function, patients are likely to die as a result of liver failure unless they receive a liver transplant^[4]. The medical costs associated with alcohol abuse in the United States is estimated to be \$166 billion per year, of which more than \$30 billion is for direct medical costs^[5].

INNATE IMMUNITY AND WOUND HEALING

Activation of innate immunity is an essential response to infection or injury; the ensuing inflammatory response protects from infection, and also limits cellular and organ damage to the host organism^[6]. A well-controlled innate immune response is characterized by rapid initiation of an inflammatory response. However, this response is sustained only until the immune insult or injury is contained; at that point, the inflammatory response is terminated or resolved. The controlled and appropriate resolution of inflammation is an essential feature of the innate immune response. Despite the beneficial effects of innate immunity, a failure to appropriately regulate activation of innate immunity contributes to a number of chronic inflammatory diseases, including alcoholic liver disease (ALD)^[7]. In addition to the clear role of innate immune responses to alcoholic hepatitis, activation of the innate immune response likely contributes to all stages of liver injury, including steatosis, hepatocellular injury and fibrosis^[8].

Dysregulation in the initiation and/or resolution of the inflammatory process could contribute to the development of ALD. Indeed, there is a growing appreciation that the resolution of inflammation is an active, highly coordinated response that could potentially be manipulated *via* therapeutic interventions to enhance the resolution of inflammatory processes and treat chronic inflammatory diseases, such as ALD.

ROLE OF IMMUNE RESPONSES IN THE INITIATION AND PROGRESSION OF ALD

The innate and adaptive immune systems are 2 distinct branches of the immune response, yet these 2 components of immunity are intimately linked at many stages of an organism's response to injury or stress. Components of the innate immune response, including natural

killer (NK) and NKT cells^[9], Kupffer cells (resident hepatic macrophages)^[10] and the complement system^[11-13], as well as T-cells and antibody-dependent adaptive immune responses^[14], are involved in the hepatic response to various types of injury, including bacterial and viral infections, exposure to toxins (including ethanol), partial hepatectomy and ischemia-reperfusion.

The localized hepatic response of the innate immune system to ethanol may be distinct from the systemic innate immune response and/or localized responses of other organs to ethanol. For example, chronic alcohol consumption generally increases the susceptibility of individuals to infections^[15,15], suggesting that, despite increased inflammatory responses observed in the liver after chronic ethanol exposure, systemic immune responses are suppressed by chronic ethanol exposure. Even within the liver, there may be distinct responses of individual components of the immune response. One example is the interaction of ethanol and hepatitis C virus (HCV) infection. Chronic alcohol abuse is associated with an increased incidence of HCV infection^[16]. This decreased ability to ward off viral infections contrasts with the increased response of the liver to endotoxins. Understanding the localized and specific effects of chronic ethanol on the immune system will help to develop intervention strategies specifically directed at targets of ethanol action leading to the progression of ALD.

Initiation of inflammation: chronic ethanol-dependent sensitization of Toll-like receptor 4 (TLR4)-dependent signal transduction and cytokine production

How does ethanol initiate inflammatory responses in the liver? An important working model for ethanol-induced liver injury proposes that lipopolysaccharide (LPS)-induced tumor necrosis factor- α (TNF α) production by Kupffer cells, the resident macrophage of the liver, is critical for development of ethanol-induced liver injury. Chronic ethanol exposure alters the jejunal microflora leading to an increase in Gram-negative bacteria and/or disrupts the barrier function of the small intestine allowing access of bacterial products to the portal circulation^[17,18]. In support of this hypothesis, endotoxin or LPS, a component of the cell wall of Gram-negative bacteria, is increased in the circulation of alcoholics^[19] and murine models of chronic ethanol exposure^[20,21]. Further, if gut bacteria are diminished using antibiotics, ethanol-induced fatty liver and inflammation are attenuated^[20].

Kupffer cells and TLR4 in the initiation of inflammation: The liver contains the first capillary bed through which blood from the intestine flows. Kupffer cells are located in the hepatic sinusoids and, among other physiologic functions, clear endotoxins from the blood, but usually without discernable inflammation. Chronic ethanol exposure also increases the sensitivity of Kupffer cells to endotoxins^[10]. Therefore, in the presence of persistent ethanol exposure and increased exposure to gut-derived endotoxins, Kupffer cells become activated and contribute to liver disease.

Kupffer cells are innate immune effectors that produce several proinflammatory mediators, including reactive oxygen species (ROS) and TNF α , which promote liver injury after chronic ethanol in response to LPS^[17]. In rats depleted of Kupffer cells by gadolinium chloride treatment, ethanol-induced liver injury is attenuated^[17]. Further evidence for a role of activated Kupffer cells and LPS in the development of ethanol-induced liver injury has been revealed, as mice deficient in LPS receptor complex components TLR4 and CD14, and NADPH oxidase function (*p47^{phox}* -/- mice) are protected from ethanol-induced liver injury^[22-24]. TLR4 signaling *via* its MyD88-independent pathway (TRIF-dependent) is a critical contributor to increased steatosis and inflammation during chronic ethanol exposure^[25,26].

Ethanol and the regulation of TNF α expression:

TNF α is produced predominately by cells of monocyte/macrophage lineage and is particularly critical for ethanol-induced liver injury. TNF α concentration is enhanced in the blood of alcoholics^[27] and in the blood of rats and mice exposed to chronic ethanol^[17]. Additionally, if TNF α is blocked in mice using TNF α -specific antibodies or if TNF α signaling is prevented in TNF α receptor-deficient mice, ethanol induced liver injury is diminished^[28].

Enhanced TLR4 signal transduction by ethanol

Production of inflammatory cytokines is a highly regulated process; regulation occurs at the level of transcription, translation and secretion^[29,30]. Ethanol exposure impacts the molecular regulation of TNF- α expression at each level of control in macrophages, resulting in an enhanced initiation of inflammation in the liver^[10]. Ethanol mediates these changes in the activation of Kupffer cells by a profound dysregulation in TLR4-initiated signal transduction^[10]. Interestingly, many of the same signaling pathways targeted by ethanol in neurons, resulting in the complex behavioral effects of ethanol, are also involved in TLR4-mediated signal transduction in macrophages. Briefly, LPS binds to a cell surface receptor, CD14, which, *via* interactions with TLR4^[31], stimulates a complex array of signal transduction cascades^[32,33]. Stimulation of macrophages with LPS activates tyrosine kinases, protein kinase C, nuclear factor κ B (NF κ B), as well as members of the mitogen-activated protein kinase family, including extracellular signal-regulated kinase 1/2 (ERK1/2), p38 and c-jun N-terminal kinase^[32]. Chronic ethanol feeding generally enhances these activation pathways, including increased LPS-stimulated phosphorylation of ERK1/2 and p38^[34-36] and NF κ B activation^[37].

Role of ROS in increased cytokine expression

Increased production of ROS during chronic ethanol exposure, either by hepatocytes during ethanol metabolism^[38] and/or from Kupffer cells during ethanol exposure^[39] and in response to LPS^[40], may contribute to this sensitization in LPS-dependent signal transduction.

ROS play a critical role in the modulation/regulation of a number of signal transduction cascades^[41], including LPS-stimulated signaling pathways both in cells of the innate immune system (monocytes/macrophages, neutrophils, *etc.*) and non-immune cells^[42-44]. Indeed, we have specifically identified NADPH oxidase-derived ROS as an important contributor to LPS-stimulated ERK1/2 phosphorylation in rat Kupffer cells, particularly after chronic ethanol^[45]. Taken together, these data suggest that the chronic ethanol-induced increase in ROS is an important contributor to the dysregulation of LPS-mediated signal transduction and inflammatory cytokine production in Kupffer cells.

Clinical implications of enhanced initiation of inflammation after chronic ethanol exposure

While the role of TNF- α in the development of ethanol-induced liver injury has been well characterized in animal models^[17], clinical investigations of the therapeutic efficacy of antibodies to TNF- α (e.g. infliximab) to treat patients with acute alcoholic steatohepatitis have generated variable results^[46,47]. There is particular concern about side effects of completely inhibiting TNF- α function. For example, since TNF- α is a critical component of immunity, infectious disease is a primary concern during anti-TNF- α therapy^[47,48]. Moreover, TNF- α is required for normal liver regeneration; hepatocyte proliferation in response to injury is impaired in mice lacking TNF- α receptors^[49]. Therefore, therapeutic strategies to normalize, rather than eliminate, TNF- α expression in ALD are more likely to be safe and effective. Interventions that re-establish normal homeostatic control of initiation and resolution of innate immune responses in liver would allow for maintenance of normal immune function and repair of hepatocyte injury during treatment to resolve ALD.

RESOLUTION OF INFLAMMATION: ROLE OF ANTI-INFLAMMATORY MEDIATORS

The controlled and appropriate resolution of inflammation is an essential feature of the innate immune response; failure to terminate an inflammatory response likely contributes to a number of chronic inflammatory diseases, including ALD^[7]. Importantly, the resolution of inflammation is an active, highly coordinated response. Indeed, inflammatory signals initiate the induction of negative regulators. During ethanol exposure, despite high expression of inflammatory mediators, the resolution phase is not functionally appropriate. Understanding the mechanisms for the failure to resolve inflammation, and the identification of effective anti-inflammatory pathways that can be upregulated in the context of chronic ethanol exposure are likely to lead to therapeutic strategies to treat, as well as prevent, ALD.

The mechanisms for resolution of an inflammatory response are complex, requiring the elimination of infiltrating neutrophils, as well as a normalization of

macrophage numbers and activity^[50]. Within the context of this review, we will focus our attention on the identification of specific intrinsic pathways that normalize the increased pro-inflammatory activity of Kupffer cells after chronic exposure to ethanol. In order for a particular anti-inflammatory pathway to have potential therapeutic value, it must remain functional after chronic ethanol exposure, must target the specific pro-inflammatory pathways enhanced by ethanol and must contribute to the prevention of hepatocellular injury. Finally, the pathway must be amenable to therapeutic intervention.

Ethanol and adiponectin

Recent studies have explored the potential of adiponectin, an abundant 30 kDa adipokine with potent anti-inflammatory properties, as a useful anti-inflammatory to exploit in the treatment of ALD. Treatment of mice with supra-physiological concentrations of adiponectin during chronic ethanol exposure prevented the development of liver injury, decreasing both steatosis and TNF- α expression in the liver^[51]. You *et al.*^[52] have recently reviewed the molecular mechanisms for the anti-steatotic effects of adiponectin in liver. In hepatocytes, activation of a SIRT1-AMPK (sirtuin 1-AMP-activated protein kinase) pathway appears to be critical in decreasing ethanol-induced lipid accumulation in the liver^[52].

Adiponectin also normalizes LPS-induced TNF- α production in primary cultures of Kupffer cells after chronic ethanol exposure^[53]. Interestingly, the anti-inflammatory effects of adiponectin appear to be mediated by either globular adiponectin (gAcrp) or full-length adiponectin^[53]. This may be due, at least in part, to the ability of macrophages to cleave full-length adiponectin to its globular form^[54]. gAcrp signals primarily *via* adiponectin receptor 1 in Kupffer cells^[55]. In contrast to the predominant role of AMPK in mediating the effects of adiponectin in hepatocytes^[52], gAcrp activates multiple signaling pathways in macrophages, including the mitogen-activated protein kinase family members, NF κ B and protein kinase A^[56]. Together, activation of these signaling pathways culminates in the expression of anti-inflammatory mediators, including interleukin (IL)-10^[56].

Despite the efficacy of adiponectin in decreasing LPS-mediated responses, both in mouse models and primary cultures of Kupffer cells, the development of adiponectin for therapeutic interventions in patients with ALD is likely of limited utility, because of the high concentration of adiponectin in the circulation, as well as the complex oligomeric structure of adiponectin. Therefore, investigators have begun to focus either on mechanisms to increase endogenous adiponectin expression, such as treatment of mice with rosiglitazone^[57], as well as on the identification of downstream molecular targets of adiponectin in the liver. Recent studies in Kupffer cells have identified an adiponectin-mediated IL-10/heme oxygenase-1 (HO-1) pathway involved in the anti-inflammatory effects of adiponectin in macrophages that may be more amenable to pharmacological intervention.

IL-10/HO-1 pathway

IL-10 is an immunomodulatory cytokine with potent anti-inflammatory properties. IL-10 decreases production of pro-inflammatory cytokines, including TNF- α and IL-1 β ^[58]. While little is known about the regulation of IL-10 expression and activity in the liver in response to chronic ethanol, studies show that impaired expression of IL-10 contributes to inflammation in alcoholic cirrhotics^[59] and IL-10 deficient mice are more sensitive to ethanol-induced liver injury^[60]. Drechsler *et al.*^[61] found that treatment with IL-10 reduced the acute effects of ethanol in monocytes in an HO-1 dependent mechanism.

We have recently identified an IL-10/HO-1 mediated pathway that can be activated in Kupffer cells after chronic ethanol feeding and suppresses LPS-stimulated TNF- α expression^[55]. IL-10 mediates its anti-inflammatory functions *via* induction of IL-10-inducible genes, including HO-1, suppressor of cytokine signaling 3 and BCL3^[58]. There is a growing appreciation that HO-1, in particular, is an important downstream mediator of the anti-inflammatory effects of IL-10 in macrophages^[58]. Interestingly, induction of HO-1 by Kupffer cells after chronic ethanol is particularly robust^[55]; siRNA knockdown or chemical inhibition of HO-1 in Kupffer cells demonstrates that HO-1 is critical to the suppression of TLR4-stimulated TNF- α expression in response to either adiponectin or IL-10 after chronic ethanol exposure^[55].

HO-1 plays an important anti-inflammatory role in a number of chronic inflammatory diseases, and also has anti-apoptotic and anti-proliferative properties^[62]. HO-1 is a 288 amino acid protein that is anchored in the endoplasmic reticulum, although localization of a cleaved form of HO-1 has also been observed in the nucleus under some conditions^[63]. HOs catalyze the initial and rate limiting step in the oxidative degradation of heme, yielding equimolar amounts of biliverdin IX α , carbon monoxide (CO), and free iron^[64]. Three isoforms of the HO protein have been identified, which are encoded by separate genes. HO-2 and HO-3 are constitutive forms, while HO-1 (also known as heat shock protein 32) is an inducible isozyme, with high expression levels in spleen and Kupffer cells^[65]. HO-1 is a stress-responsive protein whose expression is upregulated by a broad spectrum of inducers, including heme, heavy metals, nephrotoxins, cytokines, endotoxins and oxidative stress.

Surprisingly, acute and chronic ethanol exposure do not increase HO-1 expression in Kupffer cells or in the liver^[55]. However, this may be dependent on the age of the animals studied^[66,67]. While ethanol does not increase HO-1 expression, several recent studies suggested that induction of HO-1, as well as increasing concentrations of its downstream mediator CO, prevents ethanol-induced inflammation in the intestine^[68] and liver^[55], as well as oxidative damage to hepatocytes^[69] and hepatocyte apoptosis. Importantly, induction of HO-1 in mice after chronic ethanol exposure by treatment with cobalt-protoporphyrin-IX normalizes LPS-induced TNF α expression in the liver^[55].

HO-1: downstream anti-inflammatory mediators

Induction of HO-1 plays a key role in mediating cellular protection against the insult of oxidants both *in vitro* and *in vivo*^[62]. *In vivo* expression of HO-1 has potent protective effects against atherogenesis, acute cardiac ischemic failure, ischemia/reperfusion injury in liver, as well as hyperoxia-induced liver injury^[62]. The exact mechanisms involved in the anti-inflammatory effects of HO-1 are poorly understood. Recent studies suggested that 2 of the break-down products of heme generated by HO-1, i.e. CO and biliverdin (and its breakdown product bilirubin), are each potential mediators of HO-1 activity^[62]. Release of free iron by HO-1 typically induces the expression of ferritin, which can also have protective properties. In Kupffer cells from ethanol-fed rats, we find that treatment with the CO donor, CORM-2 (CO-releasing molecule), was sufficient to decrease LPS-stimulated TNF- α expression^[55], suggesting CO has strong anti-inflammatory effects in Kupffer cells after chronic ethanol feeding.

CO is also an important regulator of a number of signaling pathways that regulate hepatic metabolism and inflammatory responses, including MAPK family members, peroxisome proliferator-activated receptor- γ , Egr-1 and adenosine 2A receptor^[70-72]. Each of these signaling pathways is also a target of chronic ethanol in hepatocytes and/or Kupffer cells.

CONCLUSION

Considering the strong association between ethanol-mediated activation of the innate immune system and ALD, it is critical to understand the mechanism(s) by which ethanol disrupts the intricately regulated innate immune response. Characteristically, the innate immune response involves both a rapid, yet transient, activation; the transient nature of the innate immune response contributes to an appropriate resolution of inflammation^[50,73]. In the past, studies have focused primarily on the impact of ethanol on activation of the innate immune response *via* production of pro-inflammatory mediators. However, recent studies suggest that ethanol must also impair the “anti-inflammatory” responses elicited during inflammation, thus delaying and/or impairing the resolution of the inflammatory response. Impaired resolution of inflammation contributes to chronic inflammatory states^[6]. Recent studies have demonstrated that induction of HO-1 in primary cultures of Kupffer cells isolated from rats after chronic ethanol exposure normalizes chronic ethanol-induced sensitization to LPS^[55]. Importantly, HO-1 induction also normalizes LPS-stimulated TNF- α expression, as well as hepatocyte apoptosis, in an *in vivo* mouse model of chronic ethanol exposure^[55]. We hypothesize that the development of therapeutic agents to regulate these HO-1-activated pathways will enhance the resolution of inflammation during ALD and prevent progression of early stages of liver injury.

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Impact of alcohol on hepatitis C virus replication and interferon signaling

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Abstract

Hepatitis C virus (HCV) is one of the main etiological factors responsible for liver disease worldwide. It has been estimated that there are over 170 million people infected with HCV worldwide. Of these infected individuals, approximately 75% will go on to develop a life long necroinflammatory liver disease, which over decades, can result in serious complications, such as cirrhosis and hepatocellular carcinoma. Currently there is no effective vaccine and whilst antiviral therapies have been improved, they are still only effective in approximately 50% of individuals. HCV infection stands as a major cause of global morbidity and suffering, and places a significant burden on health systems. The second highest cause of liver disease in the western world is alcoholic liver disease. Frequently, HCV infected individuals consume alcohol, and the combined effect of HCV and alcohol consumption is deleterious for both liver disease and response to treatment. This review discusses the impact of alcohol metabolism on HCV replication and the negative impact on interferon (IFN)- α treatment, with a particular focus on how alcohol and HCV act synergistically to increase oxidative

stress, ultimately leading to exacerbated liver disease and a reduction in the efficacy of IFN- α treatment. A better understanding of the complicated mechanisms at play in hepatocytes infected with HCV and metabolizing alcohol will hopefully provide better treatment options for chronic hepatitis C individuals that consume alcohol.

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Key words: Alcohol metabolism; Hepatitis C virus; Reactive oxygen species; Interferon signaling

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INTRODUCTION

Hepatitis C virus (HCV) has emerged as a significant human pathogen worldwide and is now the most common cause of significant liver disease in many countries^[1]. Although the disease is typically not severe in the acute phase, more than 70% of infected individuals develop a persistent infection that, over the course of 2-3 decades, can result in progressive hepatic fibrosis and loss of functioning hepatocyte mass^[2,3]. A proportion of infected individuals will develop cirrhosis and eventually hepatocellular carcinoma (HCC)^[4,5]. This progressive liver disease is thought to arise as a result of the chronic inflammatory response to clear HCV infected hepatocytes.

cytes, resulting in an environment that is favorable for the fibrogenic process^[6].

There are numerous clinical studies that suggest a strong epidemiological link between the consumption of alcohol and accelerated liver disease in HCV infected individuals^[7-9]. The majority of alcohol metabolism takes place in hepatocytes, which is also the primary site of HCV replication, and thus it is logical that interactions between the two will occur, both at the clinical and molecular level. The majority of studies have concluded that HCV infected individuals that consume alcohol show a strong propensity for accelerated liver disease, and that alcohol metabolism and concurrent HCV infection act synergistically to facilitate this rapid disease progression^[7-11]. In fact, excessive alcohol consumption is now a recognized co-factor in liver disease progression, and persons infected with HCV are recommended to limit their alcohol intake^[12]. Despite strong epidemiological evidence, the molecular mechanisms by which alcohol consumption exacerbates chronic hepatitis C (CHC) remain unclear. Furthermore, the interactions between alcohol metabolism, HCV, and the host antiviral immune response are also unknown. Clearly, the relationship between alcohol and HCV is complex, and the mechanisms responsible for accelerated disease progression are most likely not related to a single factor, but are the result of alterations to hepatocyte homeostasis, production of cytokines, and modification of the immune system.

This review will focus on the role that alcohol metabolism has on HCV RNA replication and discuss the potential molecular mechanisms responsible, with a particular focus on oxidative stress. The effect of alcohol on interferon (IFN)- α action and its modulation of signal transduction pathways will also be discussed.

ALCOHOL CONSUMPTION ACCELERATES HCV RELATED LIVER DISEASE

One of the first reports documenting the role of alcohol consumption on CHC progression was published by Seeff *et al*^[5], in which they reported that two thirds of HCV positive patients that died from end stage liver disease were chronic consumers of alcohol. Poynard *et al*^[4] extended this study, showing that the consumption of 50 g/d of alcohol increased the rate at which fibrosis progresses in HCV infected individuals. They also identified three independent risk factors that were associated with increased rates of fibrosis: age greater than 40 years at time of infection, being male, and consuming more than 50 g of alcohol per day. At the virological level, Pessione *et al*^[8] found a direct dose dependent correlation between increasing HCV RNA levels and increasing levels of alcohol consumption. The mechanism for this increase was not established, but it was postulated that the increase in HCV RNA could be due to a direct effect of alcohol increasing viral replication or through reduced clearance of the virus by the immune system. One of the most comprehensive studies investigating

the effect of alcohol on HCV disease progression was performed by Corrao *et al*^[9], in which they studied a large cohort of 417 patients. The most striking finding of this study was a comparison of the associated risk factors for developing cirrhosis between HCV infected patients that abused alcohol and those that abstained. The risk factor for developing cirrhosis in patients that were HCV positive but did not consume alcohol was 9, compared to a significantly higher risk factor of 147 for those patients that abused alcohol. This study added further weight to the hypothesis that HCV and alcohol metabolism synergistically contribute to exacerbated liver disease.

There have been a number of studies that have documented a clear link between excess alcohol consumption and an increased risk of HCC development. It has been suggested that HCV infected individuals that consume alcohol show a 100-fold increase in their risk of developing HCC^[13-18]. Clearly, chronic consumption of alcohol in HCV infected individuals is a dangerous mix, with significant clinical implications.

ALCOHOL AND HCV INDUCE OXIDATIVE STRESS

Reactive oxygen species (ROS) are defined as small highly reactive oxygen-containing molecules that cause oxidative stress when the rate at which they are produced is greater than the rate at which they are removed, leading to a disturbance in the pro-oxidant/anti-oxidant balance. Cytochrome P450-2E1 (CYP2E1) metabolism of alcohol stimulates the microsomal production of ROS, such as superoxide anions (O_2^-), hydroxyl radicals (OH^\bullet), 1-hydroxyethyl radicals (CH_3CHOH^\bullet), lipid hydroperoxides (LOOH), and (when iron levels increase due to alcohol metabolism) the production of ferryl radicals^[19]. Oxidative stress can potentially lead to cellular damage that can play a role in a variety of pathological conditions^[20]. The generation of hepatic oxidative stress in CHC is now well established, and most likely a consequence of HCV proteins disrupting mitochondrial and hepatocyte organelle function, in addition to the inflammatory response directed towards HCV infected hepatocytes. Under normal conditions, oxidative stress exists in a state of equilibrium with cellular antioxidants that scavenge ROS and prevent cellular injury. However, when cellular antioxidant mechanisms are overwhelmed through chronic oxidative stress or disease processes, oxidative stress production continues unchecked, with pathological consequences. This chronic exposure of the liver to oxidative stress in CHC has significant clinical implications, as it is well documented that oxidative stress is a mediator of hepatic inflammation, fibrosis, and the development of HCC^[21].

One of the most significant mediators of oxidative stress in the liver is the metabolism of alcohol by the enzyme CYP2E1. Metabolism of alcohol can also occur *via* the other main alcohol metabolizing enzyme

alcohol dehydrogenase (ADH). Whilst ADH-mediated metabolism of alcohol does produce ROS, CYP2E1-mediated metabolism of alcohol produces levels of ROS that greatly exceed that of the ROS produced by ADH. Alcohol metabolism not only directly produces ROS, but it also creates an environment that is favorable for oxidative stress. It is becoming increasingly clear that oxidative stress plays a prominent role in the pathogenesis of alcohol-induced liver disease^[22] and CHC^[21]. It is therefore not difficult to envisage the potentially explosive situation where oxidative stress produced by HCV and alcohol leads to a synergistic exacerbation of liver disease.

HCV INFECTION INDUCES A STATE OF OXIDATIVE STRESS

While clinical studies have suggested that markers of oxidative stress are increased in CHC, it was the development of mice transgenic for the HCV core protein that clearly demonstrated that HCV directly induces a state of oxidative stress^[23]. Mice expressing either the HCV core or the complete HCV polyprotein developed pathologies consistent with those observed in human HCV infection^[23,24], such as steatosis and development of HCC. Prior to HCC development, the HCV core-expressing mice showed a marked increase in lipid peroxidation and activation of the anti-oxidant system, suggesting that the expression of HCV core is sufficient to induce oxidative stress in the mouse liver and initiate HCC through DNA damage and modulation of signaling cascades^[23]. It was subsequently shown *in vitro* that HCV core expression results in increased generation of ROS and expression of antioxidant enzymes^[25-27]. Mechanistically, it was shown that this increase in oxidative stress was due to interactions between HCV core and destabilisation of the mitochondrial electron transport chain and that this was further enhanced in the presence of alcohol^[28,29].

In addition to the core protein, the HCV nonstructural protein non-structural 5A (NS5A) has also been demonstrated to increase cellular ROS, albeit through a different mechanism to that of the HCV core. HCV NS5A localizes to the endoplasmic reticulum (ER) and lipid droplets, and is part of the HCV replication complex that results in the formation of altered cytoplasmic membrane structures, known as the membranous web. It has been postulated that this change in the membrane structure results in ER stress and the unfolded protein response, leading to the release of ER Ca²⁺ stores and resulting in the formation of oxidative stress^[30]. Expression of ectopic NS5A results in oxidative stress, and NS5A-induced transcriptional activation can be blocked by the treatment of cells with the free radical scavenger pyrrolidine-2,4-dicarboxylate acid and N-acetyl-cysteine (NAC)^[31], suggesting that NS5A induces a state of oxidative stress in the cells. However, these studies should be interrupted with caution, as they are reliant on ectopic

over-expression of HCV proteins in the absence of the complete repertoire of HCV proteins and RNA replication. However, Huh-7 cells harboring the HCV replicon do induce a state of oxidative stress^[32,33]. Thus, it is logical to hypothesize that HCV replication and alcohol metabolism lead to a synergistic increase in hepatic oxidative stress that contributes to accelerated liver disease.

ALCOHOL MODULATES HCV REPLICATION

As previously outlined, there is clinical evidence to suggest that alcohol metabolism increases HCV replication and modulates the host response to HCV^[4,7,8]. While the exact molecular mechanisms are unclear, there have been a number of postulated mechanisms, such as (1) an alcohol-induced increase in HCV RNA replication; (2) enhancement of HCV quasispecies complexity; (3) modulation of the immune system; and (4) synergistic increase in ROS. However, pinpointing the precise mechanism of how alcohol and HCV interact in the laboratory has been hampered by the lack of a small animal model of HCV pathogenesis and, until recently, the inability to culture the virus. However, the recent development of a fully permissive cell culture system for HCV has been a significant advancement for the study of HCV biology^[34]. This is further compounded by the fact that hepatocyte-derived cell lines (including the Huh-7 cell line that is permissive for HCV replication) do not express the alcohol metabolizing enzymes ADH and CYP2E1 in culture. However, numerous studies have been conducted using non-CYP2E1/ADH hepatocyte derived cell lines to determine the impact of alcohol on HCV replication, often with conflicting conclusions. To overcome this limitation, hepatocyte derived cell lines have been engineered to express CYP2E1^[19], including Huh-7 cell lines that support HCV replication and metabolize alcohol^[32]. These cells provide a useful tool to study the molecular interactions between alcohol metabolism and HCV.

There are conflicting reports surrounding the role of alcohol metabolism on HCV replication *in vitro*, which most likely reflects the different model systems used in different laboratories. Using HCV replicon cell lines that constitutively express CYP2E1 (replicon cells constitutively replicate HCV RNA under the control of an antibiotic selection marker, but do not produce infection virus particles), it was shown that physiological concentrations of alcohol (0-100 mmol/L) resulted in a CYP2E1-dependent increase in HCV RNA levels, 72 h following alcohol stimulation^[32]. This alcohol-induced increase in replication was blocked in the presence of the anti-oxidant NAC, strongly suggesting that oxidative stress plays a central role in this alcohol-induced effect. Furthermore, consistent with the data suggesting that HCV induces a state of oxidative stress alone, NAC reduced HCV replication by 50% in both HCV replicon cell lines and Huh-7 cells infected with HCV cell

culture derived virus (JFH-1) (Beard MR, personal communication). Therefore, it is conceivable that HCV uses oxidative stress to its replicative advantage. Moreover, when the infectious HCV JFH-1 virus was used to infect CYP2E1 expressing Huh-7 cells, treatment with alcohol also resulted in a significant increase in HCV (JFH-1) replication (Beard MR, personal communication). Collectively, these results suggest that CYP2E1-mediated metabolism of alcohol results in an increase of HCV replication, at least *in vitro*, and are consistent with the clinical observations described earlier. In contrast, CYP2E1-independent, alcohol-induced increases in HCV replication have been reported using the HCV replicon system^[35,36]. Conversely, it was shown that a single dose of acute ethanol exposure inhibits HCV replication^[37]. There are several methodological issues that explain these apparent discrepancies. Firstly, Huh-7 cells are different between laboratories and it is possible that low basal levels of CYP2E1/ADH exist. Secondly, there are significant differences in these studies in regards to experimental design that could account for the differences noted. For example, significant differences may occur depending on whether experimental conditions mimic acute or chronic exposure to ethanol. Acute alcohol metabolism results in a rapid increase in cellular ROS and it is possible that this burst of oxidative stress can inhibit replication^[38,39]. This correlates with unpublished findings in our laboratory, where the treatment of HCV replicon cells and HCVcc (JFH-1) infected Huh-7 cells with H₂O₂, to induce an acute burst of oxidative stress, results in a decrease in HCV replication, although chronic exposure to alcohol increases HCV replication. We have also shown in our laboratory that there is a bi-phasic effect of alcohol metabolism on HCV replication, suggesting that perhaps moderate levels of oxidative stress stimulate replication whereas more pronounced levels of oxidative stress could repress replication^[32].

The molecular mechanism whereby ROS modulates HCV replication is unknown. However, ROS have the ability to act as potent second messengers and activate cellular transcription factors, such as STAT3, nuclear factor κ B, NF-AT, and AP-1^[51,40]. Interestingly, it has been reported that ROS induced by HCV replication *in vitro* can activate the transcription factor STAT3, which can, in turn, lead to the stimulation of STAT3-dependent genes that are capable of creating a cellular environment favourable for HCV replication^[41]. Our laboratory has also shown this HCV/ROS increase in STAT3 activation. Expression of a constitutively active STAT3 molecule increases HCV replication, while conversely, specific inhibitors of STAT3 (AG490 and STA-21) cause significant decreases in replication (Beard MR, unpublished results). Clearly, the role of STAT3 and STAT3-dependent gene expression warrants further investigation.

However, not all studies implicate ROS in the increase in HCV replication following alcohol stimulation. Seronello *et al.*^[42] suggested that metabolism of alcohol modulates host lipid metabolism, thus, potentiating

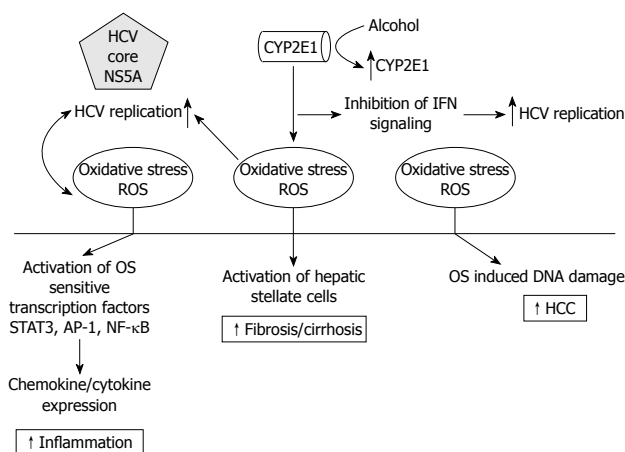


Figure 1 Proposed model of hepatitis C virus (HCV)/alcohol interactions in hepatocytes. Metabolism of alcohol by cytochrome P450-2E1 (CYP2E1) and HCV replication leads to a synergistic induction of oxidative stress in the cell. Oxidative stress inhibits interferon (IFN)- α signalling and also leads to increased rates of inflammation, cirrhosis, and hepatocellular carcinoma (HCC). NS5A: Non-structural 5A; NF- κ B: Nuclear factor κ B; ROS: Reactive oxygen species.

HCV replication. Obviously, the interactions between HCV and alcohol metabolism is a complex and multifactorial process (Figure 1), and further investigations are required to ascertain the role of the alcohol-induced modulation of HCV replication.

EFFICACY OF IFN- α IN THE PRESENCE OF ALCOHOL METABOLISM

IFN- α 2b/Ribavirin combination therapy is the current and only treatment strategy for CHC. Whilst the exact mode of IFN- α action is not well understood, IFN- α therapy is thought to result in the induction of interferon-stimulated genes (ISGs), many of which have antiviral activity. The binding of IFN to its cellular receptor results in rapid autophosphorylation and activation of receptor-associated Janus activated kinases (JAKs), TYK2 and JAK1, and activation of the JAK/STAT signaling cascade^[43]. TYK2 and JAK1 phosphorylate tyrosine residues on the cytoplasmic tail of the receptor, this provides docking sites for the signal transducer and activator of transcription (STATs), which are latent cytoplasmic transcription factors that transduce signals from the cell surface to the nucleus, where they directly regulate transcription. STAT1 is phosphorylated at tyrosine residue 701 (Y-701) and STAT2 at tyrosine residue 690 (Y-690). Once phosphorylated, STAT1 and STAT2 then form active heterodimers and translocate to the nucleus where they directly activate transcription *via* the multi component transcription factor ISG factor 3, which binds to the interferon-stimulated response element (ISRE), a cis-acting DNA element found in the promoters of the majority of type I IFN genes. Within the nucleus, STAT-1 is further phosphorylated on serine residue 727, resulting in an increase in the transcriptional activation ability of this complex. The end step of this signaling cascade cul-

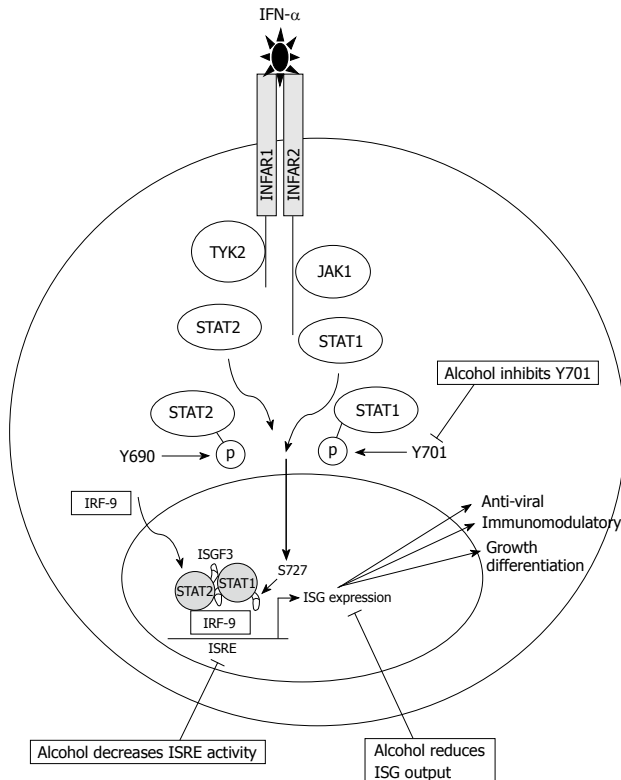


Figure 2 IFN- α signal transduction. Binding of IFN- α to its cognate receptor on the cell surface results in activation of the Janus activated kinase (JAK)/STAT signaling pathway culminating in the production of interferon-stimulated genes (ISGs), many of which have anti-viral properties that act to limit HCV infection. Alcohol inhibits Y701, decreases interferon-stimulated response element (ISRE) activity, and subsequently dampens the ISG response.

minates in the transcriptional activation of hundreds of ISGs. These produce anti-viral proteins capable of limiting HCV replication in hepatocytes and modulating the immune response (Figure 2). As mentioned previously, it is a well established clinical observation that alcohol consumption reduces the efficacy of IFN treatment^[7]. The molecular basis that underlies the reduced anti-viral capacity of IFN- α in the presence of alcohol metabolism remains to be established; however, evidence suggests that alcohol might directly inhibit the actions of IFN- α in patients at the signaling level.

In combination with the effect of alcohol on HCV replication and accelerated disease progression, alcohol metabolism also decreases the efficacy of IFN- α . Patients who consume alcohol do not respond effectively to IFN- α therapy and for this reason alcohol consumption is contraindicated during IFN- α treatment. There have been numerous studies confirming that alcoholics do not respond well to IFN- α therapy. Mochida *et al*^[44] showed that less than 10% of alcoholic patients responded to IFN- α therapy. Loguercio *et al*^[45] showed a direct relationship between alcohol consumption and response to treatment, with the numbers of patients achieving a sustained virological response decreasing as alcohol consumption increased. Safdar *et al*^[7] recently published their findings that alcohol abuse decreases response to IFN treatment in HCV patients and there-

fore, it is recommended that HCV infected patients abstain from alcohol consumption whilst on treatment. A number of studies have shown that alcohol metabolism directly interferes with IFN signaling, indicating that the consumption of alcohol could directly inhibit IFN- α treatment in patients. However, there has been some recent evidence indicating that the high non-compliance rate of alcoholics adhering to treatment programs could account for the reduced response rates of alcoholic patients in the literature^[46]. Whilst this is certainly a contributing factor, it does not detract from the strong *in vitro* data showing that alcohol has a direct inhibitory effect on IFN- α signaling. These studies have implications not only for IFN- α treatment, but also for the activity of endogenously produced type I and II IFN's (IFN- α , β , and γ , respectively) that might result in a weakened host response to HCV infection.

ALCOHOL AND IFN- α SIGNALING

There are a number of reports investigating the effects of HCV on IFN- α signaling, suggesting that HCV negatively impacts on IFN- α signaling^[47-51]; however, there are a limited number of studies investigating the combined effects of HCV and alcohol metabolism on IFN- α signaling. Insights into the effect of alcohol metabolism on IFN- α signaling can be gleaned from the study conducted by Osna *et al*^[52], in which the effects of alcohol metabolism by ADH and CYP2E1 on IFN- γ signal transduction were investigated. This study documented a decrease in STAT1 tyrosine phosphorylation in the presence of alcohol metabolism, suggesting that alcohol can effectively dampen the IFN signaling cascade. The most comprehensive study investigating the effects of alcohol and HCV replication on IFN signaling was conducted by Plumlee *et al*^[57]. This study showed that acute treatment of HCV genomic replicon cells with ethanol lead to the inhibition of the anti-HCV effects of IFN and interestingly, caused a decrease in STAT1 tyrosine phosphorylation, but induced STAT1 serine phosphorylation. This study also showed induction of ISRE promoter activity in the presence of alcohol, which is somewhat contradictory to their finding that IFN signaling was abrogated. It has also been documented that the oxidative stress generated *via* the treatment of Huh-7 cells with H₂O₂, disrupted the JAK-STAT signaling pathway specifically, by blocking STAT1, STAT2, JAK1, and TYK2 tyrosine phosphorylation^[53]. More recently it has been shown that alcohol metabolism decreases the efficacy of IFN- α *in vitro* in genomic replicon cells that express CYP2E1^[32]. In this study, alcohol metabolism preferentially inhibited STAT1 tyrosine phosphorylation at residue 701 (Y701), the critical residue required for STAT1 heterodimerisation with STAT2^[32]. These findings suggest that a decrease in STAT1-Y701 phosphorylation due to alcohol metabolism would decrease downstream ISG expression and, in part, explain the reduced efficacy of IFN- α in HCV positive patients that consume alcohol. Consistent with

this finding, a decrease in ISRE activity in the presence of alcohol metabolism was also observed^[32] and a number of anti-viral ISGs have been shown to be downregulated by alcohol metabolism (Beard MR, unpublished findings). Collectively, these studies suggest that alcohol can specifically inhibit IFN- α at the molecular level.

Clearly, the factors at play in the alcohol-induced suppression of IFN signaling in the background of HCV replication are multifactorial and complicated in an infected patient. It is also possible that alcohol can inhibit other components of the cellular innate immune response, such as components of the cellular recognition of viral pathogen-associated molecular patterns, and by activation of the toll-like-receptor-3 and the retinoic acid-induced gene-1 pathways. Another possibility is that alcohol might modulate SOCS-1 and -3, which are part of a negative feedback loop that acts to suppress IFN-signaling. It has been shown using a concanavalin-A model of hepatitis in mice that activated STAT3 plays an important role in inducing SOCS-3^[54]. Interestingly, as outlined previously, we have been able to demonstrate that both alcohol metabolism and HCV replication are capable of activating STAT3, and it appears that this increase is due to oxidative stress.

CONCLUSION

In summary, it is well established clinically that chronic alcohol consumption in the setting of CHC is a dangerous combination leading to exacerbated liver disease. While the factors that lead to increased liver disease are complex, it seems that a common thread throughout many investigations is the generation of oxidative stress by both alcohol metabolism and HCV replication. Thus, it is a logical conclusion that HCV and alcohol metabolism act synergistically to accelerate disease progression *via* oxidative stress. In an attempt to understand these processes we have developed a model to portray these interactions (Figure 1). Evidence suggests that this oxidative stress synergy impacts on HCV replication and the anti-viral action of IFN- α , although we are waiting for the development of a small animal model to confirm these *in vitro* observations. Defining the molecular mechanisms and complex interactions between HCV and alcohol will further our understanding of the pathogenic role alcohol plays in CHC development and hopefully lead to novel therapeutic strategies and patient management.

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Alcohol, nutrition and liver cancer: Role of Toll-like receptor signaling

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pro-inflammatory response is an important mechanism of liver oncogenesis. It provides a nutritional approach, which could prevent HCC from developing in many chronic liver diseases.

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Abstract

This article reviews the evidence that ties the development of hepatocellular carcinoma (HCC) to the natural immune pro-inflammatory response to chronic liver disease, with a focus on the role of Toll-like receptor (TLR) signaling as the mechanism of liver stem cell/progenitor transformation to HCC. Two exemplary models of this phenomenon are reviewed in detail. One model applies chronic ethanol/lipopolysaccharide feeding to the activated TLR4 signaling pathway. The other applies chronic feeding of a carcinogenic drug, in which TLR2 and 4 signaling pathways are activated. In the drug-induced model, two major methyl donors, S-adenosylmethionine and betaine, prevent the upregulation of the TLR signaling pathways and abrogate the stem cell/progenitor proliferation response when fed with the carcinogenic drug. This observation supports a nutritional approach to liver cancer prevention and treatment. The observation that upregulation of the TLR signaling pathways leads to liver tumor formation gives evidence to the popular concept that the chronic

INTRODUCTION

What is the mechanism that explains how alcohol abuse increases the risk of hepatocellular carcinoma (HCC) in hepatitis C, hepatitis B, diabetes^[1], hemochromatosis^[2], and α -1 antitrypsin deficiency^[3]? Evidence is emerging that the synergism is due to an activation of a common pathway in which Toll-like receptor (TLR) signaling induces pro-inflammatory cytokine production through nuclear factor (NF)- κ B activation and growth factors through activation of activator protein 1 (AP-1). A model of chronic ethanol feeding has been used as follows. Transgenic mice with hepatitis C non-structural 5A (NS5A), the NS5A protein of hepatitis C virus (HCV), were fed ethanol chronically. This induced HCC formation and resembled clinical alcohol abuse and hepatitis C progression to HCC^[4].

Likewise, an experimental model of chronic drug toxicity, which induces HCC, resembles clinical drug-induced HCC^[5]. These experimental models are reviewed to help understand how the TLR signaling pathway leads to HCC formation, as a mechanism that is shared by the clinical and experimental models.

TLR SIGNALING PATHWAY IN CHRONIC LIVER DISEASE

There is emerging evidence that upregulation of the TLR signaling pathway occurs in many chronic liver diseases. Many different cell types in the liver express TLRs^[6]. Kupffer cells express TLR2, 3, 4 and 9. Hepatocytes express TLR2, 3, 4 and 5. Stellate cells express TLR4 and 9. Biliary epithelium expresses TLR2, 3, 4 and 5. Sinusoidal endothelium expresses TLR4. TLR-mediated signals result in hepatitis B, hepatitis C, alcoholic liver disease, non-alcoholic liver diseases, primary biliary cirrhosis, primary sclerosing cholangitis, hepatic fibrosis, ischemic-reperfusion injury and liver allograft rejection^[6].

HEPATITIS B

TLR2 is downregulated in hepatocytes, Kupffer cells and peripheral blood monocytes in hepatitis-B-antigen-positive patients^[6]. In hepatitis-B-e-antigen-negative patients, TLR2 expression and tumor necrosis factor (TNF)- α production is upregulated and probably mediated by pre-core proteins^[6].

HEPATITIS C

The HCV core and NS3 protein activate TLR2/TLR1 and TLR2/TLR6 on monocytes to produce inflammatory cytokines^[6]. Patients with hepatitis C lack tolerance to lipopolysaccharide (LPS). Marked upregulation of TLR2 and TLR4 have been observed in patients with chronic hepatitis C and were detected in hepatocytes, Kupffer cells, and peripheral monocytes. TLR2-mediated activation by hepatitis C may contribute to the pro-inflammatory cytokine activation^[7].

ALCOHOL LIVER DISEASE

Alcohol ingestion decreases the intestinal mucosal barrier to LPS, which activates TLR4 on Kupffer cells, which leads to pro-inflammatory cytokine production^[8]. Chronic alcohol consumption activates other TLRs, such as TLR1, 2 and 6-9, which further increases the TNF- α response to LPS in mice^[6]. Human monocytes exposed to ethanol for 7 d develop hypersensitivity to LPS through decreased IRAK-M expression and activation of NF- κ B and extracellular signal-regulated kinase (ERK)^[9]. IRAK-M is part of the cascade that activates NF- κ B and mitogen-activated protein kinase, initiated by TLR4 signaling.

CIRRHOSIS

TLR4 expression is downregulated in monocytes from patients with Child-Pugh C cirrhosis, which indicates LPS tolerance, because the expression returns to normal after antibiotic therapy^[6].

HCC

Mice deficient in TLR4 and MyD88 develop fewer and smaller liver tumors after treatment with a chemical carcinogen, which implies that TLR4-MyD88 signaling is involved in the development of liver tumors^[6]. This suggests that chronic inflammation that results from hepatitis C and TLR-induced increase in NF- κ B activation and TNF α production promotes tumor growth^[10].

ROLE OF TLR4 SIGNALING IN ETHANOL-FACILITATED TUMOR FORMATION IN THE NS5A TRANSGENIC MOUSE MODEL OF HCC CARCINOGENESIS

Ethanol feeding upregulates the TLR4-dependent, MyD88-independent signaling pathway in mice^[11], presumably by increasing endotoxemia due to gut leakage of LPS. TLR-4 upregulation in Kupffer and stellate cells occurs in mice fed ethanol for 1 mo using the intragastric tube feeding model^[12].

Machida *et al*^[4] have taken advantage of upregulation of the TLR4 signaling pathway induced by ethanol, to accelerate HCC formation in NS5A transgenic (NS5A Tg) mice. NS5A Tg mouse livers already overexpressed TLR4, but ethanol and LPS feeding increased the TLR4 signaling due to the endotoxemia caused by ethanol. LPS challenge killed 30% of the NS5A Tg mice, but not the wild-type mice or the NS5A Tg/TLR4^{-/-} mice. The NS5A Tg mice overreacted to LPS, with the development of massive hemorrhagic, necrosis, and inflammation. The TAK1-TRAF6 and TAK1-IRAK1 interactions in NS5A Tg mice given LPS were enhanced, but not enhanced in the wild-type or TLR^{-/-} mice. Phosphorylation of Jun N-terminal kinase (JNK) and I κ B α , two further downstream components of TLR4 signaling, was increased by LPS.

To determine whether TLR upregulation in the NS5A Tg mice was due to overexpression by Kupffer cells or hepatocytes, the Kupffer cells were depleted by injection of liposome-encapsulated clodronate 2 d before LPS injection. This reduced TLR4 activation by 35% in the NS5A Tg mice, and did not reduce the mortality, whereas, in wild-type mice, it reduced the TLR4 activation by 70% to 80%.

Machida *et al*^[4] have tested human liver from patients with hepatitis C, including those who abused alcohol. NS5A was increased in both groups of hepatitis C patients. NS5A expression in the liver from hepatitis C patients was about one-third of the level seen in the NS5A

Tg mice. TLR4, p-JNK, and p-1KB α were upregulated in the hepatitis C livers, which confirmed the clinical importance of TLR4 activation in NS5A Tg mice.

The question was: were the NS5A Tg mice more susceptible to alcohol-induced liver damage? The NS5A Tg mice were fed ethanol for 4 wk by intragastric tube together with wild-type, TLR4^{-/-} and TLR4^{-/-} NS5A Tg mice. The NS5A Tg mice fed ethanol developed higher alanine aminotransferase levels, and spotty liver necrosis with inflammatory cell infiltration. These liver injury markers did not occur in the TLR4^{-/-}/NS5A Tg mice fed ethanol, which confirmed the pathogenic role of TLR4 in this mouse model. LPS levels in the ethanol-fed mice were equally elevated. The liver injury, induced by ethanol feeding, was abrogated by feeding the antibiotics polymyxin B and neomycin, when ethanol-feeding-induced injury was augmented by feeding LPS weekly and ethanol daily. These results indicate the importance of endotoxin in activated TLR4 signaling in alcohol-fed NS5A Tg mice. Lipid peroxidation was increased in the alcohol-fed NS5A Tg mice, compared to the wild-type mice fed ethanol.

The NS5A Tg mice and the same three control groups, wild-type, TLR4^{-/-} and TLR4^{-/-} NS5A Tg mice, were fed the Lieber deCarli diet that contained ethanol for 12 mo to induce liver tumor formation. Liver tumors developed only in the ethanol-fed NS5A Tg mice. TLR4 signaling induced by NS5A and alcohol feeding was documented by showing an increased TAK1 interaction with TRAF6 and by elevation of pJNK and pIKB, which occurred only in the ethanol-fed mice. It was concluded that alcohol and NS5A synergistically induced liver tumors by enhancing the expression of TLR signaling.

To determine the molecular mechanisms that cause synergism between alcohol feeding and NS5A Tg mice, microarray analysis of livers was performed^[4]. The non-tumor areas of the NS5A Tg mice fed ethanol were compared with wild-type controls. Of the 83 genes that were changed > 4-fold, for example Nanog, TLR4 and interferon (IFN)- α 4 were upregulated. By immunofluorescent antibody staining, Nanog was colocalized with the stem cell markers CD133 and CD49f, which suggested that cancer stem cells were present in the non-tumor livers of the NS5A Tg ethanol-fed mice. The TLR4^{-/-} NS5A Tg mice fed ethanol did not develop Nanog-positive stem cells, which implied that the mechanism of stem cell and tumor induction was dependent on TLR4 signaling. To support this idea, NS5A Tg Huh7 cells treated with LPS upregulated the expression of Nanog, and this was reduced in TLR4 knockdown cells using shRNA. Overexpression of TLR4 in Huh7 cells increased the expression of Nanog in the presence of LPS^[4]. LPS increased the reporter activity in NS5A transduced Huh7 cells. The results indicate that the promoter of the Nanog gene is directly activated by TLR4 and that Nanog is a novel downstream gene of TLR4 signaling.

Further studies indicated that Nanog mediated the TLR-dependent liver tumor formation by hepatic progenitor cells^[4], using the hepatic progenitor cell transplant

model. Hepatoblasts were isolated from p53-deficient embryonic liver infected with a retroviral vector that expressed TLR4 or Nanog along with green fluorescent protein (GFP). The cells were transplanted into wild-type mice *via* the spleen. These mice were given three injections of CCl₄ to stimulate the establishment of the progenitor cells. After this, the mice were repeatedly injected with LPS several times a week for 25 wk. Liver tumors formed after 48-52 d, as judged by GFP imaging *in vivo* and by autopsy. Direct Nanog transduction in hepatic progenitor cells also produced tumors, but less than when LPS was injected subcutaneously into nude mice and GFP imaging was performed over 88 d. The TLR4-transduced mice began to form tumors 40 d after LPS treatment. These cells were p53^{-/-}^[4]. Silencing Nanog expression by Nanog shRNA delayed tumor formation. It was concluded that Nanog increases HCC formation by the TLR4-transduced progenitor cells, but Nanog alone does not confer the full oncogenic potential^[4].

If Nanog activation is not the whole story, what else is required to transform progenitor cells into HCC-promoting cells? It turned out that TLR4-dependent, Nanog-expressing HCC stem cells exhibited defective transforming growth factor (TGF)- β signaling^[13]. The TGF- β signaling pathway inhibits liver cell regeneration. It has been shown that the TGF- β defective pathway in mice insufficient for β 2 spectrin (β 2SP) leads to spontaneous development of HCC. Cancer stem cells from alcohol-fed NS5A Tg mice were examined for growth in soft agar. Their lentiviral cDNA library was created and tested for transformation of the oval cell line and screening for oncogenic genes. GFP-labeled cancer stem cells were injected into nude mice that were repeatedly injected with LPS after the initial stem cell injection, and tumor formation was followed by GFP imaging. The interaction of the TGF- β pathway with the TLR4-dependent oncogenic activity was studied. The cancer stem cells had upregulation of Nanog and sex determining region Y-box 2 (Sox-2). Tumors were formed in the nude mice, and knockdown of Nanog prevented tumor formation. The cancer stem cells were defective in the expression of TGF- β 1 and β 2SP. The TLR4 response element promoter activity under TLR4 activation by LPS E2F1 was induced by LPS in the cancer stem cells. E2F1 is a transcriptional activator for Nanog and is inhibited by TGF- β . The authors conclude that heightened TLR4 activation in the Nanog-positive cancer stem cells is associated with and interactive with the defective TGF- β tumor suppressor pathway for oncogene activity of Nanog-positive cancer stem cells^[13].

ROLE OF TLR4/2 SIGNALING PATHWAY IN CHEMICAL-CARCINOGEN-INDUCED LIVER TUMOR MODEL

Oliva *et al.*^[5] have developed a chemically-induced mouse model of liver tumor formation, which recently has been shown to be associated with TLR-4/2 activation^[14]. In

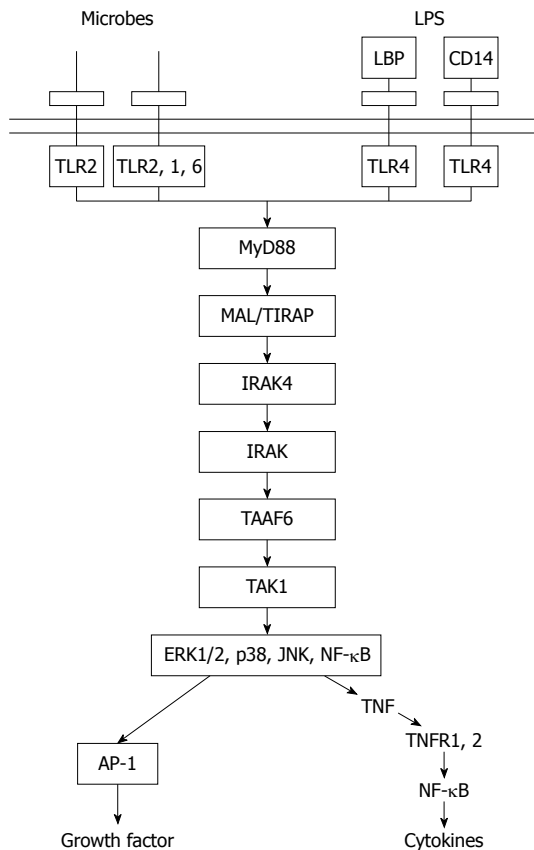


Figure 1 Main relationship between Toll-like receptor (TLR)2 and 4, their adaptors, protein kinases, which are linked to them and downstream signaling effects^[26]. LPS: Lipopolysaccharide; LBP: LPS binding protein; CD14: Cluster of differentiation 14; ERK: Extracellular signal-regulated kinase; JNK: Jun N-terminal kinase; NF-κB: Nuclear factor κB; AP-1: Activator protein 1; TNF: Tumor necrosis factor.

this model of experimental carcinogenesis, mice were fed diethyl 1,4-dehydro-2,3,6-trimethyl-3,5-pyridine decarboxylate (DDC). 0.1% DDC was fed *ad libitum* in the diet for 10 wk, at which time a large percentage of hepatocytes had become transformed into stem cell/progenitors. These cells stained positive for stem cell markers (Ubd, OV-6, GSTP), and formed Mallory-Denk bodies. When the drug was withdrawn, these stem cell/progenitors persisted in small numbers scattered throughout the liver lobules. When the drug was reintroduced after 1 mo withdrawal, the stem cell/progenitors proliferated with a growth advantage over the intervening normal hepatocytes^[5,15-17], which indicated that the stem cell/progenitors had been epigenetically changed^[5,18-20]. The replication of stem cell/progenitors was prevented by feeding S-adenosylmethionine (SAm) or betaine as methyl donors^[5,19,20]. The replication of the stem cell/progenitors was stimulated by refeeding DDC or other liver toxins. In primary liver cultures, stem cell/progenitor formation was associated with NF-κB and AP-1 activation, as well as phosphorylation of p38 JNK and ERK^[17,21-25]. All of this could be the result of increased TLR4/2 (Figure 1), according to signaling microarray analysis data mining of livers of control mice compared with mice refed DDC for 7 d and those fed DDC with SAm (4 g/kg body

weight^[14]. TLR2 and TLR4 were both upregulated and this was prevented by SAm. RT-PCR confirmed the up-regulation of TLR2/4 expression, and this was prevented by SAm. Levels of MyD88 adapter protein were increased, but the TLR2/4-TRIF-1RF3 pathway (MyD88-independent pathway) was not upregulated, which differs from the case when ethanol is fed^[11]. TRAF6 tended to be upregulated and this was prevented by SAm feeding. TRAF6 is down stream from the MyD88/IRAK complex. TRAF6 leads to activation of NF-κB and cytokine upregulation, and indirectly to the activation of p38, JNK and ERK, which initiates growth of hepatocytes (Figure 1). With an increase in NF-κB activation, TNFα (R21 and 12a) and IFN-γ (21 and 2) receptor expression was upregulated, coupled with the proliferation of stem cell/progenitors when DDC was refed, and SAm prevented these changes^[14,27].

Other signaling pathways such as STAT3, Wnt NOTCH, hedgehog and TGFβ, which are involved in stem cell renewal, differentiation and survival may also be involved in stem cell/progenitor transition to cancer stem cells found in HCC^[28].

CONCLUSION

There are increasing numbers of examples of the TLR signaling pathway in which it plays a key role in activating stem cell/progenitor proliferation and conversion to cancer-stem-cell-based liver tumor formation. Two examples were given in detail, one driven by chronic ethanol and LPS stem cell/progenitor feeding, and the other by chronic drug feeding. These two examples provide proof of the principle that TLR signaling pathway activation is required for stem cell/progenitor activation and transformation, and supports the concept that inflammation leads to cancer formation. Gene knockout of TLR4 expression prevented tumor formation in the first example and SAm prevented all the steps leading to pro-inflammatory activation, including TLR signaling in the second example, therefore, it is likely that blocking TLR signaling would prevent tumor formation prophylactically by feeding SAm.

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Effects of ethanol on the proteasome interacting proteins

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Abstract

Proteasome dysfunction has been repeatedly reported in alcoholic liver disease. Ethanol metabolism end-products affect the structure of the proteasome, and, therefore, change the proteasome interaction with its regulatory complexes 19S and PA28, as well as its interacting proteins. Chronic ethanol feeding alters the ubiquitin-proteasome activity by altering the interaction between the 19S and the 20S proteasome interaction. The degradation of oxidized and damaged proteins is thus decreased and leads to accumulation of insoluble protein aggregates, such as Mallory-Denk bodies. Ethanol also affects the immunoproteasome formation. PA28a/b interactions with the 20S proteasome are decreased in the proteasome fraction isolated from the liver of rats fed ethanol chronically, thus affecting the cellular antigen presentation and defense against pathogenic agents. Recently, it has been shown that ethanol also affects the proteasome interacting proteins (PIPs). Interaction of the proteasome with Ecm29 and with deubiquitinating enzymes Rpn11, UCH37, and Usp14 has been found to decrease. However, the two UBL-ubiquitin-associated domain (UBA) PIPs p62 and valosin-containing protein are upregulated when the proteasome is inhibited. The increase of these UBL-UBA

proteins, as well as the increase in Hsp70 and Hsp25 levels, compensated for the proteasome failure and helped in the unfolding/docking of misfolded proteins. Chronic alcohol feeding to rats causes a significant inhibition of the proteasome pathway and this inhibition results from a decreases of the interaction between the 20S proteasome and the regulatory complexes, PIPs, and the ubiquitin system components.

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Key words: Alcoholic liver diseases; Proteasome; Proteasome interacting proteins

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INTRODUCTION

Proteasome dysfunction is well established in alcoholic liver injury. However, the mechanism by which ethanol feeding causes proteasome dysfunction is still unclear. Proteasome chymotrypsin-like activity decrease has been the major finding to explain the proteasome dysfunction and accumulation of misfolded and ubiquitinated proteins in the liver of chronic ethanol-fed animals. Previous studies reported by the author^[1,2] have shown that the dysfunction of the proteasome system in alcoholic

liver disease is caused by structural changes in the α type subunits of the proteasome. However, the dysfunction of the proteasome pathway in alcoholic liver disease is more complicated. There are multiple levels of proteasome activity and specificity regulation. The ubiquitin system and the proteasome interacting proteins (PIPs) are part of the regulation of proteasome activity and specificity. The effects of ethanol feeding on the ubiquitin system and the proteasome interacting protein are still unknown.

PROTEASOME SYSTEM

The proteasome system is a sophisticated, selective, and highly specific proteolytic pathway. It includes the 20S proteasome, also called the catalytic core particle (CP), the regulatory complexes, and interacting proteins. The catalytic core is formed by 28 subunits, arranged into four heteroheptameric rings made of seven subunits each ($7\alpha7\beta7\beta\alpha$). The α -type subunits form the external rings, and the β subunits the internal rings.

CP BINDING TO THE 19S REGULATORY COMPLEX

The CP of the proteasome system binds to the regulatory complex 19S to form the 26S proteasome (Figure 1). The 26S proteasome is involved in the ubiquitin proteasome pathway (UPP), and is responsible for the ubiquitin-targeted protein degradation^[5] (Figure 1).

Cellular key proteins, such as cyclins, cyclin-dependent kinase inhibitors, I κ B, hypoxia-inducible factor-1 α , Nrf2, and p53, are substrates for the 26S proteasome, and their ubiquitin-dependent degradation is highly controlled^[4-6].

The UPP is also responsible for the clearance of misfolded and oxidized proteins. Therefore, proteasome failure is the cause of numerous diseases associated with poor clearance of these often deleterious proteins.

There are two major steps involved in the ubiquitin-proteasome-dependent degradation pathway: (1) the enzymatic polyubiquitination of protein substrates; and (2) the docking and recognition by the 26S proteasome prior to degradation. A cascade of enzymes, including the ubiquitin-activating E1 enzymes, the ubiquitin-carrier protein E2 enzymes, and the ubiquitin-protein ligases E3, which conjugate the ubiquitin residues to the target protein substrate for degradation, are responsible for the ubiquitination of the protein substrate^[7]. There are at least four different E1s, 24 different E2s, and at least 100 E3s ligases^[8].

Several rounds of ubiquitination yield an ubiquitin chain that includes at least four ubiquitin residues, which designates the target protein for degradation by the 26S proteasome. Once the polyubiquitinated protein is delivered to the 26S proteasome, the polyubiquitin chain is freed, and the ubiquitin residues are recycled. Several

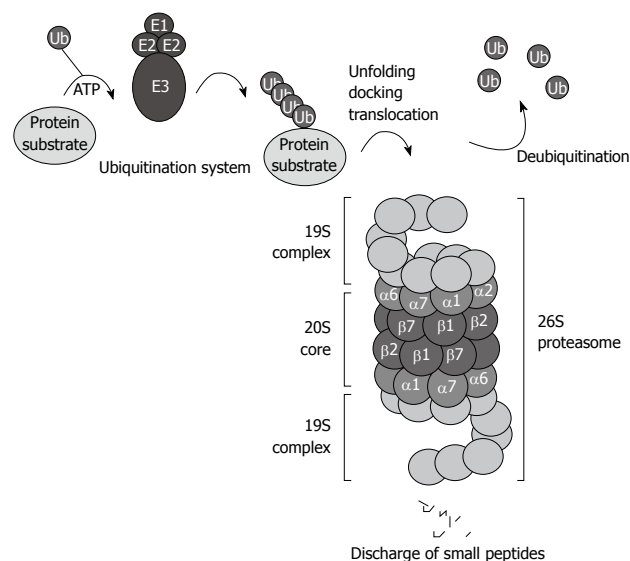


Figure 1 Schematic illustration of the ubiquitin proteasome pathway. The 26S proteasome consists of the 20S core capped with the 19S regulatory complexes that recognize ubiquitinated protein substrates designated for proteolysis.

deubiquitinating enzymes play an important regulatory role in regenerating the ubiquitin protein^[9], and in certain cases, represent a rate-limiting step for proteasome-mediated protein degradation^[10]. When change occurs and interferes with these rounds of ubiquitination, deleterious proteins accumulate and cause cellular dysfunction. For instance, mutation of the E3 ligases and misreading of ubiquitin have been reported to modify the ubiquitin system level and cause a significant inhibition of proteasome activity^[11].

This great diversity in the ubiquitination system and in the interactions of the CP with its regulatory complexes reflects the complexity and the specificity of the UPP. Disruption of the UPP has been implicated in a wide range of human diseases. Therefore, the proteasome and ubiquitination components are highly attractive targets for pharmaceutical intervention.

CP BINDING TO THE PA28 REGULATORY COMPLEX

The 20S proteasome can also bind to the regulatory complex PA28 (also known as REG and 11S) to form the immunoproteasome (Figure 2). PA28 is a heteromeric complex of 28-kDa subunits. It binds to the cylinder end of the 20S, thus opening the gate channel to the catalytic chamber^[12,13].

The immunoproteasome forms under the influence of high levels of cytokines, such as interferon (IFN) γ ^[14]. The immunoproteasome formation also consists of a replacement of the catalytic β subunits of the 20S proteasome, i.e. chymotrypsin-like (β 5), trypsin-like (β 1), and peptidylglutamyl peptide-hydrolase, recently called caspase-like activity (β 2), by the immunoproteasome sub-

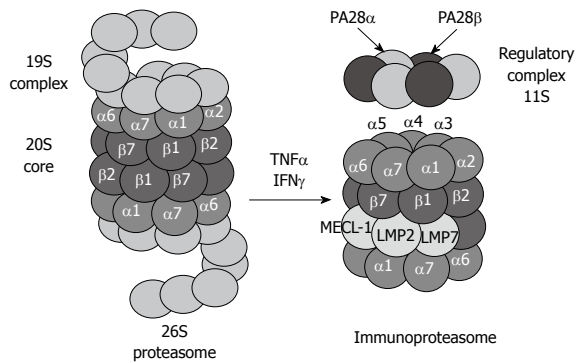


Figure 2 Tumor necrosis factor (TNF) α and interferon (IFN) γ induce formation of immunoproteasome subunits. LMP7, LMP2 and MECL-1 subunits replace the constitutive catalytic subunits $\beta 5$, $\beta 1$ and $\beta 2$, respectively, which shift the catalytic properties of the proteasome to generate MHC-I-binding peptides.

units LMP7, LMP2, and MECL-1, respectively^[15,16]. This replacement is required for the cleavage site specificity of the immunoproteasome for efficient antigen processing and presentation by major histocompatibility complex class I molecules^[17,18]. However, despite the induction of the immunoproteasome subunits, if the α subunits are modified, for instance by chronic ethanol feeding^[1,2], the binding between the catalytic core 20S and the regulatory complex PA28 is blocked, and antigen presentation is thus lowered, which alters the host defense.

CP BINDING TO THE PA200 REGULATORY COMPLEX

The 20S proteasome CP also associates with other activating complexes such as the HEAT-repeat protein PA200 (the homologue of yeast Blm10)^[19], which also opens the gate and stimulates peptide entry. PA200 is present within hybrid complexes (19S-20S-PA200), and is an ATP-independent proteasome regulatory complex. It is known to be involved mainly in the nucleus and DNA repair^[20].

In summary, the role of these different regulatory complexes, binding to the 20S proteasome CP, is to open the 20S proteasome gate at the α subunits, then to confer specificity to the proteolytic activity of the catalytic chamber formed by the β subunits. Numerous studies have focused on the effects of chronic ethanol feeding on the ubiquitin-proteasome pathway (26S proteasome activity) and the consequence of its dysfunction in liver cells^[21,22]. These effects still need further understanding. Substantial studies need to be undertaken to elucidate the effects of chronic ethanol feeding on the immunoproteasome and the nuclear proteasome, with respect to host defense and the epigenetic mechanism regulation.

WHAT ARE THE PIPS?

As much as the proteasome is complex, it does not work alone. There is an orchestra of proteins involved in the

regulation of the proteasome activity. An increasing number of PIPs have been reported, which indicates that the 20S proteasome CP is a dynamic structure that interacts with specific proteins for specific functions. Therefore, any interference with the 20S proteasome and with its interacting proteins would affect the proteasome specific functions.

It has been a decade since Verma *et al*^[23] have reported and analyzed PIPs. These authors used a one-step affinity method to purify intact 26S proteasome and its interacting proteins from budding yeast cells, and reported the existence of PIPs. These newly discovered PIPs were classified in four groups that include proteasome subunits, chaperone, transcription, and ribosomal proteins. New methods to purify the proteasome were then developed. Scanlon *et al*^[24] have used the GST UBL (ubiquitin-like domain as an affinity chromatography matrix), and purified the proteasome from human cell lines. These authors have classified the 26S PIPs into four groups of proteins that include de-ubiquitinases, ubiquitin ligases, ubiquitin domain-containing proteins and conjugating/ubiquitin, and proteins involved in DNA repair. Using the GST UBL matrix to purify the PIPs has helped find proteins dominantly related to the proteasome and ubiquitination systems; probably because of the affinity of UBL for the ubiquitin-proteasome pathway.

Among identified PIPs are a number of abundant cellular proteins, such as heat shock proteins (Hsps), elongation factors, and ribosomal proteins. The Hsps interaction with the proteasome is highly specific because they are induced to compensate for the proteasome failure, when the proteasome activity is decreased. In higher eukaryotes, it has been shown that Hsc70/Hsp70 members facilitate the delivery of aggregation-prone substrates for degradation by interacting with the proteasome through an adaptor protein^[25,26]. Along with Hsp70, Hsp27 assists in the unfolding of the proteins designated for degradation by the proteasome^[27]. In addition, Hsp90 family members have been suggested to play a role in the proteasome structural integrity and assembly through their interactions with the 26S proteasome^[28].

Other proteins work upstream of the ubiquitin system to recognize, unfold, shuttle, dock, and deubiquitinate the protein substrate designated for degradation. The chaperone system Bip/PDI is associated with endoplasmic reticulum (ER)-associated degradation^[29]. Other proteins that contain a ubiquitin-associated domain (UBA), such as Rad23 and p62, are involved in carrying and docking the protein substrates at the proteasome^[30]. These interacting proteasome partners differ in their biological roles, and are chosen to interact with the proteasome according to their specific cellular function. Therefore, different chaperone members may play distinct roles in modulating protein degradation by the proteasome.

A great number of yet-to-be-identified proteins can mediate ubiquitin recognition at the proteasome. UBL-UBA domain-containing proteins associate with

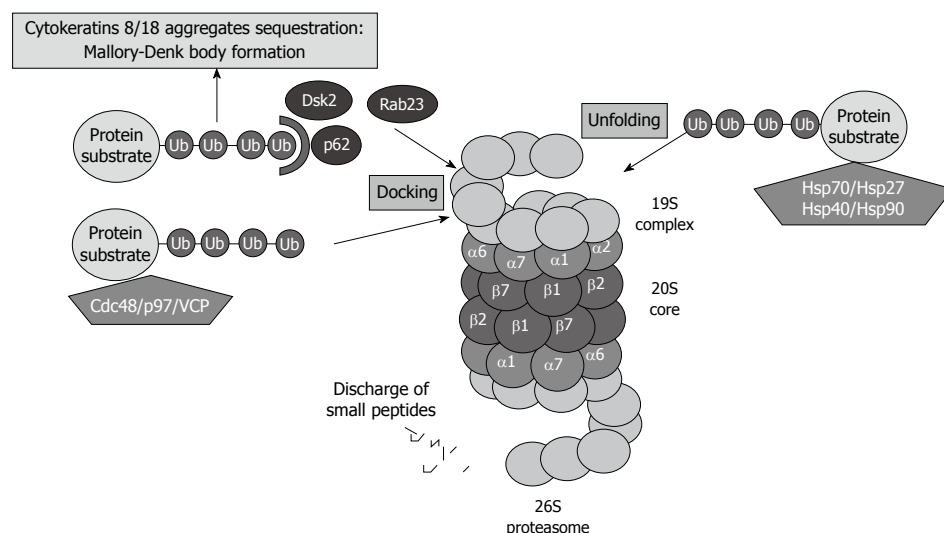


Figure 3 Proteasome interacting proteins (PIPs) involved in unfolding and docking of protein substrates designated for degradation by the proteasome. VCP: Valosin-containing protein.

substrates designated for degradation, as well as with subunits of the proteasome, thus regulating the proper turnover of proteins. The best known UBL-UBA proteins of PIPs are Cdc48/p97/valosin-containing protein (VCP), which present misfolded ER-proteins to the proteasome. P62, also called sequestosome 1, is also involved in presenting ubiquitinated proteins to the proteasome (Figure 3).

VCP and P62 have been reported to be involved in cytokeratin 8/cytokeratin 18 aggregate sequestration and Mallory-Denk body (MDB) formation in alcoholic liver disease^[31] (Figure 3). Both proteins are significantly up-regulated when the proteasome activity is inhibited using PS-341, thus indicating that these proteins play a crucial role in proteasome activity^[32].

The 26S stabilizing protein, Ecm29, has been found in the fraction of the purified proteasome, and is well established as a PIP. Ecm29 tethers the proteasome CP to the 19S regulatory particle, and confers stability of 19S-20S binding in yeast^[33]. It may play a crucial role in the 26S proteasome dysfunction in alcoholic liver disease^[34].

More recently, Kautto *et al*^[35] have developed a rapid method of 26S proteasome isolation using chromatography, and have identified over 100 proteins in the proteasome purified fraction, including the 26S proteasome and 32 proteasome subunits. 14-3-3-like proteins have been identified to interact with the proteasome, and also have been classified as PIPs. In our laboratory, when chromatography and high salt concentration purification was used, only a few proteins were identified by mass spectrometry, which indicates that the salt disrupted the proteasome interactions with its associating proteins. The identified proteins included the 14-3-3 proteins, the kinases protein kinase A (PKA) and transglutaminase, and the phosphatases PP2A and PP1^[36].

The role and function of the 14-3-3 proteins, in the regulation of proteasome function, remain to be elucidated. This protein has been identified by mass spectrometry in the 20S fraction purified chromatographically with a high salt gradient, which reflects the strength

of 14-3-3 interaction with the 20S proteasome. 14-3-3, which is a major scaffolding protein and a phospho-binding protein in the cell, plays a major role in cellular mechanisms, such as signal transduction and regulation of transcription factors^[37-39]. A pilot study has shown that chronic ethanol feeding increases the interaction of 14-3-3 with the 20S proteasome, probably to regulate the 20S proteasome ratio of phosphorylation/dephosphorylation to modulate the proteasome activity changes due to ethanol feeding^[36].

The proteins kinases associated with the 20S proteasome, such as casein kinase II^[40], transglutaminase (TG2), and PKA also co-isolated with the 20S proteasome through multiple chromatographic steps and high salt concentration, as well as the phosphatases PP2A and PP1^[41,42]. These proteins regulate the 20S proteasome activity *via* phosphorylation/dephosphorylation and are believed to regulate also the 20S proteasome binding to its regulatory complexes. TG2 has been found in the fraction of highly purified 20S proteasome^[36], and is known to be responsible for stabilizing macromolecular assemblies^[43,44]. It is possible that TG2 is involved in the stabilization of the proteasome macromolecules *via* its kinase activity. These kinases and phosphatases are crucial for the function of the different types of proteasomes because they regulate the phosphorylation of the α type subunit of the proteasome, thus determining the binding of the 20S proteasome to its regulatory complexes (Figure 4).

Similarly to PKA, TG2 and PP2A, the enzyme δ -aminolevulinic acid dehydratase (ALAD) has been identified by mass spectrometry in the highly purified 20S proteasome fraction^[45]. ALAD, also called porphobilinogen synthase, is a cytosolic sulfhydryl-containing enzyme that catalyzes the condensation of two molecules of aminolevulinic acid (ALA). It has been reported that blood ALAD activity is significantly decreased when rats are chronically fed ethanol, which indicates that ethanol feeding causes an alteration in blood^[46] as well as liver ALAD activity^[47].

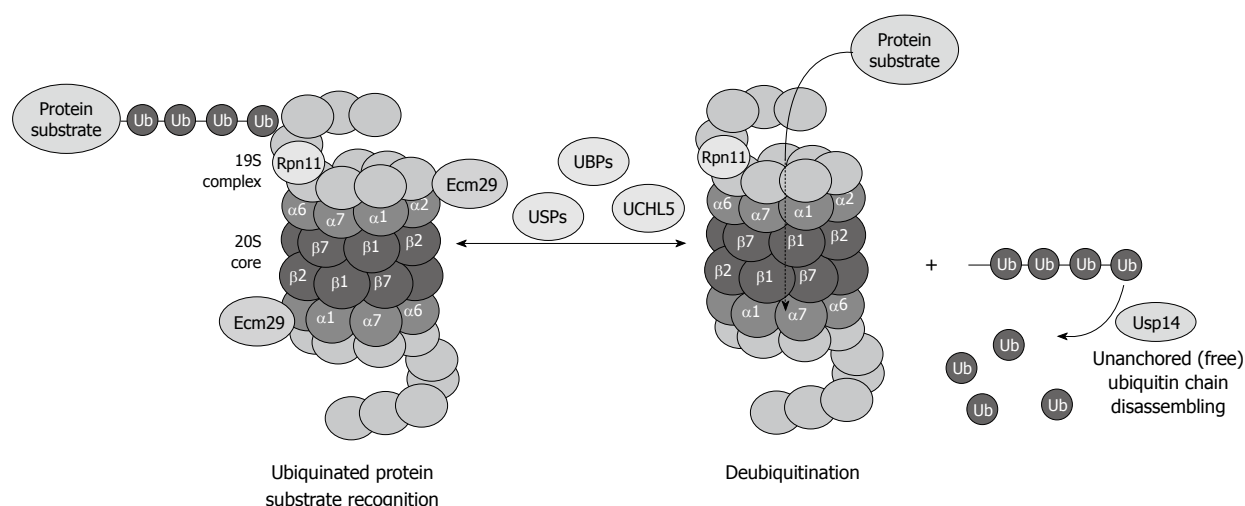


Figure 5 Diagram showing the important role of the deubiquitinases in the process of proteasome function and removal of ubiquitinated protein substrates, as well as recycling of free ubiquitin.

enhanced proteolytic activity, and are expressed constitutively by professional antigen-presenting cells, including B cells^[69]. It is well known that the host defense is reduced in alcoholic liver disease^[64]. This is possibly due to the failure of the immunoproteasome assembly. Recently, it has been shown that chronic ethanol feeding causes a decrease in the binding between the 20S catalytic core and the regulatory complex PA28; binding that is required for the immunoproteasome function, despite the induction of the immunoproteasome subunits^[34]. Results from the latest study have indicated that, similar to the mechanism that causes 26S proteasome dysfunction, the immunoproteasome is also altered in alcoholic liver disease. Post-translational modifications of α -type subunits are the key mechanism that regulates the binding of the 20S proteasome catalytic core to its regulatory complexes 19S or PA28. Phosphorylation/dephosphorylation of these subunits is known to regulate their interaction with the 19S ATPases subunits, and thus, 26S formation^[70]. Chronic ethanol feeding alters α -type subunit phosphorylation^[1]. It is possible that the immunoproteasome assembly is regulated *via* the interaction between the 20S and the α and β subunits of the PA28 regulatory complex.

Studies to date have focused on the changes that can occur either to the ubiquitin system, or to the proteasomes subunits themselves. However, there is increasing evidence that this pathway is also regulated by other proteins that are just as important as the ubiquitin-proteasome pathway elements^[71], and the regulatory complexes 19S, PA28, or PA200.

It is now well established that PIPs are significantly involved in the regulation of proteasome activity. However, the effect of chronic alcohol feeding on these PIPs remains to be investigated. A change in the proteasome interaction with its interacting proteins or modulators, concomitant with the proteasome inhibition due to ethanol feeding, could result in significant inclusion body

formation, a decrease in the anti-inflammatory and immune responses, and apoptotic conditions that lead to liver cell injury. Therefore, it is important to determine the effects of ethanol on the proteasome activity, especially the effects of alcohol feeding on the PIPs. The UBA-UBL docking proteins p62 and VCP have been shown to be induced by chronic ethanol feeding, which reflects the failure of the proteasome system to clear the accumulated damaged proteins^[31,32]. Similar to the UBA-UBLA proteins, Hsps are also affected by alcohol intake^[72], and are generally upregulated when the proteasome is inhibited, mainly to compensate for proteasome failure^[31,32].

Recently, the effect of chronic ethanol feeding has been investigated on PIPs, and it has been shown that chronic ethanol feeding affects PIPs^[34]. Most importantly, the level of Ecm29, a PIP known to stabilize 20S proteasome interaction with the 19S regulatory complexes^[73], is decreased after ethanol feeding^[34]. Proteasomes that lack Ecm29 are prone to dissociate from their regulatory complex 19S^[74] and most likely from the regulatory complexes PA28a/b and PA200. However, the mechanism of the ethanol effect remains unknown. It is possible that the oxidative stress caused by alcohol-induced detoxifying CYP2E1 is one of the mechanisms that modifies the proteasome subunits^[57] and impedes binding between the CP and Ecm29^[74].

The deubiquitination system is also affected by chronic ethanol feeding. Enigmatically, the three deubiquitinases Rpn11, Usp14 and UCHL5 (Figure 5), are decreased in the 26S proteasome fraction that is purified from ethanol-fed animals^[34]. Why the deubiquitination requires at least three enzymes and why alcohol feeding causes a decrease in the three enzymes is not known. However, a decrease in the proteasome activity and the deubiquitination process leads to serious cellular dysfunction that is reflected by accumulation of the ubiquitinated proteins that aggregate and form MDBs.

CONCLUSION

Chronic ethanol feeding modifies the structure of the proteasome subunits, but also alters proteasome interaction with its proteins partners, thus contributing to serious dysfunction in liver cells. However, we are still at the very beginning of understanding the effects of chronic ethanol feeding on the proteasome pathway. The focus has been to determine the post-translational modifications of the 20S proteasome α type subunits caused by ethanol feeding, because modification of these subunits regulates the 26S proteasome and immunoproteasome formation. As Ecm29 is a key protein involved in 26S proteasome formation, and because it plays a crucial role in stabilizing these proteasomal macromolecules, ethanol-induced Ecm29 downregulation merits further analysis. The determination of the effect of chronic ethanol feeding on the ubiquitination/deubiquitination system to maximize clearance of the altered and ubiquitinated proteins and prevent MDB formation associated with alcoholic liver disease should also be the focus of future research.

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Alcohol-induced alterations of the hepatocyte cytoskeleton

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Abstract

The hepatocyte cytoskeleton consists of three filamentous networks: microtubules, actin microfilaments and keratin intermediate filaments. Because of the abundance of the proteins that comprise each system and the central role each network plays in a variety of cellular processes, the three filament systems have been the focus of a host of studies aimed at understanding the progression of alcohol-induced liver injury. In this review, we will briefly discuss the hepatic organization of each cytoskeletal network and highlight some components of each system. We will also describe what is known about ethanol-induced changes in the dynamics and distributions of each cytoskeletal system and discuss what is known about changes in protein expression levels and post-translational modifications. Finally, we will describe the possible consequences of these cytoskeletal alterations on hepatocyte function and how they might contribute to the progression of liver disease.

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Key words: Ethanol; Hepatotoxicity; Tubulin; Actin; Keratin

INTRODUCTION

The hepatocyte cytoskeleton consists of three major filamentous networks: microtubules, actin and cytokeratins. Recent genomics and proteomics studies have revealed that components of each of these networks are altered in ethanol-treated hepatocytes and/or liver. For example, the expression levels of many different genes encoding for cytoskeletal proteins are changed in ethanol-treated systems (Table 1). Similarly, cytoskeletal protein expression levels are altered by ethanol exposure (Table 2). However, it is important to note that changes in gene expression do not necessarily correlate with changes in protein expression. Furthermore, ethanol metabolites have been shown to form adducts with various proteins of the cytoskeleton (Table 3). More recently, it has become apparent that many post translational modifications of the natural repertoire are also induced by ethanol exposure (Table 3). These alcohol-induced alterations strongly suggest that cytoskeleton structure and function is impaired in ethanol-treated hepatocytes. Because the cytoskeleton is vital to innumerable cellular processes, these alterations likely have profound effects on proper hepatocyte function and lead to the progression of alcoholic liver injury. In this review, we will describe what is

known about specific alterations of components of the three cytoskeletal networks and discuss how they may contribute to ethanol-induced hepatotoxicity.

TUBULIN

Microtubules are made of repeating units of α - and β -tubulin heterodimers that form protofilaments, which in turn assemble into hollow tubes consisting of 13 protofilaments arranged in parallel. Microtubules exist as both dynamic and stable polymers. The latter population is characterized by a longer half-life, resistance to microtubule poisons (e.g. cold and nocodazole) and by specific post-translational modifications on the α -tubulin subunit^[22]. These modifications include the removal of a carboxy-terminal tyrosine, polyglutamylation, polyglycylation and acetylation of lysine 40^[22]. In hepatocytes, cell surface polarity is reflected in the asymmetric organization of microtubules. Unlike in non-polarized cells where microtubules emanate from a juxta-nuclear microtubule organizing center, in polarized cells, there is accumulating evidence that microtubules are instead (or additionally?) organized from sites at or near the apical plasma membrane (Figure 1). The emanating microtubules are oriented with their minus ends at the apical surface and their plus ends attached to or near the basolateral membrane^[23]. Because microtubules are central to multiple cellular processes including organelle placement, mitosis and vesicle motility *via* motor proteins, they have been the subject of a host of studies examining the effects of chronic ethanol exposure on hepatic function.

Acetaldehyde adduction

As ethanol is metabolized, acetaldehyde is produced. This highly reactive metabolite can readily covalently modify proteins, DNA and lipids^[21,24-33]. Many proteins have been shown to be modified by acetaldehyde including tubulin, actin, calmodulin, hemoglobin, hepatic enzymes and plasma proteins^[21,29-31,33,34]. In general, acetaldehyde is thought to form stable adducts with the ϵ -amino group of lysine residues^[32,35,36]. The hypothesis is that these cumulative covalent modifications disrupt the normal functioning of hepatic proteins leading to cell injury.

One of the best-studied target proteins for acetaldehyde is α -tubulin^[32]. *In vitro*, soluble tubulin dimers purified from either bovine brain^[18,19] or rat liver^[20] were found to be much more highly adducted than pre-formed microtubules (Table 3). Adduction occurred preferentially on the α -tubulin subunit at a highly reactive lysine^[18,19]. Further examination revealed that adduction of this highly reactive lysine on α -tubulin drastically impaired *in vitro* microtubule polymerization^[18,19]. Assays using low acetaldehyde: tubulin dimer levels further revealed that impaired microtubule formation occurred at substoichiometric amounts of acetaldehyde (0.2 mol acetaldehyde/mol tubulin) suggesting that small levels of adduction can have far reaching effects on microtu-

Table 1 Ethanol-induced changes in cytoskeleton-associated protein gene expression

Gene product	Expression levels	System	EtOH exposure	Ref.
α -tubulin	Increased	Rat liver	Chronic (IG)	[1]
β -tubulin	Increased	Rat liver	Chronic (IG)	[1]
β -tubulin	Decreased	Rat liver	Chronic	[2]
Kinesin 2c	Increased	Mouse liver	Acute	[3]
Tau	Decreased	Rat liver	Chronic	[2]
α E integrin	Increased	Rat liver	Chronic (IG)	[4]
PDZ and LIM domain protein 1	Decreased	Rat liver	Chronic	[5]
Thymosin β -like protein	Decreased	Rat liver	Chronic	[2]
Myosin 1b	Decreased	HepG2 (+ CYP2E1)	Acute	[4]
Myosin 1E	Decreased	Rat liver	Chronic	[2]
Myosin light chain 2	Decreased	Rat liver	Chronic	[2]
Myosin light chain 3	Decreased	Rat liver	Chronic	[2]
Titin	Decreased	HepG2 (+ CYP2E1)	Acute	[4]
Myosin VII A and Rab interacting protein 1	Increased	Rat liver	Chronic (IG)	[6]
ZO-2	Decreased	Rat liver	Chronic	[5]
Cadherin 17	Increased	Rat liver	Chronic (IG)	[4]

IG: Intragastric.

Table 2 Ethanol-induced changes in cytoskeletal protein expression levels

Protein	Expression levels	System	EtOH exposure	Ref.
α -tubulin	No change	WIF-B cells, rat liver, isolated hepatocytes	Chronic	[7,8]
α -tubulin	Decreased	Rat liver	Chronic	[9]
Dynein	No change	Rat liver	Chronic	[7]
Kinesin	No change	Rat liver	Chronic	[7]
Actin	No change	WIF-B cells, rat liver	Chronic	[10,11]
Cortactin	No change	WIF-B cells, Rat liver	Chronic	[11]
Vinculin	No change	Rat liver	Chronic	[10]
FAK	No change	Rat liver	Chronic	[10]
Paxillin	No change	Rat liver	Chronic	[10]
RhoA	No change	Rat liver	Chronic	[12]
Rac	Increased	Rat liver	Chronic	[12]
Cdc42	Increased	Rat liver	Chronic	[12]
α 1 integrin	Increased	Rat liver	Chronic	[10,13]
α 5 integrin	Increased	Rat liver	Chronic	[10,13]
β 1 integrin	Increased	Rat liver	Chronic	[10,13]
Keratin 8 ¹	No change	Rat liver	Chronic	[14]
Keratin 18 ¹	No change	Rat liver	Chronic	[14]

¹Please see the text for a description of changes in keratins 8 and 18 protein expression levels that are associated with Mallory-Denk bodies in ethanol-fed human and mouse hepatocytes.

bule function^[37]. More recently it was shown that tubulin purified from ethanol-fed rat livers displayed impaired polymerization relative to control^[7]. Although consistent with an acetaldehyde-induced impairment, the presence of adducts on the purified tubulin was not confirmed.

Impaired microtubule polymerization has also been examined in isolated hepatocytes from alcohol-fed rats^[7]. After removing nocodazole (a reversible microtubule

Table 3 Ethanol-induced cytoskeletal protein modifications

Modification	Protein	System	EtOH exposure	Ref.
Lysine acetylation	α -tubulin	WIF-B cells, rat liver	Chronic	[8]
	β -actin	Rat liver	Chronic	[11]
	Cortactin	Rat liver	Chronic	[11]
Dephosphorylation	Keratin 8 ¹	Rat liver	Chronic	[15]
	Keratin 18 ¹	Rat liver	Chronic	[15]
Phosphorylation	Keratin 8	Rat liver	Acute	[16]
	Keratin 18	Rat liver	Acute	[16]
Cysteine oxidation	50 kDa dynactin subunit	Mouse liver	Chronic	[17]
	α -actin	Mouse liver	Chronic	[17]
	β -actin	Mouse liver	Chronic	[17]
	Keratin 1	Mouse liver	Chronic	[17]
	Keratin 2	Mouse liver	Chronic	[17]
	Keratin 9	Mouse liver	Chronic	[17]
	Keratin 10	Mouse liver	Chronic	[17]
	Keratin 14	Mouse liver	Chronic	[17]
Lysine adduction by acetaldehyde	Keratin 16	Mouse liver	Chronic	[17]
	α -tubulin	<i>In vitro</i> ²	N/A	[18-20]
	MAPS ³	<i>In vitro</i> ⁴	N/A	[18]
	Actin	<i>In vitro</i> ⁵	N/A	[21]

¹Please see the text for a description of the multiple post-translational modifications on keratins 8 and 18 that are associated with Mallory-Denk bodies in ethanol-fed human and mouse hepatocytes; ²Purified from bovine brain or rat liver; ³Microtubule associated protein and motor fraction; ⁴Purified from bovine brain; ⁵Purified from rabbit skeletal muscle.

depolymerizing agent), microtubule regrowth was monitored morphologically and was found to be significantly impaired in ethanol-treated hepatocytes. Ethanol-treated WIF-B cells exhibited a similar tubulin phenotype where microtubule regrowth after nocodazole washout was impaired^[8]. Although the formation of tubulin-adducts has not been defined *in vivo*, these results are consistent with the effects of acetaldehyde on tubulin assembly *in vitro*.

Acetaldehyde has also been shown to form adducts on a purified fraction of microtubule associated proteins (MAPs) and motors at levels 1.5-fold more than tubulin^[18] (Table 3). Although the consequences of these modifications have not been explored, vesicle motility in isolated hepatocytes from ethanol-fed rats was found to be significantly decreased suggesting alcohol-induced motor dysfunction^[38]. Although the microtubule activated ATPase activities of kinesin or dynein purified from ethanol-exposed livers was not altered^[38], this does not exclude the possibility that their microtubule binding properties are altered *in vivo* thereby leading to decreased motility. This is consistent with our recent findings that histone deacetylase 6 (HDAC6) binding to endogenous microtubules was impaired in ethanol-treated WIF-B cells and that this impairment partially required ethanol metabolism^[39]. Measuring HDAC6 tubulin deacetylase activity further revealed that ethanol did not impair HDAC6's ability to bind or deacetylate exogenous tubulin suggesting that tubulin from ethanol-treated cells was modified (acetaldehyde adducted?) thereby preventing HDAC6 binding^[39]. Similarly, tubulin modifications may prevent

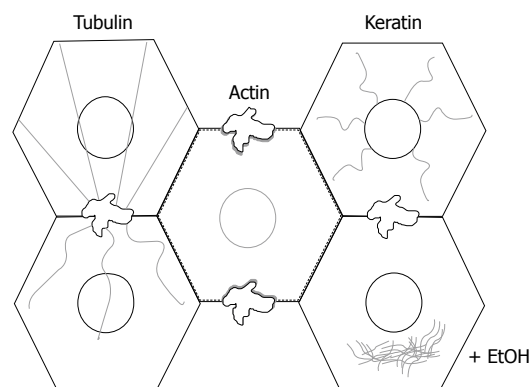


Figure 1 Ethanol alters cytoskeletal organization in hepatocytes. In control cells (top left cell), microtubules emanate from sites at or near the apical plasma membrane with their plus ends attached to or near the basolateral plasma membrane. In the presence of ethanol (bottom left cell), microtubules appear thicker, shorter and more gnarled. In contrast, ethanol does not alter actin filament organization (middle cell). Both control and ethanol-exposed actin filaments form a dense sub-cortical web at both the apical and basolateral plasma membranes. Keratin filaments normally form dense cortical networks originating from the apical and basolateral plasma membrane in hepatocytes (top right cell). In the presence of ethanol, keratin filaments accumulate in dense cytoplasmic inclusions known as Mallory-Denk Bodies (bottom right cell).

motor or MAP binding leading to impaired vesicle motility or altered microtubule dynamics.

Acetylation

Although tubulin polymerization was impaired in ethanol-treated WIF-B cells, when microtubules were examined morphologically, they resembled the so-called “stable” polymers (Figure 1). Antibodies to acetylated lysine 40 on α -tubulin confirmed their identity morphologically and revealed biochemically that ethanol-treated cells had approximately three-fold more acetylated α -tubulin than control cells. Consistent with increased acetylated α -tubulin levels, microtubules in ethanol-treated WIF-B cells were more stable. We further confirmed these results in livers from ethanol-fed rats indicating the findings have physiologic importance^[8]. Because microtubule hyperacetylation and stability increased with increased time of ethanol exposure or concentration, and was prevented by 4-methylpyrazole and potentiated by cyanamide, we conclude that increased acetylation requires alcohol metabolism and is likely mediated by acetaldehyde^[8]. Thus, ethanol metabolism impairs tubulin polymerization, but once microtubules are formed they are hyperstabilized.

Possible consequences of tubulin modifications

Because microtubules are central to multiple cellular processes, changes in their dynamics will likely alter hepatic function. An active area of research has been aimed at understanding the relationship between protein trafficking and alterations in microtubule dynamics. Not only is protein trafficking microtubule-dependent, the trafficking of many hepatic proteins is also impaired by ethanol^[40-43]. Thus, the observed alcohol-induced defects in protein trafficking may be explained by changes

in microtubule dynamics, altered tubulin/MAP/motor post-translational modifications or impaired interactions between microtubules and other proteins. Although the precise relationship between acetaldehyde-modified tubulin and defects in protein trafficking is not known, a link between tubulin acetylation and impaired protein trafficking is emerging. Of particular interest are studies performed in WIF-B cells that used a novel microtubule depolymerizing drug, 201-F^[44]. Poüs *et al.*^[44] showed that this drug specifically depolymerized deacetylated microtubules; only acetylated populations remained. This implies that 201-F is a specific poison for dynamic microtubules. They further examined specific protein transport steps and found that secretion and transcytosis are dependent on dynamic (deacetylated) microtubules while delivery of basolateral glycoproteins to the sinusoidal surface is dependent on stable (acetylated) microtubules. Can the defects in protein trafficking in ethanol-treated cells be explained by increased acetylation and increased stability of microtubules?

To test this possibility, we compared the trafficking of selected proteins in control cells and cells treated with ethanol or the HDAC6 inhibitor, trichostatin A (TSA). Importantly, TSA led to increased microtubule acetylation and stability to the same extent as ethanol^[45]. Both treatments led to the impaired clathrin internalization of asialoglycoprotein receptor and aminopeptidase N^[45]. Interestingly, the internalization of 5'nucleotidase a GPI-anchored protein, was not impaired indicating the effect was selective (i.e. only certain internalization mechanisms were impaired) and specific (i.e. the changes were due to altered microtubule dynamics)^[45]. Furthermore, we determined that albumin secretion was impaired in TSA-treated cells. Thus, increased microtubule acetylation and stability, in part, can explain ethanol-induced defects in protein trafficking. It remains to be determined the specific mechanism by which tubulin acetylation impairs protein trafficking and whether tubulin acetaldehyde adducts also contribute to the impairments observed.

ACTIN

Actin is a ubiquitous cytoskeletal protein that exists as both a monomer (G-actin) and a filamentous polymer (F-actin). These dynamic filaments are the primary component of many cellular structures including stress fibers in adherent cells, the contractile ring in dividing cells and lamellipodia at the leading edge of migrating cells. The actin cytoskeleton has a unique organization in many polarized cells. In general, actin microfilaments extend to the basolateral membrane and form attachments through interactions with proteins of zonulae adherens, tight junctions and focal adhesions. At the apical surface, actin is found as the core filament of microvilli and also as a dense sub-cortical web^[46-48] (Figure 1). At the basolateral domain, the actin-associated proteins, fodrin and ankyrin, form a scaffold that restricts the movements of certain integral membrane proteins, thereby stabilizing

the basolateral protein population^[46] (Figure 1). Actin microfilaments are involved in a host of cellular processes ranging from cytokinesis to establishment of epithelial cell polarity. Interestingly, actin is also known to be important in regulating clathrin vesicle fission, vesicle trafficking *via* myosin motor proteins and cell attachment and spreading - important processes known to be impaired by ethanol exposure. Thus, the effects of ethanol metabolism on the actin cytoskeleton deserve close examination.

Acetaldehyde adduction

Like tubulin, actin contains several reactive lysines making it an attractive candidate for acetaldehyde-adduct formation. When the covalent binding of radiolabeled acetaldehyde to purified actin was examined, stable adducts were formed under both reducing and non-reducing conditions^[21] (Table 3). Interestingly, globular (G) actin formed considerably more adducts with acetaldehyde than filamentous (F) actin, and the monomer efficiently competed for acetaldehyde adduction when co-incubated with albumin, another known adducted protein. However, despite the readily adducted G actin, actin polymerization was not impaired^[21]. This does not rule out the possibility that adduction can lead to altered actin dynamics *in vivo* or impair binding of actin to its binding partners or myosin motors that may explain some of the observed defects in cell spreading and protein trafficking described below.

Hepatocyte attachment and spreading

Although actin adduction does not alter actin polymerization, ethanol has been shown to impair the function of actin-regulated processes. Among these are the observed defects in hepatocyte-extracellular matrix (ECM) attachment and subsequent cell spreading. Initially, hepatocyte attachment occurs when the transmembrane adhesion proteins, integrins, bind to ECM components including fibronectin, collagen, and laminin. Integrin clustering leads to the formation of focal adhesions and induces actin reorganization *via* the activation of Rho family GTPases. Focal adhesion turnover and actin dissociation from integrins are required for subsequent cell spreading. In isolated hepatocytes from ethanol-fed livers, a significant increase in the expression of α_1 , α_5 and β_1 integrins was observed^[10,13] (Table 2). Despite the increased levels of these ECM receptors, decreased hepatocyte attachment to their ECM ligands was observed and subsequent cell spreading was significantly impaired^[13,49,50]. These somewhat disparate findings suggest that integrin overexpression was compensating for decreased ECM attachment. Furthermore, this defect was found to be more prominent in the perivenous hepatocytes, sites where alcoholic liver injury predominates^[49].

Because actin cytoskeleton rearrangement is required for cell ECM attachment and subsequent cell spreading, a more recent study examined the downstream Rho GTPases in isolated hepatocytes from ethanol-fed rats.

This family of small molecular weight GTPases regulates actin rearrangement, including the formation of lamellipodia (*via* Rac), filopodia (*via* Cdc42) and stress fibers (*via* RhoA). Like for integrin expression levels, both Rac and Cdc42 levels were increased in isolated hepatocytes from ethanol-fed rats suggesting another possible compensatory mechanism to regain proper adhesion^[12] (Table 1). However, despite the increased total protein levels of these two GTPases, the activated GTP-bound forms of Rac and Cdc42 were significantly decreased in the presence of ethanol^[12]. Because no change in GTPγS binding to either GTPase was observed, the authors suggest that GTP/GDP exchange by either the guanine nucleotide exchange factors or activating proteins is impaired, not GTP binding itself. Furthermore, when activation profiles of Rac and Cdc42 were examined during cell spreading, decreased Rac activation was observed only for the first 24 h. In contrast, decreased activation of Cdc42 persisted^[12]. This might be due, in part, to the additional role that Cdc42 plays in the establishment of cell polarity such that its activity is required longer. In contrast, RhoA activation or protein expression levels were not changed by ethanol exposure at steady state or during cell spreading assays. This might be explained by the absence of stress fibers in hepatocytes. Together, these studies suggest that decreased hepatocyte ECM attachment and spreading is due to altered Rac and Cdc42 GTPase activity that results in altered actin reorganization such that cell spreading is impaired.

Other actin cytoskeleton modifications

More recently, ethanol has been shown to induce additional protein modifications besides the well characterized acetaldehyde adduction. For example, a recent study examined the widespread CYP2E1-mediated cysteine oxidation in ethanol-fed mouse livers. Over 90 cytosolic proteins were found to be cysteine-oxidized including α and β actin and a dynactin subunit (Table 3). Although the functional consequences of this modification are not currently known^[17], it is attractive to postulate that the modified cysteine residues lead to altered actin dynamics leading to impaired hepatocyte function.

Our recent proteomics studies have identified β-actin as one of 40 other non-nuclear rat liver proteins that is hyperacetylated upon ethanol-exposure^[11,51]. We confirmed its ethanol-induced hyperacetylation and further determined that cortactin, a known actin binding protein, was also hyperacetylated in these samples^[11] (Table 3). Although the acetylated lysine(s) has not been identified, the possible functional consequences of this modification may be gleaned from other studies. Previous work from our lab and others has found that ethanol impairs clathrin-mediated endocytosis, secretion and delivery of newly synthesized membrane proteins to the basolateral membrane^[40-43]. In addition, studies using TSA (a histone deacetylase inhibitor), have linked these impairments to increased protein acetylation^[45]. Since both actin and its binding partner, cortactin, are likely required for clathrin-

vesicle formation at the plasma membrane and TGN^[52,53], an intriguing possibility is that actin hyperacetylation may contribute to the observed alcohol-induced defects in protein trafficking. In general, cortactin is thought to promote actin polymerization at sites of vesicle formation and recruit dynamin (a GTPase required for vesicle fission) to the necks of budding vesicles^[52,53]. At present, the exact mechanism by which cortactin, actin and dynamin function to promote vesicle release is not yet completely elucidated. However, acetylation of cortactin is known to prevent its association with actin and alters its subcellular localization^[54]. From these results, we propose that alcohol-induced hyperacetylation leads to decreased interactions between actin and cortactin such that cortactin is no longer recruited to sites of clathrin-vesicle formation thereby inhibiting associations with dynamin and subsequent vesicle fission. We are currently testing this exciting possibility.

In a recent system-wide survey for lysine acetylation, approximately 200 proteins were found to be acetylated including many cytoskeletal proteins^[55]. In addition to γ-actin, this list included many known actin-binding proteins, suggesting a possible general regulatory role for this modification on actin function. For example, profilin (involved in microfilament elongation) and cofilin and thymosin (involved in actin filament destabilization) were among those identified, suggesting acetylation may regulate actin dynamics. The screen also detected moesin, an ERM family member, required for actin assembly at the apical plasma membrane, suggesting that lysine acetylation may also regulate the formation of the cortical actin web in hepatocytes. However, it is not yet known whether ethanol induces hyperacetylation of these or other actin binding proteins. Nonetheless, we predict that many actin-dependent processes are regulated by lysine acetylation and that ethanol exposure leads to increased hyperacetylation thereby provoking altered regulation. Clearly, these exciting hypotheses need to be rigorously tested.

KERATIN

In general, the cytokeratin intermediate filament system is composed of polymerized dimers consisting of one acidic (Type I) and one basic (Type II) keratin subunit. Although over 50 keratin isoforms have been identified, hepatocytes express only keratin 8 (Type II) and keratin 18 (Type I). The developing embryonic liver also expresses keratin 19 (Type I), and some very low levels of this isoform may be also expressed in adult hepatocytes^[56]. In most polarized epithelial cells, keratin filaments form dense apical cortical networks^[57]. In contrast, apical and basolateral cortical keratin filaments are observed in hepatocytes^[57,58] (Figure 1). The polarized distribution of the keratin filaments have led some researchers to suggest that they play an important role in the establishment and maintenance of epithelial cell polarity and in polarized secretion^[57]. Unlike for microtubules and actin, there are no specific motor proteins

associated with intermediate filament systems suggesting this filament system does not support vesicle motility directly. However, several intermediate filament associated proteins (IFAPs) have also been identified that are thought to modulate filament assembly and associations with actin filaments and microtubules^[59]. These associations are required for the polarized distribution of both actin and microtubules suggesting the role of keratins in regulating polarity and polarized protein trafficking is likely indirect by providing a polarized scaffold for filament orientation^[57].

Keratin post-translational modifications

For almost 100 years, Mallory-Denk bodies have been recognized as a pathological marker in patients with alcoholic liver disease. Because there have been a number of excellent recent reviews^[56,59-62] written about the composition and formation of these inclusions, we will only discuss them briefly here. Morphologically, Mallory-Denk bodies are dense cytoplasmic inclusions formed of fibrillar keratin, chaperones, components of the protein degradation machinery and other proteins^[56,59-62] (Figure 1). Interestingly, the keratin filaments in these inclusions are highly post-translationally modified. They are hyperphosphorylated, transamidated (*via* transglutaminase 2), ubiquitinated and partially degraded^[59,62]. In the alcoholic, Mallory-Denk bodies are thought to form in response to oxidative stress that triggers many interrelated cellular responses including the upregulation of keratin expression (keratin 8 > keratin 18) and its subsequent post-translational modification^[60,62]. Other hepatic proteins are misfolded in response to oxidative stress and associate with chaperones. Damaged proteins are also ubiquitinated and targeted for degradation *via* the proteasome or autophagosomes. The accumulated proteins overwhelm the degradative machinery that is already compromised by ethanol treatment^[63] resulting in the formation of dense inclusions. Whether Mallory-Denk bodies contribute to the progression of alcoholic liver disease, are inert or are hepatoprotective is currently not clear^[59,62].

Another open question is the extent to which keratin intermediate filament function is impaired before the appearance of Mallory-Denk bodies. In general, Mallory-Denk bodies are formed at the expense of an intact keratin filament system. Do keratins present in the intact filament system display lower levels of the various post-translational modifications before inclusion formation? Does this alter keratin function? A few studies performed in rat hepatocytes suggest this is the case. In isolated hepatocytes after acute ethanol exposure both keratins 8 and 18 were found to be hyperphosphorylated^[16] (Table 3). In ethanol-fed rats, the keratin network was also found to be disrupted with increased cytosolic staining observed^[58]. One attractive hypothesis is that keratin phosphorylation leads to altered associations with IFAPs thereby leading to altered keratin function. However, it is important to point out that rats do not form

Mallory-Denk bodies, so whether these changes occur in humans will be important to determine. It is also not known whether keratins 8 or 18 or other hepatic IFAPs are targets for adduction by ethanol metabolites. It is interesting to point out that a number of non-hepatocyte keratin isoforms were found to be cysteine-oxidized in chronically-fed mouse livers (Table 3) suggesting the intermediate filament networks in other cell types may be altered upon ethanol exposure. Clearly, this hypothesis merits further attention.

CONCLUSION

In this review, we have briefly discussed the hepatocyte cytoskeleton and the known ethanol-induced impairments in its structure and function. In general, elements of the actin, microtubule and keratin filament networks undergo changes in distributions, expression levels or post-translational modifications upon exposure to ethanol. While the direct effects of these alterations are still under investigation, it is attractive to speculate that they lead to profound changes in hepatic function. Continued research in this field will not only increase our understanding of the pathogenesis of alcoholic liver disease, but may also provide novel therapeutic approaches to treatment.

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Hepatoprotective effects of *S*-adenosyl-L-methionine against alcohol- and cytochrome P450 2E1-induced liver injury

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Abstract

S-adenosyl-L-methionine (SAM) acts as a methyl donor for methylation reactions and participates in the synthesis of glutathione. SAM is also a key metabolite that regulates hepatocyte growth, differentiation and death. Hepatic SAM levels are decreased in animal models of alcohol liver injury and in patients with alcohol liver disease or viral cirrhosis. This review describes the protection by SAM against alcohol and cytochrome P450 2E1-dependent cytotoxicity both *in vitro* and *in vivo* and evaluates mechanisms for this protection.

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Key words: Cytochrome P450 2E1; *S*-adenosyl-L-methionine; Ethanol; Toxic hepatitis; Oxidative stress

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INTRODUCTION

S-adenosyl-L-methionine (SAM) is the principal biological methyl donor, the precursor of aminopropyl groups utilized in polyamine biosynthesis and, in the liver, SAM is also a precursor of glutathione (GSH) through its conversion to cysteine *via* the transsulfuration pathway^[1,2]. SAM is important for the metabolism and regulation of nucleic acids and for the structure and function of membranes and many other cellular constituents^[3,4]. SAM is particularly important for opposing the toxicity of free radicals generated by various toxins, including alcohol^[1,2]. Abnormalities in SAM metabolism have been well recognized in liver diseases and in various neurological disorders. SAM is synthesized in the cytosol of every cell, but the liver plays a central role in the homeostasis of SAM as the major site of its synthesis and degradation^[3-5]. Methionine adenosyltransferase (MAT) is the enzyme responsible for the synthesis of SAM using methionine and ATP. SAM can transfer its methyl group to a large variety of acceptor substrates. After methyl transfer, SAM is converted to *S*-adenosylhomocysteine (SAH). SAH is a potent competitive inhibitor of transmethylation reactions, and is removed by hydrolysis to homocysteine plus adenosine. In the liver, there are 3 pathways that metabolize homocysteine. One is the transsulfuration pathway, which converts homocysteine to cysteine. Cysteine is often rate-limiting for GSH synthesis hence methionine metabolism *via* SAM and transsulfuration is very important in regulating GSH levels in the liver. The other 2 pathways that metabolize homocysteine resynthesize methionine from homocysteine, methionine synthase and betaine-homocysteine methyltransferase. This cycle is shown in Figure 1.

In mammals, 2 different genes, MAT1A and MAT2A, encode for 2 homologous MAT catalytic subunits, $\alpha 1$ and $\alpha 2$ ^[6-8]. MAT1A is expressed only in the liver while MAT2A encodes for a catalytic subunit ($\alpha 2$) found in a native MAT isozyme (MAT II), which is widely distributed^[4]. MAT2A predominates in the fetal liver and is progressively replaced by MAT1A during development^[9]. Expression of MAT2A is associated with rapid growth of the liver. A switch in the gene expression from MAT1A to MAT2A in liver cancer occurs^[10-12]. MAT isozymes differ in kinetic parameters and in their regulatory properties so that a switch in MAT expression is likely to affect the steady state SAM level and methylation. Apart from the presence of MAT1A and 2A in liver parenchymal cells, both are also present in hepatic macrophages and endothelial cells, whereas stellate cells only express MAT2A^[13].

Rodents fed diets deficient in lipotropes, such as choline and methionine, develop steatosis, which can proceed to non-alcoholic steatohepatitis (NASH) and to fibrosis and cirrhosis^[14]. A decrease in SAM synthesis in the liver results in a decrease in hepatic GSH levels^[15]. MAT1A knockout mice develop steatosis and hyperplasia^[16]. Liver injury causes a decrease in SAM concentration largely because of decreased MAT1A activity^[17,18]. Prooxidant conditions decrease liver MAT1A activity as a critical thiol residue (cysteine 121) becomes oxidized by reactive oxygen species (ROS) or nitrosylated by nitric oxide^[19,20]. Impairment of SAM synthesis is believed to play an important role in hepatic injury induced by various agents, and indeed there is a considerable literature, which shows that exogenous administration of SAM can protect against injury induced by CCl₄, acetaminophen, galactosamine, cytokines, thioacetamide, and ischemia-reperfusion^[21-28]. The decrease in liver GSH produced by various hepatotoxins was prevented by SAM; conversely, MAT and SAM synthesis is regulated by GSH e.g. treatment with l-buthionine sulfoximine (BSO), which lowers GSH, decreases MAT1A activity *in vivo* or in hepatocytes, probably because of oxidation of cysteine 121^[29,30]. Interestingly, MAT1A mRNA and protein declined during 12 h of rat hepatocyte culture, whereas MAT2A mRNA levels increased.

The effects of ethanol on SAM concentrations are somewhat variable. Baboons fed ethanol chronically had decreased hepatic levels of SAM and GSH, and administration of SAM elevated these and protected against liver injury^[31]. Rats fed the Lieber-DeCarli diet showed no or a small decrease in SAM after 4 wk but a more substantial decrease occurred after 8 wk on the diet^[32,33]. Mini pigs fed ethanol for 1 year had no change in SAM levels^[34]. Rats fed ethanol in the intragastric infusion model for 9 wk had an increase in MAT1A and MAT2A mRNA but only MAT2A protein was elevated^[35]; these changes were associated with a 40% fall in SAM levels. Depletion of mitochondrial GSH appears to be an important sensitizing factor for susceptibility to tumor necrosis factor- α (TNF- α) toxicity after chronic ethanol feeding^[36]. This

depletion results from a decrease in transport of GSH into the mitochondria and can be corrected by administration of SAM^[37]. A carrier transport system for SAM entry into the mitochondria has been characterized^[38]. The correction by SAM appeared to reflect an increase in fluidization of the mitochondrial membrane^[37]. In isolated hepatocytes, SAM prevented the decrease in GSH caused by ethanol^[39] and in perfused rat liver studies, SAM prevented the decline in GSH and oxygen consumption and liver damage produced by ethanol^[40].

Cytochrome P4502E1 (CYP2E1), an ethanol-inducible form of P450, is of interest because of its ability to metabolize and activate many important toxicological substrates including ethanol, carbon tetrachloride, acetaminophen, and N-nitrosodimethylamine to more toxic products^[41-43]. Whereas most ethanol is oxidized by alcohol dehydrogenase, CYP2E1 assumes a more important role in ethanol oxidation at elevated concentrations of ethanol and after chronic consumption of ethanol^[44,45]. The major interest in CYP2E1 reflects the ability of this enzyme to oxidize ethanol, to generate reactive products from ethanol oxidation, e.g. acetaldehyde and the 1-hydroxyethyl radical, to activate various agents (CCl₄, acetaminophen, benzene, halothane, halogenated alkanes, alcohols) to reactive products, to generate ROS, and to be "induced" by ethanol^[41-48].

CYP2E1 from rat and rabbit liver exhibits enhanced NADPH oxidase activity as it appears to be poorly coupled with NADPH-cytochrome P450 reductase^[49,50]. Microsomes from ethanol-treated rats, in which CYP2E1 is predominantly induced, displayed elevated rates of production of superoxide and hydrogen peroxide^[49,51-54]. Increases in formation of ROS after ethanol treatment are prevented by anti-CYP2E1 IgG thus linking them to induction of CYP2E1^[55]. There is considerable interest in the role of oxidative stress and ethanol generation of ROS in the mechanisms by which ethanol is hepatotoxic^[56,57]. A major advance has been the development of the intragastric model of ethanol feeding in which prominent induction of CYP2E1 occurs and in which significant alcohol liver injury occurs^[58-60]. In these models, the ethanol-induced liver pathology has been shown to correlate with CYP2E1 levels and elevated lipid peroxidation. Chlormethiazole (CMZ), an inhibitor of CYP2E1, prevented the elevation of lipid peroxidation and partially blocked the ethanol-induced liver pathology^[61].

Understanding the biochemical and toxicological properties of CYP2E1 is important for many reasons, even apart from its role in contributing to alcohol-induced liver injury, since CYP2E1 is induced under a variety of pathophysiological conditions such as fasting, diabetes, obesity, and a high-fat diet^[62-66]. Besides ethanol, CYP2E1 can be induced by drugs such as isoniazid, hydrocarbons such as trichloroethylene, benzene, chloroform, and solvents such as DMSO, acetone, and pyridine^[41-45]. NASH causes steatosis, liver cell injury, inflammation, and variable necrosis. NASH is associated with obesity, type 2 diabetes, and hyperlipidemia, conditions in

which CYP2E1 is induced. Increased CYP2E1 protein, activity, and mRNA levels were observed in a rat nutritional model of NASH^[67]. CYP2E1 was also increased in patients with NASH^[68]. It was concluded that induction of CYP2E1 is involved in the pathogenic mechanisms for NASH^[67,68]. SAM was recently shown to attenuate NASH produced in rats fed with high fat diets by decreasing CYP2E1 mRNA and protein levels^[69]. These decreases in CYP2E1 were accompanied by a lowering of oxidative stress^[69]. In view of the induction of CYP2E1 under a variety of conditions and by many chemicals, further understanding of how levels of CYP2E1 can be downregulated within the cell and how CYP2E1-dependent toxicity can be prevented is important. The goal of this report is to evaluate the effects of SAM on CYP2E1 metabolism and toxicity.

ANTIOXIDANT EFFECTS OF SAM IN VITRO

Our laboratory has investigated the possible direct antioxidant effects of SAM in *in vitro* systems^[70]. This study focused on the effect of SAM on the reactions of Fe²⁺ with dioxygen and Fe²⁺ with H₂O₂, as these are considered to be the most important routes of initiation of biological free radical oxidations. Aerobic HEPES-buffered solutions of Fe²⁺ spontaneously oxidize and consume O₂ with concomitant production of ROS and oxidation of substrates to radical products, e.g. ethanol to hydroxyethyl radical. SAM inhibited this oxidation of ethanol and inhibited aerobic Fe²⁺ oxidation and consumption of O₂. SAM did not regenerate Fe²⁺ from Fe³⁺ and was not consumed after incubation with Fe²⁺. SAM less effectively inhibited aerobic Fe²⁺ oxidation in the presence of competing chelating agents such as EDTA, citrate, and ADP. The effects of SAM were mimicked by SAH, but not by methionine or methylthioadenosine. SAM did not inhibit Fe²⁺ oxidation by H₂O₂ and was a relatively poor inhibitor of the Fenton reaction. Lipid peroxidation initiated by Fe²⁺ in liposomes was associated with Fe²⁺ oxidation; these 2 processes were inhibited by SAM. However, SAM did not show significant peroxyl radical scavenging activity. SAM also inhibited the nonenzymatic lipid peroxidation initiated by Fe²⁺ + ascorbate in rat liver microsomes. These results suggest that SAM inhibits alcohol and lipid oxidation mainly by Fe²⁺ chelation and inhibition of Fe²⁺ autoxidation. This could represent an important mechanism by which SAM exerts cellular protective actions and reduces oxidative stress in biological systems.

IN VITRO INHIBITION OF CYP2E1 CATALYTIC ACTIVITY BY SAM

We studied the possible *in vitro* interactions of SAM and its metabolites SAH, 5'-deoxy-5'-(methylthio)adenosine (MTA) and methionine with cytochrome P450 enzymes,

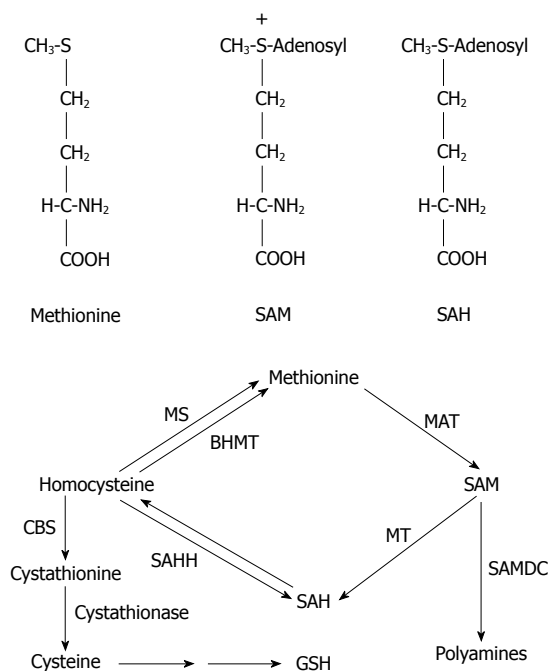


Figure 1 Structure of S-adenosyl-L-methionine (SAM), S-adenosylhomocysteine (SAH), and methionine and hepatic methionine metabolism. BHMT: Betaine homocysteine methyltransferase; CBS: Cystathionine β -synthase; GSH: Glutathione; MAT: Methionine adenosyltransferase; MS: Methionine synthase; MT: Methyltransferase; SAHH: SAH hydrolase; SAMDC: SAM decarboxylase.

in particular CYP2E1^[71]. SAM (but not SAH, MTA or methionine) produced a type II binding spectrum with liver microsomal cytochrome P450 from rats treated with acetone or isoniazid to induce CYP2E1. Binding was less effective for control microsomes. SAM did not alter the carbon monoxide binding spectrum of P450, nor denature P450 to P420, nor inhibit the activity of NADPH-P450 reductase. However, SAM inhibited the catalytic activity of CYP2E1 with typical substrates such as p-nitrophenol, ethanol, and dimethylnitrosamine, with an IC₅₀ of around 1.5-5 mmol/L. SAM was a non-competitive inhibitor of CYP2E1 catalytic activity and its inhibitory actions could not be mimicked by methionine, SAH or MTA. However, SAM did not inhibit the oxidation of ethanol to α -hydroxyethyl radical, an assay for hydroxyl radical generation. In microsomes engineered to express individual human P450s, SAM produced a type II binding spectrum with CYP2E1-expressing, but not with CYP3A4-expressing microsomes, and SAM was a weaker inhibitor against the metabolism of a specific CYP3A4 substrate than a specific CYP2E1 substrate. SAM also inhibited CYP2E1 catalytic activity in intact HepG2 cells engineered to express CYP2E1. These results suggest that SAM interacts with cytochrome P450s, especially CYP2E1, and inhibits the catalytic activity of CYP2E1 in a reversible and non competitive manner. However, SAM is a considerably weaker inhibitor than other typical CYP2E1 inhibitors such as diethyldithiocarbamate or 4-methylpyrazole and it is unclear whether inhibition of CYP2E1 activity by SAM plays a significant role in the protection by SAM against

the hepatotoxicity produced by toxins which require activation by CYP2E1 e.g. acetaminophen, thioacetamide, carbon tetrachloride.

HEPATOCYTES STUDIES

The ability of SAM *in vitro* to protect against CYP2E1-dependent toxicity was studied in pyrazole-treated rat hepatocytes, with high levels of CYP2E1^[72]. Toxicity was initiated by the addition of arachidonic acid (AA) or by depletion of glutathione after treatment with BSO. In pyrazole hepatocytes, SAM (0.25-1 mmol/L) protected against AA but not BSO toxicity. SAM elevated GSH levels, thus preventing the decline in GSH caused by AA, and SAM prevented AA-induced lipid peroxidation. SAM analogs such as methionine or SAH which elevate GSH, also protected against AA toxicity. MTA, which cannot produce GSH, did not have a protective effect. The toxicity of BSO was not prevented by SAM and the analogs because GSH cannot be synthesized. In pyrazole hepatocytes, SAM prevented the decline in mitochondrial membrane potential produced by AA. These results suggest that the ability of SAM to prevent the decline in GSH produced by AA blunts the AA initiation of lipid peroxidation and ROS production and the subsequent loss of hepatocyte viability.

The exposure of hepatocytes to ethanol caused c-Jun amino-terminal kinase (JNK) activation, c-Jun phosphorylation, Bid fragmentation, cytochrome c release and procaspase 3 cleavage; these effects were diminished by SP600125, which caused a significant decrease in ethanol-induced apoptosis^[73]. SAM exerted an antioxidant effect maintaining glutathione levels and decreasing ROS generation, without a significant effect on JNK activity, and SAM prevented cytochrome c release and procaspase 3 cleavage. The JNK signaling cascade is a key component of the proapoptotic signaling pathway induced by ethanol. JNK activation may be independent from ROS generation, since SAM, which exerted antioxidant properties, did not have a significant effect on JNK activity. JNK pathway modulator agents and SAM may be components of promising therapies for alcoholic liver disease treatment.

Kharbanda *et al*^[74] compared the effects of betaine and SAM, on ethanol-induced changes of methionine metabolism and hepatic steatosis. Wistar rats were fed ethanol or a control Lieber-Decarli liquid diet for 4 wk, and metabolites of the methionine cycle were measured in isolated hepatocytes. Hepatocytes from ethanol-fed rats had a 50% lower intracellular SAM/SAH ratio and almost 2-fold greater homocysteine release into the media compared with controls. Supplementation with betaine or SAM in the incubation media increased the SAM/SAH ratio in hepatocytes from both control and ethanol-fed rats and attenuated the ethanol-induced increase in hepatocellular triglyceride levels by approximately 20%. On the other hand, only betaine prevented the increase in generation of homocysteine in the incubation media

under basal and methionine-loaded conditions. SAM can correct only the SAM/SAH ratio and the methylation defects and may in fact be detrimental after prolonged use because of its propensity to increase homocysteine release. Both SAM and betaine are effective in increasing the SAM/SAH ratio in hepatocytes and in attenuating hepatic steatosis; however, only betaine can effectively methylate homocysteine and prevent increased homocysteine release by the liver^[74].

DECLINE OF SAM BY CYCLOLEUCINE TREATMENT POTENTIATES CYP2E1 TOXICITY IN HEPATOCYTES

Cycloleucine is an inhibitor of MAT and prevents the conversion of 5'-methylthioadenosine to SAM through the methionine salvage pathway^[75,76]. SAM levels were lower in hepatocytes isolated from pyrazole-treated rats than control hepatocytes. Mato *et al*^[4] found that defective formation of SAM is associated with an impaired synthesis of GSH and that reduced synthesis of SAM and GSH can both act together in a self-perpetuating cycle, where a reduction in hepatic GSH leads to an inhibition of MAT activity *via* a mechanism involving ROS. A critical cysteine residue required for MAT1A activity is oxidized by ROS, with a subsequent decline in MAT1A activity and levels of SAM^[19,20]. Pyrazole increases the expression of CYP2E1, which produces ROS during its catalytic cycle. We speculate that CYP2E1-derived ROS inhibit the MAT1A which results in lower SAM levels in the hepatocytes isolated from pyrazole-treated rats^[77]. We therefore studied whether lowering of SAM levels in hepatocytes by treatment with cycloleucine would increase CYP2E1-dependent toxicity^[77].

Treatment with 5 mmol/L cycloleucine decreased SAM levels to 50% of initial values in pyrazole or saline hepatocytes. This more modest decline in SAM levels allowed the pyrazole hepatocytes to remain viable and thus permitted evaluating the effect of an added prooxidant such as AA on cell viability when SAM levels were further lowered by cycloleucine treatment.

Pyrazole hepatocytes that express elevated CYP2E1 were more sensitive to 20 mmol/L cycloleucine or 5 mmol/L cycloleucine plus 20 μ mol/L AA treatment, and exhibited significant cell death compared with that of control hepatocytes. Cell nuclear morphology, DNA ladder and caspase 3 cleavage revealed that cycloleucine or cycloleucine plus AA treatment induced apoptosis in pyrazole hepatocytes to a much greater extent than in control hepatocytes. The lactate dehydrogenase leakage assay indicated that necrosis may also be occurring and contributing to the pyrazole hepatocyte death^[77]. The CYP2E1 inhibitor, CMZ, protected pyrazole hepatocytes from cycloleucine or cycloleucine plus AA cytotoxicity by decreasing the elevated CYP2E1 activity, maintaining SAM levels and decreasing ROS levels. It is important to emphasize the very effective hepatoprotective effects

of SAM since toxicity is observed only when SAM levels are lowered to undetectable levels. Decreases of “only” 70% to 80% as found with non-treated pyrazole hepatocytes or cycloleucine-treated control hepatocytes did not result in hepatotoxicity even after 3 d in culture.

A likely explanation as to why cycloleucine or cycloleucine plus AA toxicity is increased in the pyrazole hepatocytes is elevated oxidant stress, as ROS is generated *via* the induction of CYP2E1 in the endoplasmic reticulum, and ROS detoxification is reduced as a result of the decline in SAM. The antioxidant Trolox could rescue pyrazole hepatocytes from cycloleucine or cycloleucine plus AA induced cell death. Trolox also decreased the elevated ROS and intracellular $O_2^{\cdot-}$ produced by cycloleucine in the pyrazole hepatocytes, indicating that enhanced ROS production appears to be central to the mechanism leading to the death of pyrazole hepatocytes^[77].

Trifluoroperazine, an inhibitor of the mitochondrial membrane permeability transition, effectively protected pyrazole hepatocytes from toxicity induced by cycloleucine or cycloleucine plus AA treatment, which suggest that a decrease in mitochondrial membrane potential contributes to the potentiation of cell death caused by cycloleucine treatment in the pyrazole hepatocytes.

SAM levels were lowered in animal models of alcoholic liver injury and human alcoholic liver disease^[25,26]. In these situations the CYP2E1 levels are elevated and liver injury is potentiated by diets enriched in polyunsaturated fatty acids^[59,60]. A decrease in SAM (with or without addition of AA) and elevated CYP2E1 expression combine to lead to an increased oxidative stress in pyrazole hepatocytes, which plays a crucial role in execution of downstream events leading to apoptosis.

MAT1A KNOCKOUT MOUSE

The MAT1A knockout mouse has been a most valuable contribution to research^[16,78,79]. Genomic profiling *via* microarrays revealed changes in genes involved in cell proliferation, differentiation and the acute phase response. Of significance for this review is the observation that CYP2E1 mRNA and activity was increased in the MAT1A knockout mouse. Conversely, CYP4A10 and 4A14 were decreased^[79], in agreement with the observations in the CYP2E1 knockout mouse that there is reciprocal expression of CYP2E1 compared to CYP4A10^[67]. Toxicity by CCl_4 was more severe in the MAT1A knockout mouse perhaps because of the 70% decrease in SAM and the increase in CYP2E1; toxicity was prevented by the CYP2E1 inhibitor diallylsulfide. Importantly, the decrease in MAT activity in wild type mice produced by CCl_4 was also prevented by diallylsulfide and the authors suggested that CYP2E1-derived ROS may inactivate MAT during liver injury, and this may sensitize the liver to further oxidative injury^[79]. We recently found that protection by SAM against CYP2E1-dependent toxicity was associated with a decrease in CYP2E1 levels and activity (discussed below). These results suggest possible interac-

tions/modulation between CYP2E1 and MAT1A, which are worthy of more detailed investigations.

ANTI-FIBROGENIC EFFECTS OF SAM AND PROTECTION AGAINST ALCOHOL-DEPENDENT TOXICITY *IN VIVO*

To study the anti-fibrogenic actions of SAM, transgenic mice harboring the -17 kb to +54 bp of the collagen α_2 promoter (COL1A2) cloned upstream from the β -gal reporter gene were injected with CCl_4 to induce fibrosis and coadministered with either SAM or saline^[80]. Control groups received mineral oil. SAM lowered the pathology in the CCl_4 livers, and decreased the elevated levels of collagen and trichrome staining. Elevated β -galactosidase activity indicated activation of the COL1A2 promoter in isolated stellate cells from the CCl_4 -treated mice and such activation was repressed by *in vivo* treatment with SAM. SAM also prevented the increase in lipid peroxidation and transforming growth factor- β (TGF- β) and the decline in GSH produced by CCl_4 . Incubation of primary stellate cells with SAM downregulated basal levels of collagen and TGF- β stimulation of collagen protein and smooth muscle actin. SAM repressed basal and TGF- β -induced reporter activity in stellate cells transfected with COL1A2 promoter deletion constructs. SAM blocked TGF- β induction of the -378 bp region of the COL1A2 promoter and prevented activation of ERK and binding of Sp1 to the TGF- β -responsive element. These observations reveal antifibrogenic actions of SAM^[80]. *In vitro*, SAM was found to blunt activation of hepatic primary stellate cells in culture as shown by decreased levels of collagen and smooth muscle actin, and a decrease in DNA synthesis stimulated by platelet-derived growth factor and a decrease in stellate cell contractility^[81].

Chronic ethanol consumption by baboons (50% of energy from a liquid diet) for 18 to 36 mo resulted in significant depletion of hepatic SAM concentration and levels of GSH^[82]. These depletions were corrected with SAM administration. There was a significant correlation between hepatic SAM and GSH level. This attenuation by SAM of the ethanol-induced increase in plasma glutamic dehydrogenase was associated with a decrease in the number of giant mitochondria (assessed in percutaneous liver biopsy specimens), with a corresponding change in the activity of succinate dehydrogenase, a mitochondrial marker enzyme. Succinate dehydrogenase activity was increased in liver homogenates of animals fed ethanol, probably reflecting the increased mitochondrial mass. SAM decreased succinate dehydrogenase levels. SAM supplementation also significantly lessened the ethanol-induced increase of plasma aspartate aminotransferase^[82].

Esfandiari *et al*^[83] reported that feeding micropigs with ethanol at 40% of total calories with folate-deficient diets for 14 wk increased, and supplemental SAM maintained, levels of liver and plasma triglyceride. Serum

adiponectin, liver transcripts of adiponectin receptor-1, and phosphorylated adenosine monophosphate kinase- β were each reduced by ethanol feeding and were sustained at normal levels by SAM supplementation of the ethanol diets. Ethanol feeding activated and SAM supplementation maintained control levels of endoplasmic reticulum stress-induced transcription factor SREBP-1c (sterol regulatory element-binding protein-1c) and its targeted transcripts of lipid synthesizing enzymes acetyl-CoA carboxylase, fatty acid synthase, and glycerol-3-phosphate acyltransferase. SAM attenuated oxidative liver injury in micropigs fed ethanol with a folate-deficient diet^[84]. The elevated transcripts and protein levels of CYP2E1 and activities of NADPH oxidase and inducible nitric oxide synthase were lowered upon supplementation of the diet with SAM. Thus, SAM protected against ethanol toxicity by decreasing oxidative enzymes such as CYP2E1. Liver mRNA and protein levels of CYP2E1 correlated positively to SAH or homocysteine but negatively to the SAM/SAH ratio in minipigs fed ethanol plus a folate-deficient diet, and the authors concluded that induction of abnormal methionine metabolism is associated with activation of CYP2E1^[85].

The protective effect of SAM against rat liver steatosis induced by chronic ethanol ingestion was investigated by Feo *et al*^[86]. SAM given during ethanol treatment prevented steatosis and accelerated recovery from steatosis when given after ethanol withdrawal. It also caused a slight inhibition of blood ethanol concentration in both acutely and chronically intoxicated rats. About 30% inhibition of alcohol dehydrogenase, but not of the microsomal ethanol oxidation system, occurred in rats subjected to acute ethanol toxicity as well as in normal rats as a consequence of SAM treatment. Ethanol induced a drastic decrease of GSH liver content. SAM treatment almost completely reconstituted the liver GSH pool. It is suggested that, although SAM induced a small inhibition of ethanol metabolism in the liver, its antisteatotic effect could largely depend on its role as a modulator of the GSH liver content^[86].

Bailey *et al*^[87] found that alcohol feeding to rats for 5 wk caused a significant decrease in mitochondrial state 3 respiration and the respiratory control ratio whereas SAM administration prevented these alcohol-mediated defects and preserved hepatic SAM levels. SAM treatment prevented alcohol-associated increases in mitochondrial superoxide production, mitochondrial DNA damage, and inducible nitric oxide synthase induction, without a significant lessening of steatosis. SAM also prevented alcohol-mediated losses in cytochrome c oxidase subunits, which resulted in partial preservation of complex IV activity. SAM treatment attenuated the upregulation of the mitochondrial stress chaperone prohibitin. Although SAM supplementation did not alleviate steatosis by itself, SAM prevented several key alcohol-mediated defects to the mitochondria genome and proteome that contribute to the bioenergetic defect in the liver after alcohol consumption^[87]. Supplementation of SAM to ethanol diets

which were fed to rats prevented dissociation of hepatic mitochondrial ribosomes and elevated respiratory rates with glutamate/malate or with succinate as substrates^[88]. Thus, SAM is protective against ethanol-induced mitochondrial dysfunction.

Male C57BL/6 mice received ethanol (5 g/kg body weight) by gavage every 12 h for a total of 3 doses. SAM (5 mg/kg body weight) was administered ip before ethanol administration^[28]. Acute ethanol administration caused prominent microvesicular steatosis with mild necrosis and an elevation of serum ALT activity. SAM treatment significantly attenuated the liver injury. In association with the hepatocyte injury, acute alcohol administration induced significant decreases in both hepatic SAM and mitochondrial GSH levels along with enhanced lipid peroxidation. SAM treatment attenuated hepatic SAM and mitochondrial GSH depletion and lipid peroxidation following acute alcohol exposure^[28].

SAM PROTECTS AGAINST CYP2E1-DEPENDENT TOXICITY *IN VIVO*

As CYP2E1-mediated toxicity may play a role in alcoholic liver injury and toxicity of many hepatotoxins, whereas SAM can prevent alcoholic liver injury and toxicity of hepatotoxins, it is of interest to study whether and how SAM can affect CYP2E1-mediated toxicity in the liver *in vivo*. We recently reported that induction of CYP2E1 in mice by treatment with pyrazole increased the hepatotoxicity caused by Fas agonistic Jo2 antibody^[89]. Increased hepatotoxicity in the pyrazole/Jo2-treated mice was associated with increased oxidative and nitrosative stress in association with decreased GSH and other antioxidant levels. CMZ, an inhibitor of CYP2E1, prevented the synergistic toxicity of Jo2 antibody in pyrazole-treated mice. We investigated the effect of SAM on agonistic Jo2 Fas-induced hepatotoxicity following induction of CYP2E1 by pyrazole pretreatment *in vivo* and explored a possible relationship among oxidative stress, hepatic apoptosis, and homeostasis of SAM metabolism^[90]. Suboptimal administration of Jo2 Fas antibody combined with pyrazole pretreatment caused severe hepatotoxicity as determined by elevations in serum transaminase levels and histopathology. Exogenous administration of SAM (50 mg ip/kg body weight every 12 h for 3 d) significantly decreased serum transaminases and ameliorated morphological changes of the liver. Addition of SAM elevated hepatic SAM and total glutathione levels and inhibited CYP2E1 activity. SAM also lowered the elevated oxidative stress (lipid peroxidation, protein carbonyls, and superoxide production) and nitrosative stress (induction of inducible nitric oxide synthase and 3-nitrotyrosine adducts) and lowered increases in caspase-8 and -3 activation produced by the pyrazole plus Jo2 treatment. SAM did not prevent the increase in serum TNF- α levels or the decrease in catalase activity in this model. These results indicate that SAM can have an important hepatoprotective role as an effective

reagent against Fas plus CYP2E1-induced hepatotoxicity by lowering oxidative and nitrosative stress.

A study was carried out^[91] to investigate the effect of SAM on the enhanced hepatotoxicity induced by Fas agonistic Jo2 antibody plus acute ethanol administration in mice. Acute ethanol plus Fas produced toxicity under conditions in which the ethanol or the Jo2 alone had no effect. SAM attenuated this elevated hepatotoxicity. Levels of SAM and activity of MAT1A were decreased by the ethanol plus Jo2 treatment but restored after administration of SAM. The ethanol plus Jo2 treatment elevated levels of CYP2E1, iNOS and TNF- α : these increases were blunted by SAM. SAM also lowered the elevated oxidative and nitrosative stress produced by ethanol plus Jo2. Calcium-induced mitochondrial swelling was elevated by ethanol plus Jo2 and lowered by SAM. The activation of JNK by the ethanol plus Jo2 combined treatment was also prevented by SAM. It was suggested that SAM protects against the acute ethanol plus Jo2 toxicity by restoring MAT activity and levels of SAM, preventing the increases in CYP2E1, iNOS, JNK and TNF- α , which thereby lowers oxidative/nitrosative stress and protects against loss of mitochondrial function^[91].

Obese mice have been used extensively as animal models to study human obesity. We have recently observed that pyrazole-induced CYP2E1 promotes liver injury in ob/ob mice, compared with saline-treated ob/ob mice or with lean controls treated with pyrazole^[92]. We investigated whether SAM can prevent this CYP2E1-mediated toxicity to the obese mouse liver^[93]. Pyrazole treatment of ob/ob mice for 2 d caused necrosis, steatosis, and elevated serum transaminase and triglyceride levels compared with saline-treated ob/ob mice. Administration of SAM (50 mg/kg body wt ip every 12 h for 3 d) prevented the observed pathological changes as well as the increase of apoptotic hepatocytes, caspase 3 activity, and serum TNF- α levels. SAM administration inhibited CYP2E1 activity but not CYP2E1 content. The pyrazole treatment increased lipid peroxidation, 4-hydroxynonenal and 3-nitrotyrosine protein adducts, and protein carbonyls. These increases in oxidative and nitrosative stress were prevented by SAM. Treatment of ob/ob mice with pyrazole lowered the endogenous SAM levels, and these were elevated after SAM administration. Mitochondrial GSH levels were very low after pyrazole treatment of the ob/ob mice; this was associated with elevated levels of malondialdehyde and 4-hydroxynonenal and 3-nitrotyrosine protein adducts in the mitochondria. All these changes were prevented with SAM administration. SAM protected against pyrazole-induced increase in serum transaminases, necrosis, triglyceride levels, caspase-3 activity, and lipid peroxidation even when administered 1 d after pyrazole treatment. In summary, pyrazole induced necrotic changes and apoptosis in livers of obese mice, and SAM pretreatment abrogated these pathological changes. Important mechanisms involved in the protective actions of SAM are the blunting of the increased catalytic activity of CYP2E1, possibly the decline in

TNF- α levels, and the lowering of the elevated oxidative/nitrosative stress produced by SAM treatment. SAM also protected pyrazole-treated obese mice from CYP2E1-generated oxidative and nitrosative stress in the mitochondria, especially the depletion of mitochondrial GSH. Therefore, SAM is effective in protecting against pyrazole-induced oxidative and nitrosative stress and liver injury in obese mice by lowering CYP2E1-generated oxidative/nitrosative stress in the liver and in the mitochondrial compartment^[93].

A model of chronic alcohol-induced liver injury using Cu, Zn-superoxide dismutase deficient mice (SOD^{-/-}) was developed^[94]. Feeding the homozygous SOD^{-/-} mice, but not the wild type mice, with ethanol resulted in liver injury characterized by extensive centrilobular necrosis and inflammation, and increased transaminase levels. Liver injury in the SOD^{-/-} mice was associated with induction of CYP2E1, and increased protein carbonyls, lipid peroxidation aldehydic products and 3-nitrotyrosine protein adducts, indicative of enhanced oxidative and nitrosative stress. SAM protected SOD^{-/-} mice from alcohol-induced liver injury. SAM lowered the serum ALT and AST levels, decreased steatosis, necrosis and inflammation as compared to alcohol alone in treated SOD1 knockout mice (unpublished data).

MITOCHONDRIAL SAM

Normal intra-mitochondrial SAM concentrations play a pivotal role in mitochondrial functions because methylation reactions are required for the methylation of RNA and proteins, and they function as intermediates in the biosynthesis of lipoic acid, ubiquinone and biotin^[95]. Since mitochondria have a relatively large pool of SAM^[96], and the enzyme required for SAM synthesis (MAT) is present only in the cytosol and not in the mitochondria^[97] a specific SAM transporter is needed to maintain normal mitochondrial SAM levels. The human mitochondrial SAM carrier exhibited a high transport affinity for SAM and was found to be expressed in all human tissues examined and was localized to the mitochondria. The physiological role of the SAM carrier is probably to exchange cytosolic SAM for mitochondrial SAH^[98]. It has been reported that increased cytosolic SAH caused a decrease in SAM concentration in the mitochondria in rat hepatocytes^[38]. Furthermore, studies by Colell *et al*^[99] demonstrated that exogenous SAM supplementation prevented the depletion of the mitochondrial GSH pool induced by alcohol consumption and protected hepatocytes from alcohol-consuming rats from TNF- α -induced hepatotoxicity. Bailey *et al*^[87] showed that SAM treatment prevented mitochondrial dysfunction induced by chronic alcohol consumption in rats. Song *et al*^[100] examined the effects of chronic alcohol consumption on hepatic SAM and SAH levels in both the cytosol and mitochondria, and found that mitochondrial SAM was significantly decreased in a mouse model of alcohol liver disease. Supplementation of SAM to rats fed alcohol chronically has been shown

to replenish the mitochondrial GSH levels because of normalization of the microviscosity of the mitochondrial inner membrane. Because of the instrumental role of GSH in mitochondria in hepatocyte survival against inflammatory cytokines, its repletion by SAM feeding may underlie the potential therapeutic use of this hepatoprotective agent in the treatment of alcohol-induced liver injury^[36,37]. Mitochondrial GSH plays an important role in maintaining a functionally competent organelle. SAM administration resulted in a significant increase in the basal cytosol and mitochondrial GSH in both periportal and perivenous cells from both pair-fed and ethanol-fed groups. When hepatocytes isolated from ethanol-fed rats which were supplemented with SAM were incubated with methionine plus serine or N-acetylcysteine, mitochondrial GSH increased in parallel with cytosolic GSH, an effect not observed in cells from ethanol-fed rats not treated with SAM. Feeding equimolar N-acetylcysteine raised cytosolic GSH but did not prevent the mitochondrial GSH defect. In addition, SAM feeding resulted in significant preservation of cellular ATP levels, mitochondrial membrane potential and the uncoupler control ratio of respiration for mitochondria. Thus, these effects of SAM suggest that it may be a useful agent to preserve the disturbed mitochondrial integrity in liver disease caused by alcoholism through maintenance of mitochondrial GSH transport^[37,99].

CONCLUSION

A scheme by which SAM is envisioned to protect against CYP2E1 hepatotoxicity is shown in Figure 2. SAM, at high concentrations, inhibits CYP2E1 catalytic activity, lowering formation of ROS. SAM has antioxidant actions, especially against iron potentiation of ROS production. ROS lower GSH levels, whereas SAM increases GSH levels by providing cysteine *via* the transsulfuration pathway. ROS cause mitochondrial dysfunction and membrane destabilization, events blunted by SAM. Mild or transient oxidant stress produced by CYP2E1 can upregulate cellular protective factors such as GSH, glutathione transferase, heme oxygenase-1, γ glutamyl cysteine ligase *via* activation of Nrf-2^[46-48]. The effect of SAM on this upregulation would be important to evaluate especially in view of methylation reactions as epigenetic regulators.

SAM was first described in 1952 and has been available in the United States as an over the counter supplement since 1999; in Europe, it is a prescription medicine^[101]. SAM has shown protective effects against alcohol and CYP2E1 induced cytotoxicity *in vitro* or in animal models. Although studies in experimental animal models of liver injury support a therapeutic role for SAM, Rambaldi *et al*^[102] identified 9 randomized clinical trials including a heterogeneous sample of 434 patients with alcoholic liver diseases and could not find evidence supporting or refuting the use of SAM in patients with alcoholic liver diseases. Therefore, more long-term, high-quality

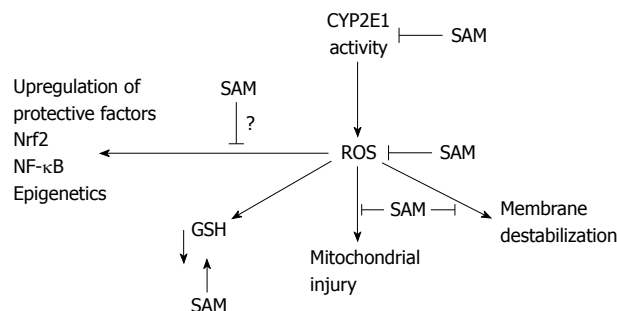


Figure 2 Proposed mechanism by which SAM protects against CYP2E1-hepatotoxicity. ROS: Reactive oxygen species; CYP: Cytochrome P450; NF- κ B: Nuclear factor κ B; Nrf2: Nuclear factor erythroid 2-related factor 2.

ity randomized trials on administration of SAM to these patients are needed before SAM may be recommended for clinical use. The mechanisms of abnormal SAM metabolism leading to liver injury and the mechanisms of pharmacologic actions of SAM deserve more research.

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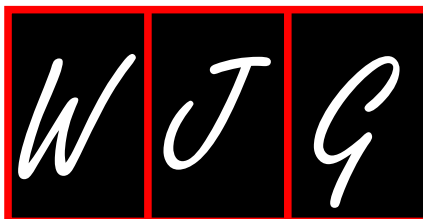
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Alcoholic liver injury: Influence of gender and hormones

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Abstract

This article discusses several subjects pertinent to a consideration of the role of gender and hormones in alcoholic liver injury (ALI). Beginning with an overview of factors involved in the pathogenesis of ALI, we review changes in sex hormone metabolism resulting from alcohol ingestion, summarize research that points to estrogen as a cofactor in ALI, consider evidence that gut injury is linked to liver injury in the setting of alcohol, and briefly review the limited evidence regarding sex hormones and gut barrier function. In both women and female animals, most studies reveal a propensity toward greater alcohol-induced liver injury due to female gender, although exact hormonal influences are not yet understood. Thus, women and their physicians should be alert to the dangers of excess alcohol consumption and the increased potential for liver injury in females.

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INTRODUCTION

Much work has been done in recent years to provide insight into the pathogenesis of alcoholic liver injury (ALI). Liver injury from alcohol has many components, including intrahepatic events [such as hepatocyte alcohol metabolism, generation of reactive oxygen species (ROS) that result in cellular oxidative stress, loss of protective enzymes and transporters] and extrahepatic stimuli, such as gut-derived endotoxin, induced by ethanol exposure. These factors can act separately or in concert to trigger common pathways involving an inflammatory cytokine cascade. These cytokines bring about interplay between different functional cell types in the liver, i.e. hepatocytes, Kupffer cells, stellate or Ito cells, mononuclear cells, and neutrophils. The outcome of such interplay between biochemical alterations and changes in stimuli is the induction of early metabolic dysfunction in the form of fatty liver, followed possibly by an inflammatory response (alcoholic hepatitis), and progression to a more progressive liver disease (fibrosis and cirrhosis). The current model of ALI pathogenesis involves roles of different cytokines that induce various transcription factors, which in turn modulate expression of different genes (reviewed)^[1-9]. Contributing to this injury is mitochondrial dysfunction, with resultant reduced cellular ATP

levels, increased oxidative stress brought about by mitochondrial injury and altered activities of enzymes such as CYP2E1 and NADPH oxidase, along with a decrease in protective antioxidant enzymes such as superoxide dismutase and glutathione peroxidase^[8,9].

The aims of this review are to examine the relationship between sex hormones, changes in hormone levels due to alcohol exposure, and potential roles for sex hormones in the pathogenesis of ALI. To explore such relationships, evidence from both human studies and animal models will be considered. Human studies include collection of serum for hormone analysis under a variety of patient populations, including long-term heavy drinkers and alcoholics, and normal subjects challenged with acute doses of alcohol.

Most of the biochemical mechanisms of alcohol-related injury have been discovered using animal models, particularly rodent models. While studies in human populations are critical to determine demographics, risks, and outcomes, human studies have a number of drawbacks, such as the difficulty of reliable reporting of alcohol intake by subjects, genetic differences in ability to metabolize alcohol, coexisting disease, nutritional deficiencies, and individual timelines in progression of disease. In animal models, factors such as alcohol dose, age, sex, and dietary composition can be controlled by the investigator to limit differences among experimental and control groups. From such studies, it is becoming clear that certain dietary factors are critical in the development of ALI. Further, use of animal models allows testing of potential therapeutic agents prior to testing in human populations.

ANIMAL MODELS AND DIETARY FACTORS

The first diet protocol used widely to study ALI was that of Lieber and DeCarli^[10]. This liquid diet is easy to administer and provides nutritional equivalence by pair-feeding, in which the control animal of the pair receives the exact number of calories consumed by the experimental animal the previous day. This model typically results in alcohol-induced fatty liver, but not the degree of fibrosis and/or inflammation observed in human liver disease. Thus, this model is not appropriate for studying factors involved in generation of cirrhosis. More recently, an enteral feeding model, that of Tsukamoto and French (T/F)^[11,12], has been used to induce inflammation and fibrogenic changes more like that seen in human disease. However, major concerns regarding this model are that alcohol is administered 24 h a day, surgery is required for gastric tube placement, infection is a risk, and the equipment to maintain diet administration is expensive. The original T/F diet has been modified by several groups to produce more or less liver injury, based on the type of fat present. For example, Nanji *et al*^[13-15] showed that addition of medium-chain saturated fatty acids result in prevention or reversal of ALI, presumably due to

decreases in lipid peroxidation and CYP2E1 induction. Other studies showed that polyunsaturated fats result in more severe injury^[16]. Of interest with respect to sex, these diets result in an increase of fat, inflammation, and necrosis in females but not males on the control diets containing no ethanol, suggesting that diet alone may have sex-specific effects^[17,18]. Tipoe *et al*^[19] have now refined this dietary approach by developing a diet that results in significant liver injury particularly in females; the details of this fish oil-containing diet have recently been published. Our studies with this diet have shown that female rats fed alcohol have significant elevations of serum markers such as aspartate aminotransferase, alanine aminotransferase, and endotoxin, and much more fatty change and inflammatory foci in their livers than male rats fed the same diet (manuscript in preparation). Thus, the roles of dietary components, along with differences in response to ethanol between the sexes, have only begun to be elucidated.

ALCOHOL INGESTION CHANGES SEX HORMONE LEVELS IN BOTH SEXES

Clinical observations indicate that females are at greater risk than males for liver injury due to several different causes^[20]. One of these causes is ALI; women develop severe ALI and cirrhosis at lesser intake of alcohol and fewer years of exposure^[1]. While exact mechanisms remain obscure, these findings strongly suggest an involvement of sex hormones in the pathophysiology of alcohol-induced liver disease. Much research has shown that chronic alcohol ingestion changes dramatically the hormonal milieu of blood and the liver in both sexes. The liver is a key player in this interplay, because in addition to being the site of steroid hormone metabolism, the liver is responsive to sex hormones. Our laboratory has studied the effect of sex hormones on the liver for more than 25 years; we performed much of the characterization of sex steroid receptors in human and rat liver, and our work has examined the mechanism(s) of alcohol-induced alterations in hepatic sex hormone function and metabolism.

Because the liver itself is an end organ for sex hormone actions, an alcohol-induced disruption in sex hormone status can result in a self-perpetuating and detrimental pattern of sex hormone metabolism. Our studies and those of others have identified specific mechanisms by which sex hormone homeostasis changes in both male and female rats, reviewed in this section and the next. Newer information on the effect of alcohol on the gut barrier function also enhances our understanding of the relationships between sex hormones and injury to the liver.

Chronic alcohol ingestion results in significant alterations in sex hormone levels and function; the spectrum of sexual dysfunction has been reviewed^[21-25]. Alcoholic men often display phenotypic changes due to an inability to maintain appropriate hormone balance, displaying low

serum testosterone and elevated estrogen levels. Such changes have been reproduced in rat models as well. Our studies reveal that in male rats with chronic alcohol exposure, key hepatic enzymes involved in hormone homeostasis are profoundly affected. Alcohol-fed male rats demonstrated a significant reduction in serum testosterone, and reduced hepatic activity of two androgen-dependent estrogen-clearing enzymes [estrogen sulfotransferase (EST) and estrogen 2-hydroxylase (E2-OHase)], resulting in increased serum estradiol levels^[26-29]. Decreases in serum testosterone, due to alcohol's toxicity to the testes, occurs early in alcohol exposure, whereas increases in estrogen levels are evident upon longer periods of feeding, thus hypogonadism precedes liver feminization. The studies emphasize that the disruption of the androgen-estrogen balance resulting from chronic alcohol ingestion in male rats can drastically affect the hepatic route and rapidity of sex steroid metabolism^[26].

Endocrine changes in chronic alcoholic females have been less well studied. Alcoholic females in their reproductive years also display profound abnormalities in hormone homeostasis and reproductive potential. Chronic alcoholic women may have menstrual cycle disorders, including amenorrhea, anovulatory or irregular cycles, and luteal phase dysfunction^[21,23,24,30-33]. Fertility is often (but not always) impaired^[31]. Onset of menopause is earlier in these women^[32]. Hypothalamic and pituitary dysfunction is also observed, with luteinizing hormone (LH) and follicle stimulating hormone (FSH) release suppressed, although hyperprolactinemia is common. Estrogen levels in the amenorrheic women may be reduced or normal^[30-33], and may differ among ethnic groups^[34]. In contrast, other studies showed that acute alcohol ingestion in nonalcoholic women, especially when administered simultaneously with hypothalamic/pituitary stimulation, results in increased estrogen levels^[35]. Whether this increase is due to increased synthesis or decreased hepatic clearance is not known, but the latter is suggested by the results of a study showing that women taking oral contraceptives had higher estrogen levels than control subjects after an acute dose of alcohol^[36]. Rat studies have shown that chronic alcohol ingestion leads to lower estrogen levels, and in anovulatory cycles characterized by reduced progesterone^[37], similar to findings demonstrated in premenopausal alcoholic women. Not all chronic studies in rats show a change in estrogen levels, however; after 18 mo of alcohol treatment, estradiol levels in one study remained normal^[38]. In postmenopausal chronic alcoholic women, however, estrogen levels appear to be higher (approximately 2-fold) than in controls, and even higher when cirrhosis is present^[32,33]. In these women, pituitary hormones (LH, FSH) are lower than in controls, although prolactin is elevated^[33]. In postmenopausal women on hormone replacement therapy, an acute dose of alcohol results in dramatically increased estrogen levels, presumably as a result of decreased hepatic metabolism of estrogens^[39]. Beyond this study, the effect of chronic alcohol ingestion on hepatic metabolism of sex steroids in fertile or postmenopausal women does not appear to

have been studied in any detail. All of these results taken together suggest that if estrogen (and progesterone) status is important in development of ALI, then the impact of hormones can differ, depending on menopausal/postmenopausal status and hormone replacement therapies, when alcohol ingestion is part of the equation.

IS ESTROGEN A PATHOGENIC COFACTOR IN ALI?

Some evidence suggests that estrogen may be an important pathogenic cofactor in development of alcohol liver injury. For example, females are more susceptible to ALI than males, both in human studies^[40-42] and in certain animal models of ALI^[43]. Further, female sex is considered as a risk factor for development of more profound and irreversible types of ALI^[42]. Estrogens are also implicated in other types of liver disease. In acute fatty liver of pregnancy, estrogen results in of mitochondrial injury^[44] by decreasing β -oxidation of fatty acids, altering the function of mitochondrial proteins and enzymes in mitochondrial uptake, and causing ultrastructural changes such as mitochondrial enlargement. These changes lead to fat accumulation in the liver, in the form of micro/macrovacular steatosis^[45]. Of interest, estrogen also mediates mitochondrial ROS generation in breast cancer cells, resulting in increases in proliferation signals^[46]. It is known that mitochondrial damage is a major apoptosis-triggering event, which is necessary for further development of ALI^[47-49]. In an enteral feeding rat model (T/F), Thurman^[8] documents an increased susceptibility in female rats, showing that females had an increased pathology score, neutrophil infiltration, and increased levels of circulating endotoxin; the authors concluded that increased endotoxin, LPS, and Kupffer cell activation are responsible for the increased ALI in these rats. Further, it has been shown that the sensitivity of Kupffer cells to endotoxin is increased by very high doses of estradiol (a weak estrogen with high levels in pregnancy)^[50]. In a recent study in humans, estrogen treatment was shown to increase inflammatory cytokine production^[51]. Other reports stated that estrogen treatment enhances ALI in ovariectomized females^[52]; however, no data comparing control rats treated with estrogen were presented, so it is difficult to factor out the effects of estrogen alone from the effects of the combined treatment (alcohol plus estrogen). None of these studies determined whether the effect of estrogen is at the level of the liver, the gut, or both. This factor is important, since it is clear that sterilizing the gut with antibiotics prevents much of the ALI in male rats so treated^[53]. In a Lieber/DeCarli model, post-pubertal females have been shown to incur more alcohol-induced oxidative injury than males, as evidenced by increased levels of malonaldehyde^[54]. There is also evidence that estrogens induce potentially more damaging immunological responses^[55], due to estrogen's effects on macrophages^[56], although such effects may be dose-dependent^[57,58]. Estrogen receptors (ER) have been

identified in both Kupffer cells and sinusoidal endothelial cells^[59] as well as in hepatocytes^[27], although the levels of ERs are about 10-fold lower in the nonparenchymal cell types. These latter cells, along with hepatocytes, are major producers of those cytokines shown to have crucial roles in pathogenesis of ALI^[60,61], tumor necrosis factor (TNF)- α , transforming growth factor (TGF)- β , and interleukins, and they also attract PMNs, which produce additional cytokines and ROS, all of which are contributing pathogenic factors in ALD^[1]. One report notes that injury in a low-carbohydrate alcohol feeding model is greater in females^[62], and that such injury is partially prevented by administration of an antiestrogen toremifene^[63]. The antiestrogen primarily reduced inflammation and necrosis, reduced TNF- α release from isolated Kupffer cells, enhanced selenium-glutathione peroxidase activity, and reduced CYP2E1 activity, as compared to the control groups. Lastly, there is known cross-signaling between estrogen and growth factor (EGF, TGF- β , HGF) intracellular pathways. The estrogen appears to act through nuclear and possibly cell-surface ERs, and post-transcriptional regulation of signaling elements, with impact on cell growth, regeneration and fibrosis^[64-67]. Since similar changes are observed in alcohol-induced liver injury, the conclusion was reached that estrogen must play a role in this injury. It is also possible that males might also be susceptible to estrogenic influences, since hyperestrogenization is common with chronic alcohol ingestion, noted above. Our studies^[28] showed that androgen-responsive functions in male rat liver are altered during chronic alcohol exposure, with feminization of male rat liver as a consequence of lowered testosterone levels and elevation of estrogen in liver and serum. The latter appears to be due to a time-dependent reduction in activity of androgen-controlled estrogen metabolizing liver enzymes E2-OHase and EST^[26]. In males, and in both the Lieber/DeCarli^[26] and the intragastric models^[29], the greatest liver injury is observed under circumstances of high estrogen and low testosterone. However, the serum estradiol level in male rats, regardless of alcohol intake, is always several-fold (7-10 \times) lower than in females. Thus, if estrogen is a cofactor in ALI, then it stands to reason that females should be more affected by this hormone than males. In addition, in both female rats and women, progesterone, the "counter-hormone" to estrogen, is reduced with alcohol consumption, since ovulation is less likely to occur. Thus, the estrogen present is less opposed by progesterone. It should also be noted that no studies have been done to determine if the other female sex hormone, progesterone, has any role in causing, or preventing, liver injury.

DIFFERENTIAL GENE EXPRESSION IN FEMALE RATS FED ETHANOL

Several studies have shown that the livers of female rats fed alcohol express different gene sets than males in response to injury. Tadic *et al*^[68] showed that a microar-

ray analysis in the Lieber/DeCarli model of alcohol exposure, female rats fail to up-regulate a number of hepatoprotective genes in response to alcohol, and that those genes that regulate various compensatory metabolic pathways also do not respond appropriately. Using the same model and with addition of lipopolysaccharide treatment, Banerjee *et al*^[69,70] also showed differential expression of genes involved in oxidative stress and inflammation. Sharma *et al*^[71] found similar changes in genes involving oxidative stress and metabolic adaption. Donohue *et al*^[72] demonstrated a lack of induction of hepatoprotective enzymes and increased lysosomal leakage in female rats fed an ethanol- and fish-oil containing diet. While these studies reveal differences in response to metabolic stress in females caused by alcohol, it is not clear how many of these changes are due directly to hormone influences.

RELATIONSHIP OF GUT INJURY TO ALI

The intestinal barrier to antigens, food materials, bacteria, invasive organisms, such as viruses, is a gatekeeper that is critical in maintaining the health of the host. The barrier is a continuous epithelial cell layer, connected by tight junctions that may permit passage of small amounts of materials under normal circumstances, but in pathological conditions, become leaky, permitting the inappropriate passage of particles, bacteria, macromolecules, and immunogenic substances. Stress, infection, chemical insult, surgery, and burns are among the conditions that result in gut injury. The response of the intestine is to secrete fluid and ions to wash away the causative agent, and an immune response involving recruitment of inflammatory cells, and mucus secretion. Key to host protection, however, is maintenance of tight junction integrity in the gut. The tight junctions are highly specialized complexes at the apical end of epithelial cells, and form the actual barrier between the intestine and the blood. Tight junctions are characterized by complex interactions among a group of transmembrane proteins including claudins, occludins, and junction adhesion molecules that interact with other proteins, such as the family of zonula occludens proteins. Complex interactions among these proteins involve anchoring the protein complexes with the actin cytoskeleton. Loss of the appropriate interactions among members of this complex can lead to loss of integrity, and result in a gut that is leaky to bacteria, bacterial products such as endotoxin and other toxins, immunogenic food particles, and other substances^[73,74].

The evidence linking gut-derived endotoxin in the blood as a factor in ALI is compelling. Endotoxins, for example, lipopolysaccharide originating from the cell wall of gram-negative bacteria that populate the gut, are maintained almost exclusively in the lumen as a result of the intestinal barrier afforded by a healthy gut mucosa. However, in circumstances such as stress, trauma, or other gut injury (such as chronic alcohol ingestion),

the mucosal barrier becomes more permeable, allowing endotoxin to cross the mucosa and enter the portal circulation. In the healthy liver, Kupffer cells maintain a constant surveillance and will sequester and degrade endotoxin. However, if Kupffer cell function is impaired, or if levels of endotoxin are excessive, an inflammatory response can result. In addition, endotoxin will be taken up by the resident macrophage population within the intestinal mucosa, triggering an inflammatory response that can lead to further impairment of mucosal barrier function. Endotoxin is linked to ALI in that clinical observations show that endotoxin levels are high in patients with ALI^[1,73,74]. Animal studies show that circulating endotoxin and subsequent liver injury can be eliminated by sterilization of the bowel with antibiotics^[53] and reduced by dietary strategies such as oat fiber^[75] and zinc supplementation^[76] that presumably help maintain barrier function. While the appearance of endotoxin in the circulation implies the possibility of gut injury, very few studies address directly the mechanism of alcohol injury to the gut beyond nutrient uptake considerations. Several studies in Caco-2 cell monolayers, which are derived from human epithelial colorectal adenocarcinoma, show that alcohol^[77] and its first metabolic product, acetaldehyde^[78] can result in disrupted tight junctions and increased paracellular permeability in these cells. Acetaldehyde treatment results in loss of tight junctions because it results in redistribution of tight junction proteins^[78], an effect that may be mediated by a tyrosine kinase-dependent mechanism. Increased endotoxin in rat alcohol feeding models have been shown, and reports document a greater endotoxin level in female rats fed alcohol, and more liver injury as well^[17,43]. Our recent studies using the Nanji rat alcohol feeding model have indicated that young female rats show significantly more gut inflammation, injury, and loss of barrier function due to alcohol ingestion than do male rats (submitted for publication).

Once endotoxin is released by the intestine to the blood, it is bound with high affinity to lipopolysaccharide binding protein (LBP), an acute phase protein synthesized in the liver. The LBP binds endotoxin to its N-terminus through its lipid A moiety, whereas its carboxy terminus is a ligand for the CD14 receptor on the Kupffer cell membrane. Binding of the LBP-LPS to CD14 triggers the activation of Toll-like receptor 4, with subsequent induction of pro-inflammatory transcription factors (NF- κ B), the production of pro-inflammatory mediators such as cytokines [TNF α , interleukin (IL)-6, IL-18], and the production of COX-2-derived prostaglandins^[79]. The crucial role of the Kupffer cell in this process is illustrated by experiments in an alcohol feeding model where treatment with GdCl₃, an inactivator of cells of macrophage lineage, significantly reduced liver injury^[8].

EFFECT OF SEX HORMONES ON GUT PERMEABILITY AND ENDOTOXIN LEVELS

Very little is known about possible direct effects of fe-

male sex hormones on gut permeability. A recent report shows that increased intestinal permeability is a factor in intrahepatic cholestasis of pregnancy^[80]; this finding is relevant in that pregnancy is characterized by high levels of estrogen and progesterone. A few studies have examined the effects of hormones on liver-enterotoxin interaction. When female rats are treated with a sublethal dose of endotoxin *via* tail vein, those rats also treated with a combination of synthetic estrogen and progesterone identical to that in oral contraceptives showed pronounced gut permeability and liver injury, with increased TNF α production by Kupffer cells^[81]. However, these studies used very high doses of hormones, and the hormones were only used as a combination, and not separately. In an alcohol model, Enomoto *et al*^[50] showed that treatment of female rats with very high doses of estriol, a very weak estrogen, resulted in increased endotoxin levels and increases in Kupffer cell TNF α and CD14 content. Because these effects were blocked with oral nonabsorbable antibiotics, the authors concluded that the estriol treatment resulted in increased gut permeability. In another study using burn and trauma/hemorrhagic models, it was shown that estrogen decreased markers of gut injury, whereas testosterone increased susceptibility^[82]. It should be noted that the mechanisms in these models may differ from that in ours, since in gut trauma models injury to the mucosal barrier is acute and severe, whereas in alcohol models the injury is chronic. Thus, hormonal changes in the acute gut trauma models may be entirely different than in the chronic alcohol models. In Caco-2 cells, Asai *et al*^[83] showed that estradiol treatment potentiated alcohol-induced apoptosis, leading to speculation that estrogen might increase gut permeability *in vivo*. With respect to the liver, an interesting study using cultured hepatic stellate cells showed that estradiol treatment reduced ROS generation through the NADPH oxidase system, and also attenuated TGF β 1 expression, cell activation, MAPK pathways, as well as fibrogenic responses. In contrast, progesterone treatment increased ROS generation, TGF β 1 expression, and stellate cell proliferation^[84]. Thus, a potential role of progesterone in ALI should be considered.

FUTURE RESEARCH AND CLINICAL IMPLICATIONS

Future work will foster further understanding of the roles of sex in susceptibility to and in the pathogenesis of ALI. The role of sex hormones, both estrogen and progesterone, remain to be elucidated in this injury. Furthermore, the influences of age and menopausal status are not clear. Are postmenopausal women who are not using hormone replacement less susceptible to ALI? Another emerging area of interest is whether dietary supplements such as fiber and probiotics can alter the course of gut injury and thus liver injury. Obviously, much work needs to be done to understand the interplay of these factors. However, it remains prudent to advise

women that they are at greater risk for alcohol-induced injury, and that intake should be limited to one drink per day. Further, caution should be exercised with heavy use of alcohol in the setting of oral contraceptives or hormone replacement therapy. Since heavy alcohol use interferes with hepatic metabolism of hormones, circulating levels of hormones may increase, thus also placing women at risk for hormone-responsive tumors of the breast and reproductive tract.

CONCLUSION

The evidence outlined herein strongly points to greater risk for women for developing severe ALI and cirrhosis than men. Although the exact roles of female hormones have not yet been proven or elucidated, women should exert caution by limiting their alcohol intake to one drink or less per day to avoid the complications of alcohol-induced liver injury.

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Time-series gene expression profiles in AGS cells stimulated with *Helicobacter pylori*

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correlated with several important immune response and tumor related pathways.

CONCLUSION: Early infection may trigger some important pathways and may impact the outcome of the infection.

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Key words: *Helicobacter pylori*; Gene expression; Microarray; Time-series

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Abstract

AIM: To extend the knowledge of the dynamic interaction between *Helicobacter pylori* (*H. pylori*) and host mucosa.

METHODS: A time-series cDNA microarray was performed in order to detect the temporal gene expression profiles of human gastric epithelial adenocarcinoma cells infected with *H. pylori*. Six time points were selected to observe the changes in the model. A differential expression profile at each time point was obtained by comparing the microarray signal value with that of 0 h. Real-time polymerase chain reaction was subsequently performed to evaluate the data quality.

RESULTS: We found a diversity of gene expression patterns at different time points and identified a group of genes whose expression levels were significantly

INTRODUCTION

Helicobacter pylori (*H. pylori*) have been shown to be the principal cause of acute and chronic gastritis and a major risk factor in gastric cancer development. A chronic inflammatory process induced by the pathogen is thought to be the cause of tumor development. It is well known that *H. pylori* binding to epithelial cells can induce tyrosine phosphorylation of host cell proteins and rearrangement of the cytoskeleton, which may contribute to inflammation and oncogenic transformation^[1]. *H. pylori* colonization to the mucosa may also induce a systemic immune response and be susceptible to Ab-dependent complement-mediated phagocytosis and killing. Infected epithelial cells may also induce a mucosal inflammation under a mechanism of autoantibody-mediated destruction^[2]. Some host factors like interleukin (IL)-1 β , tumor necrosis factor (TNF)- α ,

and IL-10 may influence the disease outcome. One investigation on nuclear factor (NF)- κ B signaling pathway and iNOS suggests that NF- κ B activation may play an important role in protecting mucosal cells from apoptosis through upregulating iNOS^[3]. Many previous studies have performed expression profiling to investigate host changes induced by *H. pylori* infection. These studies have provided some useful and significant information and shed some light for exploring the potential mechanism of *H. pylori* infection and host immunity^[4-10]. However, none of them is designed based on a time-series scheme, the global and sequential profile of *H. pylori* infection that may be involved in the pathogenetic mechanism by which *H. pylori* infects and contributes to gastric carcinogenesis remains poorly understood. In this study, human gastric epithelial adenocarcinoma cells (AGS) co-cultured with an *H. pylori* 26695 strain at different time points were separated and analyzed by a whole genome Illumina microarray. Computer-assisted bioinformatics analysis was conducted to analyze the differential gene expression pattern.

MATERIALS AND METHODS

H. pylori and AGS cell co-culture

H. pylori strain 26695 was routinely cultured for 24 h on Columbia agar plates (Oxoid) containing 5% goat blood under microaerophilic conditions at 37°C, following a wash in sterile PBS and estimation of the quantity of bacteria by OD600. The human gastric epithelial adenocarcinoma cell line AGS (ATCC CRL 1739) was cultured in RPMI 1640 without antibiotic or antifungal agents, and supplemented with 4 mmol/L L-glutamine and 10% fetal calf serum (Gibco) at 37°C in a humidified atmosphere of 5% CO₂. A monolayer of AGS cells grown to 80% confluence was co-cultured with *H. pylori* at a multiplicity of infection of 300:1 in culture media for 0.5, 1, 2, 4, and 6 h.

RNA isolation

Co-culture was stopped at each time point and followed by washing three times with PBS. Total RNA was isolated using Trizol extraction (Gibco/BRL). The quality of the RNA was verified by 1% agarose gel containing ethidium bromide.

Microarray expression profiling and data analysis

Illumina Human-6 v2 BeadChips used for this study contains probes for well characterized genes, gene candidates and splice variants for a total number of 48000 features. The "Detection Score > 0.99" was used to determine the expression. It was a statistical measure in the BeadStudio software, which was computed based on the Z-value of a gene relative to that of the negative controls. The data were normalized using a cubic spline method, which was generally used as a normalization algorithm in BeadStudio. The differentially expressed genes in different time point were identified using the Illumina custom error model implemented in BeadStudio. DiffScore, the expression difference score, takes into account background noise

and sample variability^[11]. The formula for the calculation of the DiffScore is: $DiffScore = 10 \operatorname{sgn}(\mu_{\text{cond}} - \mu_{\text{ref}}) \log_{10}(p)$. The differentially expressed genes with a $|DiffScore| > 13$ were selected for further analysis. The genes with a fold change > 1.5 were integrated and hierarchically clustered using Mev_4_0 (Multiple Experiment Viewer, TIGR). Gene enrichment in KEGG pathways (Kyoto Encyclopedia of Genes and Genomes) and Gene Ontology (GO) were accomplished with Onto-Tool (Pathway Express, OE2GO)^[12,13], and co-expression gene clustering by short time-series expression miner (STEM, Carnegie Mellon University)^[14] with a maximum number of model profiles set as 245, and a maximum unit change in model profiles between time points set at 2. Four interesting co-expression profiles were selected for further analyses. To obtain an optimized GO distribution, we also took all differentially expressed genes including those with a fold change < 1.5 as input for STEM analysis, and chose four profiles for GO enrichment using OE2GO. For pathway level analysis, those genes with a fold change > 1.5 were imported into Pathway-Express to obtain the significantly perturbed pathway list and gene mapping. This program was based on an impact analysis that included the classical statistics but also considered other crucial factors such as the magnitude of each gene's expression change, their type and position in the given pathways, their interactions, *etc.* The IF of a pathway is calculated as the sum of the following two terms:

$$IF(P_i) = \log(1/p_i) + \frac{\sum_{g \in P_i} |PF(g)|}{|\Delta E| \cdot N_{de}(P_i)}$$

$$PF(g) = \Delta E(g) + \sum_{u \in U_{Sg}} \beta_{ug} \cdot \frac{PF(u)}{N_{ds}(u)}$$

Then a simplified network construction was completed based on the genes enriched and mapped to KEGG pathways using STRING (version 8.2)^[15], which is a known Predicted Protein-Protein Interactions Database (<http://string.embl.de/>).

Real-time polymerase chain reaction for confirmation of microarray results

Real-time reverse-transcriptase polymerase chain reaction (Q-RT-PCR) validation of microarray results was carried out for the *GFPT2* gene at the five time points which were significantly altered according to the microarray data. RNA samples of different time points were prepared as previously described in RNA isolation. Briefly, 2 g total RNA of each sample was used for cDNA synthesis. Real time PCR was performed on the Rotor-Gene RG-3000 Real-Time Thermal Cycler with the SYBR Premix Ex Taq™ (TakaRa) and *GAPDH* was used as an internal control. The relative quantification of mRNA expression at each time point was calculated and compared with that of the untreated AGS cells as control. The primers of selected gene for RT-PCR were: (1) *GFPT2* forward primer (5'-GACAAGCAGATGCCCCGTCAT-3') and reverse primer (5'-AACTTGGAACTTTTCAGTATCGTCCTT-3'); and (2) *GAPDH* forward primer

(5'-AGAAGGCTGGGGCTCATTTG-3') reverse primer
(5'-AGGGGCCATCCACAGTCTTC-3').

RESULTS

Definition of differentially expressed genes

Microarray hybridization results showed that about 3577 genes in total ($P < 0.05$, DiffScore > 13 , named dataset1 in this study) expressed differentially compared with 0 h group. This dataset was generated by taking an integration and alignment for the gene list of different time points using Microsoft Excel software, and the repeated genes were thus excluded. Rows were gene names and columns were differential expression values in different time points. Those genes without fold changes in some time points were set as a value equal to 0. The gene numbers at each time point for the 808 genes ($P < 0.05$, a fold change > 1.5 , named dataset2 in this study) are listed in Table 1 and were selected for further emphatic analysis.

Microarray data analysis

Taking dataset2 as input, hierarchical cluster analysis showed some differentially expressed genes down-regulated at 4 h and up-regulated at 6 h (Figure 1A and B). Eighty of the most differentially expressed genes were extracted by sorting their fold change and were hierarchically clustered as shown in Figure 1C. Immunity and tumor-related genes were labeled with triangles and circles, respectively. Ten significant profiles were obtained by STEM and four interesting profiles were shown with genes in detail (Figure 2 and Table 2). However, GO analysis did not provide significant terms. Taking dataset1 as input, the GO analysis results for the four profiles clustered are listed in Table 3 and Figure 3. Table 4 shows the GO distribution change of each time point by up-regulation and down-regulation, respectively. Analysis of KEGG pathways revealed many enrichment-related pathways including cell adhesion molecules, MAPK signaling, p53 signaling, and TGF- β signaling pathways, complement and coagulation cascades, and epithelial cell signaling in *H. pylori* infection. The top four significantly perturbed pathways are listed in Table 5. Related networks extracted from significant pathways are shown in Figure 4.

Real-time PCR confirmation of microarray results

Relative expression levels of each time point were consistent with that of the microarray profile except at 0.5 h, for which a little higher fold-change was obtained in microarray data.

DISCUSSION

Some previous studies have reported that *H. pylori* type I strains that harbor the *cag* pathogenicity island (PAI) and *cagA* are associated with increased bacterial virulence and a more severe inflammatory response in

Table 1 Number of different genes expressed at different time points compared with those of control AGS cells

Time point (h)	Up-regulation (n)	Down-regulation (n)	Total
0.5	109	209	318
1	140	242	382
2	151	203	354
4	126	291	417
6	198	156	354

$P < 0.05$, fold-change > 1.5 , dataset2.

gastric epithelial cells. These virulence factors have also been considered to be associated with induction of interleukin through an NF- κ B-dependent pathway in host mucosa^[16]. In addition, host protein phosphorylation, cytoskeletal rearrangement, and differential activation of MAP kinases have been described in host cells after infection of type I strains^[1]. Although *CagA* and *Cag* PAI are considered to be factors highly involved in the development of gastritis and carcinoma, more complex as yet undiscovered mechanisms may exist between *H. pylori* and host cells. We aimed to take a global view of gene expression profiles of host response to infection in a time-series interaction model, which may help understand the pathogenesis of *H. pylori* related diseases.

Considering that only genes with fold changes > 1.5 were included in the analysis, the number of differential genes was only 808. This may lead to an ignorance for many important genes. Therefore, we initiated co-expression clustering analysis using STEM for both the 3577 differentially expressed genes (dataset1) and 808 genes (dataset2) with fold-change > 1.5 . For the 808 genes, four significant clusters showed four different co-expression profiles (Figure 2). One hundred and twenty-six genes down-regulated at 4 h were clustered into profile 123, but no significant GO terms were enriched for these genes. In profile 3, some genes related to tumors were consistently down-regulated. For instance, *cdkn1c* had consistently decreased expression of these genes, which may be involved in promotion of tumor formation. Profile 144 was mainly involved in factors regulating cell bioactivity and morphology such as *rflb*, *gdf15*, *sqstm1* and *adm2*. DNA-damage-inducible transcript and *csf2* also had increased gene expression at 4 and 6 h, suggesting that some potential mechanisms for cell differentiation and damage may be triggered beginning at 4 h. Hierarchically clustered results also showed two gene clusters with down-regulation at 4 h and up-regulation at 6 h. Analysis of all differentially expressed genes showed four interesting profiles whose GO distributions included nucleic acid binding, regulation of transcription, oxido-reductase activity *etc.* For the GO distribution of dataset1, profile 71 and profile 83 showed a similar co-expression profile as well as GO terms including nucleus, nucleic acid binding *etc.* (Table 3, Figure 3B and D). However, profile 83 showed an obvious and continuous up-regulated gene cluster. Profile 111 and 108 mainly focused on cell surface and showed a down-regulated

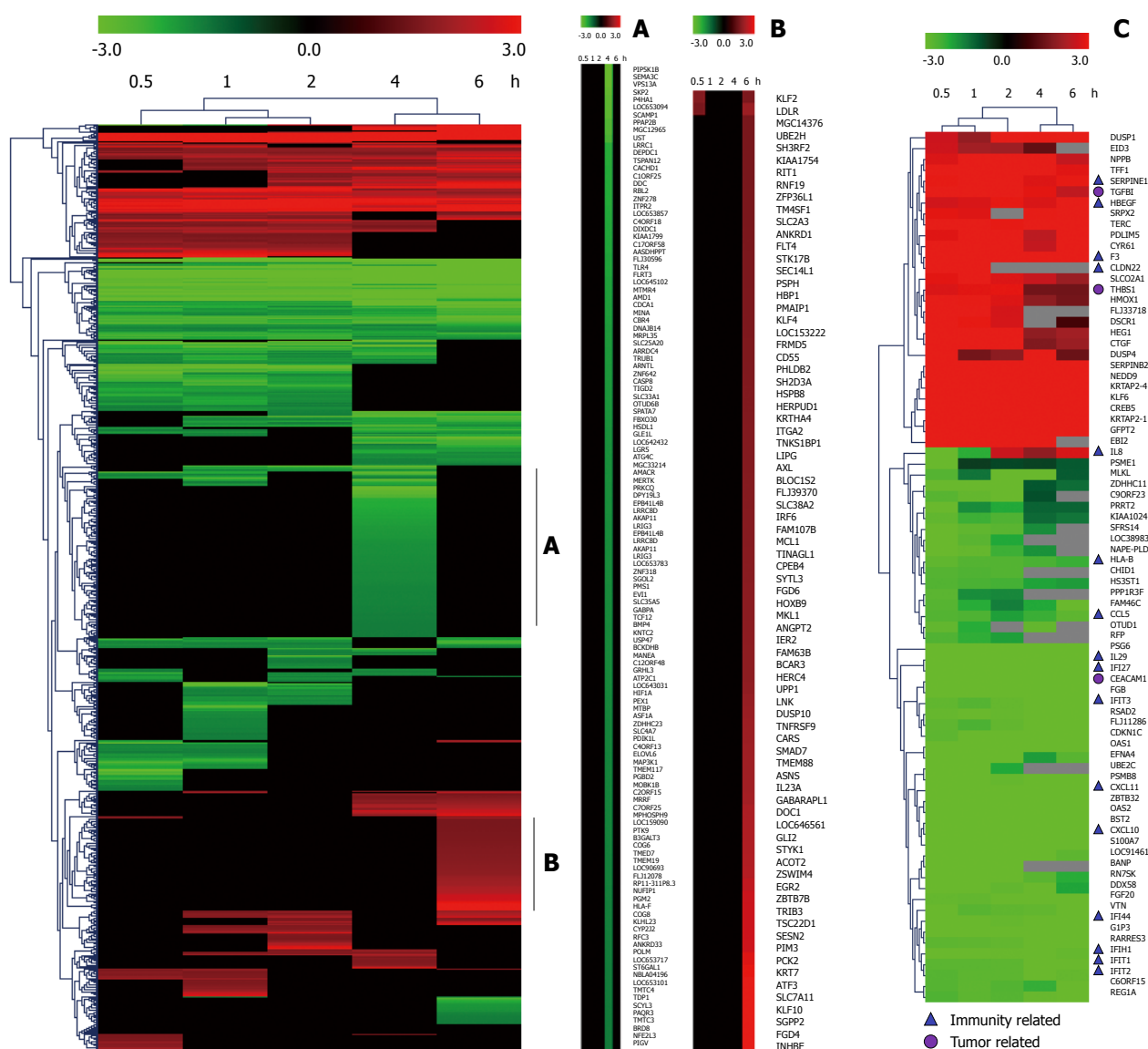


Figure 1 Hierarchical cluster analysis of time-series gene expression alteration after infection of *Helicobacter pylori* at 5 time points. Genes that significantly changed during infection were included in hierarchical clustering analysis using average linkage and Euclidean dissimilarity methods. Significant clusters A and B show the details of genes including name of the gene down-regulated at 4 h and up-regulated at 6 h. Eighty of the most differentially expressed genes were clustered in C. Immunity and tumor related genes are labeled.

gene cluster (Figure 3A and C). All profiles illustrated an obvious expressional change at 4 h. Statistically significant changes in gene ontology at each time point showed that apoptosis appeared from 1 h in up-regulated genes. At the same time, in down-regulated genes, chemokine activity became the most significant term (Table 4). This seemed consistent with results of the pathway analysis, which showed that the P53 signaling pathway became the most significantly perturbed pathway at 1 h in up-regulated genes. In down-regulated genes, the cytokine-cytokine receptor interaction pathway became more significant. Genes involving immune response and other responses to viruses were at the top of the GO list of down-regulated genes. This suggested an inhibition of immune response by *H. pylori* during early infection. Tumor-related pathways like P53 and MAPK may play an important role in determining the development of

special phenotype and disease outcomes according to the results of pathway analysis. For the top 80 differentially expressed genes, 43 (54%) were related to immunity (29, 36%) and tumor development (14, 18%). Many immune factor-related down-regulated genes showed a consistently increasing expression levels. The cell adhesion molecules (CAM) pathway was the most significantly perturbed pathway at several time-points. The increased expression of CAM induced by *H. pylori* may contribute to cell adhesion, invasion and cell proliferation in gastric epithelial cells^[17].

From the reconstructed simplified pathway, we can inspect some important nodes with several interaction edges like *stat1*, *stat2*, *fos*, *csf2*, *pdgfb* and *ccl5* genes. These genes may be the trigger and linker of the pathway net during early infection, which however requires further studies. From Figure 4 and the expression value of each

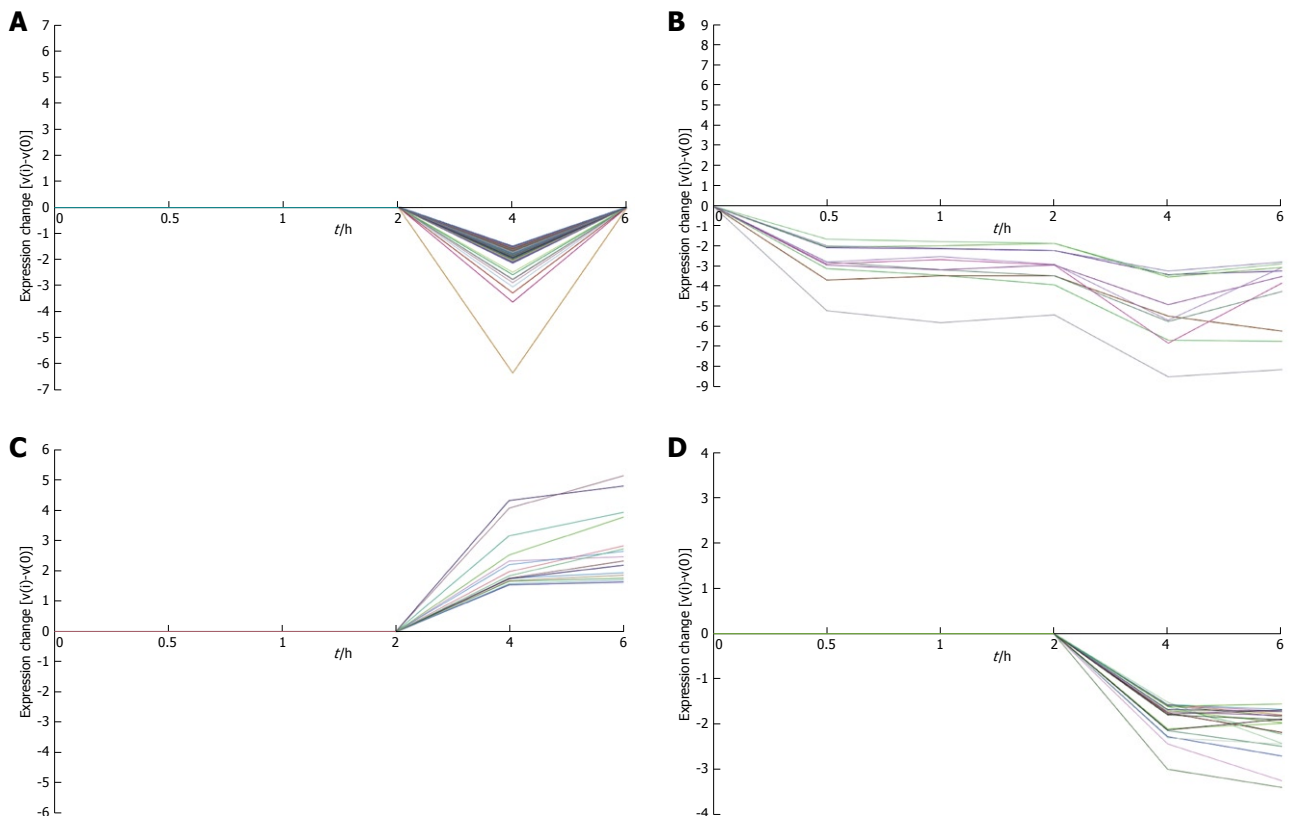
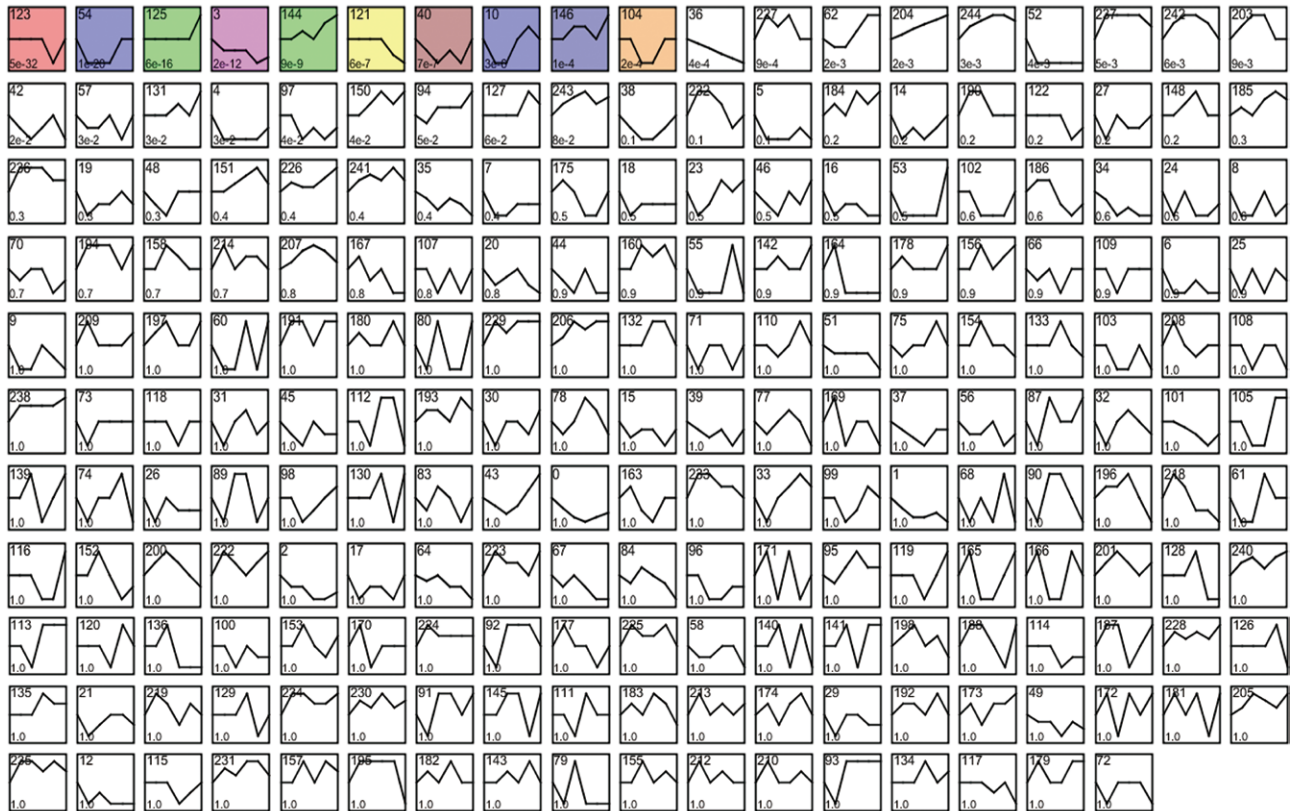


Figure 2 Short time-series expression miner (STEM) clustering of the differentially expressed genes. All profiles are ordered based on the *P* value significance of the number of genes assigned vs expected. A: Profile 123 (0, 0, 0, 0, -1, 0): 126.0 genes assigned, 37.8 genes expected, *P*-value = 5.4E-32 (significant); B: Profile 3 (0, -2, -2, -2, -4, -3): 11.0 genes assigned, 0.4 genes expected, *P*-value = 1.9E-12 (significant); C: Profile 144 (0, 0, 1, 0, 2, 3): 16.0 genes assigned, 2.5 genes expected, *P*-value = 8.5E-9 (significant); D: Profile 121 (0, 0, 0, 0, -2, -3): 21.0 genes assigned, 5.7 genes expected, *P*-value = 6.3E-7 (significant).

gene, we could learn that most immunity-related genes were down-regulated while many tumor-related genes

were up-regulated. *Il-24* is an important oncogene and could inhibit specifically the tumor growth. The protein

Table 2 Description of selected clustered genes from short time-series expression miner (STEM) using dataset2 as input

Cluster ID	Symbol									
Profile 123	C4ORF18	USP47	CYP2J2	LGR5	FLRT3	LOC643031	TMEM117	CACHD1	C12ORF48	MTMR4
	RBL2	ZDHHC23	TTC13	NUFIP1	FLJ30596	AASDHPPT	C2ORF15	PGBD2	LRR8C8D	EVI1
	SKP2	ZNF318	VPS13A	AMACR	ST6GAL1	AMD1	ELOVL6	PGM2	SLC35A5	CBR4
	EPB41L4B	C1ORF25	C1GALT1	ATG4C	MERTK	FANCL	LRIG3	RHPN1	PIP5K1B	SEMA3C
	P4HA1	LOC653094	SCAMP1	PPAP2B	MGC12965	UST	LRR1C	DEPDC1	DDC	ZNF278
	ITPR2	LOC653857	DIXDC1	KIAA1799	C17ORF58	TLR4	LOC645102	CDCA1	MINA	DNAJB14
	MRPL35	SLC25A20	ARRDC4	TRUB1	ARNTL	ZNF642	CASP8	TIGD2	SLC33A1	OTUD6B
	SPATA7	FBXO30	HSDL1	GLE1L	LOC642432	MGC33214	PRKCQ	DPY19L3	AKAP11	LOC653783
	SGOL2	PMS1	GABPA	TCF12	BMP4	KNTC2	BCKDHB	MANEA	GRHL3	ATP2C1
	HIF1A	PEX1	MTBP	ASF1A	SLC4A7	PD1K1L	C4ORF13	MAP3K1	MOBK1B	MRRF
	C7ORF25	MPHOSPH9	LOC159090	PTK9	B3GALT3	COG6	TMED7	TMEM19	LOC90693	FLJ12078
	RP11-311P8.3	ZNF181	COG8	KLHL23	RFC3	NBLA04196	LOC653101	TMTC4	TDP1	SCYL3
	PAQR3	TMTC3	BRD8	NFE2L3	PIGV	TSPAN12				
	PSG6	FBG	CEACAM1	CDKN1C	IFIT3	RSAD2	PSG7	FLJ11286	BTN3A2	STAT1
	FLJ20035									
	EHD2	RELB	COL16A1	GDF15	GNA15	LETM2	STX11	FOSL1	LOC647512	SQSTM1
	C12ORF59	ADM2	DDIT3	CHAC1	CSF2	DDIT4				
	ZC3HAV1	PSG9	LYZ	FGG	PSG2	PAGE4	REG4	GAD1	PPM1H	TMEM70
	LRP8	PAQR8	SH3BGRL	MYLIP	ROR1	CSORF14	SUSD4	MGC3265	CADPS2	IDUA
	EPSTI1									

Table 3 Statistically significant changed gene ontology of the four selected profiles

Profile	GO name	n	Corrected P value	Function code
111	Apical part of cell	2	0.00842	CC
71	Nucleic acid binding	12	2.7E-4	MF
	Zinc ion binding	23	0.00308	MF
	Regulation of transcription	22	0.01027	BP
	Myeloid cell differentiation	2	0.01577	BP
	Nucleus	39	2.9E-4	CC
108	Intracellular	23	3.5E-4	CC
	Small GTPase binding	2	0.01173	MF
	Oxido-reductase activity	6	0.02544	MF
	GPI anchor biosynthetic process	2	0.02591	BP
	Female pregnancy	3	0.02622	BP
	Golgi membrane	5	0.03987	CC
	Cell surface	3	0.03987	CC
83	DNA binding	6	0.00577	MF
	Metal binding	6	0.03346	MF
	Nucleus	10	0.01029	CC

Corrected P value < 0.05, derived from dataset1.

encoded by this gene can induce apoptosis selectively in various cancer cells. Overexpression of this gene has been shown to lead to elevated expression of several GADD family genes, which correlates with the induction of apoptosis^[18-20]. In this study, we examined *il-24* levels which gradually increased more than two-fold from 2 to 6 h. At 6 h, there was a ten-fold change, indicating that after perturbation of P53 and MAPK, *il-24* may participate in maintaining the immune defense against invading pathogens. We also examined an increased level of *gadd45* which can stimulate DNA excision-repair *in vitro* and inhibits entry of cells into S phase. This gene is a member of a group of genes whose transcript levels are increased following stressful growth arrest and treatment with DNA-damaging agents. In the network, both *c-Fos*

and *c-Jun*, two genes considered to mediate inflammation and carcinogenesis, have been found to be up-regulated, which is consistent with the results of this study^[21].

We also analyzed expression profiles of some other important infection-related genes that were reported previously and may play an important role in *H. pylori*-induced diseases, although these genes were not clustered into a special profile in this study using the current analytical tools. MMP is a mucosal matrix metalloproteinase. Previous studies have demonstrated elevated MMP-9 levels in *H. pylori*-infected gastric mucosa, and eradication of *H. pylori* can significantly decrease MMP9 expression levels consistently^[22,23]. MMP1 has been the subject of studies of inflammatory gene profiles in gastric mucosa^[2,24]. MMP7 has been reported to be up-regulated in gastric cancer tissues^[25,26]. However, few studies have reported on MMP24. In this study, the profile of MMP24 showed a consistent and increased level from 1 to 6 h, which suggested a similar function with MMP9 during *H. pylori* infection. Some other genes with similar expression profiles are *il-27ra*, *il-32*, *il-23a*, *il-11*, *il-8* and *ccl20*. This gene cluster showed down-regulation or no change at the first two or three time points and up-regulation in the last two or three time points. *Il-29*, *ccl5*, *cxc10* and *cxc11* showed a consistent down-regulation at all time points with high fold-change. Expression of these genes suggested that the immune defense system may be suppressed during the first 1 or 2 h of *H. pylori* infection and some tumor-related genes and pathways were activated. After this short interaction and competition for about 2 h, the immune defense system may have regained the advantage with increasing expression levels of inflammatory and tumor suppressor factors. CagA translocation might occur 30 min after infection and may be at its maximum level in a time range of about 4-5 h^[27,28]. In this study, the differentially expressed genes significantly increased at the time point of 4 h. This also

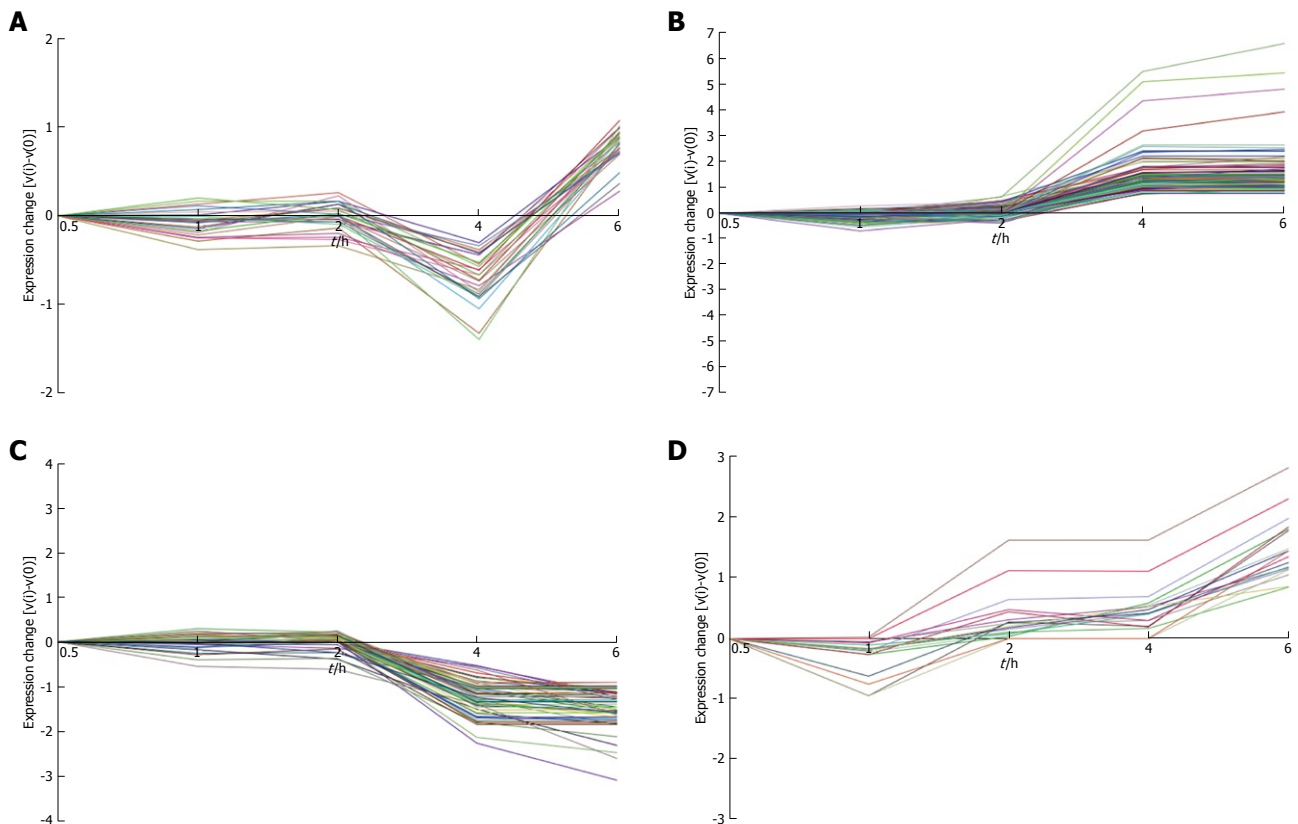
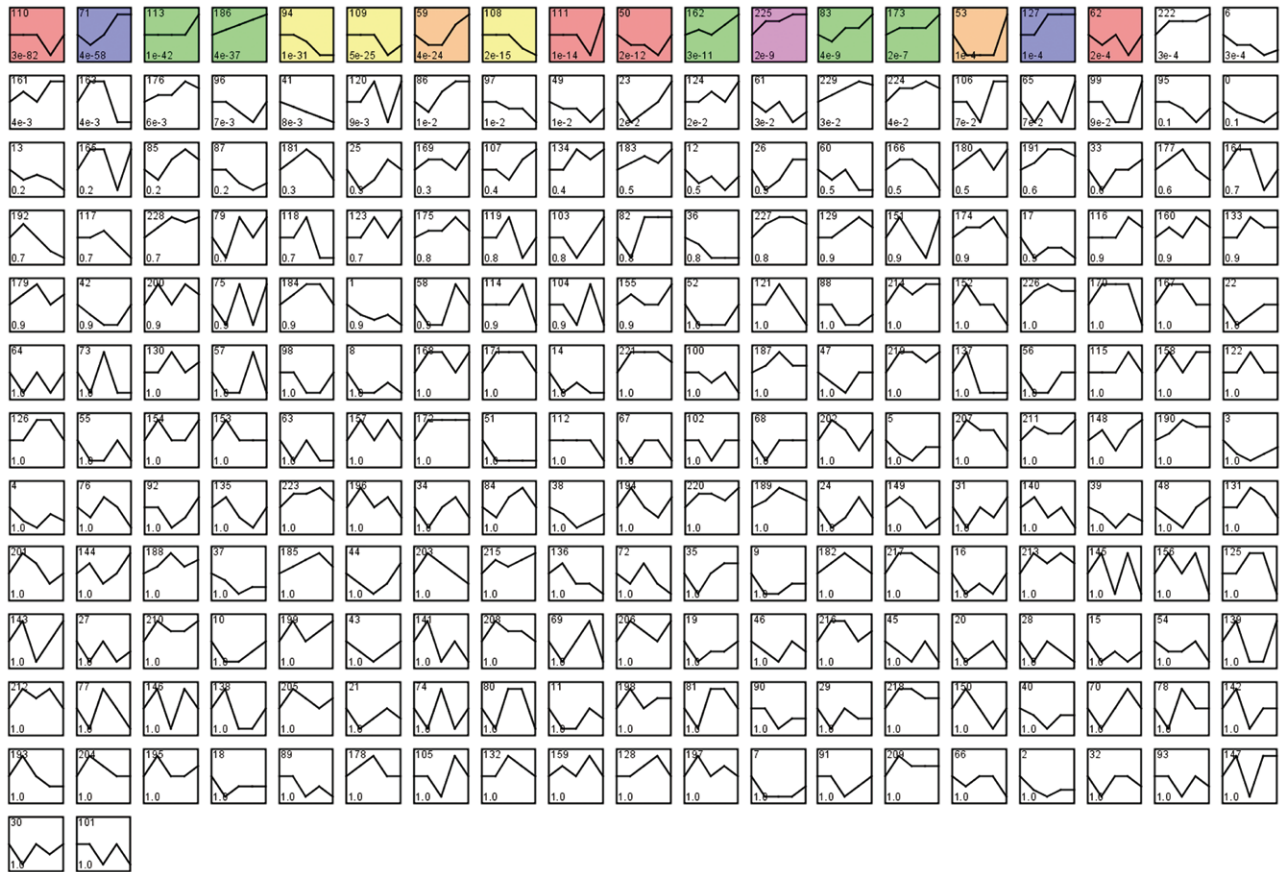


Figure 3 STEM clustering of all the 3577 differentially expressed genes labeled by accession number. All profiles were ordered based on the *P* value significance of the number of genes assigned vs expected. A: Profile 111 (0, 0, 0, -1, 1): 28.0 genes assigned, 4.2 genes expected, *P*-value = 1.2E-14 (significant); B: Profile 71 (0, -1, 0, 2, 2): 123.0 genes assigned, 19.0 genes expected, *P*-value = 4.4E-58 (significant); C: Profile 108 (0, 0, 0, -2, -3): 57.0 genes assigned, 16.2 genes expected, *P*-value = 1.5E-15 (significant); D: Profile 83 (0, -1, 1, 1, 3): 17.0 genes assigned, 2.7 genes expected, *P*-value = 4.3E-9 (significant).

Table 4 Statistically significant changed gene ontology at each time point

Time point (h)	Up-regulation					Down-regulation				
	GO ID	GO name	Genes	P value	Code	GO ID	GO name	Genes	P value	Code
0.5	GO:0008201	Heparin binding	5	7.1E-4	MF	GO:0006955	Immune response	20	0.00000	BP
	GO:0008134	Transcription factor binding	4	0.01585	MF	GO:0009615	Response to virus	10	0.00000	BP
	GO:0003700	Transcription activity	10	0.02835	MF	GO:0008150	Biological process	15	0.00896	BP
	GO:0008083	Growth factor activity	4	0.03882	MF	GO:0007267	Cell-cell signaling	10	0.00966	BP
	GO:0005576	Extracellular region	15	0.02875	CC	GO:0006935	Chemotaxis	6	0.01581	BP
	GO:0005634	Nucleus	28	0.03452	CC	GO:0006954	Inflammatory response	8	0.01581	BP
						GO:0008285	Negative regulation of cell proliferation	7	0.03430	BP
						GO:0007275	Multicellular organismal development	16	0.03576	BP
						GO:0008009	Chemokine activity	7	0.00000	MF
						GO:0046870	Cadmium ion binding	3	0.00194	MF
						GO:0016779	Nucleotidyl transferase activity	5	0.02486	MF
						GO:0005576	Extracellular region	37	0.00000	CC
						GO:0005615	Extracellular space	14	2.0E-4	CC
1						GO:0005634	Nucleus	56	7.0E-4	CC
	GO:0008201	Heparin binding	5	0.00265	MF	GO:0008009	Chemokine activity	6	3.5E-4	MF
	GO:0003700	Transcription factor activity	13	0.00886	MF	GO:0046870	Cadmium ion binding	3	0.00264	MF
	GO:0005515	Protein binding	38	0.01716	MF	GO:0003677	DNA binding	26	0.00264	MF
	GO:0045766	Positive regulation of angiogenesis	3	0.01125	BP	GO:0046872	Metal ion binding	36	0.01144	MF
	GO:0001558	Regulation of cell growth	6	0.01502	BP	GO:0008270	Zinc ion binding	34	0.02041	MF
	GO:0006915	Apoptosis	8	0.02591	BP	GO:0003674	Molecular function	15	0.02257	MF
	GO:0008285	Negative regulation of cell proliferation	6	0.02591	BP	GO:0003676	Nucleic acid binding	13	0.02257	MF
	GO:0005634	Nucleus	36	0.00597	CC	GO:0016779	Nucleotidyl transferase activity	4	0.02571	MF
	GO:0005575	Cellular component	10	0.02160	CC	GO:0005515	Protein binding	61	0.03204	MF
						GO:0003704	Specific RNA polymerase II transcription factor activity	3	0.04080	MF
						GO:0009615	Response to virus	10	0.00000	BP
						GO:0006955	Immune response	18	0.00000	BP
						GO:0006355	Regulation of transcription DNA-dependent	39	4.0E-5	BP
						GO:0006350	Transcription	31	4.5E-4	BP
						GO:0008150	Biological process	18	0.00348	BP
						GO:0007267	Cell-cell signaling	11	0.00480	BP
						GO:0006954	Inflammatory response	8	0.03385	BP
						GO:0045087	Innate immune response	5	0.04274	BP
2						GO:0005634	Nucleus	71	0.00000	CC
						GO:0005576	Extracellular region	37	1.1E-4	CC
						GO:0005615	Extracellular space	13	0.00474	CC
						GO:0005622	Intracellular	31	0.01344	CC
						GO:0005575	Cellular component	15	0.03381	CC
	GO:0003700	Transcription factor activity	18	1.4E-4	MF	GO:0009615	Response to virus	10	0.00000	BP
	GO:0008201	Heparin binding	5	0.00193	MF	GO:0006955	Immune response	16	0.00000	BP
	GO:0043565	Sequence-specific DNA binding	10	0.01819	MF	GO:0008150	Biological process	18	6.6E-4	BP
	GO:0008083	Growth factor activity	5	0.01885	MF	GO:0007267	Cell-cell signaling	10	0.01111	BP
	GO:0005178	Integrin binding	3	0.02722	MF	GO:0006954	Inflammatory response	8	0.01911	BP
	GO:0008134	Transcription factor binding	4	0.02722	MF	GO:0045087	Innate immune response	5	0.02866	BP
	GO:0008009	Chemokine activity	3	0.02849	MF	GO:0007565	Female pregnancy	5	0.03928	BP
	GO:0046872	Metal ion binding	23	0.04806	MF	GO:0005576	Extracellular region	36	1.0E-5	CC
	GO:0045944	Positive regulation of transcription from RNA polymerase II promoter	7	0.00234	BP	GO:0005615	Extracellular space	13	0.00113	CC
	GO:0006955	Immune response	10	0.00470	BP	GO:0005634	Nucleus	51	0.00899	CC
	GO:0008285	Negative regulation of cell proliferation	7	0.00681	BP	GO:0046870	Cadmium ion binding	3	0.01145	MF
	GO:0000122	Negative regulation of transcription from RNA polymerase II promoter	6	0.00681	BP	GO:0016831	Carboxy-lyase activity	3	0.02198	MF
	GO:0006915	Apoptosis	9	0.00713	BP	GO:0030674	Protein binding bridging	4	0.04373	MF
	GO:0006954	Inflammatory response	7	0.00769	BP					
	GO:0001558	Regulation of cell growth	5	0.00914	BP					
	GO:0009611	Response to wounding	3	0.01457	BP					
	GO:0005615	Extracellular space	12	8E-5	CC					
	GO:0005634	Nucleus	42	2.4E-4	CC					
	GO:0005576	Extracellular region	22	4E-4	CC					
	GO:0030173	Integral to Golgi membrane	3	0.02101	CC					

4	GO:0008083	Growth factor activity	8	1.0E-5	MF	GO:0009615	Response to virus	12	0.00000	BP
	GO:0005125	Cytokine activity	6	3.7E-4	MF	GO:0007565	Female pregnancy	9	1.6E-4	BP
	GO:0046983	Protein dimerization activity	6	0.00123	MF	GO:0006955	Immune response	17	5.0E-4	BP
	GO:0005100	Rho GTPase activator activity	3	0.00268	MF	GO:0001525	Angiogenesis	7	0.02671	BP
	GO:0008201	Heparin binding	4	0.00826	MF	GO:0007267	Cell-cell signaling	10	0.02671	BP
	GO:0003700	Transcription factor activity	13	0.00826	MF	GO:0008150	Biological process	19	0.02928	BP
	GO:0008047	Enzyme activator activity	3	0.01045	MF	GO:0016477	Cell migration	5	0.03984	BP
	GO:0005178	Integrin binding	3	0.01447	MF	GO:0005576	Extracellular region	45	0.00000	CC
	GO:0016563	Transcription activator activity	4	0.02237	MF	GO:0005577	Fibrinogen complex	3	6.0E-4	CC
	GO:0005515	Protein binding	33	0.03960	MF	GO:0005615	Extracellular space	14	0.00843	CC
	GO:0043565	Sequence-specific DNA binding	8	0.03960	MF	GO:0031093	Platelet α granule lumen	4	0.01203	CC
	GO:0006955	Immune response	11	2.4E-4	BP	GO:0016020	Membrane	61	0.03962	CC
	GO:0006915	Apoptosis	9	0.00440	BP	GO:0005794	Golgi apparatus	15	0.03962	CC
	GO:0030183	B cell differentiation	3	0.01798	BP					
	GO:0045944	Positive regulation of transcription from RNA polymerase II promoter	5	0.03323	BP					
	GO:0000079	Regulation of cyclin-dependent protein kinase activity	3	0.03704	BP					
	GO:0007050	Cell cycle arrest	4	0.03704	BP					
	GO:0008284	Positive regulation of cell proliferation	5	0.03704	BP					
	GO:0007267	Cell-cell signaling	6	0.03704	BP					
	GO:0001558	Regulation of cell growth	5	0.04074	BP					
6	GO:0005515	Protein binding	65	0.00000	MF	GO:0046870	Cadmium ion binding	3	0.00135	MF
	GO:0003700	Transcription factor activity	24	1.0E-5	MF	GO:0003674	Molecular function	13	0.00852	MF
	GO:0008083	Growth factor activity	8	3.5E-4	MF	GO:0008009	Chemokine activity	4	0.00852	MF
	GO:0003714	Transcription co-repressor activity	7	3.5E-4	MF	GO:0003950	NAD+ADP-ribosyl transferase activity	3	0.01169	MF
	GO:0005125	Cytokine activity	8	3.5E-4	MF	GO:0030674	Protein binding bridging	3	0.04041	MF
	GO:0005100	Rho GTPase activator activity	4	3.5E-4	MF	GO:0009615	Response to virus	12	0.00000	BP
	GO:0003700	Transcription factor activity	7	6.2E-4	MF	GO:0006955	Immune response	16	0.00000	BP
	GO:0046983	Protein dimerization activity	7	6.9E-4	MF	GO:0007565	Female pregnancy	9	0.00000	BP
	GO:0008270	Zinc ion binding	32	0.00504	MF	GO:0008150	Biological process	14	0.00612	BP
	GO:0046872	Metal ion binding	32	0.00827	MF	GO:0007267	Cell-cell signaling	8	0.00676	BP
	GO:0005085	Guanyl-nucleotide exchange factor activity	5	0.02023	MF	GO:0006952	Defense response	5	0.00728	BP
	GO:0043565	Sequence-specific DNA binding	11	0.03272	MF	GO:0030168	Platelet activation	3	0.01864	BP
	GO:0008201	Heparin binding	4	0.03502	MF	GO:0051258	Protein polymerization	3	0.03966	BP
	GO:0005178	Integrin binding	3	0.04652	MF	GO:0005576	Extracellular region	44	0.00000	CC
	GO:0006915	Apoptosis	13	0.00173	BP	GO:0005615	Extracellular space	13	6.0E-5	CC
	GO:0006950	Response to stress	7	0.00173	BP	GO:0005577	Fibrinogen complex	3	6.0E-5	CC
	GO:0007050	Cell cycle arrest	6	0.00788	BP	GO:0031093	Platelet α granule lumen	4	8.1E-4	CC
	GO:0045944	Positive regulation of transcription from RNA polymerase II promoter	7	0.01021	BP					
	GO:0045740	Positive regulation of DNA replication	3	0.01720	BP					
	GO:0008360	Regulation of cell shape	4	0.02121	BP					
	GO:0008285	Negative regulation of cell proliferation	8	0.02486	BP					
	GO:0000122	Negative regulation of transcription from RNA polymerase II promoter	7	0.02486	BP					
	GO:0009611	Response to wounding	3	0.02698	BP					
	GO:0030183	B cell differentiation	3	0.02698	BP					
	GO:0007229	Integrin-mediated signaling pathway	5	0.02698	BP					
	GO:0006954	Inflammatory response	7	0.02698	BP					
	GO:0007179	Transforming growth factor β receptor signaling pathway	4	0.02698	BP					
	GO:0043066	Negative regulation of apoptosis	4	0.02698	BP					
	GO:0006935	Chemotaxis	5	0.04499	BP					
	GO:0007010	Cytoskeleton organization and biogenesis	5	0.04841	BP					
	GO:0006955	Immune response	10	0.04843	BP					
	GO:0005576	Extra cellular region	31	9.0E-5	CC					
	GO:0005615	Extra cellular space	14	6.6E-4	CC					
	GO:0005622	Intracellular	29	0.00843	CC					
	GO:0005737	Cytoplasm	40	0.03660	CC					

ONTO-TOOLS/OE2GO was used to identify the differentially expressed GO terms based on the hypergeometric distribution and corrected *P* value (< 0.05). The GO identified number (GOID), GO term name (GO name), the number of genes changed within each functional gene category, *P* values are listed. GO terms with at least 3 genes changed and corrected *P* values < 0.05 are listed in Table 4.

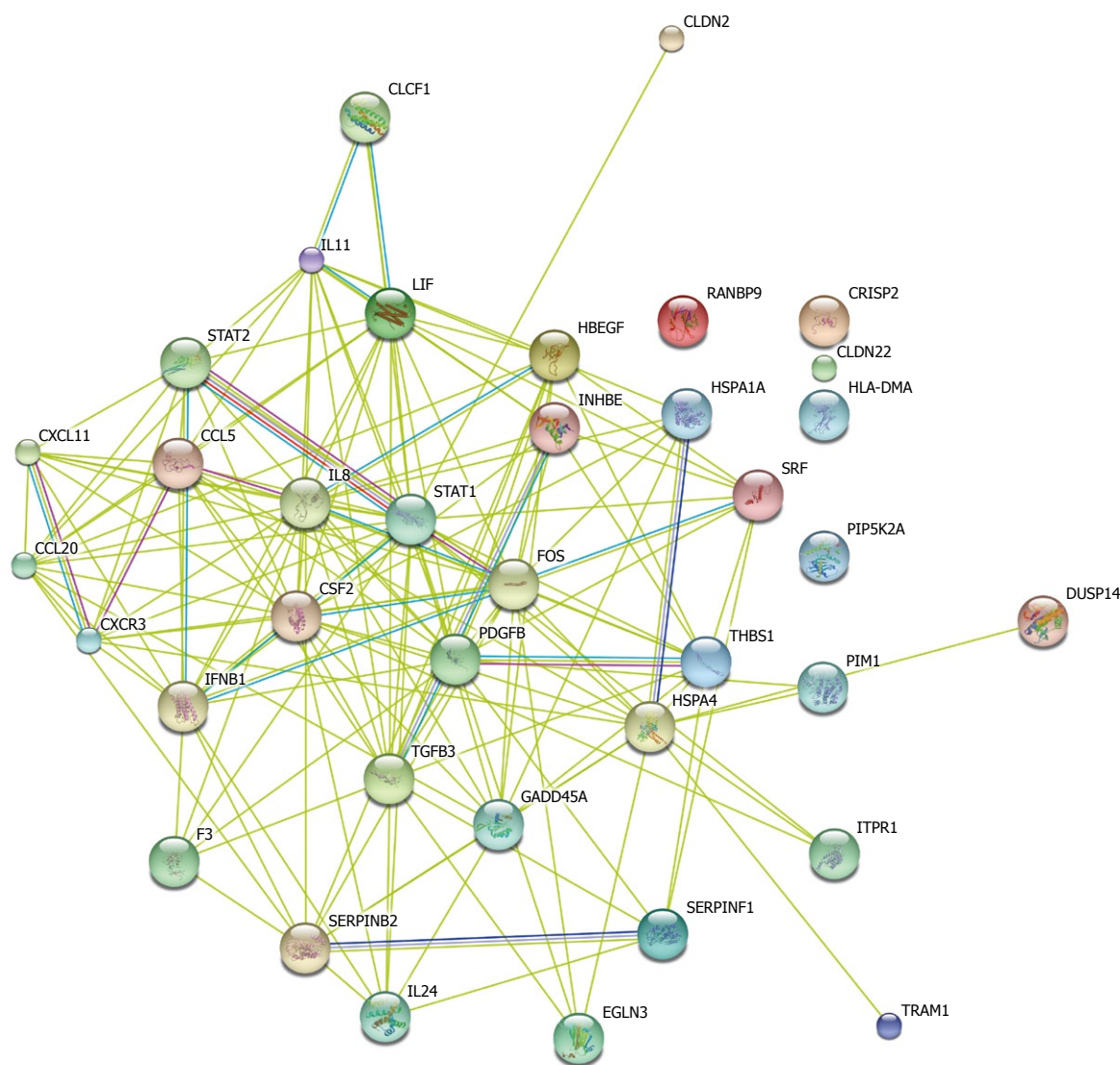


Figure 4 A simplified gene network extracted from significant pathways using STRING database.

Table 5 Top four significantly perturbed pathways at each time point					
Time point	0.5 h	1 h	2 h	4 h	6 h
Gene mapping	CAM	P53	MAPK	CAM	CAM
	MAPK	TGF	ECHP	CY-CY	CY-CY
	P53	MAPK	RCC	MAPK	JAK-STA
	TGF	CCC	P53	JAK-STA	MAPK
Down-regulation	APP	APP	APP	Phos	APP
	Toll	CY-CY	CY-CY	APP	CY-CY
	CY-CY	Toll	Toll	Toll	Toll
	NKMC	Mela	Mela	Mela	Mela

suggested that it might be an important turning point between infection and host response. Although a model system of the AGS cell line infected with *H. pylori* was used to explore the host response^[5,29], it should be noted that this is an isolated cell culture system, and cannot account for the varied effects of conditions in a human stomach. Therefore, the speculation generated from this study represents a valuable, but a simplified view of the

situation. More researches are required to confirm these findings. In addition, we also compared our results with the genes with significant change after *H. pylori* infection in another report^[30]. Several genes in that report are consistent with our results in dataset1 like *socs2*, *stat6*, *ccl4*, *cxcl2*, *bla-dma*, *hsph1*, *plat*, *ifitm1*, *alox5*, *thr4*, *faim3*, *cd47*, *ifngr1* and *il8*.

Only part of these genes showed a high fold change > 1.5 in differential expressions, including *il8*, *faim3*, *thr4*, *alox5*, *bla-dma*, *cxcl2* and *ccl4*.

In summary, the results from this sequential expression microarray have extended previous studies that were limited to the comparison of normal and diseased tissues. We took a global view on the genes and pathway net related to *H. pylori* infection, several co-expressional profiles and important new genes like *mmp24* and *il-24* involved in immune response and tumorigenesis during *H. pylori* infection were also identified. Our study also suggested that the outcome of *H. pylori* infection is probably involved in a complex mechanism, and is associated with a number of immune factors. Formation of tumors may be a result

of an imbalance between bacterial attack and immune defense of host. We speculate that this competition may occur at 1-2 h after infection, and 4 h may be a first time point at which the balance is upset.

COMMENTS

Background

It has been indicated that *Helicobacter pylori* (*H. pylori*) infection may highly contribute to gastritis and carcinogenesis in the past two decades since it was recovered from human gastric mucosa in 1983, and many studies have focused on identification of both bacterial factors and host determinants that may contribute to the pathogenic mechanism.

Research frontiers

Gene expression microarray has been widely used in identifying genes associated with *H. pylori* infection and gastric tumor. However, the time-series gene expression profile of *H. pylori* infection remains unexplored. In this study, the authors extended the knowledge of the dynamic interaction between *H. pylori* and host mucosa using a high density human gene microarray and flexible bioinformatics analysis.

Innovations and breakthroughs

Several important genes that have not been reported previously and a pathway net related to *H. pylori* infection were discovered by the sequential microarrays. Based on the co-expressional profile analysis during infection, a new speculation for the pathogenic mechanism has been set up.

Applications

This study has provided a systemic view of expression profile of time-series *H. pylori* infected AGS cells. The new identified genes and pathway net as well as the hypothesis could help researchers in this field further understand the potential mechanism associated with *H. pylori* infection and carcinogenesis, and provide important information for prevention and control of *H. pylori* related diseases.

Peer review

The scientific and innovative contents as well as readability in this manuscript reflect the advanced levels of the clinical and basic researches in gastroenterology both at home and abroad.

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Combined MELD and blood lipid level in evaluating the prognosis of decompensated cirrhosis

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MELD ≥ 18 and TC ≤ 2.8 mmol/L were independent risk factors for prognosis of decompensated cirrhosis. Survival analysis showed that MELD ≥ 18 combined with TC ≤ 2.8 mmol/L can clearly discriminate between the patients who would survive and die in 1 year.

CONCLUSION: MELD ≥ 18 and TC ≤ 2.8 mmol/L are two important indexes to predict the prognosis of patients with decompensated cirrhosis. Their combination can effectively predict the long-term prognosis of patients with decompensated cirrhosis.

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Key words: Cirrhosis; Model of end-stage liver disease; Blood lipid; Prognosis; Survival time

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Abstract

AIM: To evaluate the prognostic value of the combined model for end-stage liver disease (MELD) and blood lipid level in patients with decompensated cirrhosis.

METHODS: A total of 198 patients with decompensated cirrhosis were enrolled into the study. The values of triglyceride (TG), cholesterol (TC), high density lipoproteins (HDL) and low density lipoprotein (LDL) of each patient on the first day of admission were retrieved from the medical records, and MELD was calculated. All the patients were followed up for 1 year. The relationship between the change of blood lipid level and the value of MELD score was studied by analysis of variance. The prognostic factors were screened by multivariate Cox proportional hazard model. Draw Kaplan-Meier survival curves were drawn.

RESULTS: Forty-five patients died within 3 mo and 83 patients died within 1 year. The levels of TG, TC, HDL and LDL of the death group were all lower than those of the survivors. The serum TG, TC, HDL and LDL levels were lowered with the increase of the MELD score. Multivariate Cox proportional hazard model showed that

INTRODUCTION

Cirrhosis is a common disease and the major cause of death in China. There are liver function failure, portal hypertension and a variety of complications during decompensated period, leading to a high mortality. The true and objective judgments on end-stage liver disease conditions are helpful for clinicians to choose appropriate treatment protocols for individual patients. With continuous emergence of new treatments for liver diseases in recent years, the evaluation system used for the severity and prognosis of patients with decompensated

cirrhosis is needed urgently. Model for end-stage liver disease (MELD) established in recent years is a new method to determine the reserved liver function. It has shown many advantages in clinical use, especially in liver transplantation^[1,2]. But the model does not contain all prognostic factors, thus it has become a hotspot in the studies to improve the predictive power of MELD score by combination with other evaluation indexes^[3-5]. The liver is the vital organ to synthesize blood lipid and lipoprotein. Whether it is injured seriously or otherwise determines the level of blood lipids and apolipoprotein to a large extent. Some papers have shown that the lipid level was an important indicator to reflect the liver damage, which can be used to determine the condition and estimate the prognosis of the patients^[6]. In this study, we observed the value of four indexes of triglyceride (TG), cholesterol (TC), high density lipoproteins (HDL) and low density lipoprotein (LDL) to judge the prognosis of patients with decompensated cirrhosis. We also observed the value of combined MELD and TC in evaluating the prognosis of patients with decompensated cirrhosis.

MATERIALS AND METHODS

Patients

A total of 198 patients with decompensated cirrhosis treated in Department of Gastroenterology of Shanghai East Hospital from October 2005 to June 2008 were evaluated, and their medical profiles were retrospectively analyzed. The clinical diagnoses were all based on the program of 2000 for the prevention and treatment of virus hepatitis established in Xi'an congress^[7]. We excluded patients with past or current hepatocellular carcinoma, diseases affecting blood lipid such as hypertension, diabetes, cardiovascular and cerebrovascular disease, kidney disease and so on, use of lipid-regulating drugs recently, admission to hospital repeatedly, incomplete case records and lost to follow-up. The patients aged 26-88 years (61.4 ± 10.5 years), including 122 (61.6%) men and 76 (38.4%) women.

Clinical data

Baseline laboratory results of all the patients obtained at admission (i.e. bilirubin, creatinine, INR, TG, TC, HDL and LDL) were retrieved from the medical records. All the patients were followed up for 1 year. The outcome was assessed based on the 3- and 12-mo mortalities.

Calculation of MELD

All the prognostic models were calculated based on laboratory results obtained on the first day of admission. The MELD equation was used to calculate the severity score: $9.6 \times \log_e [\text{creatinine (mg/dL)}] + 3.8 \times \log_e [\text{bilirubin (mg/dL)}] + 11.2 \times \log_e (\text{INR}) + 6.43$ ^[8].

Statistical analysis

All statistical analyses were conducted with the SPSS for Windows version 13 release. The measurement data be-

tween two sets were compared by Student's *t* test. The data between multiple sets were compared by analysis of variance. The factors affecting the prognosis were screened by the Cox proportional hazards model. The survival curve was established by Kaplan-Meier analysis and compared by Log rank test. $P < 0.05$ was accepted as significant.

RESULTS

Clinical features in the survival group and the death group

Forty-five patients died (22.7%) at 3 mo and 83 patients died at 1 year (41.9%) among the 198 patients with cirrhosis. The scores of MELD (23.3 ± 9.82), TG (0.80 ± 0.29 mmol/L), TC (2.63 ± 1.04 mmol/L), HDL (0.93 ± 0.46 mmol/L) and LDL (1.67 ± 0.77 mmol/L) in the death group at 3 mo were significantly different statistically compared with those in the survival group (13.6 ± 6.40 , 0.86 ± 0.36 mmol/L, 3.22 ± 1.07 mmol/L, 1.30 ± 0.60 mmol/L and 1.88 ± 0.71 mmol/L) (Table 1). The scores of MELD (22.90 ± 8.90), TG (0.76 ± 0.26 mmol/L), TC (2.77 ± 1.00 mmol/L), HDL (1.06 ± 0.62 mmol/L) and LDL (1.66 ± 0.69 mmol/L) of the death group at 1 year were also significantly different statistically from the survival group (12.42 ± 5.29 , 0.94 ± 0.39 mmol/L, 3.36 ± 1.11 mmol/L, 0.93 ± 0.46 mmol/L and 1.67 ± 0.77 mmol/L) (Table 1).

Relationship between lipid levels and MELD score in patients with cirrhosis

The 198 cases of cirrhosis were divided into three groups according to MELD score: < 11 (group 1), $11 \leq$ or < 21 (group 2), and ≥ 21 (group 3). The levels of TG, TC, HDL and LDL declined with increasing MELD score. The TC and HDL were all statistically significant among the three groups (Table 2). There was no statistically significant difference in the level TG and LDL between group 1 and group 2, but between group 2 and group 3 (Table 2).

Multivariate Cox regression analysis in patients with cirrhosis

The prognostic factors (MELD ≥ 21 , TG ≤ 0.8 mmol/L, TC ≤ 2.8 mmol/L, HDL ≤ 1.2 mmol/L and LDL ≤ 1.6 mmol/L) of 198 patients with cirrhosis were included into the multivariate Cox proportional hazard model for regression analysis. As a result, MELD ≥ 21 and TC ≤ 2.8 mmol/L were independent risk factors for prognosis of decompensated cirrhosis. The risk ratios (HR) were 2.69 and 0.68 (Table 3).

Kaplan-Meier fractional survival curves of combined MELD and TC

Based on the results of multivariate Cox regression analysis, the MELD ≥ 21 combined with TC ≤ 2.8 mmol/L was divided into four groups. The 1-year survival curve

Table 1 Clinical features of 198 cirrhotic patients at 3-mo and 1-yr follow-ups (mean \pm SD)

Clinical features	Follow-up at 3 mo			Follow-up at 1 yr		
	Survival group	Death group	P value	Survival group	Death group	P value
MELD	13.6 \pm 6.40	23.3 \pm 9.82	0.000 ^a	12.42 \pm 5.29	22.90 \pm 8.90	0.000 ^a
TG (mmol/L)	0.86 \pm 0.36	0.80 \pm 0.29	0.036 ^a	0.94 \pm 0.39	0.76 \pm 0.26	0.003 ^a
TC (mmol/L)	3.22 \pm 1.07	2.63 \pm 1.04	0.006 ^a	3.36 \pm 1.11	2.77 \pm 1.00	0.001 ^a
HDL (mmol/L)	1.30 \pm 0.60	0.93 \pm 0.46	0.013 ^a	1.37 \pm 0.50	1.06 \pm 0.62	0.021 ^a
LDL (mmol/L)	1.88 \pm 0.71	1.67 \pm 0.77	0.010 ^a	2.04 \pm 0.74	1.66 \pm 0.69	0.020 ^a

^aP < 0.05, the death group *vs* the survival group. MELD: Model for end-stage liver disease; TG: Triglyceride; TC: Cholesterol; HDL: High density lipoproteins; LDL: Low density lipoprotein.

Table 2 Relationship between lipid levels and MELD score in 198 patients with cirrhosis (mean \pm SD)

	Group 1 (MELD < 11)	Group 2 (11 \leq MELD < 21)	P value (groups 1 and 2)	Group 3 (MELD \geq 21)	P value (groups 2 and 3)
TG (mmol/L)	1.01 \pm 0.46	0.96 \pm 0.33	0.089	0.80 \pm 0.31	0.021 ^b
TC (mmol/L)	3.68 \pm 1.23	3.03 \pm 0.91	0.006 ^d	2.62 \pm 1.18	0.002 ^d
HDL (mmol/L)	1.97 \pm 0.73	1.34 \pm 0.83	0.026 ^d	0.76 \pm 0.55	0.018 ^d
LDL (mmol/L)	1.89 \pm 0.44	1.71 \pm 0.44	0.092	1.42 \pm 0.86	0.032 ^b

^bP < 0.05, group 2 *vs* group 3; ^dP < 0.05, group 1 *vs* group 2 and group 2 *vs* group 3.

Table 3 Multivariate Cox regression of 198 patients with decompensated cirrhosis

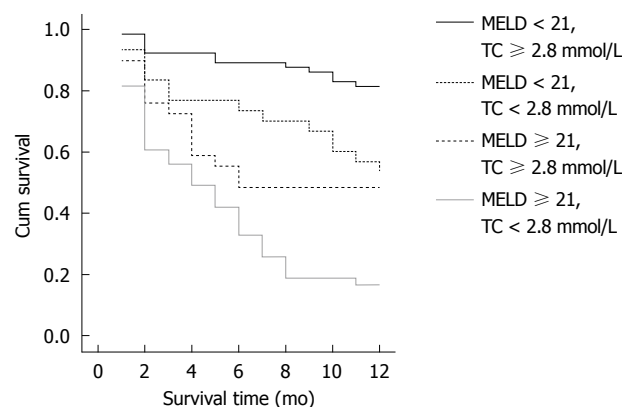
	HR	95% CI	P
MELD \geq 21	2.69	1.58-4.58	< 0.001
TC \leq 2.8 mmol/L	0.68	0.31-1.43	0.020

HR: Hazard ratios.

was set up by Kaplan-Meier (Figure 1). It showed that MELD \geq 21 combined with TC \leq 2.8 mmol/L can distinguish between the patients who would survive and die in 1 year (Figure 1).

DISCUSSION

MELD was created by Malinchoc *et al*^[9] of Mayo Clinic, America in 2000, which was initially used to predict survival following elective placement of transjugular intrahepatic portosystemic shunt to prevent bleeding and cure ascites. Later, MELD score has been proven to effectively reflect the severity of pathogenetic condition and prognosis status of different liver diseases, and it has become the recognized standard to judge the severity of pathogenetic condition for end-stage liver disease. Many studies have reported that MELD was indeed a good indicator in determining the condition of end-stage liver disease such as cirrhosis, hepatitis, liver transplantation^[1,10]. However, Angermayr *et al*^[11] thought that the judging ability of MELD is not superior to CTP score. Earlier reports confirmed that the MELD scoring system had an advantage in assessment of liver transplanted patients, but recent studies showed that the system was still not perfect, which is not suitable for all patients with liver transplantation^[12,13].

**Figure 1** One-year Kaplan-Meier survival curves of 198 cirrhotic patients.

MELD score is still controversial for its ability to determine the pathogenetic condition of end-stage liver disease. Currently, scholars are combining MELD with other indicators in an attempt to estimate pathogenetic condition of liver diseases more accurately^[14]. The liver is the main site to synthesize, store, transport and decompose lipids, including synthesis and secretion of endogenous lipoprotein; synthesis of rate-limiting enzyme of lipoprotein metabolism to regulate mutual conversion and metabolism between a variety of lipoprotein; and absorbing and removing metabolites of lipoprotein through the lipoprotein receptors on the hepatocytes surface to maintain equilibrium of TC and TG metabolism^[15]. The liver cannot esterify fatty acid to synthesize phosphatidyl choline required by TG and TC esterification. The reduced synthesis of TC acyl-transferase can decrease the serum TG and TC levels. In addition, damaged hepatocytes, abnormal synthesis of apolipoprotein A by liver and/or the reduced synthesis

of phosphatidylcholine TC acyltransferase may decrease the levels of high-density and very low-density lipoproteins. It has been reported that the Child-Pugh score of patients with cirrhosis was high, the higher severity of the liver damage, the lower the blood lipid level, which is an important indicator to reflect liver damage. The blood lipid level can be used to determine the condition and estimate the prognosis of the patients^[16]. In this paper, the patients with cirrhosis were divided into low-risk, medium-risk and high-risk groups according to the MELD score. It was also observed that the blood lipid levels in the patients were decreased with increasing MELD score. There was no significant difference in the two indicators (TG and LDL) for cirrhosis between low-risk and medium-risk groups. Considering that extrahepatic tissues of patients may still have some degree of synthesizing ability in early stage of cirrhosis, and there is blood lipid elimination disorder in the cirrhotic patients, it is not likely for blood lipid level to reduce. But with a further reduction in cytoactivity of hepatocytes, blood lipid will significantly reduce. Habib *et al*^[6] have studied 248 cases of cirrhosis and found that the HDL, TC and VLDL level of deaths in 90, 180, 365 d were all lower than that of the survivals. This result also showed that all serum lipids of the patients who died within 3 mo and 1 year were all significantly lower than that of survivals.

The liver plays a leading role in the synthesis of TC and maintain the balance in TC metabolism. It can control the dynamic equilibrium of TC *in vivo* through participation in the process of uptake, synthesis, intracellular transport, esterification or hydrolysis of TC, and rotation to the circulating blood, and synthesizing and secreting bile acid. TG oxidation (hydrolysis) is inhibited in patients with hepatitis. On the other hand, due to appetite loss and short-term starvation, the liver metabolism mobilized a large number of stored fat, which can raise the TG levels. The liver function was severely damaged during cirrhosis. The hepatocytes lack of energy result in fatty acid oxidation and TG levels decrease. The liver is not only the organ that synthesizes most TC in human body by up to 60%-80%, but also the site for TC transformation and excretion. In liver disease, the level of serum TC is reduced because of decreased TC synthesis in the liver and blocked esterification. With deterioration of liver cell damage, ALT level increased and the serum TC further decreased. Liver function is seriously and irreversibly damaged during cirrhosis, resulting in significant decrease of TC level. So the level of serum TC is meaningful to determine the severity of acute liver disease, which can reflect the acute processes, it is particularly helpful in severe hepatitis judgment. Selcuk *et al*^[17] analyzed 99 dead patients with cirrhosis and found that their TC levels were all lower than normal. Li *et al*^[18] selected six indicators including TC by Logistic regression analysis as the prognostic indicator of patients with chronic severe hepatitis. In our study, MELD ≥ 21 and TC ≤ 2.8 mmol/L were selected by multivariate Cox proportional hazard model as the independent prognos-

tic factors of patients with cirrhosis. HR was 2.69 and 0.68. MELD and the TC values are corresponding to the severity of illness. Kaplan-Meie survival curve showed that MELD ≥ 21 combined with TC ≤ 2.8 mmol/L can distinguish between the patients who would survive and die in 1 year, which may provide more information in clinical treatment. In addition, 90% of the HDL *in vivo* is synthesized by the liver. The change in serum HDL concentration is closely related to the severity of the liver disease. The progressive reduction of HDL is the performance indicating deteriorated condition, while the level of HDL would gradually increase with the condition improved. Thus, serum HDL is the ideal target for liver disease diagnosis and prognostic evaluation^[19]. Habib *et al*^[6] found that HDL was the marker of liver function and prognostic factor of nonalcoholic cirrhosis. After liver transplantation, the mortality of patients was significantly correlated with decreased serum HDL. Especially the risk of death in cirrhotic patients increased by 3.4% as the serum HDL became lower than 30 mg/dL. However, we did not find that HDL is an independent prognostic factor in cirrhotic patients by Cox regression mode. Larger series of patients is needed to verify the findings.

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COMMENTS

Background

Cirrhosis is a common disease and the major cause of death in China. The true and objective judgments on end-stage liver disease conditions is helpful for clinicians to choose different treatment protocols suitable for individual patients.

Research frontiers

Model for end-stage liver disease (MELD) established in recent years is a new method to determine reserved liver function. It has shown many advantages in clinical use, especially in liver transplantation. But the model is not perfect. It is a hotspot in the studies to improve the predictive power of MELD score by combination with other evaluation indexes. This study has shown that the lipid level is an important indicator to reflect the degree of liver damage, which can be used to determine the condition and estimate the prognosis of the patients.

Innovations and breakthroughs

In some studies, the lipid level has been used to determine the condition and estimate the prognosis of patients with liver damage. In this study, the authors observed four indexes of triglyceride, cholesterol (TC), high density lipoproteins and low density lipoprotein to judge the prognosis of 198 patients with decompensated cirrhosis through retrospective analysis, and observed the value of MELD combined with TC in evaluating the prognosis of patients with decompensated cirrhosis as well.

Applications

MELD combined with cholesterol can be used to judge the pathogenetic condition and prognosis of patients with decompensated cirrhosis.

Terminology

Multivariate Cox proportional hazard model is a multivariate technique for analyzing the effect of two or more metric and/or nonmetric variables on survival.

Peer review

The aims of this paper are to see whether modification of MELD can improve accuracy. The rationale for use of lipids needs to be strengthened. The authors should consider using some of the modifications of MELD such as MELD-Na.

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Effects on the pouch of different digestive tract reconstruction modes assessed by radionuclide scintigraphy

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Abstract

AIM: To determine the effect of three digestive tract reconstruction procedures on pouch function, after radical surgery undertaken because of gastric cancer, as assessed by radionuclide dynamic imaging.

METHODS: As a measure of the reservoir function, with a designed diet containing technetium-99m (^{99m}Tc), the emptying time of the gastric substitute was evaluated using a ^{99m}Tc -labeled solid test meal. Immediately after the meal, the patient was placed in front of a γ camera in a supine position and the radioactivity was measured over the whole abdomen every minute. A frame image was obtained. The emptying sequences were recorded by the microprocessor and then stored on a computer disk. According to a computer processing system, the half-emptying actual curve and the fitting curve of food containing isotope in the detected region were depicted, and the half-emptying actual curves of the three reconstruction procedures were directly compared.

RESULTS: Of the three reconstruction procedures, the half-emptying time of food containing isotope in the Dual Braun type esophagojejunal anastomosis proce-

dure (51.86 ± 6.43 min) was far closer to normal, significantly better than that of the proximal gastrectomy orthotopic reconstruction (30.07 ± 15.77 min, $P = 0.002$) and P type esophagojejunal anastomosis (27.88 ± 6.07 min, $P = 0.001$) methods. The half-emptying actual curve and fitting curves for the Dual Braun type esophagojejunal anastomosis were fairly similar while those of the proximal gastrectomy orthotopic reconstruction and P type esophagojejunal anastomosis were obviously separated, which indicated bad food conservation in the reconstructed pouches.

CONCLUSION: Dual Braun type esophagojejunal anastomosis is the most useful of the three procedures for improving food accommodation in patients with a pouch and can retard evacuation of solid food from the reconstructed pouch.

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Key words: Isotope γ -scintigraphy; $T_{1/2}$ time; Digestive tract reconstruction; ^{99m}Tc -DTPA; Emptying time

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INTRODUCTION

Patterns and functions of digestive tract reconstruction

after gastrectomy play a vital role in the quality of life of patients. As to which reconstruction after gastrectomy provides the least complications and best quality of life, much research has been carried out. Most of these studies focus on the function of the pouch built by surgery, including volume of the pouch, storage time of food in the pouch, and emptying mode of the food. In previous studies, most judgments were made from the subjective experience of the inspector, which cannot reveal the true state of the reconstructed pouch function. There is a long way to go, as regards further research, before we know which kind of reconstruction mode will provide patients with a good quality of life with the least problems after gastrectomy is performed.

Radionuclide dynamic imaging is the gold standard for measuring emptying time of the gastrointestinal tract^[1,2]. It has been used to measure gastric emptying function when there is abnormal digestive function with normal anatomy. This is a simple technique and its best advantage is that there is seldom impact from the inspector or subject, and results reflect the actual empty time of the pouch based on objective facts. The method is simple and non-invasive, requiring a single oral administration of the radionuclide tracer. The meal used in the procedure does not alter the normal physiology of the gut. It is possible with this imaging to judge whether a reconstructed pouch matches normal physiological function. The aim of the present study was to quantify pouch emptying time of a solid meal in patients who had undergone gastrectomy surgery by calculating their half-emptying time ($T_{1/2}$) values.

MATERIALS AND METHODS

Patients

This study was conducted in patients who had undergone total and proximal gastrectomy for gastric cancer after a period of 6-36 mo, and who were surviving without cancer. Total number was 34 cases, and the average age of the patients was 28-72 years. Three operation procedures were conducted as follows: proximal gastrectomy orthotopic reconstruction, 10 cases; total gastrectomy P type esophagojejunal anastomosis, 11 cases; total gastrectomy Dual Braun type esophagojejunal anastomosis, 13 cases. Clinical data are displayed in Table 1.

Methods

Meal preparation: The solid test meal was prepared by mixing 1 mCi non-absorbable containing technetium-99m (^{99m}Tc)-DTPA with 100 g flour, a 50 g raw egg and 80 mL clear water (thus containing protein 12.9 g, fat 15.4 g, carbohydrate 72.9 g); this was mixed to a paste. A pancake was heated in a microwave oven for 3 min. Each test meal offered 1814 kJ of calorific energy.

Scintigraphic measurements: Patients were supplemented with potassium chlorate 15 min before scanning, in order to block out hypothyroid and gastric mucosa influence and speed up the excretion of remnant ^{99m}Tc.

Table 1 Patient clinical data

Clinical data	Reconstruction mode		
	Proximal gastrectomy (n = 10)	P type (n = 11)	Dual-Braun (n = 13)
Gender			
Male	7	8	8
Female	3	3	5
mean age (yr)	61	57	59
General type			
Borrmann I	0	0	0
Borrmann II	6	4	4
Borrmann III	4	7	9
Borrmann IV	0	0	0
Staging			
Early cancer	1	0	2
I B	2	2	3
II	4	4	3
III A	2	4	4
III B	1	1	1

Emission images were taken by a γ camera. All the patients ate the test meal within a 5 min period. Immediately after the meal, the patient was placed in front of a γ camera in a supine position, representing 0 min, and radioactivity was measured over the whole abdomen. The whole detection process took 90 min, and a frame image was collected approximately every minute. One frame image was consequently obtained which was stored in a computer disk, to measure the period and image of nuclide attenuation. Areas of interest corresponding to the gastric substitute were outlined, and the radioactivity was counted on each image and expressed as the percentage of ingested activity. After revising the half life of ^{99m}Tc and calculating every emission count of every image, the half-emptying actual curve and fitting curve of food containing isotopes in the detected region were depicted according to the computer analyzing processing system.

Two regions from each patient were investigated as follows: upper esophagus anastomosis, detecting reflux; food accommodation region after reconstruction, detecting half-emptying time of food containing the nuclide.

Statistical analysis

The half-emptying actual curve and fitting curve of the radionuclide were analyzed, and the actual half-emptying time of food containing the nuclide was compared among the three different reconstruction procedures using group comparison. Data analyzed among groups: differences in age and postoperative time among groups were tested by variance analysis, and there was no significance among groups; variance analysis of the actual half-emptying time compared between groups was conducted by Mann-Whitney test, and results were considered to be significant at $P < 0.05$.

RESULTS

No differences in esophagus reflux were found during the experiment between the three groups and no increment of isotope radioactivity was detected in the lower

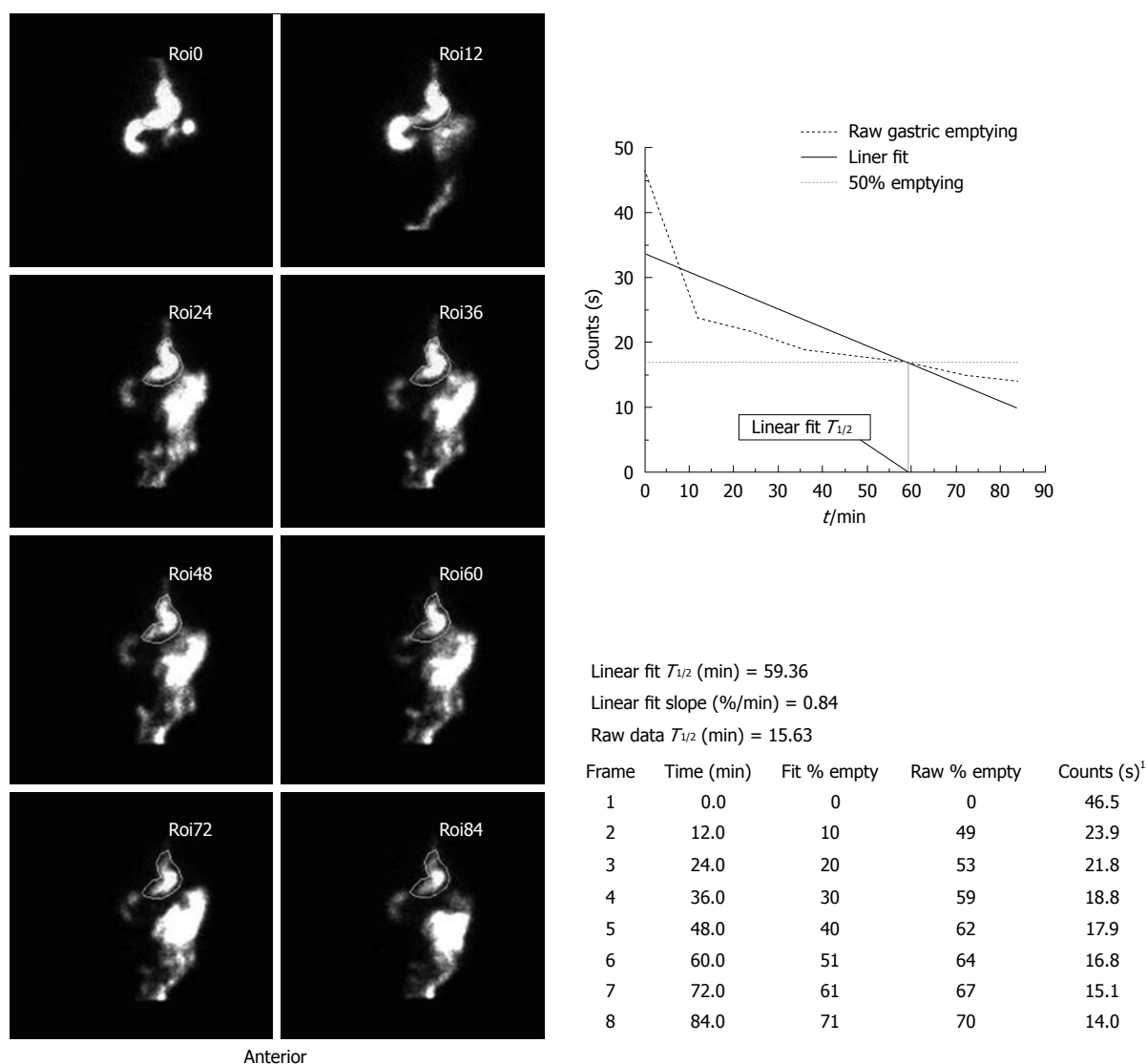


Figure 1 Proximal gastrectomy, 36 mo after operation. Raw data $T_{1/2}$ was 15.63 min, linear fit $T_{1/2}$ was 59.36 min. ¹Anterior only.

esophagus. Increments of isotope radioactivity were detected in the pouch region among some patients, which revealed that the actual curve of half-emptying descended initially and then ascended.

Emptying time of different reconstruction procedures

Proximal gastrectomy: The half-emptying time of the isotope markedly decreased (30.07 ± 15.77 min) with great significance compared to the Dual Braun procedure (51.86 ± 6.43 min). The actual and fitting curves were rather separated. After dietary intake for 20 min, time approached half-emptying, showing a poor food capacity in the remnant stomach (Figure 1).

P type reconstruction: Half-emptying time (27.88 ± 6.07 min) was close to that of the proximal procedure. This reconstruction had a significantly shorter transit time compared to the Dual Braun procedure. The actual and fitting curves were rather separated, which showed a poor accommodation of food in the pouch (Figure 2).

Dual Braun type esophagojejunal anastomosis: Emptying time of this procedure was 51.86 ± 6.43 min, not far from normal (60.00 ± 10.00 min), and its actual and fitting curves were fairly similar, which revealed that the reconstructed pouch could play a role in retarding the excretion of food (Figure 3).

Of the three kinds of reconstruction mode, the differences in emptying time have significant statistical relevance when compared among groups (Table 2).

Characteristics of food discharge from pouch

As seen from emptying images of patients having the proximal gastrectomy procedure and the total gastrectomy P type reconstruction procedure, a strong isotope development was found outside the detected region with the increasing of time, which revealed that food could not be discharged evenly from the pouch, but in an integral transferred manner. However, the double circles of the Dual Braun reconstruction procedure could retard food emptying (Figure 4).

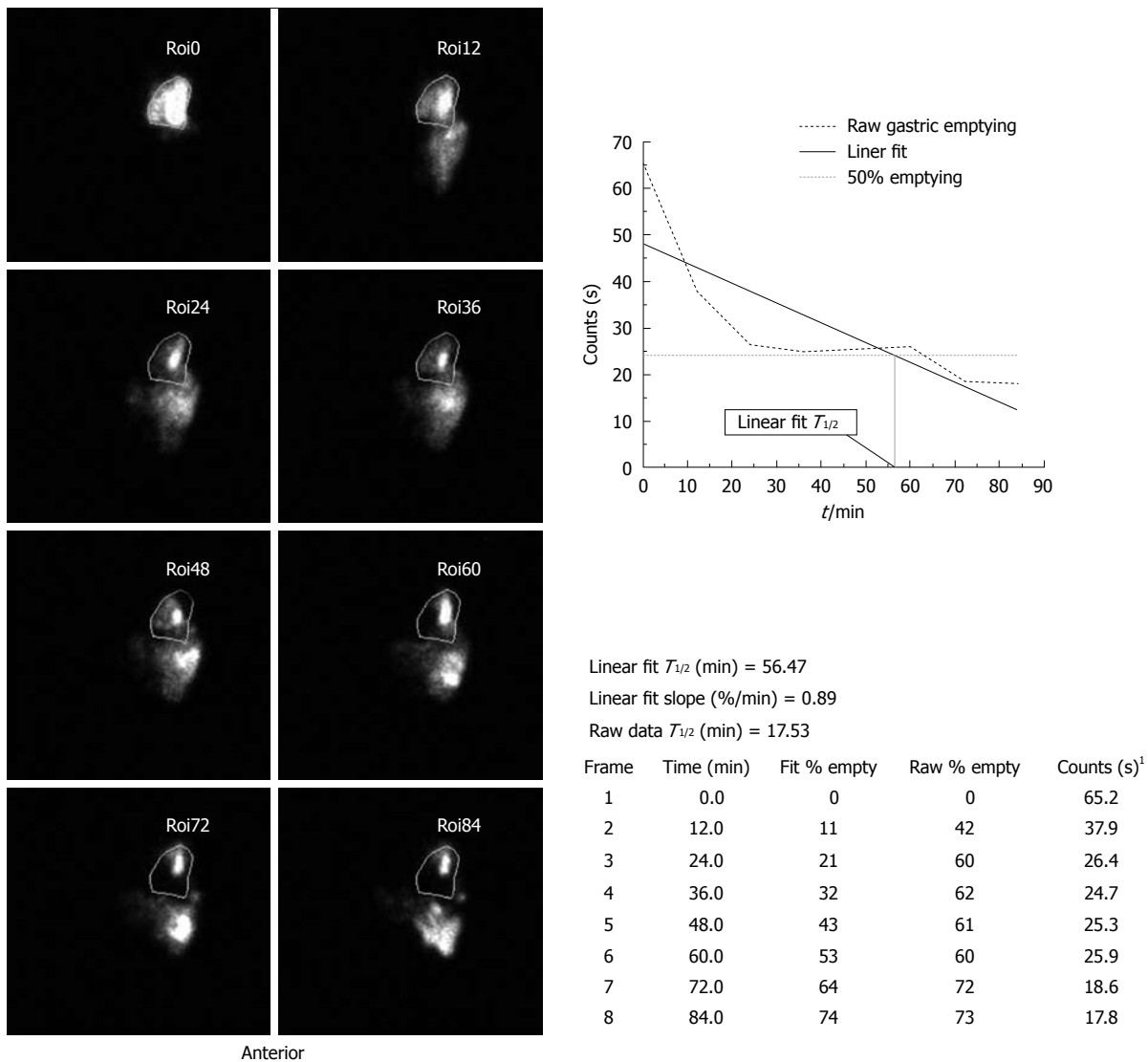


Figure 2 P type reconstruction, 24 mo after operation. Raw data $T_{1/2}$ was 17.53 min, linear fit $T_{1/2}$ was 56.47 min. ¹Anterior only.

Table 2 Emptying times of the three kinds of reconstruction (mean \pm SD)

Reconstruction mode	n	Time after operation (mo) ²	Raw data $T_{1/2}$ (min)	Linear fit $T_{1/2}$ (min)	P ¹
Proximal gastrectomy	10	16	35.16 \pm 13.51	69.86 \pm 11.71	0.706 ^a
P type reconstruction	11	22	29.66 \pm 6.07	67.33 \pm 8.75	0.002 ^b
Dual-Braun reconstruction	13	24	52.66 \pm 10.33	65.77 \pm 11.43	0.001 ^c

¹SPSS 11.0 software; ²There were no statistical differences between groups regarding operation time. ^aProximal gastrectomy *vs* P type; ^bProximal gastrectomy *vs* Dual-Braun reconstruction; ^cP type *vs* Dual-Braun reconstruction.

DISCUSSION

In order to improve the quality of life of patients post-operatively, functions of digestive tract reconstructions after gastrectomy have been frequently studied by many

researchers. A consensus was reached that radionuclide dynamic imaging is the most desirable method among a host of techniques used to detect effectiveness of digestive tract reconstruction. This method is simple and easy to perform; it is a non-invasive examination without any pain and is preferred by patients. Moreover, the results are not affected by subjective factors, and thus reflect pouch efficacy after digestive tract reconstruction objectively.

It has been over 100 years since Schlatter reported the first successful total gastrectomy with reconstruction. During that period, gastric carcinoma surgical techniques evolved into extended resection and ultra-extended resection. Currently, these have been converted into diminution and rational therapeutic protocols. Though many operation protocols have changed with time, they all tightly stick to the principles of radical treatment, aiming to provide security and functionality after gastric carcinoma. In recent years, studies regarding functionality of digestive tract reconstruction after gastrectomy have been progressively increasing. Different methods

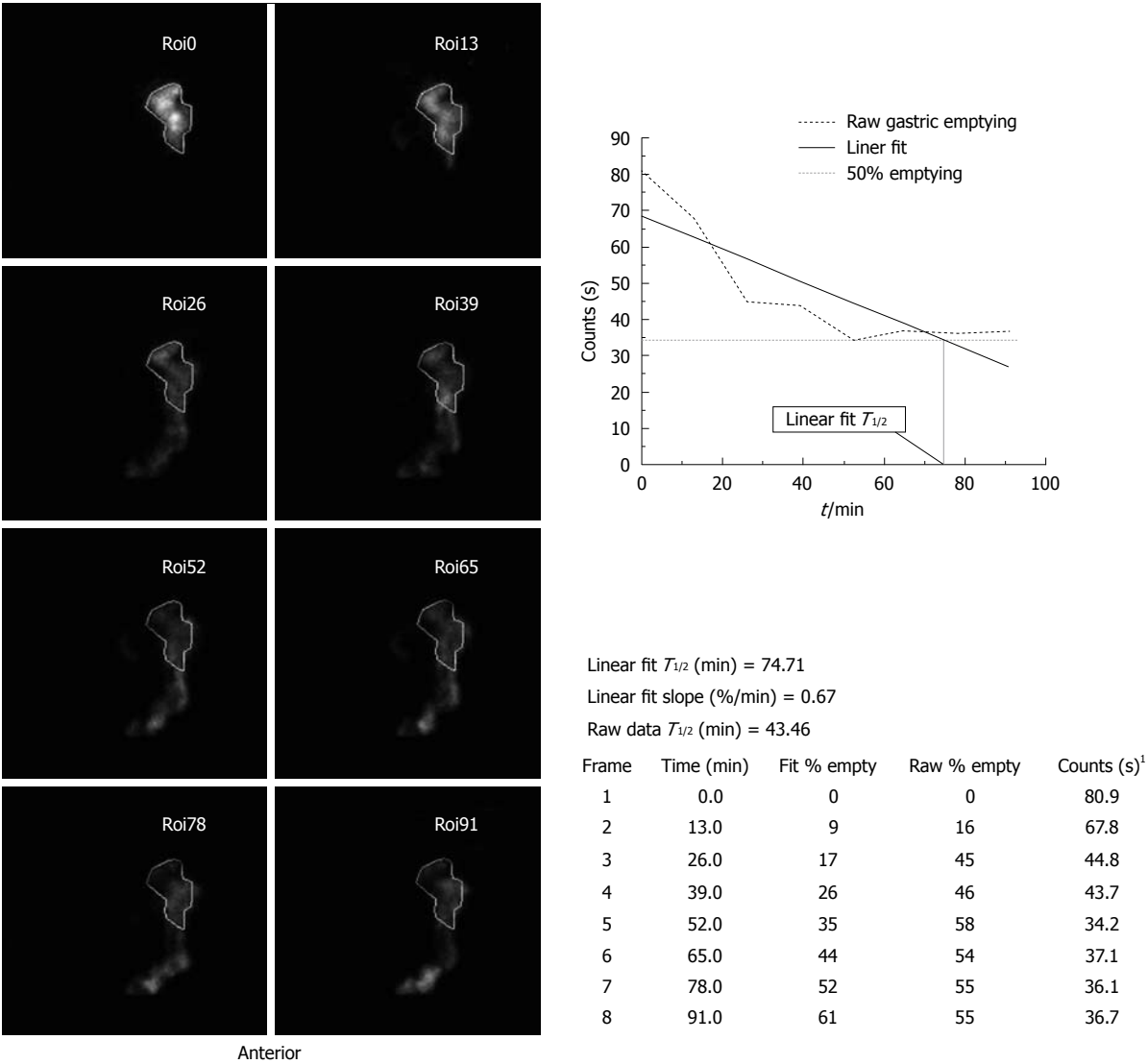


Figure 3 Dual-Braun reconstruction, 17 mo after operation. Raw data $T_{1/2}$ was 43.46 min, linear fit $T_{1/2}$ was 74.71 min. ¹Anterior only.

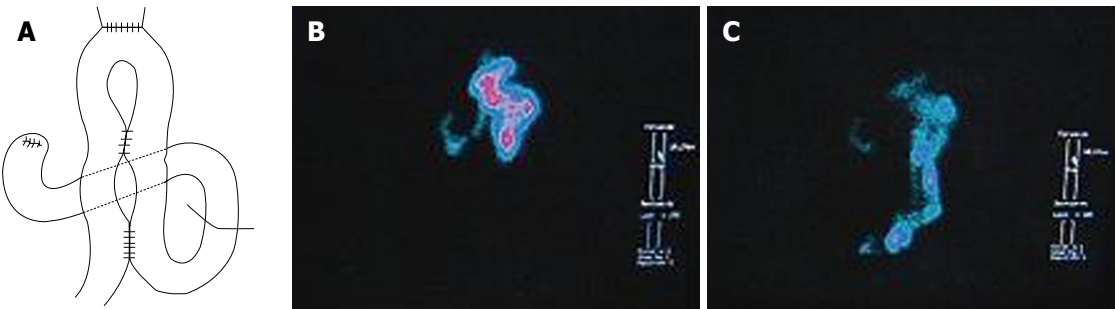


Figure 4 Dual-Braun reconstruction (A) and duodenal reflux seen at examination (B, C).

have their own disadvantages and advantages when viewed from different perspectives^[3-12]. To determine the optimum reconstruction procedure after gastrectomy in terms of the quality of life of patients, we conducted a controlled trial to compare the pouch effectiveness of the three reconstruction procedures by radionuclide dynamic imaging.

We have previously reported a study of the same

three kinds of reconstruction procedure, regarding the quality of life after operation^[13]. We found Dual-Braun reconstruction provided good quality of life compared to the other two construction methods. Results showed patients put on more bodyweight and had less meal frequency. We considered that it could be the pouch that had good function with regard to digestion, so we performed this study in the same patients and confirmed

that Dual Braun reconstruction can increase storage time in the pouch and empty food in a very even manner, nearly at normal levels. Therefore we proposed: (1) Proximal gastrectomy with esophagojejunal anastomosis partly preserves normal gastric tissue with easy operation, allowing a normal physiological pathway of food transit. Due to the small volume of remnant stomach and destruction of pylorus nerves, the remnant pylorus loses its routine function. As seen from the observations, food containing radionuclide was excreted fast from the remnant stomach, and thus incapable of mixing with digestive juice completely, which affects the intake of nutritional substances. The syndrome occurring when food intake of patients decreased and emptying times increased with a resultant weight loss postoperatively, was obvious clinically. The incidence of reflux was also higher postoperatively. All the results were confirmed in our subsequent review of patients; and (2) Total gastrectomy is a common resection used for gastric carcinoma, and its complications are still a cardinal factor affecting the quality of life of patients. Modification of reconstruction methods plays a positive role in avoiding postoperative complications; however, no modified protocols have received a consensus of approval. Among many modifications of pouch reconstruction and capacity, we compared P type esophagojejunal anastomosis and Dual Braun type esophagojejunal anastomosis, which has been advocated recently. In our subsequent review, we found that there was no difference in pouch capacity (food intake) between the two procedures, but from radionuclide dynamic imaging, there was an obvious difference in emptying time. One case even showed that the half-emptying time of nuclide was less than 1 min and emptying time was 3 min, as measured 24 mo after a P type procedure. The body weight of the patient decreased from 80 to 63 kg postoperatively. The patient did not undergo complete lymph node dissection and had no steatorrhea postoperatively, and had no symptoms of celiac plexus injury. Weight loss was mainly ascribed to inflexible function of the pouch in which food transited fast through the upper jejunum and nutritional substance intake was poor. The original intention of the Dual Braun procedure was to reduce reflux postoperatively and to promote functionality of the pouch. Double circles were placed after reconstruction, and the intestinal wall internal plexus was not damaged due to retention of the continuity of the jejunum, which retarded the food excretion from pouch to some extent. The half-emptying time of food containing nuclide was longer and the actual and fitting curves were similar, which showed the superiority of this reconstruction procedure.

A Dual Braun pouch provides a slower transit of food directly below the esophagus, while a double circle slows down the passage at that part of the digestive tract where the food can be mixed with bile and pancreatic juice arriving from the Y limb. This may provide a better utilization of digestive enzymes and probably leads to an improved digestion.

There was reflux noted in almost all patients after reconstruction methods of total and proximal gastrectomy, but there was no obvious reflux of food containing nuclide detected in our observations. After subsequent review, main reflux did not occur immediately after food intake, but during the period beginning from 6 min after food intake. This difference between experimental observation and clinical investigation is mainly ascribed to different detecting conditions. Our results were not in line with some other experimental reports.

Radionuclide solid gastric emptying studies have been performed clinically since 1976^[14]. Many investigations published since then have confirmed the clinical utility of such studies for diagnosis of a variety of gastric motility disorders, but most of the radionuclide scintigraphy research was not in relation to reconstructed gastric tract^[15-18].

The latest paper using radionuclide imaging to detect gastric emptying was published by Bernstine *et al*^[19]; their study was to clarify whether laparoscopic sleeve gastrectomy to treat morbid obesity causes changes in gastric emptying.

Total gastrectomy is accompanied by a host of complications postoperatively, and some symptoms (i.e. malignant anemia) are unable to be avoided by operation. However, reconstruction procedures show a positive inhibitory effect on such symptoms. There are about 60 digestive tract reconstruction protocols at present; however, new prospective clinical trials should be undertaken and optimum pouch reconstruction protocols further investigated in order to ameliorate the quality of life of patients.

COMMENTS

Background

There are more than 60 kinds of reconstruction mode after gastrectomy. The purpose of the different modes is to diminish the complications, such as reflux and dumping syndrome, after operation and improve the quality of life. However, there is no single type of reconstruction mode commonly accepted by all researchers. Radionuclide solid gastric emptying studies have been performed clinically since 1976. Many investigations published since then have confirmed the clinical utility of such studies for diagnosis of a variety of gastric motility disorders. Nevertheless, there is a remarkable lack of standardization of this type of study and no generally applicable reference values. In particular, radionuclide studies have seldom been used in the reconstructed digestive tract.

Research frontiers

The latest paper using radionuclide imaging to detect gastric emptying was published by Bernstine *et al*; their study was to clarify whether laparoscopic sleeve gastrectomy to treat morbid obesity causes changes in gastric emptying. Other research was focused on different reconstruction procedures and there has not been a study comparing emptying between different reconstruction modes.

Innovations and breakthroughs

The authors use the radiolabeled solid food to detect and compare the functions of the postoperative gastric tract, to contrast the pouch function between three kinds of reconstructed pouch. Compared with other research, the authors have used simultaneous patient follow up data to demonstrate their results.

Applications

The authors have concluded through their study that the Dual-Braun procedure was a good mode of reconstruction; it has a comparative emptying time to normal digestive tract (gastric). Radionuclide scintigraphy is a very useful

method to detect the emptying time of the reconstructed pouch, is very simple and non-invasive, and can be used in other kinds of pouch.

Peer review

This manuscript is original, has scientific value, is well written and has clinical value for the readers.

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Role of *BMP3* in progression of gastric carcinoma in Chinese people

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system, the LOH frequency was higher in gastric carcinoma at stages III and IV than in gastric carcinoma at stages I and II ($P = 0.01$), which was also significantly correlated with lymph node metastasis and clinicopathological characteristics of gastric carcinoma. Methylation of bone morphogenetic protein 3 (*BMP3*) gene promoter was detected in 64.44% of gastric carcinoma tissue samples. However, no statistical significance was observed between promoter region methylation and carcinoma differentiation. Interestingly, the *BMP3* gene methylation rate was 71.05% and 28.58%, respectively, in MSI positive and negative cases ($P = 0.031$), suggesting that *BMP3* genetic instability and promoter methylation are initiated during gastric carcinogenesis. LOH was detected mostly in the late stages of gastric carcinoma, indicating that gastric carcinoma at late stages has a higher infiltration and a poorer prognosis.

CONCLUSION: Promotor region methylation of the *BMP3* gene may cause gastric carcinoma in Chinese people.

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Abstract

AIM: To investigate the relation between gastric cancer and microsatellite instability (MSI), loss of heterozygosity (LOH) and promoter region methylation.

METHODS: Fifty primary gastric carcinoma specimens were collected from patients with no family history of cancer. In addition, normal tissues were also collected from patients as controls. DNA was extracted by polymerase chain reaction for single-strand conformation polymorphism, bisulfite DNA sequencing, and methylation-specific band analysis.

RESULTS: The positive rate for MSI and LOH in gastric carcinoma was 16% and 20%, respectively. According to the tumor, node and metastasis staging

Key words: Bone morphogenetic protein 3 gene; Gastric carcinoma; Microsatellite instability; Loss of heterozygosity; Methylation

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Chen XR, Wang JW, Li X, Zhang H, Ye ZY. Role of *BMP3* in progression of gastric carcinoma in Chinese people. *World J Gastroenterol* 2010; 16(11): 1409-1413 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v16/i11/1409.htm> DOI: <http://dx.doi.org/10.3748/wjg.v16.i11.1409>

INTRODUCTION

Gastric carcinoma is one of the leading causes of cancer-related death in China. Its incidence and mortality are increasing in Chinese people. Research has been focused on the development of cancer and its progression, by studying epigenetics and tumor suppressor genes. It was reported that microsatellite instability (MSI) and loss of heterozygosity (LOH) are associated with gastric cancer^[1-5]. In addition, gene promoter region hypermethylation can activate certain tumor suppressor genes. Most of the MSI and LOH studies have been focused on *p53*^[6], *p16* and fragile histidine triade genes^[7] with less efforts made on the role of bone morphogenetic protein (BMP) 3 epigenetics in tumor suppressor genes.

BMP3, belonging to the transforming growth factor β superfamily proteins, plays an important role in embryonic development by inducing and patterning early skeletal formation. It has been recently reported that *BMP3* is associated with tumor development and progression^[8]. However, no report is available on the role of *BMP3* in gastric carcinoma development. The present study was to investigate the D4S2922 and D4S2964 loci in 4q21 region of the *BMP3* gene by examining their MSI, LOH as well as the promoter region methylation and to reveal the relation between *BMP3* gene and gastric carcinogenesis.

MATERIALS AND METHODS

Gastric carcinoma specimens

Fifty gastric carcinoma samples were obtained from patients with no family history of cancer and normal tissue samples were also collected from patients as controls. The study was approved by the Ethics Committee and patient families before chemotherapy and radiotherapy.

DNA extraction and polymerase chain reaction (PCR)

DNA was extracted from gastric carcinoma and normal tissue samples using a QIAGEN kit according to its manufacturer's instructions. PCR for D4S2922 was performed using the primers (sense: 5'-TGCTTATGCAAGAG-GTTGTTTC-3' and antisense: 5'-AAAGGGCAGT-TAGGGATGCT-3') and for D4S2964 using the primers of (sense: 5'-CTTCCTTCCCAACTCACACA-3' and antisense: 5'-GGTCATTTCATGCAATCCACA-3'). Thirty-five PCR amplification cycles were performed at 94°C for 1 min (denaturation), at 59°C for 1 min (annealing), and at 72°C for 1 min (extension) in a 25 μ L reaction buffer containing 1.0 mmol/L MgCl₂ and PCR buffer (QIAGEN PCR kit). The PCR products were resolved in 2% agarose gels. The fragment size of D4S2922 and D4S2964 was 230 bp and 164 bp, respectively.

Single-strand conformation polymorphism (SSCP) analysis

For SSCP analysis, 3 μ L of each amplification product was added to 3 μ L denaturing buffer containing 98% formamide and 0.09% bromophenol blue. The samples

were heat-denatured at 98°C for 10 min and then placed on ice for 10 min. The denatured DNA was electrophoresed on 8% non-denaturing polyacrylamide gels (140 V for 2 h, 4°C). SSCP patterns on the gels were visualized with silver staining.

Bisulfite DNA sequencing analysis

Using a CpGenome™ fast DNA modification kit (Chemicon International, Inc.), genomic DNA (1 μ g) in a volume of 100 μ L was denatured with 7 μ L of 3 mol/L NaOH freshly prepared at 37°C for 10 min, 550 μ L of freshly prepared DNA modification reagent was then added and the reaction was continued at 55°C for 16-20 h. The modified DNA was cooled on ice for 5 min before 750 μ L binding buffer was added. After centrifugation, the products were washed with 750 μ L 1 \times washing buffer and denatured with 50 μ L 20 mmol/L NaOH/90% EtOH for 10 min, followed by an additional 750 μ L 1 \times washing buffer. The eluted products were stored at -20°C for use.

Methylation-specific PCR analysis

Primers (sense: 5'-GGAGTTTAATTTTGGTTT-TTGTT-3' and antisense: 5'-ATCAACTCCCAACATCACTACA-3') were used for the nonmethylated specific *BMP3* gene, yielding a PCR product of 73 bp. Primers (sense: 5'-GTTTAATTTTCGGTTTCGTCGTCGT-3' and antisense: 5'-GTCGACTCCCGACGTCG TACG-3') were used for the methylated specific *BMP3* gene, yielding a PCR product of 70 bp. After an initial preheating step at 94°C for 10 min, 40 cycles of PCR were performed at 94°C for 30 s, at 64°C for 45 s, at 72°C for 75 s, and a final extension at 72°C for 10 min. The amplified PCR products were examined on 8% agarose gels.

Band analysis

PCR-SSCP showed one main band of the D17S396 locus in normal lymph node genomic DNA. The presence of two main bands indicated that a sample could be further evaluated in LOH analysis. LOH was considered when less than 50% bands or a lower band density was found in tumor tissue samples than in normal tissue samples, while MSI was considered when more than 50% bands or a band migration was observed in tumor tissue samples.

Statistical analysis

All data were analyzed with SPSS version 16.0 by one-way ANOVA and *t*-test. *P* < 0.05 was considered statistically significant.

RESULTS

Correlation between BMP3 genetic instability and clinicopathological features of gastric carcinoma

Both D4S2922 and D4S2964 from tumor and normal tissue samples were successfully amplified and the bands on gel images were heterozygous for the allele. Com-

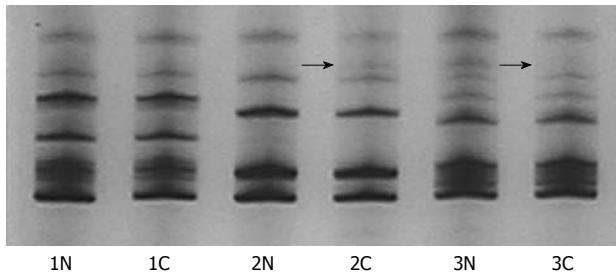


Figure 1 Polyacrylamide gel electrophoresis (PAGE) of bone morphogenetic protein 3 (*BMP3*) gene. No difference was found between tumor tissue (1C) and normal tissue (1N). Compared with normal tissue (2N), positive MSI was represented as an added allele band (arrow) in tumor tissue (2C). Compared with normal tissue (3N), positive LOH was represented as lacking an allele band (arrow) in tumor tissue (3C).

Table 1 Relation of genetic instability and methylation of *BMP3* gene with clinicopathological features of gastric cancer

Clinicopathological features	MSI		LOH	
	%	(+/n)	%	(+/n)
Differentiation degree	16.00	(8/50)	20.00	(10/50)
High differentiation	25.00	(2/8)	0.00	(0/8)
Middle differentiation	8.69	(2/23)	21.74	(5/23)
Low differentiation	21.05	(4/19)	26.32	(5/19)
Serosa infiltration				
Positive	17.15	(6/35)	22.86	(8/35)
Negative	13.33	(2/15)	13.33	(2/15)
Lymph node metastasis				
Positive	12.12	(4/33)	30.30	(10/33)
Negative	23.53	(4/17)	0.00 ^b	(0/17)
TNM stage				
I + II	15.16	(5/33)	6.06	(2/33)
III + IV	17.65	(3/17)	47.05 ^c	(8/17)

^b $P < 0.001$ vs positive lymph node metastasis; ^c $P = 0.01$ vs TNM stages I + II. MSI: Microsatellite instability; LOH: Loss of heterozygosity; *BMP3*: Bone morphogenetic protein 3; TNM: Tumor, node and metastasis.

pared with the normal tissue samples, MSI was considered when more bands were found in gastric carcinoma tissue samples and LOH was considered when less bands were observed in gastric carcinoma tissue samples (Figure 1). A *BMP3* was considered positive when the two loci showed at least one MSI or one LOH.

In the present study, 16.00% of the gastric carcinoma tissue samples were positive for MSI of the *BMP3* gene (Table 1). However, MSI was not correlated with tumor differentiation, infiltration, lymphatic metastasis and tumor, node and metastasis (TNM) staging. The frequency of LOH was 20.00% (Table 1). Interestingly, LOH was significantly related with lymphatic metastasis and TNM staging of gastric carcinoma. The frequency of LOH was not correlated with differentiation and infiltration of gastric carcinoma and much higher in gastric carcinoma with lymph node metastasis than in gastric carcinoma without lymph node metastasis (30.30% vs 0.00%, $P < 0.05$). The LOH detection rate was higher in tumors at stages III and IV than in tumors at stages I and II (47.05% vs 6.06%, $P < 0.01$).

Table 2 Relation between *BMP3* gene methylation and clinicopathological features of gastric cancer

Clinicopathological features	Methylation frequency		<i>P</i> value
	%	(+/n)	
Differentiation degree	64.44	(29/45)	0.273
High	75.00	(6/8)	
Middle	68.18	(15/22)	
Low	53.33	(8/15)	
Serosa infiltration			0.275
Positive	59.38	(19/32)	
Negative	76.92	(10/13)	0.663
Lymph node metastasis			
Positive	62.07	(18/29)	0.844
Negative	68.75	(11/16)	
TNM stage			0.031 ^a
I + II	62.50	(10/16)	
III + IV	65.52	(19/29)	0.290
MSI			
Negative	71.05	(27/38)	0.290
Positive	28.58	(2/7)	
LOH			0.290
Negative	68.57	(24/35)	
Positive	50.00	(5/10)	

^a $P < 0.05$.

Relation between *BMP3* gene promoter region methylation and clinicopathological features of gastric carcinoma

The methylation status in promoter region of *BMP3* gene was analyzed by methylation-specific PCR (Table 1). Positively methylated *BMP3* promoter region was found in 45 of the 50 gastric carcinoma tissue samples, with a methylation rate of 64.44%. The frequency of methylation in normal tissue was 53.49% (43/50) with no statistical difference between gastric carcinoma and normal tissue samples ($P > 0.05$). In addition, *BMP3* gene promoter region methylation was not correlated with tumor differentiation, infiltration, lymphatic metastasis and clinical TNM staging.

Correlation between gastric carcinoma gene *BMP3* instability and its promoter region methylation

The frequency of *BMP3* gene promoter region methylation was much lower in MSI negative than in MSI positive cases (28.58% vs 71.05%, $P < 0.05$, Table 2). However, the frequency of *BMP3* gene promoter region methylation was 68.75% and 50.00%, respectively, in LOH negative and positive cases with no statistical difference.

DISCUSSION

Microsatellites are the short sequences of DNA, usually 2-6 base pairs in a row along a DNA molecule. MSI is mutations in genes, whereby repair of damaged DNA causes microsatellite-associated regions to become longer or shorter. LOH in a cell represents the loss of normal function in one allele of a gene in which the other alleles have been inactivated. MSI and LOH play an

important role in tumor development and progression. LOH occurs when the remaining functional allele in a somatic cell of the offspring becomes inactivated due to mutation. No normal tumor suppressor is produced, thus resulting in tumorigenesis^[9].

Microsatellites were first reported in 1981 by Miesfeld *et al*^[10] and further elucidated in 1993 by Aaltonen *et al*^[11] who revealed a higher MSI frequency in hereditary non-polyposis colorectal cancer cells. MSI has been reported in colon, gastric, cervical, breast, prostate and pancreatic carcinomas^[12-17].

Alexander *et al*^[18] investigated the sporadic colon carcinoma cases and found that MSI occurs in early stages with a better prognosis, suggesting that MSI can serve as an early diagnostic index for gastric and colon carcinomas. Our previous report also demonstrated that the frequency of MSI is higher in D17S396 loci of the *nm23H1* gene in gastric and colon carcinomas at stages I and II with a better prognosis than in those at stages III and IV with a worse prognosis, suggesting that MSI can serve as an early diagnostic index for gastric and colon carcinomas^[19]. In this study, however, no statistical significance was found between MSI of *BMP3* gene and tumor differentiation, infiltration, lymphatic metastasis and tumor TNM staging.

Berney *et al*^[20] showed that the LOH frequency of *nm23H1* gene is significantly correlated with tumor infiltration and metastasis. Candusso *et al*^[21] reported that LOH is more frequently found in late stage tumors, often with lymphatic metastasis. In our study, the LOH frequency of *BMP3* gene was much higher in gastric carcinomas at stages III and IV with lymphatic metastasis than in those at stages I and II without lymphatic metastasis, suggesting that LOH occurs more often in late tumor stages with lymphatic metastasis and can thus serve as an index for the evaluation of malignancy, metastasis and prognosis of gastric carcinomas.

DNA methylation is believed to be closely associated with tumor development and progression. Reduced methylation and hypermethylation in gene promoter regions can inactivate tumor suppressor genes, leading to tumor development. In the present study, the methylation level in promoter region of *BMP3* gene in gastric carcinomas was high, leading to the occurrence of gastric carcinoma. Loh *et al*^[8] reported that *BMP3* gene methylation is associated with MSI in colon cancer, indicating that the higher the methylation is, the higher the MSI frequency is. In this study, the level of methylation was higher in MSI negative than in MSI positive gastric carcinoma cases, which is not consistent with the findings of Loh *et al*^[8]. Further study is needed to verify the differences.

In conclusion, genetic instability of the *BMP3* gene and promoter region methylation occur in gastric carcinomas, thus affecting tumor characteristics through different pathways. LOH is observed more frequently in late stage tumors with infiltration. Hypermethylation in promoter region of *BMP3* gene may result in gastric carcinoma in Chinese people.

COMMENTS

Background

Gastric carcinoma is one of the leading causes of cancer-related death in China. Its incidence and mortality are increasing in Chinese people. Microsatellite instability (MSI) and loss of heterozygosity (LOH) are known to be associated with gastric cancer. Moreover, gene promoter region hypermethylation can inactivate certain tumor suppressor genes.

Research frontiers

Genetic instability and gene promoter region methylation play an important role in gene mutation, gene inactivation, and carcinogenesis. However, the role and mechanism of bone morphogenetic protein 3 (*BMP3*) in cancer development are poorly characterized. The relation between the development of gastric cancer and the number of altered microsatellite loci (D4S2922 and D4S2964), LOH, and promoter region methylation was elucidated in the present study.

Innovations and breakthroughs

In the present study, the methylation levels in promoter region of *BMP3* gene in gastric carcinoma was high, which may lead to gastric carcinoma and is not consistent with the reported findings. The methylation level was higher in MSI negative than in MSI positive gastric carcinoma.

Applications

MSI and LOH of the *BMP3* gene can serve as an index for the evaluation of malignancy, metastasis and prognosis of gastric carcinoma.

Terminology

MSI is a condition manifested as damaged DNA due to defects in normal DNA repair process. LOH in a cell represents the loss of normal function in one allele of a gene in which the other allele has been inactivated.

Peer review

The paper is well-written and interesting. *BMP3* gene, MIS, LOH and promoter region methylation were evaluated in patients with gastric adenocarcinoma, thus making an additional contribution to studies on the role of MIS and LOH in carcinogenesis.

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Interferon- α induced severe thrombocytopenia: A case report and review of the literature

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Abstract

We report a case of severe thrombocytopenia following pegylated interferon- α 2a (Peg-IFN- α 2a) treatment of hepatitis C virus infection and summarize the clinical characteristics of 16 cases of IFN- α induced severe thrombocytopenia and its immune-mediated mechanism. Discontinuation of IFN- α and early administration of immunosuppressants are the effective therapy for IFN- α induced severe thrombocytopenia.

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Key words: Interferon- α ; Severe thrombocytopenia; Chronic hepatitis C

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INTRODUCTION

Interferon- α (IFN- α) and pegylated IFN- α 2a (Peg-IFN- α 2a) are the effective antiviral drugs for chronic liver diseases. However, IFN- α is associated with a number of side effects, including mild thrombocytopenia, a common adverse effect largely ascribed to bone marrow suppression. IFN- α treatment-associated severe thrombocytopenia, like immune thrombocytopenia or thrombotic thrombocytopenic purpura, has rarely been reported in the literature. Here, we report a patient who developed immune thrombocytopenia 3 mo following Peg-IFN- α 2a (Pegsys, Roch) treatment and report the clinical features of severe thrombocytopenia.

CASE REPORT

A 54-year-old female was diagnosed as chronic hepatitis C virus (HCV) infection in 1998. Laboratory test showed that her serum anti-HCV and HCV RNA were positive, viral genotype was 1b, and serum alanine aminotransferase (ALT) level was 60-100 U/L. The patient was treated with standard recombinant IFN- α 2a (Roch, 3 MU), 3 times per week, at a clinic in 2003. Hepatic cirrhosis was excluded before treatment. Her auto-antibodies including antinuclear antibody (ANA), anti-smooth muscle antibody (ASMA) and anti-thyroid antibody were negative. HCV RNA turned negative 1 mo after treatment with IFN- α 2a. Three months later, IFN- α 2a was discontinued due to adverse effects, including poor appetite, fatigue, nausea and mild gingival bleeding. Her platelet (PLT) count was slightly decreased to $80 \times 10^9/L$. Six months later, her HCV RNA was positive again and she administered no antiviral drugs.

In May 2008, the patient felt mild fatigue. Laboratory test showed that the levels of HCV RNA, ALT, and AST were 1.5×10^5 copies/L, 67 U/L (normal < 40 U/L), and 42 U/L (normal < 40 U/L), respectively, while her PLT count was $110 \times 10^9/L$ and the aforementioned auto-antibodies were negative. One month after treatment with

Peg-IFN- α 2a (180 μ g, *sc*, once a week), her HCV RNA turned negative with normal ALT and peripheral blood cell count. After discharged from hospital, she continued to administer the same dose of Peg-IFN- α 2a and was followed up. Three months later, the patient was admitted to our hospital again due to severe gingival bleeding, fatigue, poor appetite, and nausea. Laboratory test on admission showed that her haematocrit was 29.2%, leucocyte count was 3.23×10^9 /L (including 76.5% of polymorphonuclear cells, 15.2% of lymphocytes, and 6.8% of monocytes), PLT count was 2×10^9 /L, ALT was 53.6 U/L, AST was 44.9 U/L, total bilirubin was 5.2 μ mol/L, prothrombin time was 10.5 s, activated partial thromboplastin time (APTT) was 30.9 s, fibrinogen was 2.82 g/L and HCV RNA was negative. Bone marrow aspirate showed a large number of megakaryocytes in her hypercellular marrow with few granules, scanty cytoplasm and no PLT around. Indirect immunofluorescence showed a high anti-platelet IgG titer (1:1280, normal < 1:80) and negative ANA and ASMA. Complements C₃ and C₄ were 0.79 g/L (range: 0.88-2.01 g/L) and 0.13 g/L (range: 0.16-0.47 g/L), respectively. B-mode gray scale ultrasonography showed no splenomegaly.

She was diagnosed as immune-mediated thrombocytopenia with Peg-IFN- α 2a highly suspected as its cause. Peg-IFN- α 2a was discontinued and two units of PLT was transfused on the day at admission. The PLT count was then increased to 27×10^9 /L, but decreased to 1×10^9 /L on the second day with a mild fever caused by rapid destruction of PLT. Immunoglobulin (400 mg/kg) and intravenous methylprednisolone (1 mg/kg per day) were administered during the following 5 d. On day 7, PLT count was increased to 33×10^9 /L, and methylprednisolone was replaced with prednisone (30 mg/d). Two weeks later, her PLT count was increased to 107×10^9 /L, and prednisone was withdrawn 1 mo later. Her PLT count remained normal during the follow-up, but her HCV RNA turned positive 3 mo after discharge. She has not received any other antiviral therapy since then.

DISCUSSION

In this case, Peg-IFN- α 2a was considered the cause of autoimmune thrombocytopenia due to the following reasons^[1]. First, thrombocytopenia presented following Peg-IFN- α 2a treatment and recovered after the drug was discontinued. Second, Peg-IFN- α 2a was the only candidate drug used before the onset of thrombocytopenia. Third, etiologies unrelated with drugs, such as splenomegaly, viral infection, acute hepatitis, and aplastic anemia, were excluded. Fourth, re-exposure to Peg IFN- α 2a resulted in recurrence of thrombocytopenia. Fifth, anti-PLT antibody was positive and bone marrow aspirate showed signs of megakaryocytic hyperplasia. All these factors suggest that immune-mediated mechanism is involved in thrombocytopenia.

IFN- α is one of the drugs inducing thrombocytopenia. Based on its pathogenesis, drug-induced throm-

bocytopenia is usually due to bone marrow suppression, immune-mediated destruction, and PLT aggregation^[2]. Acute thrombocytopenia can often present as immune-mediated thrombocytopenia or PLT aggregation thrombocytopenia, whereas a slow decline of PLT often indicates a thrombocytopenia due to marrow suppression. In this case, the number of thrombocytes was rapidly decreased to 1×10^9 /L after PLT transfusion due to acute damage of PLTs. Elevated level of anti-PLT antibody and decreased level of complements C₃ and C₄ support that immune-mediated mechanism is involved in the pathogenesis of thrombocytopenia.

Only few reports are available on IFN- α -induced severe thrombocytopenia (Table 1). A PubMed search with the key words 'interferon α ' and 'thrombocytopenia' yielded 16 reports. Common IFN- α (9 cases) and Peg-IFN- α 2a (8 cases) were found to be associated with IFN- α -induced immune-mediated thrombocytopenia.

The mean age of the patients was 44.06 ± 14.27 years (range: 20-73 years). No significant difference was observed in gender. All the patients were infected with HCV. Serotype or genotype was examined in 7 cases. Of them, 4 had serotype 1b, 2 had genotype 3 and 1 had genotype 4. Since only a small number of cases were examined, whether the serotype 1b is more susceptible to immune-mediated thrombocytopenia than other types of thrombocytopenia needs to be further studied.

The median time from administration of IFN- α to the onset of thrombocytopenia was 3.6 mo (range, 1-36 mo). Fifteen out of 17 (83%) cases had epistaxis, gingival bleeding, oral mucosa bleeding, petechia, skin ecchymosis of trunk or lower extremities but no severe internal organ bleeding. The average PLT count was $(4.8 \pm 3.1) \times 10^9$ /L. The anti-platelet antibody or platelet-associated IgG was positive in 12 and negative in 3 of the 17 (67%) cases, respectively, but not detected in 2 cases. Bone marrow aspirates showed signs of megakaryocytic hyperplasia with decreased platelet count in 16 patients. Besides the discontinuation of IFN- α /Peg-IFN- α 2a and administration of immunosuppressants (Table 1), two cases received platelet transfusion and ten cases received immunoglobulin simultaneously. The PLT count of all patients was gradually increased within 2 wk and recovered finally with no severe bleeding or death occurred, indicating that IFN- α induced severe thrombocytopenia can be reversed by discontinuing IFN- α /Peg-IFN- α 2a and giving immunosuppressant in time.

Interestingly, all the 17 cases had hepatitis C virus (HCV) infection rather than hepatitis B or other virus infections. Extrahepatic manifestations including thrombocytopenia were more frequently observed in HCV infection rather than in other virus infection^[3]. HCV-related immune thrombocytopenia has been reported by Nakajima *et al*^[4]. It was also reported that IFN- α can exacerbate thrombocytopenia by triggering the production of auto-antibodies in patients with HCV infection^[5]. However, no patient with thrombocytopenia or positive anti-platelet antibodies has been reported in the literature

Table 1 Clinical characteristics of IFN- α -induced severe thrombocytopenia

Report source	Sex/ age (yr)	Liver disease/ treatment	HCV genotype/ serotype	Baseline/ Lowest PLTs (\times 10^3 /mL)	Duration of IFN- α treatment	Bleeding tendency	Antiplatelets antibodies /PAIgG	Mgk in bone marrow	Treatment	Outcome
Shrestha <i>et al</i> ^[8]	M/41	HCV/IFN α	NR	6	NR	Yes	Negative	Increased	NR	CR
Dourakis <i>et al</i> ^[9]	M/39	HCV/IFN α	NR	→/14	8 mo	Yes	NR	Increased	Steriods/IvIg	CR
Dourakis <i>et al</i> ^[9]	F/64	HCV/IFN α	NR	→/10	6 mo	Yes	Positive	Increased	Steriods/IvIg	CR
Tappero <i>et al</i> ^[10]	F/NR	HCV/IFN α 2a	NR	→/11	2 mo	NR	NR	NR	Steriods	CR
Jiménez-Sáenz <i>et al</i> ^[11]	M/46	HCV/IFN α 2b	NR	→/3	3 yr	Yes	Positive	Increased	Steriods/IvIg	CR
Pockros <i>et al</i> ^[12]	M/61	HCV/IFN α	1b	→/9	4 mo	Yes	Positive	NR	Steriods	CR
Sagir <i>et al</i> ^[13]	M/45	HCV/Peg-IFN α 2b	NR	147/9	10 wk	Yes	Negative	Increased	Steriods	CR
Sevastianos <i>et al</i> ^[14]	F/38	HCV, compensated cirrhosis/Peg-IFN2b	Group 4	141/5	4 wk	Yes	Positive	Increased	Steriods/IvIg	CR
Fujii <i>et al</i> ^[15]	F/24	HCV/IFN α	NR	→/1.1	4 wk	Yes	Positive	Increased	Steriods/IvIg	CR
Dimitroulopoulos <i>et al</i> ^[16]	F/20	HCV/IFN α con-1	3a	→/11	28 wk	No	Positive	Increased	Steriods/IvIg	CR
Medeiros <i>et al</i> ^[17]	M/40	HCV/IFN α /PegIFN α 2a	NR	→/6	6 mo	Yes	Positive	No performed	Steriods/IvIg	CR
Nakajima <i>et al</i> ^[4]	M/47	HCV/IFN α 2a	1b	75/18	8 wk	Yes	Positive	Increased	IFN Discontinued	Not CR
Lambotte <i>et al</i> ^[18]	F/73	HCV/Peg-IFN α 2a	1b	100/4	2 mo	Yes	Positive	Peripheral origin of the pancytopenia	Steriods/ IvIg/PT	CR
Demirtur <i>et al</i> ^[19]	F/40	HCV/Peg-INF	NR	217/6	13 wk	Yes	Positive	Increased	Danazol/IvIg	CR
Elefsiniotis <i>et al</i> ^[20]	M/27	HCV/Peg-IFN α 2b	NR	150/10	48 wk (6 mo after IFN discontinued)	Yes	Positive	Increased	Steriods	CR
Alves Couto <i>et al</i> ^[21]	M/44	HCV/Peg-IFN α 2b	Group 3	164/2	16 wk	No	Negative	Increased	Steriods	CR
Our hospital	F/54	HCV/Peg-IFN α 2a	1b	100/1	3 mo	Yes	Positive	Increased	Steriods/ IvIg/PT	CR

F: Female; M: Male; IFN: Interferon; Peg: Pegylated; NR: Not reported; →: Normal; PAlGg: Platelet associated IgG; Mgk: Megakaryocytosis; IvIg: Intravenous Immunoglobulin; PT: Platelets transfusion; CR: Completed resolved.

before interferon treatment, indicating that interferon may increase the incidence of ITP in HCV-infected patients.

IFN- α has been widely used not only in treatment of viral infections but also in treatment of malignancies, skin diseases, and myeloproliferative disorders. IFN- α induced thrombocytopenia also occurs not only during antiviral treatment but also during treatment of other malignant diseases such as chronic myeloid leukemia^[6], and renal cell carcinoma^[7]. It is, therefore, essential for clinicians to recognize the disorder early and give patients appropriate treatment for a favorable prognosis.

In conclusion, severe immune-mediated thrombocytopenia may occur during IFN- α /Peg-IFN- α 2a treatment, especially in chronic HCV-infected patients. Discontinuation of IFN- α and administration of immunosuppressant is the key to the avoidance of severe bleeding or death due to thrombocytopenia.

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A case of occult insulinoma localized by pancreatic dynamic enhanced spiral CT

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INTRODUCTION

Insulinomas are the most common category of pancreatic endocrine tumors, with an annual incidence of 1-4 cases per million people. Most are intrapancreatic, benign and solitary. Therefore, they have an excellent prognosis after surgical resection. However, the localization diagnosis of insulinomas still poses a challenge to surgeons and radiologists. Eighty to ninety percent of insulinomas are < 2 cm in size and the lesions are distributed equally throughout the head, body and tail of the pancreas^[1]. Thus, localization diagnosis before operation is important.

In the present case, the tumor was occult and could not be found by either abdominal enhanced spiral computed tomography (CT) or ultrasonography. Therefore, we tried a new method of CT scanning and localized the tumor. This paper introduces the method.

CASE REPORT

A 38-year-old man presented with a typical Whipple's triad. He often suffered from symptoms of hypoglycemia such as hunger, sweating, palpitations, loss of consciousness and syncope. When these symptoms occurred, the blood glucose level was always below 2.48 mmol/L, and the symptoms could be relieved after being given glucose. These symptoms had lasted 14 years. During that time, he had been to many hospitals for treatment, but none of them

Abstract

Insulinomas are the most common category of pancreatic endocrine tumors, with an annual incidence of 1-4 cases per million people. Most are intrapancreatic, benign and solitary. Therefore, they have an excellent prognosis after surgical resection. However, the localization diagnosis of insulinomas still poses a challenge to surgeons and radiologists. In this case, the tumor was occult and could not be found by either abdominal enhanced spiral computed tomography (CT) or ultrasonography. Therefore, we tried a new method of CT scanning and localized the tumor.

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Key words: Insulinoma; Computed tomography; Localization diagnosis; Dynamic enhanced scan; Enhancement value

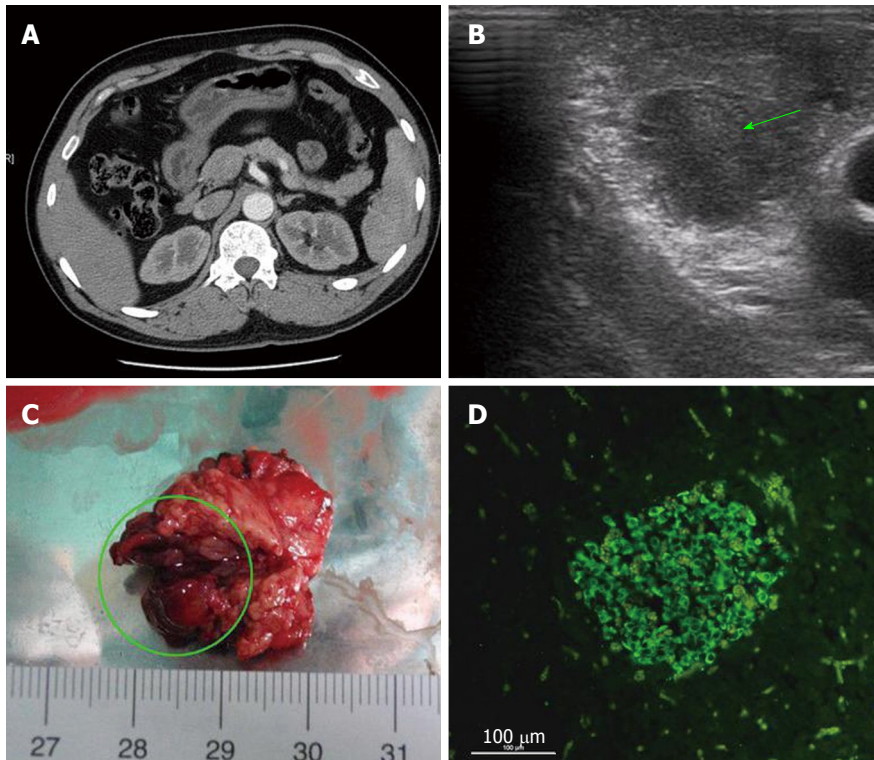
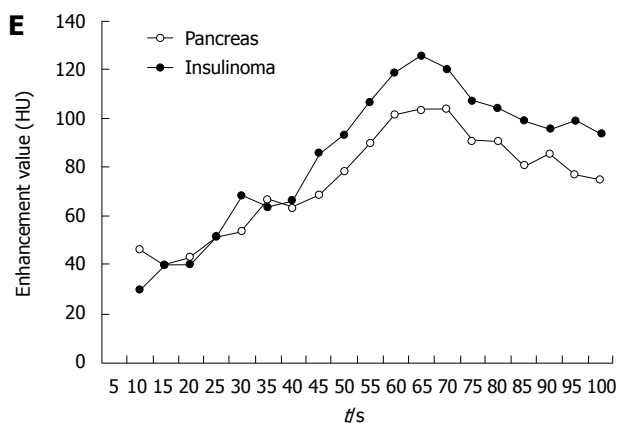


Figure 1 Clinical images related to this case and linear analysis of pancreatic dynamic enhanced spiral CT. A: Negative result of abdominal enhanced spiral CT; B: We used intraoperative ultrasonography to detect the tumor and to determine the location relationship between the tumor and the main pancreatic duct. Fortunately, the tumor (green arrow) did not adjoin the main pancreatic duct. Therefore, we performed simple tumor resection; C: The insulinoma (green circle) (1.4 cm × 1.4 cm × 1.2 cm) was resected; D: Microscopic image of the insulinoma after immunostaining (× 400); E: Enhancement value of the pancreas and insulinoma at each time point after the onset of contrast material injection. The enhancement value peak of the tumor appeared at 65 s. As the interval of tumor-to-pancreas contrast was not obvious (mostly it is < 20 HU), the insulinoma was occult and difficult to find.



could localize the tumor, and no one was willing to perform an exploratory operation. This time in our hospital, we tried abdominal enhanced spiral CT (Figure 1A) and ultrasonography to localize the tumor, but neither was successful. Then, the patient underwent selective arterial calcium stimulation with hepatic venous sampling. The data from the test suggested that the tumor was in the head or neck of the pancreas. Therefore, we designed a new method of CT scanning. The CT machine we used was Lightspeed VCT 64-row detector (GE Healthcare, Milwaukee, WI, USA). We chose the pancreas as the object of scanning. We use non-enhanced CT to define the range of the pancreas. After that, we started to inject contrast material (50 mL in total) by using a technology called intra-arterial infusion^[2]. The injection rate was 4 mL/s. Ten seconds after the onset of injection, the dynamic enhanced CT scanning started. It was designed to scan the whole pancreas from top to bottom in 1 s. This was repeated for 90 s. The slice thickness was 5 mm.

The insulinoma was finally localized in the body of the pancreas (adjoining the neck) (Figure 2). Then, with the help of intraoperative ultrasonography (Figure 1B), we performed the operation successfully. The tumor was about 1.4 cm × 1.4 cm × 1.2 cm in size (Figure 1C) and was proven to be an insulinoma by immunopathology (Figure 1D). After resection of the insulinoma, the patient was followed up for 6 mo. His blood glucose level was in the normal range, and no symptoms of hypoglycemia recurred.

DISCUSSION

Some researchers have advocated that preoperative localization diagnosis is not necessary because most insulinomas can be localized by operative exploration. Others insist that preoperative localization diagnosis is necessary and valuable for surgery^[1,3]. Preoperative localization is very helpful in planning the operation: it allows the sur-

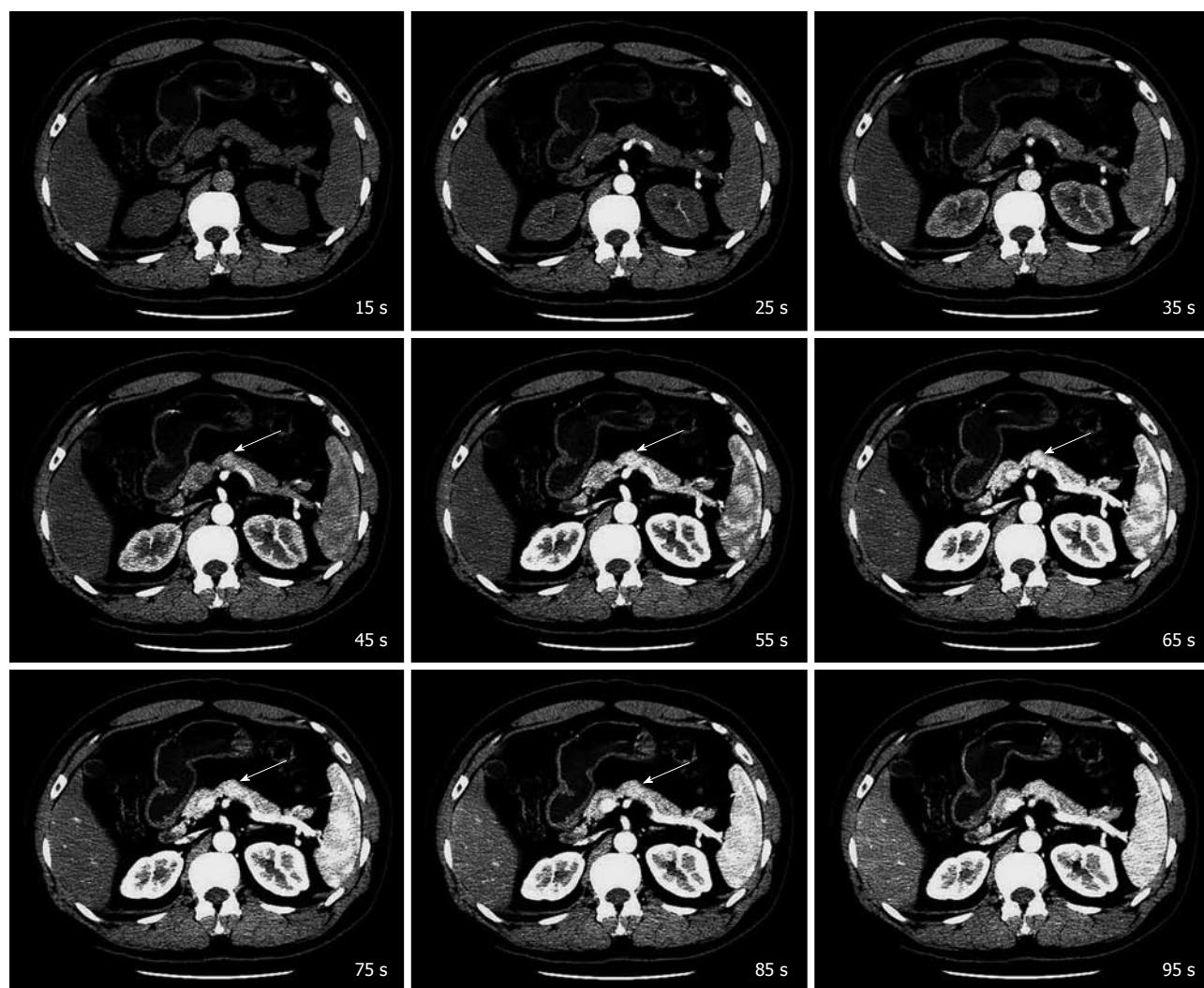


Figure 2 Images of pancreatic dynamic enhanced spiral CT showed the localization of the insulinoma (white arrows). It was in the body of the pancreas (adjoining the neck). The tumor started to enhance from 45 s after the onset of contrast material injection, and could be seen until 85 s. As the contrast between the insulinoma and the pancreas was not obvious, the tumor was difficult to find by other conventional imaging techniques.

geon to determine whether simple tumor resection or partial pancreatectomy is likely to be required. Furthermore, it avoids prolonging the duration of surgery and potential intraoperative damage to major structures.

The advent of spiral CT has improved the detection of insulinomas compared with conventional CT. The sensitivity of CT scanning is significantly higher than it was 15 years ago^[4]. A retrospective study showed that 63% of insulinomas could be detected by multiphasic spiral CT prospectively, and 83% of the lesions could be seen in retrospect^[5]. However, occult insulinomas are not rare. There is still argument about which phase is more sensitive in detecting insulinoma. Some subscribe to the arterial phase, whereas others espouse that the pancreatic phase may be more useful^[5]. Furthermore, different injection rates of contrast material may have great effects on enhancement of tumor and pancreas contrast. Also, the time point of the peak may be different^[6,7].

Therefore, when we are confronted with an occult insulinoma, how should we localize the tumor by CT

scanning? And why is abdominal dual-phase spiral CT not able to find the tumor in these patients? First of all, the interval of occult insulinoma-to-pancreas contrast is not obvious. We know that tumor delineation is based mainly on the interval of tumor-to-pancreas contrast during contrast-enhanced CT. Generally speaking, when the interval of tumor-to-pancreas contrast exceeds 30 HU, it is relatively easier to distinguish the tumor from the surrounding pancreas^[6]. However, in this case, the interval of tumor-to-pancreas contrast was less than 20 HU most of the time. The largest interval was only 21.9 HU, and appeared at 65 s (Figure 1E). Secondly, as with the individual differences, the time point of the enhancement value peak of the tumor is uncertain. Thus, it is possible that abdominal dual-phase spiral CT cannot catch the time point of the peak. For these reasons, we designed the pancreatic dynamic consecutive enhanced CT scanning. It is able to display images of each time point (containing the arterial, pancreatic and portal venous phases). Also, it supplies a large number of images for radiologists

to search the tumor. Therefore, when we are confronted with an occult insulinoma, pancreatic dynamic consecutive enhanced CT scanning may help us to localize the tumor.

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Resection of a giant hepatocellular carcinoma weighing over ten kilograms

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INTRODUCTION

Primary hepatocellular carcinoma (HCC) is the most common malignant tumor in Asia, especially in China. Liver neoplasms with diameters of ≤ 5 cm, 5-10 cm, and > 10 cm are designated as small, big, and giant HCC, respectively^[1]. However, a diameter over 20 cm is extremely rare. Here, we report a giant HCC with a diameter over 30 cm that weighed over 10 kg.

CASE REPORT

A 62-year-old man had a history of progressive abdominal uplift with unknown etiology for 2 years (Figure 1). He was admitted because of difficult defecation. A computed tomography (CT) scan revealed that the entire abdomen was filled with a huge cystic mixed lesion (Figure 2). An enhanced scan demonstrated the poorly-circumscribed lesion under the right lobe of the liver compressing the surrounding organs. The patient had no history of hepatitis, smoking, or drinking. Upon physical examination, no superficial lymphadenectasis, icteric sclera, or liver palm, and spider telangiectasias were observed. Abnormal cardiac and lung function were not observed. Blood serum study showed that the hepatitis

Abstract

The authors report a giant hepatocellular carcinoma (HCC) with a diameter over 30 cm and weight over 10 kg that was resected completely. A 62-year-old man was admitted because of continuous abdominal uplift. A computed tomography scan demonstrated that the entire abdomen was filled with a giant tumor containing both cystic and solid components with a size of 29 cm \times 22 cm. The huge tumor was successfully resected without any complication, such as massive hemorrhage or visceral injuries. The size and weight of the tumor were 35 cm \times 30 cm \times 15 cm and 10050 g, respectively. Pathological examination showed that the tumor was a well-differentiated HCC, and α -fetoprotein was positive. Postoperative syndrome, characterized by hypovolemic shock, diarrhea and urine retention, was observed and induced by abdominal decompression. This syndrome was resolved with expectant treatment. The patient was still alive without recurrence after a 27-mo follow-up.



Figure 1 Patient had continuous abdominal uplift.

B surface antibody was positive and hepatitis B surface antigen, hepatitis B e antigen HBeAg, hepatitis B e antibody, and hepatitis B core antibody were all negative. Tumor markers such as α -fetoprotein (AFP), cancer embryonic antigen, carbohydrate antigen 199 (CA199), CA125, and CA724 were detected and their values were all within the normal limits. B-type ultrasonic examination demonstrated a relatively well-circumscribed tumor that measured 30 cm \times 30 cm \times 25 cm. Preoperative glucose tolerance was normal.

Surgical resection under general anesthesia was performed in our hospital on August 30, 2007. Most of the organs in abdominal cavity, including the right kidney, duodenum, abdominal aorta, inferior vena cava, spleen, stomach, small intestine, ascending colon, transverse colon, and urinary bladder were deformed due to the compression by the giant HCC. The right lobe of the liver and diaphragm were adhered closely, the entire right lobe of liver was compressed as thin as a piece of paper. The left lobe of the liver was hypertrophied with normal appearance. Hepatic cirrhosis was not observed. This giant tumor was close to the second portal hepatic vein, and compressed the first and the third portal hepatic vein, blood supply of the tumor was from the hepatic artery, abdominal aorta, renal artery, and collateral circulations. This well-circumscribed tumor was encompassed in a pseudocapsule. The huge tumor and the right lobe of the liver were successfully resected. The injury of abdominal aorta and duodenal serosa was repaired during operation. The intraoperative course was uneventful, which took 185 min, and no intraoperative complication occurred during the operation. Intraoperative blood loss was 600 mL and blood transfusion was 1200 mL. The patient resumed breathing on his own uneventfully. His tracheal intubation was pulled out at the end of operation, and he was sent to ICU for intensive observation and treatment for 48 h.

Postoperative tumor size and weight turned out to be 35 cm \times 30 cm \times 15 cm and 10050 g, respectively (Figure 3). Grossly, the tumor had cystic degeneration and was encompassed in a pseudocapsule. Histological examination showed that the tumor was infected by a fungus and was a well-differentiated HCC (Figure 4A). Immuno-

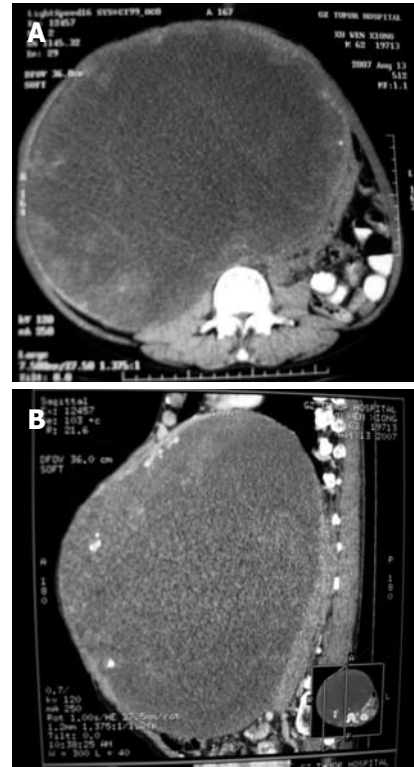


Figure 2 Computed tomography of the tumor. A: A huge hepatic carcinoma at the right lobe of the liver. The left lobe of the liver was compensatory hypertrophy; B: A huge hepatic carcinoma above the right lobe of the liver and compression of the surrounding organs.

histochemical staining showed that the tumor was AFP positive (++) (Figure 4B).

Hypovolemic shock was observed 18 h after operation and he recovered 36 h after blood and fluid intravenous transfusions. The patient was treated with fluid intravenous transfusion and total parenteral nutrition after operation in the department of abdominal surgery for 5 d. Postoperative liver function was normal, and no liver failure occurred. His catheter was removed on postoperative day 3 and he urinated without medical aid. However, urinary retention was observed on postoperative day 6, he resumed his self-urination by re-indwelling the catheter, which was pulled out 4 d later. Gastrointestinal function recovered 5 d after operation and he was given semi-liquid diet. Additionally, he had serious diarrhea 9 d after operation, which was relieved by intravenous injections of anisodamine hydrochloride 20 mg once a day and a 9 g oral dose of montmorillonite powder once a day for 4 d. Postoperative acute heart failure (CHF) occurred at day 7 after operation and the patient recovered after treatment with cardiotonics, diuresis and vascular dilation for 2 d. The right pleural effusion occurred 8 d after operation and was treated by CT guided percutaneous drainage. Other postoperative recovery was successful, and surgical incision healed well.

DISCUSSION

Studies on surgical resection of huge hepatic carcinoma have been reported since the 1950s in China. Currently, surgical resection is still the best way to achieve a long-term survival for patients with huge hepatic carcinoma, especially for patients without cardiac, renal, or lung dysfunction or coagulation disturbances^[2,3]. However, the

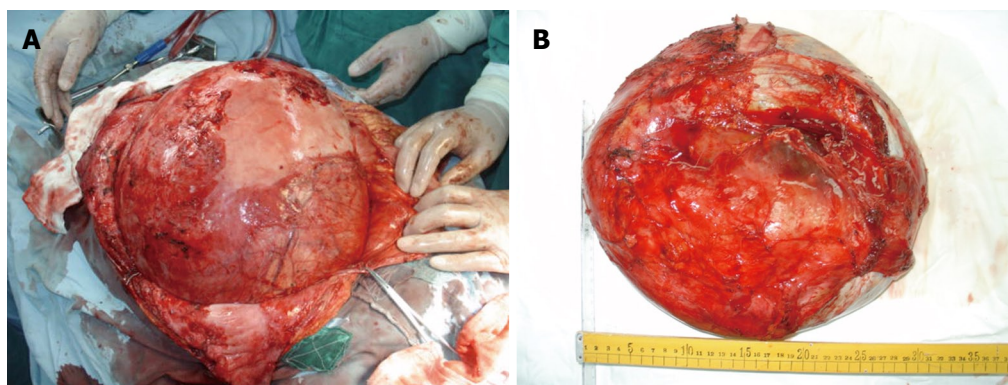


Figure 3 Imaging of the huge tumor. A: The tumor was encompassed by a pseudocapsule and compressed surrounding organs; B: Postoperative measurement of tumor was 35 cm × 30 cm × 15 cm in size and 10 050 g in weight.

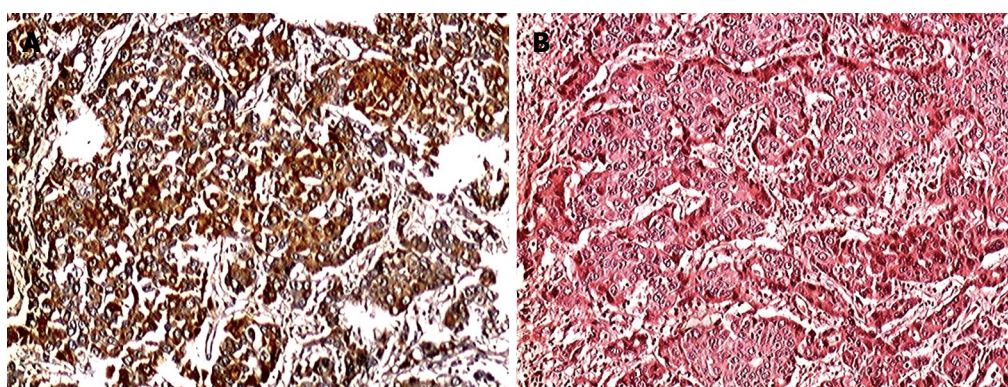


Figure 4 Pathological and biochemical examinations of the huge tumor. A: Pathological examination showed the tumor was a well-differentiated HCC. HE × 400; B: Immunohistochemical staining showed the tumor was α -fetoprotein positive (++) . HE × 400.

prevention of hemorrhages and postoperative hepatic failure are vital for a successful resection of huge hepatic carcinomas. Therefore, surgeons must be skillful at surgical techniques and have a breadth of clinical experience^[2,4,5]. In this case, resection of the huge tumor was performed because the left lobe of the liver was compensatory hypertrophy and liver function was normal.

To complete the operation uneventfully and avoid possible complications during tumor excisions depends on the patient status and clinical experience of surgeons^[2,6]. A higher incidence of complications and mortality were found during mass excisions, including hemorrhage and injury of abdominal aorta, inferior vena cava, portal hepatic vein, small intestine, *etc.*^[7,8]. In this case, the surgery was uneventful except some complications.

In this case, postoperative syndromes characterized by hypovolemic shock, urinary retention, CHF, diarrhea, and pleural effusion were observed due to serious intra-abdominal pressure from a huge liver tumor. This syndrome was relieved with expectant treatment. The optimal treatment protocol of syndromes induced by abdominal decompression after surgical resection of huge hepatic carcinomas has not been defined and further studies are needed.

To our knowledge, this tumor 10 050 g in weight is the largest malignant hepatic carcinoma found to date.

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Liver transplantation for polycystic liver and massive hepatomegaly

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TO THE EDITOR

We read with great interest the article of Jiang *et al*^[1], published in last October in *World Journal of gastroenterology* 2009 October 28; 15 (40): 5112-5113 regarding a case of liver transplantation (LT) for polycystic liver with massive hepatomegaly. After an unsuccessful attempt to defenestrate the cyst, they performed hepatectomy for the native liver during LT accomplishing a left hepatectomy followed by a right hepatectomy because the massive hepatomegaly resulting from the enlarged liver cysts caused a very small surgical space, thus making the classic dissection of the hilar structures extremely difficult. Although we agree with the authors that the polycystic liver is not only large but also rigid, and consequently the mobility and access to vascular supply are decreased, we think that performing either a right or a left partial liver resection during LT adds some risk to intra-operative bleeding and we alternatively advise a different approach.

Indeed, we have some successful experience with LT in 6 patients with massive hepatomegaly due to giant liver lesions.

The primary indications for LT in 3 patients were polycystic kidneys and liver disease (Figure 1A), and two of them required a combined liver and kidney transplantation (Figure 1B) after bilateral nephrectomy.

LT was performed due to multifocal hepatocellular carcinoma (HCC) (Figure 1C), giant hepatic hemangioma

Abstract

Liver tumor and other benign liver diseases such as polycystic liver disease can cause massive hepatomegaly and may represent an indication for liver transplantation (LT) in some instances. In this setting, LT can be extremely difficult and challenging due to its decreased mobility and access to vascular supply. Benefit from either a right or a left partial liver resection during the transplant procedure has been advocated to safely accomplish the hepatectomy of the native liver. Although we believe that partial hepatectomy adds some risk to intra-operative bleeding, we alternatively advise a different approach. We have a successful experience with LT in 6 massive hepatomegaly patients due to giant liver lesions. All the transplant procedures were performed without intermediate partial liver resection, showing that selective use of veno-venous bypass can play a significant role in the treatment of massive hepatomegaly.

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Key words: Liver transplantation; Massive hepatomegaly; Polycystic liver

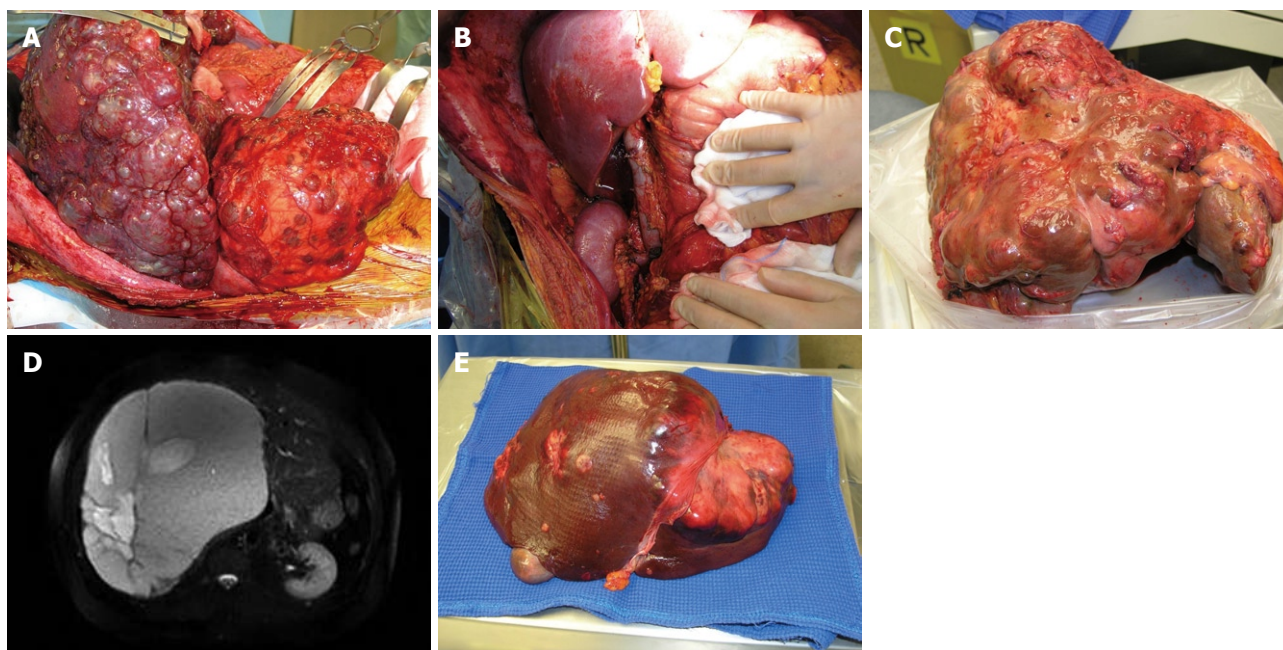


Figure 1 Liver transplantation for hepatomegaly. A: Polycystic kidney and liver disease; B: Combined liver and kidney transplantation after bilateral nephrectomy; C: Multifocal hepatocellular carcinoma (HCC); D: Giant hepatic hemangioma; E: Multiple metastases of carcinoid tumor.

(Figure 1D), and multiple metastases of carcinoid tumor (Figure 1E) for the 3 patients, respectively.

The recipient with giant multifocal HCC was obviously an exception to the Milan criteria (2) for LT in HCC patients.

After consulting our Internal Review Board, this patient was listed in a special list of potential recipients of “liver that nobody uses”^[3]. All LT procedures were performed using cadaveric grafts except in 1 living case of right lobe donation in favor of the recipient with giant hemangioma.

The weight of all the explanted livers was more than 7 kg each (range: 5.440-10.000 g) and all the hepatectomies were extremely difficult because of the massive hepatomegaly.

In the HCC recipient and living donor, venous-venous bypass was performed during hepatectomy^[4] and no systemic anti-coagulation occurred as previously described^[5]. A standard hepatectomy technique instead of venous-venous bypass was used in the other 4 cases. Cystic defenestration was performed in the 6 cases using a large incision instead of thoracotomy for the spaces.

All the recipients had an uneventful post operative course and were alive at the time when they were followed up for 12 mo.

After two-disease free years, the HCC recipient developed a single bone lesion which was treated with selective radiotherapy.

We have never performed intermediate liver resection of massive hepatomegaly, because it may increase the risk of intra-operative bleeding.

We advise venous-venous bypass whether its access to vascular structures is difficult or impossible due to massive hepatomegaly.

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Patent (list all authors)

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

Statistical data

Write as mean \pm SD or mean \pm SE.

Statistical expression

Express *t* test as *t* (in italics), *F* test as *F* (in italics), chi square test as χ^2 (in Greek), related coefficient as *r* (in italics), degree of freedom as *v* (in Greek), sample number as *n* (in italics), and probability as *P* (in italics).

Units

Use SI units. For example: body mass, *m* (B) = 78 kg; blood pressure, *p* (B) = 16.2/12.3 kPa; incubation time, *t* (incubation) = 96 h, blood glucose concentration, *c* (glucose) 6.4 ± 2.1 mmol/L; blood CEA mass concentration, *p* (CEA) = 8.6 ± 24.5 μ g/L; CO₂ volume fraction, 50 mL/L CO₂, not 5% CO₂; likewise for 40 g/L formaldehyde, not 10% formalin; and mass fraction, 8 ng/g, etc. Arabic numerals such as 23, 243, 641 should be read 23 243 641.

The format for how to accurately write common units and quantums can be found at: <http://www.wjgnet.com/wjg/help/15.doc>.

Abbreviations

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abbreviations, such as DNA, RNA, HIV, LD50, PCR, HBV, ECG, WBC, RBC, CT, ESR, CSF, IgG, ELISA, PBS, ATP, EDTA, mAb, can be used directly without further explanation.

Italics

Quantities: *t* time or temperature, *c* concentration, *A* area, *l* length, *m* mass, *V* volume.

Genotypes: *gyrA*, *arg 1*, *c myc*, *c fos*, etc.

Restriction enzymes: *EcoRI*, *HindI*, *BamHI*, *Kho I*, *Kpn I*, etc.

Biology: *H. pylori*, *E. coli*, etc.

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AIM AND SCOPE

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Intensive or not surveillance of patients with colorectal cancer after curative resection

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Abstract

It is common practice to follow patients with colorectal cancer for some years after resection and/or adjuvant treatment. Data are lacking about how often patients should be seen, what tests should be performed, and what surveillance strategy has a significant impact on patient outcome. Seven randomized trials have addressed this issue, but none had sufficient statistical power. Four published meta-analyses have established that overall survival is significantly improved for patients in the more intensive programs of follow-up. This improvement amounts to a risk difference of 7% (95% CI: 3%-12%, $P = 0.002$) in 5-year survival. This should be partly attributable to more frequent reoperation for cure of asymptomatic recurrence, or more intense follow-up, as well other factors, such increased psychosocial support and well-being, diet and lifestyle optimization, and/or improved treatment of coincidental diseases. A large-scale multicenter European study [Gruppo Italiano di Lavoro per la Diagnosi Anticipata (GILDA)] is underway to answer the question of what constitutes optimal surveillance for patients after primary therapy, based on an adequately powered study.

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Key words: Colorectal cancer; Follow-up; Meta-analysis; Recurrence; Salvage surgery

INTRODUCTION

Colorectal cancer (CRC) is one of the most common malignancies and the second leading cause of death related to cancer^[1]. Although about two-thirds of patients undergo radical surgery, up to 30%-50% of them will develop tumor relapse and die of their disease^[2]. This provided a rationale for trying to diagnose recurrence early, while resection is still feasible.

Although there is general agreement about the usefulness of postoperative patient follow-up, no consensus has been reached and there have been no clinical trials to demonstrate effectiveness. Thus, in a survey of members of the American Society of Colon and Rectal Surgeons, it was found that 31% of surgeons request computed tomography (CT) at least once during the first postoperative year after colon cancer surgery, while 53% do not employ CT at all. Similarly, 5% monitor carcinoembryonic antigen (CEA) levels monthly during the first postoperative year, while 4% never check CEA levels^[3]. This poses an important problem because there are many patients at risk and the costs of diagnosis (and treatment, if needed) are high.

OPEN QUESTIONS

An optimal level of follow-up would maximize patient welfare at the least cost. Patients are harmed if a strategy is too intensive, because they are unnecessarily exposed to radiation, and undergo uncomfortable endoscopy and blood tests. If high-intensity surveillance testing provides no improvement in duration or quality of life (QoL), society is also harmed by the waste of resources. If the strategy is not intensive enough, patients who relapse and are potential candidates for effective salvage treatment may die needlessly. Society is also harmed in this case because the costs to treat incurable CRC are high, patients are unable to work, their QoL decreases, and their children grow up without a parent^[4]. How good is the evidence for choosing which follow-up examinations to order?

Early detection of CRC recurrence is seldom useful. In this context, intensive testing leads to anxiety and financial burden, and seldom changes disease outcomes. On the contrary, to wait for signs or symptoms, rather than detecting early disease manifestations is defensible, albeit somewhat paternalistic. Physical examination virtually never discerns early hepatic, lung, or anastomotic recurrence, and CEA is only elevated in 60% of patients with recurrence. Thus, waiting for signs and symptoms seems contrary to the instinct that early detection and prompt disease management is best^[5].

WHAT IS KNOWN ON THIS TOPIC?

In an attempt to rationalize care, the concept of the randomized trial was introduced in the mid-twentieth century. However, very few of these studies have dealt with follow-up of patients with CRC, and no consensus has been reached with respect to the most effective strategy. Indeed, follow-up schedules are highly heterogeneous with regard to procedures (clinical history, physical examination, CEA monitoring, imaging techniques, and colonoscopy) and the frequency with which they are carried out.

Seven randomized controlled trials have addressed this issue, but none had sufficient statistical power^[6-12]. Thus, four meta-analyses have been published to evaluate the impact of various intensities and strategies on the outcome of patients after curative surgery for CRC^[13-16].

Overall survival is significantly improved for patients in the more intensive programs of follow-up. This improvement amounts to a risk difference of 7% (95% CI: 3%-12%, $P = 0.002$) in 5-year survival^[15]. The incidence of asymptomatic recurrence is significantly more common in patients in the more intensive follow-up program. This may explain the more frequent reoperation for recurrence in that group of patients.

The analysis of Renehan *et al*^[13] has found a larger impact on survival in trials using abdominal CT and frequent CEA determinations. The study by Jeffery *et al*^[14] had similar findings. The patients undergoing more intense follow-up have improved survival, earlier diagnosis of recurrence, and more frequent curative resection, as a result of undergoing more tests, especially liver imaging.

However, it is not clear to date which tests or frequency of visits is optimal. Also noted in these overviews are the paucity of data on complications and QoL.

One trial noted two perforations and two episodes of bleeding after polypectomy in 731 colonoscopies: a complication rate of 0.55%^[9]. This complication rate is comparable to that of other colonoscopy studies^[17].

The QoL and attitude of patients participating in follow-up programs have been investigated by Stiggelbout *et al*^[18]. Their results have indicated that regular contact with a physician reassures patients, and that visits and tests cause only slight anticipatory anxiety and other minor inconveniences^[18].

Kjeldsen *et al*^[6] have confirmed these findings in a subgroup of patients participating in a randomized trial that compared minimal to regular follow-up, and which demonstrated similar survival for both follow-up regimens. Patients were mailed the questionnaire to complete at home. Ninety-one percent returned the completed questionnaires. QoL measures and attitudes were almost the same for patients in the minimal and intensive follow-up programs, which indicated that the extra tests or inconveniences were balanced by the more frequent reassurance of health.

Other authors have postulated that improved survival with intensive follow-up is not only due to salvage surgery offering a second chance of cure, but that an additional 4%-11% gain in survival may be attributable to other factors^[19]. The following may be relevant: (1) increased psychosocial support and well-being; (2) cancer survivors tend to optimize their diet and lifestyle, although it is unclear whether these adjustments are self motivated, or a direct consequence of health-care interventions; and (3) improved treatment of coincidental disease.

CONCLUSION

Many doubts about follow-up of CRC patients must be clarified and further well-designed studies with a sufficient number of patients must be carried out. The generation of high-quality evidence for the practice of cancer patient follow-up is an important priority for the medical community, although trials are expensive to mount and take a long time to complete. In this context, two trials of high- vs low-intensity follow-up for breast cancer have been carried out, which indicates that such trials are feasible^[19]. Both employed a randomized two-arm design with a sample size of about 1500 patients. The same is likely to be necessary for trials of CRC patient follow-up; one such trial by Gruppo Italiano di Lavoro per la Diagnosi Anticipata (GILDA) completed patient recruitment in September 2006, and the results should be published during 2010. Major objectives of this study are: overall survival, better timing profile of diagnosis of recurrence, QoL, and program costs. It is hypothesized that the results of high- and low-intensity strategies will be equivalent, and it will answer the question of what constitutes optimal surveillance for patients after primary therapy, based on an adequately powered study.

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COX-2 in liver, from regeneration to hepatocarcinogenesis: What we have learned from animal models?

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tained in 3 independent models of mice expressing a COX-2 transgene specifically in the hepatocyte. Upon challenge with pro-inflammatory stimuli, the animals behave very differently, some transgenic models having a protective effect but others enhancing the injury. In addition, one transgene exerts differential effects on normal liver physiology depending on the transgenic animal model used.

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Abstract

The use of animals lacking genes or expressing genes under the control of cell-specific promoters has significantly increased our knowledge of the genetic and molecular basis of physiopathology, allowing testing of functional hypotheses and validation of biochemical and pharmacologic approaches in order to understand cell function. However, with unexpected frequency, gene knockout animals and, more commonly, animal models of transgenesis give experimental support to even opposite conclusions on gene function. Here we summarize what we learned on the role of cyclooxygenase 2 (COX-2) in liver and revise the results ob-

INTRODUCTION

Bioactive lipids, including prostaglandins and thromboxanes - collectively known as prostanoids - are involved in many physiopathological processes ranging from vascular function to gastric mucosa integrity, inflammation and development/progression of various types of oncologic processes including colorectal cancer^[1-5]. These prostanoids are synthesized from arachidonic acid by the sequential action of cyclooxygenase (COX) and a specific prostaglandin or thromboxane synthase. Two isoforms of COX exist: COX-1 that is constitutively expressed in almost all tissues and is responsible for the homeostatic synthesis of prostanoids, and COX-2 that is expressed upon response to cell stressors, such as pro-inflammatory

cytokines, growth factors and hormones^[2,6-8]. Whereas COX-1 exhibits a modest but continuous synthesis of prostanoids, COX-2 is involved in the high throughput synthesis of these bioactive lipids under pathological conditions. Mice lacking COX-1, COX-2 or both isoenzymes have been generated and these animals are fully viable despite the observation of alterations in fertility (the COX-2 deficient females are sterile) and the appearance of nephropathies during aging^[2,9,10]. In adult liver, the expression of COX-2 under rapid response to a pro-inflammatory challenge is almost restricted to the non-hepatocyte cell population. However, under chronic pro-inflammatory conditions hepatocytes express this isoenzyme and the contribution of the increased synthesis of prostanoids to liver pathology is a current subject of research^[11-13]. To better approach the study of COX-2 expression as an early cause of liver pathology, various groups engineered mice that expressed this isoenzyme specifically in the hepatocytes. The results obtained using these animal models are the subject of this review and highlight the need to interpret the data from animal models with a certain caution.

GENE TARGETING IN LIVER INJURY AND REGENERATION

The development of targeted animal models to answer key biological questions in some cases requires appraisal to understand why animals lacking the same gene but generated under different genetic backgrounds or gene-deletion strategies result in different and sometimes even opposite physiopathological conclusions. One case is liver regeneration after partial hepatectomy where only a reduced number of genes appeared to be essential for the survival following resection of two-thirds of the liver^[14-19]. Cumulative studies in this area identified about 70 genes whose expression increased following partial hepatectomy; interestingly, despite the large number of signals controlling the early steps of regeneration only a handful have been described to play a critical role in the successful outcome of the process. Models in which liver regeneration is impaired after partial hepatectomy are animals deficient of insulin-like growth factor-1-binding protein, TAB2 (transforming growth factor- β -activated kinase 1-binding protein 2)-a transforming growth factor-activated kinase-1 interacting protein involved in the early response to interleukin-1 β (IL-1 β), or animals overexpressing transforming growth factor- β (TGF- β)^[20-22]. Examples of relevant genes for regeneration are those controlling commitment to proliferation or inhibiting apoptosis, such as *c-met*, *pdck1*, *p75ntr* (the neurotrophin receptor in stellate cells), and *gadd45b*^[23-26]. Previous studies reported delayed regeneration and sometimes increased death in animals lacking *il-6*, *stat-3*, *cox-2* or *nos-2*^[2,9,27-29], but further studies notably found an attenuated impact of the deficiencies in these genes in terms of liver mass recovery and survival. One extreme example is the requirement of caveolin-1 for regeneration that in one model has been described to be “a gene

essential for liver regeneration”^[30], whereas in the commercially available caveolin-1-deficient mice this gene appears to be absolutely “dispensable” with the peculiarity of an accelerated regeneration and, therefore, being a positive condition for a rapid liver mass recovery^[31]. The reasons for these discrepancies lie with the different genetic backgrounds of both mice strains. Indeed, in those caveolin-1-deficient animals that died after partial hepatectomy, there was partial restoration of the liver mass but they died at day 4-5 post-surgery, a situation that could be overcome after administration of glucose, suggesting that a metabolic problem was the most likely defect in these animals rather than deficient cell replication and growth^[30]. Indeed, in addition to the caveolin-1-deficient mice, there are also gene-targeted animals that exhibit an accelerated early proliferation and liver mass recovery, among them animals deficient in *pai-1*, *timp-1*, *ikk2* or *socs-3*^[32-35]. Finally, there are a few models that, despite being unable to restore liver cellularity because of deficient hepatocyte proliferation/division, show liver growth and fully restored hepatic function through an hypertrophic response with multinucleated and polyploid hepatocytes^[36].

COX-2 TARGETING IN LIVER

As previously mentioned, prostaglandin (PGs) synthesis in mammals is carried out by the expression of 2 forms of cyclooxygenase. COX-1 is constitutively expressed in most tissues and has a narrow specificity for substrates, preferentially using arachidonic acid and releasing PGs that are involved in the physiological action of these lipid mediators. However, for expression of COX-2, the inducible enzyme, in various tissues, a high throughput synthesis of PGs occurs both from arachidonic acid and other polyunsaturated fatty acids. These PGs are involved in the regulation of physiopathological responses as diverse as inflammation, tumor development and progression, and cell growth^[1,3].

One interesting observation in the liver is that normal adult hepatocytes, either in primary culture or *in vivo*, fail to express COX-2 upon challenge with pro-inflammatory stimuli, including toll-like receptor ligands and combinations of tumor necrosis factor- α (TNF- α), IL-1 β and interferon- γ (IFN- γ). This lack of inducibility by pro-inflammatory mediators occurs in adult hepatocytes, but not in hepatocytes from fetal or early newborn animals or in hepatic-derived stable cell lines^[2,11,37]. Previous studies indicated that this behavior resulted from the presence of elevated levels of CCAT/enhancer binding protein- α (C/EBP- α), a transcription factor that is highly expressed in the adult hepatocyte and that interferes with the commitment of the cells to proliferate^[9]. This absence of COX-2 expression has also been observed in *in vivo* models of sepsis, where the production of PGs in the liver is accomplished by the expression of COX-2 in non-hepatocyte cells, mostly Kupffer cells and infiltrating macrophages^[11,13]. These observations reinforce the role of liver infiltration by circulating inflammatory cells

in the release of transcellular mediators, such as PGs. Despite lipopolysaccharide (LPS) or a pro-inflammatory challenge failing to induce the expression of COX-2 in hepatocytes, liver regeneration after partial hepatectomy promotes a rapid expression of COX-2 and synthesis of PGs^[11] that contribute to the regeneration onset as deduced by the impaired recovery observed after administration of selective COX-2 inhibition with COX inhibitors or from animals lacking the *COX-2* gene^[2]. Indeed, COX-2-deficient animals exhibited a full recovery of liver mass and function after partial hepatectomy with a delayed early commitment to proliferation^[2,9,11,38]. The simultaneous absence of COX-2 and other genes relevant for liver regeneration, such as nitric oxide synthase-2 resulted in an impaired liver mass recovery after partial hepatectomy leading to animal death^[39,40]. Dual deficiencies of COX-2 and other genes relevant for liver function and regeneration may help to identify targets critical for hepatocyte survival.

COX-2 TRANSGENESIS AND LIVER INJURY

More intriguing are the models of COX-2 transgenesis that lead to different end-responses without a clear reason. One example came recently when 3 groups engineered mice specifically expressing COX-2 in hepatocytes in order to investigate the role of this inducible enzyme on liver physiopathology. As previously mentioned, hepatocytes only express low levels of COX-1, the constitutive COX enzyme that is responsible for PGE₂ synthesis measured in primary cultures of hepatocytes. However, hepatocytes fail to express COX-2 after onset of inflammation as do typically inflammation-responsive cells, such as Kupffer and stellate cells, macrophages, astrocytes and microglial cells^[2,9]. Interestingly, in the case of hepatocytes, only under time-dependent progression is COX-2 expressed as a result of the drop in C/EBP α levels, among other conditions^[9]. Thus, ectopic expression of COX-2 in hepatocytes constitutes an unphysiological condition ideal for evaluating the role of PGs in liver pathogenesis. Recently, the *COX-2* gene has been expressed under the control of different specific promoters: apolipoprotein E^[38,41], transthyretin^[42,43] or the albumin-enhancer promoter^[44,45], all 3 models giving a high liver-specificity in the expression of the transgene. On analysis, after the selection of the founder colonies, it is remarkable to observe the different intrahepatic levels of PGE₂ reached under each model, as summarized in Table 1. This is despite the observation of a robust expression of the transgene by Western blotting analysis in the 3 models. More tantalizing are the effects upon LPS/D-galactosamine (D-GalN) challenge of the transgenic animals: whereas in the third model^[44], the expression of the COX-2 transgene notably enhanced the injury, in the first model^[41] a clear protection in terms of transaminases release and histological integrity of the tissue was observed. Perhaps the genetic background of the animals was also playing a role in view of the absence

Table 1 Summary of metabolic patterns and liver responses in transgenic mice with a liver-specific expression of the *COX-2* gene

	Model #1 ^[38,41]	Model #2 ^[42,43]	Model #3 ^[44,45]
PGE ₂ WT vs TG	45 vs 175 ²	30 vs 550 ²	22 vs 58 ¹
Challenge			
LPS/D-GalN	Protection	ND	Sensitization
MCD/CCL ₄	ND	Irrelevant	ND
Jo2 (FasL)	Protection	ND	Protection
Transaminases after challenge			
WT/TG (UI/L)	3750/625	500/500	400/1600
Infiltration ³	No	Yes	ND
Fibrosis ³	No	No	No
Hepatitis ³	No	Yes	ND
HCC-induction ³	ND	ND	ND

¹pg/mg of liver; ²pg/mg of protein; ³In animals aged 12-mo. COX-2: Cyclooxygenase 2; ND: Not determined; PG: Prostaglandin; LPS: Lipopolysaccharide; D-GalN: D-galactosamine; MCD: Methionine and choline-deficient diet model; HCC: Hepatocellular carcinoma; WT: Wild type; TG: COX-2 transgenic mice.

of liver apoptosis in the wild-type animals of the third model (C57BL/6) after LPS/D-GalN treatment, whereas those of a C57BL/6XDBA background (first model) exhibited a significant apoptotic response, previously described by many authors. This apoptosis was prevented after the expression of the COX-2 transgene, and was lost upon pharmacological inhibition of COX-2 with selective inhibitors. However, when animal models #1 and #3 were confronted with the Fas/FasL challenge using Jo2 as the stimulus, a very potent protection against liver injury and animal death was observed in those animals that carried the COX-2 transgene, through a mechanism that involved Src/epidermal growth factor receptor signaling^[38,45]. Finally, in a methionine and choline-deficient diet model MCD/CCL₄-induced injury, COX-2 transgenesis failed to exhibit any significant protection on liver injury (animal model #2 and reference 43).

The consequences of transgene expression over time also exhibited different patterns among the animal models: whereas at 12 mo mice of the first model did not exhibit histopathological symptoms of cell infiltration or fibrosis, the animals of model #2 developed significant inflammatory cell infiltration and hepatitis through a mechanism that appears to involve a persistent activation of nuclear factor- κ B. As there is a continuous activity of COX-2 in the hepatocyte in both models, with elevated PGE₂ synthesis, it can be concluded that other factors are required for the development of infiltration and spontaneous hepatitis. Interestingly, COX-2 does not appear to mediate the development of liver fibrosis since similar results were observed in wild-type, COX-2 knockout and COX-2 transgenic mice in an experimental model of induction of liver fibrosis^[43].

At present, there are cumulative studies supporting the proliferative and antiapoptotic role of PGs in different models of liver failure as well as after ischemia/reperfusion injury^[46,47]. This protective role has been observed even in other tissues like cardiomyocytes^[48,49]. Moreover, it is known that PGE₂ inhibits T-cell proliferation,

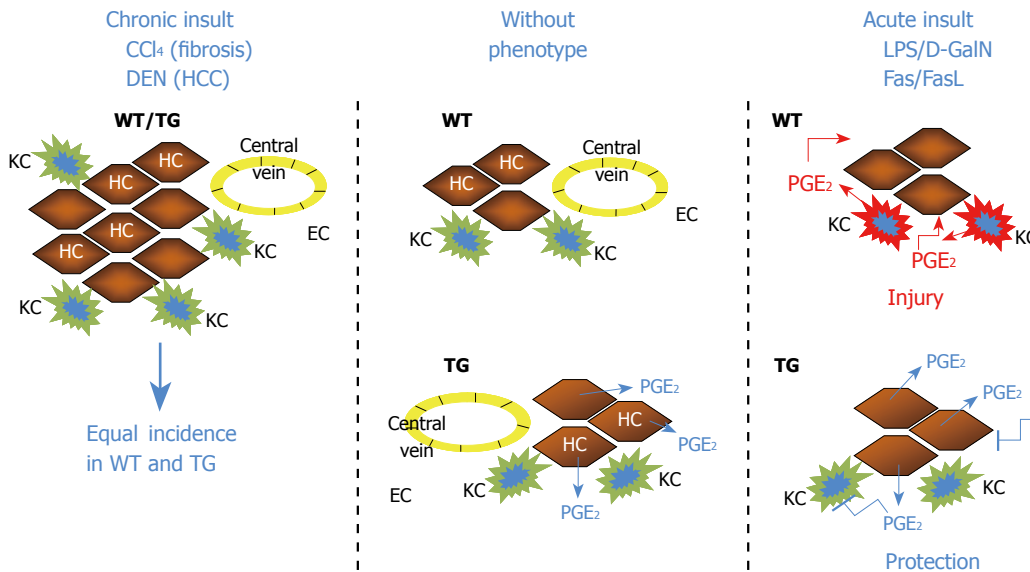


Figure 1 Schematic representation of the effects of transgenic expression of cyclooxygenase 2 (COX-2) in hepatocytes. The transgene protects against acute liver injury, but fails to alter the response to hepatocarcinogens, such as diethylnitrosamine (DEN). KC: Kupfer cell; HC: Hepatocyte; EC: Endothelial cell; HCC: Hepatocellular carcinoma; WT: Wild type; TG: COX-2 transgenic mice.

and exerts a suppressant effect on type-1 immune responses in macrophages, drastically inhibiting the production of Th1 cytokines, such as IFN- γ and TNF- α and upregulating IL-10^[4,7].

In conclusion, experiments on transgenesis need to take into account the biological activity of the expressed protein. In the case of COX-2, the availability of substrate for this enzyme (arachidonic acid) appears to be a rate-limiting step in the synthesis of PGs. Therefore, different levels of protein expression might result in similar levels of PG synthesis because of substrate restrictions for COX-2. In addition, the time of activation of the ectopic promoter of the transgene, usually after or during the perinatal transition restricts the influence of COX-2 in the embryonic steps of development, but effects on early postnatal development cannot be ruled out among the different COX-2 transgenic models considered. This is in addition to the contribution of the distinct genetic backgrounds used in these animal models. Accordingly, caution must be exercised in deducing conclusions in view of the arbitrary insertion of the transgene and the fact that the biological repercussions of this genetic event may have unexpected effects in the transcriptome, in addition to the specific actions of the protein encoded by the transgene. Scientists are innocent players in this scenario and their work should be well considered, although filtered by evidence coming from ancillary physiopathological data.

COX-2 TRANSGENESIS AND LIVER CARCINOGENESIS

Despite the constitutive presence of COX-2 in hepatocytes in the mice^[38,41-45], they failed to spontaneously develop hepatocellular tumors, and only hepatitis and fibrosis was observed in model #2^[42,43]. This is interesting because COX-2 has been frequently associated

with the presence of hepatocellular tumors (but in the “healthy” portion of the liver) and exacerbated COX-1 and COX-2 expression are frequently observed in hepatoma cell lines^[13]. In addition, targeting of the COX enzymes or the PG receptors (EP1-4) contribute to antiproliferative effects in these cultured cells^[50,51]. Tissue-specific constitutive expression of COX-2 has been reported as a positive factor for the development of carcinogenesis. In mice expressing the enzyme in mammary glands, the continuous release of PGE₂ has been reported to favor angiogenesis and development *per se* of tumorigenic foci in the mammary gland^[52,53]; these data were corroborated by pharmacological approaches based on COX-2 inhibition^[54]. In addition to this, constitutive expression of COX-2 under the control of the keratin 5 promoter markedly sensitized skin to carcinogens; for example, under these conditions the sole challenge of DMBA, without further requirement of phorbol ester administration or other skin tumor promoters was sufficient to induce epidermal hyperplasia and frequent dysplastic lesions in the skin of the transgenic animals^[55,56]. In the gastrointestinal tract, constitutive coexpression of COX-2 and microsomal PGE synthase (mPGES-1; the enzyme that is coupled to COX-2 and directs the prostaglandin synthesis towards PGE₂) under the control of the cytokeratin 19 promoter (that targets the expression of these transgenes in the epithelial cells of gastric mucosa), resulted in animals developing hyperplasia and tumors in the stomach glandular anatomy through a process in which the contribution of infiltrating inflammatory cells, mainly macrophages plays a relevant role^[57,58]. Stable expression of COX-2 in hepatocyte-like cells induced proliferation, with an increase in the proportion of cells in S-phase^[59]. Interestingly, the basal protein levels of pJNK (phosphorylated c-jun-NH2-kinase) and p53, were greater in COX-2 expressing cells

and were induced treatment with diethylnitrosamine (DEN). However, animals of model #1, challenged with DEN did not show an increased sensitivity, compared to the parental strain, in developing hepatic tumors during the following 12 mo of treatment (preliminary results). A schematic representation of these actions of COX-2 in hepatocytes and livers is summarized in Figure 1.

CONCLUSION

The data reported in this review suggest that COX-2 expression provides a continuous supply of bioactive lipids that protect the liver against acute injury. However, attention should be paid to ensure that the substrate for COX-2 is available, since arachidonic acid mobilization requires the activation of phospholipase A₂ isoenzymes, a process that is not usually accomplished under *in vivo* conditions. A noteworthy point is that COX-2 may use other unsaturated fatty acids as substrates, releasing molecules that can activate various nuclear receptors, such as peroxisome proliferator-activated receptors. In addition to this, the fact that COX-2 is not expressed in hepatocytes under pro-inflammatory conditions restricts PG synthesis to other hepatic cells, such as Kupffer cells and activated macrophages. However, in the course of progression of hepatocellular carcinomas, it cannot be excluded that there is a transient expression of COX-2 in transformed cells, as observed in many cell lines derived from hepatocellular carcinomas that express this enzyme either constitutively or after pro-inflammatory induction. Finally, the data from the models of COX-2 transgenesis in hepatocytes support the idea that by itself, COX-2 appears not to contribute to development of tumors in the full life-span of these animals.

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Nonalcoholic steatohepatitis-associated hepatocellular carcinoma: Our case series and literature review

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HCC is necessary in non-cirrhotic NASH patients as well as cirrhotic patients.

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Abstract

Recently, nonalcoholic steatohepatitis (NASH) has been considered to be another cause of liver cirrhosis and hepatocellular carcinoma (HCC). The natural history and prognosis of NASH are controversial. Accordingly, we assessed the clinicopathological features of NASH-associated HCC in our experience and reviewed the literature of NASH-associated HCC. We experienced 11 patients with NASH-associated HCC (6 male, 5 female; mean age 73.8 ± 4.9 years) who received curative treatments. Most (91%) patients had been diagnosed with obesity, diabetes, hypertension, or dyslipidemia. Seven patients (64%) also had a non-cirrhotic liver. The recurrence-free survival rates at 1, 3 and 5 years were 72%, 60%, and 60%. We also summarized and reviewed 94 cases of NASH-associated HCC which were reported in the literature (64 male; mean age 66 years). The majority of patients (68%) were obese, 66% of patients had diabetes, and 24% had dyslipidemia. Furthermore, 26% of the HCCs arose from the non-cirrhotic liver. In conclusion, patients with non-cirrhotic NASH may be a high-risk group for HCC, and regular surveillance for

INTRODUCTION

Hepatocellular carcinoma (HCC) is a common malignancy in Asia and South Africa. HCC usually develops in patients with hepatitis B, hepatitis C, and alcoholic liver disease. Recently, nonalcoholic steatohepatitis (NASH) has been considered to be another cause of liver cirrhosis and HCC. Powell *et al*^[1] reported the first case of NASH-associated HCC. Since then, several case series of NASH-associated HCC have been reported^[2-4].

The prevalence of nonalcoholic fatty liver disease (NAFLD) is 10%-30% in adults^[5] and its prevalence is increasing in Japan as well as Western countries because of the epidemic rise in obesity and diabetes mellitus (DM). NASH is part of the spectrum of NAFLD, and 20% of NASH cases are thought to slowly progress to cirrhosis^[6]. According to a previous study^[7], NASH can progress to cirrhosis and result in complications including HCC.

Almost all patients with cryptogenic cirrhosis (CC) had clinical features consistent with NASH, but a diagnosis of NASH could not be confirmed by histology (likely “burnt out NASH”)^[8]. The natural history and prognosis of NASH is controversial, because there are few reports on prospective cohort studies of NASH^[9,10]. Accordingly, it is necessary to clarify an etiology and a prognosis of NASH-associated HCC. Thus, we retrospectively assessed the clinicopathological features of NASH-associated HCC in our experience and reviewed the literature on NASH-associated HCC.

NASH-ASSOCIATED HCC IN OUR EXPERIENCE

We reviewed 797 consecutive patients treated for primary HCC at National Hospital Organization Iwakuni Clinical Center from January 1996 and September 2008. Of these, 445 with HCC initially underwent curative treatment. Curative treatment was defined as complete tumor eradication, with no residual tumor visible by computed tomography (CT), or resection of all evident tumor tissue, and no tumors detected in the remnant liver on CT scan performed 3 to 4 wk after curative treatment. Curative treatment included surgery, percutaneous radiofrequency ablation (RFA), microwave coagulation therapy (MCT), and percutaneous ethanol injection (PEI). Within this group, 11 patients were considered to have NASH based on the histology of the non-cancerous parts of the surgical specimens or biopsy specimens.

NASH was diagnosed using the following criteria^[3]: (1) histological features of steatohepatitis; (2) intake of less than 20 g ethanol per day; (3) absence of other liver diseases such as autoimmune hepatitis, drug-induced liver disease, primary biliary cirrhosis, primary sclerosing cholangitis, and metabolic liver disease such as Wilson’s disease and hemochromatosis; and (4) negative for hepatitis B surface antigen and antibody to hepatitis C virus (HCV) and/or negative for HCV RNA on polymerase chain reaction analysis.

The body mass index (BMI) was calculated as body weight in kilograms divided by the square of the height in meters (kg/m^2). The definition of DM was fasting plasma glucose level ≥ 126 mg/dL on at least two occasions, plasma glucose level of ≥ 200 mg/dL at 2 h in a 75 g oral glucose tolerance test, or the need for insulin or an oral antihyperglycemic drug to control glucose levels. The oral glucose tolerance test was undertaken by patients who had no medical history of DM.

Dyslipidemia was defined as blood total cholesterol concentration > 220 mg/dL or triglyceride > 150 mg/dL or a history of taking oral drugs for dyslipidemia.

The histological status of underlying liver disease was based upon microscopic examination of the non-cancerous part of the surgical specimen or biopsy specimen with hematoxylin-eosin and Azan staining. All liver tissue specimens were evaluated by two senior pathologists who were unaware of the laboratory data and the clinical course. Steatohepatitis was pathologically graded on quantified

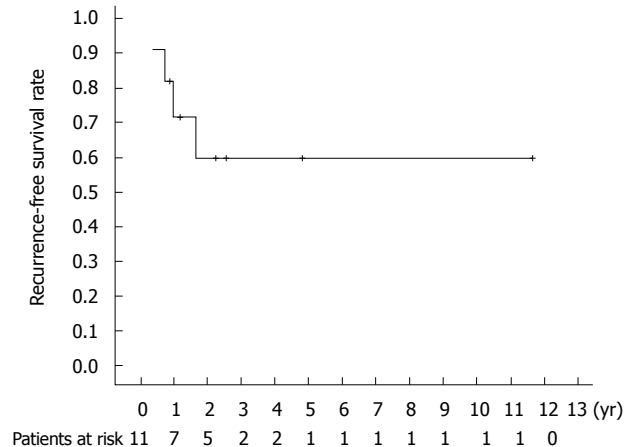


Figure 1 Recurrence-free survival rates in nonalcoholic steatohepatitis (NASH)-associated hepatocellular carcinoma (HCC).

steatosis, ballooning degeneration, and lobular inflammation to produce an NAFLD activity score (NAS)^[11]. When this score is ≥ 5 it is diagnostic for NASH. The extent of fibrosis, established by Desmet *et al.*^[12], is as follows: F0 (no fibrosis), F1 (mild fibrosis), F2 (moderate fibrosis), and F3 (severe fibrosis) were all categorized non-cirrhosis. F4 was categorized as cirrhosis.

A summary of our 11 cases with NASH-associated HCC is shown in Table 1. The mean age of patients with HCC was 73.8 ± 4.9 years. Of the 11 patients, 6 patients (55%) were male, and 5 (45%) were female. The mean BMI was $24.8 \text{ kg}/\text{m}^2$. Six patients (55%) were obese (BMI $\geq 25 \text{ kg}/\text{m}^2$), 6 (55%) had DM, 3 (27%) had dyslipidemia, and 6 (55%) had hypertension. Ten patients (91%) had obesity or at least 1 comorbid illness. The prevalence of positivity of HBV core (HBc) antibodies in our cases was 27% (3 of 11 patients). Ten patients (91%) had Child-Pugh classification A and 1 (9%) was Child-Pugh classification B, and all patients received curative treatments as outlined below. Seven patients (64%) received surgery, 3 patients (27%) received RFA, and 1 patient (9%) received MCT. Four patients (36%) had concomitant liver cirrhosis (F4), and 7 patients (64%) had a non-cirrhotic liver (F1-3). Concerning the characteristics of HCC, 8 patients (73%) had a single nodule, 3 patients (27%) had multinodular lesions, and the mean size of the largest lesion was 3.3 ± 1.3 cm (range 1.7-5.0 cm).

Patients were followed for 41.3 ± 40.0 mo (range, 9.4-151.7 mo), and no patients dropped out. Local tumor progression was not found. The recurrence-free survival rates at 1, 3, and 5 years calculated by Kaplan-Meier method were 72%, 60%, and 60% (Figure 1). All of tumor recurrences were observed within the first 2 years, and no recurrence was observed after 2 years. During the follow-up, 3 patients (27%) died as a result of HCC (2 patients) and hepatic failure (1 patient).

EPIDEMIOLOGIC TRENDS OF NASH AND NASH-ASSOCIATED HCC

HCC is the third leading cause of cancer death in world-

Table 1 Characteristics of 11 NASH patients with HCC undergoing curative treatment

Case	1	2	3	4	5	6	7	8	9	10	11
Sex (M/F)	M	F	F	M	M	M	F	F	F	F	M
Age (yr)	73	73	83	75	67	68	77	79	73	76	68
BMI	25.0	23.6	21.0	22.3	25.3	24.2	28.1	25.3	23.4	28.8	26.2
Diabetes mellitus (yes/no)	No	Yes	Yes	Yes	No	Yes	Yes	No	No	No	Yes
Dyslipidemia (yes/no)	No	Yes	No	Yes	No	No	No	No	No	No	Yes
Hypertension (yes/no)	No	Yes	No	Yes	Yes	No	Yes	Yes	No	No	Yes
Total bilirubin (mg/dL)	1.6	0.6	0.6	0.7	1.4	0.8	0.8	1.2	0.6	1.3	0.9
Albumin (g/dL)	3.9	4.4	3.2	3.8	4.4	4.3	3.5	4.1	4.4	3.8	4.9
ALT (IU/L)	73	41	22	24	73	18	19	29	41	40	57
γGTP (IU/L)	110	45	36	22	57	33	26	111	124	41	177
Prothrombin time (%)	78.1	95.8	96.3	106.6	90.9	113.4	75.4	77.2	94.0	68.4	86.0
Platelets ($\times 10^4/\mu\text{L}$)	10.1	15.0	15.1	27.8	14.3	27.2	9.3	13.9	13.0	5.5	13.4
Child-Pugh classification (A/B)	A	A	A	A	A	A	B	A	A	A	A
AFP (ng/mL)	8.7	4.6	40.6	9181.6	1.7	1.8	1957.0	7.9	13.0	8.8	16.2
DCP (mAU/mL)	31	10	2066	1765	64	10	718	19	571	106	2930
Anti-HBc (+/-)	+	-	-	-	-	+	-	-	-	+	-
Treatment (RFA/MCT/Ope)	RFA	RFA	Ope	Ope	Ope	Ope	Ope	Ope	RFA	MCT	Ope
No. of nodules (1/2/3)	3	1	1	1	1	1	1	1	3	2	1
Size of largest tumor (cm)	1.8	1.7	5.0	5.0	3.6	4.5	3.2	2.0	2.5	3.0	4.4
Stage of fibrosis (F1/F2/F3/F4)	F4	F1	F4	F1	F1	F1	F4	F4	F1	F3	F1

NASH: Nonalcoholic steatohepatitis; HCC: Hepatocellular carcinoma; M: Male; F: Female; BMI: Body mass index; ALT: Alanine aminotransferase; γGTP: γ-glutamyltransferase; AFP: α-fetoprotein; DCP: Des-γ-carboxy prothrombin; Anti-HBc: HBc antibody; RFA: Radio-frequency ablation; MCT: Microwave coagulation therapy; Ope: Operation.

wide; there are an estimated 500 000 to 1 million new cases each year resulting in 600 000 deaths annually^[13]. The major causes of cirrhosis seen in HCC are viral (hepatitis B, hepatitis C), and alcohol. HCV infection is the most prevalent risk factor for HCC in Japan and United States. In United States, the most leading etiology underlying liver disease among the patients with HCC was HCV (51%), and the second most common etiology was CC (29%)^[14].

In the majority of CC cases it is thought to be end-stage NASH because some clinical features such as obesity and diabetes in CC patients are linked to NASH. However, histology often is no longer informative when cirrhosis is already established^[15] because it has been theorized that CC often represents “burned out” NASH. Marrero *et al*^[16] studied the etiology of liver disease in 150 patients with HCC wherein NAFLD-related CC accounted for at least 13% of the cases.

CLINICOPATHOLOGICAL FEATURES

Articles were searched in Medline and Pubmed. The search terms used were NASH, nonalcoholic steatohepatitis, fatty liver, HCC, hepatocellular carcinoma, hepatoma, and liver neoplasms. We summarized and reviewed several studies and numerous case reports which explored NASH-associated HCC^[1-4,17-31]. At least 94 cases of NASH-associated HCC were reported (Table 2). Sixty-four patients were male (64%), and the age at diagnosis ranged from 35 to 89 years (mean, 66 years). The majority of patients (68%) were obese, 66% of patients had DM, and 24% had dyslipidemia. Concerning tumor characteristics, 69% of HCCs were multinodular, maximum tumor size ranged from 1.4-13 cm (mean, 3.5 cm). Furthermore,

26% of cases arose from a non-cirrhotic liver. In a case-controlled study of 34 Japanese NASH-associated HCC patients, those patients were predominantly male, had a median age of 70 years and 88% had advanced fibrosis. Older age, low level of AST, low grade of activity, and advanced fibrosis were independent predictors of developing HCC in NASH^[3].

NATURAL HISTORY AND PROGNOSTIC FACTORS

The natural history and prognosis of NASH is controversial because there are few reports on prospective cohort studies of NASH^[3,9,10].

Yatsuji *et al*^[10] reported that the 5-year HCC rate was 11.3% for NASH-cirrhosis and 30.5% for HCV-cirrhosis in Japanese patients. On the other hand, Hui *et al*^[9] reported that HCC occurred in 8 (17%) of 46 patients with HCV-cirrhosis compared with none of 23 patients with NASH-cirrhosis after 5 years follow-up in Australia.

A prospective cohort study of NASH patients in Japan^[3] showed that the 5-year cumulative incidence of HCC was 7.6%, and the 5-year survival rate was 82.8%. Concerning outcome, 26 of 137 NASH patients died, with death caused by liver failure in 7 patients, HCC in 12 patients and other causes in 7 patients. Liver-related deaths thus accounted for 19 (73%) deaths.

Malik *et al*^[4] reported that the survival rate after liver transplantation for HCC complicated NASH-cirrhosis was 88% at a mean follow-up of 2.5 years. There was no difference in 5-year survival between patients transplanted for NASH-cirrhosis with and without HCC, and no difference in 5-year survival after liver transplantation between

Table 2 Clinical features of cases with NASH-associated HCC in previous reports

Author	No. of cases	Age (yr)	Sex	Underlying disease	Liver histology	No. of nodules	Tumor size (cm)	Treatment
Powell <i>et al</i> ^[1]	1	57	F	DM	LC	Mul	NR	Ope
Zen <i>et al</i> ^[17]	1	72	F	DM	LC	Mul	1.4	NR
Orikasa <i>et al</i> ^[18]	1	67	F	DM	LC	Sol	2.6	NR
Cotrim <i>et al</i> ^[19]	1	66	M	OB, DM	LC	Sol	3.0	PEI
Bencheqroun <i>et al</i> ^[20]	1	68	M	OB	Non-LC	NR	NR	NR
Mori <i>et al</i> ^[21]	1	76	M	OB, DM	LC	Sol	1.9	RFA
Bullock <i>et al</i> ^[22]	2	64, 74	M (2)	OB (2), DM (2)	Non-LC (2)	Sol (2)	NR	Ope (2)
Cuadrado <i>et al</i> ^[23]	2	69, 74	M (2)	OB (2), DM (2)	LC, Non-LC	Sol (2)	NR	RFA (2)
Sato <i>et al</i> ^[24]	1	64	M	OB, DL	Non-LC	Sol	13.0	None
Ichikawa <i>et al</i> ^[25]	2	60, 66	M, F	OB (1), DL (1)	Non-LC (2)	Sol (2)	1.5, 2.5	Ope (2)
Ikeda <i>et al</i> ^[26]	1	69	M	OB, DM	LC	Sol	4.5	Ope
Tsutsumi <i>et al</i> ^[27]	2	46, 68	M, F	NR	LC (2)	Sol (2)	NR	NR
Hashizume <i>et al</i> ^[2]	9	45-82	M (5), F (4)	OB (5), DM (7), DL (8)	LC (6), Non-LC (3)	Sol (8), Mul (1)	1.5-7.0	Ope (6), RFA (2), TAE (1)
Hai <i>et al</i> ^[28]	2	65, 72	M (2)	OB (2), DM (2)	LC, Non-LC	Sol (2)	4.0, 6.0	Ope (2)
Maeda <i>et al</i> ^[29]	3	52-68	M (2), F (1)	NR	LC (3)	Sol (3)	1.3-5.0	Ope (3)
Kawada <i>et al</i> ^[30]	6	59-81	M (3), F (3)	OB (2), DM (3), DL (1)	Non-LC (6)	Sol (6)	1.3-5.8	Ope (6)
Hashimoto <i>et al</i> ^[3]	34	54-89	M (21), F (13)	OB (62%), DM (74%), DL (29%)	F1-2 (22%), F3-4 (88%)	Sol (24), Mul (10)	NR	NR
Malik <i>et al</i> ^[4]	17	47-72	M (12), F (5)	OB (17), DM (12)	LC (17)	Sol (8), Mul (9)	1.1-8.0	LT (17)
Chagas <i>et al</i> ^[31]	7	35-77	M (4), F (3)	OB (7), DM (3), DL (2)	LC (6), Non-LC (1)	Sol (4), Mul (3)	2.8-5.2	Ope (2), PEI (1), TAE (3), LT (1)

HCC: Hepatocellular carcinoma; DM: Diabetes mellitus; OB: Obesity; DL: Dyslipidemia; LC: Liver cirrhosis; Mul: Multiple; Sol: Solitary; Ope: Surgical operation; PEI: Percutaneous ethanol injection; RFA: Radiofrequency ablation; TAE: Transarterial embolization; LT: Liver transplantation; NR: Not reported.

HCC patients with NASH-cirrhosis and with non-NASH-cirrhosis (HCV, HBV, alcoholic, CC, and otherwise). They concluded that patients with NASH and HCC have a good outcome after liver transplantation.

Giannini *et al*^[32] reported that patients with CC had a significantly greater prevalence of advanced HCC stage, lower amenability to any treatment, and shorter survival times compared with HCV patients, because HCC in CC patients is often diagnosed at an advanced stage owing to lack of surveillance.

MECHANISMS OF NASH-INDUCED HEPATOCARCINOGENESIS

Although the mechanism of carcinogenesis in patients with NASH remains uncertain, insulin resistance and oxidative stress may be involved in carcinogenesis of NASH.

NASH is characterized by insulin resistance with hyperinsulinaemia, and the resistance is thought to be involved in hepatocarcinogenesis. Insulin-like growth factor 1 (IGF-1)^[33] significantly activated mitogen-activated protein kinase (MAPK), and increased overexpression of the c-fos and c-jun proto-oncogenes in cultured hepatoma cells^[34].

Adiponectin and leptin are associated with insulin resistance. Severe liver steatosis and fibrosis were observed in adiponectin knockout (KO) mice as compared with wild type (WT) mice^[35]. Furthermore, liver adenoma and hyperplastic nodules developed in an adiponectin KO mouse, whereas no tumor was detected in WT mice. In animal models, leptin-mediated neovascularization,

which coordinated with VEGF, produced liver fibrosis and hepatocarcinogenesis in NASH^[36].

NASH-associated insulin resistance causes inhibition of hepatic mitochondrial fatty acid oxidation and increased intracellular fatty acids may lead to oxidative DNA damage by stimulating microsomal peroxidases^[37].

Oxidative stress may also promote carcinogenesis. Trans-4-hydroxy-2-nonenal, a major electrophilic by-product of lipid peroxidation caused by oxidative stress may be an important etiological agent for HCC *via* mutation at codon 249 of the *p53* gene^[38].

Reactive oxygen species (ROS) can activate fibrosis^[39]. Furthermore, the major products of lipid peroxidation, malondialdehyde, stimulates DNA mutations^[40]. Therefore, inflammation is a risk factor for various carcinomas^[41]. Oxidative stress has inactivated the expression of *Nrf1* gene that regulates gene transcription encoding enzymatic antioxidants. Recently, in an animal model, oxidative stress inactivation of the *Nrf1* gene in the liver has been reported to spontaneously produce HCC when oxidative injury was present before tumor formation^[42].

Ishii *et al*^[43] reported that in animal models, eicosapentaenoic acid (EPA) ameliorated steatohepatitis with decreasing serum ROS, which consequently inhibited development of HCC. Medical treatment with EPA may minimize the risk of HCC development in patients with NASH.

CONCLUSION

Most (91%) patients with NASH-associated HCC in our experience had been diagnosed with obesity, diabetes,

hypertension, or dyslipidemia. CC patients had these comorbid illnesses, and CC had clinical features consistent with NASH.

Occult HBV infection might be a possible etiologic agent of HCC, and the prevalence of past/occult HBV infection *via* positivity of HBc antibody in our cases was 27%. Negativity of HBc antibody is not necessarily a required item of diagnosis for NASH, and liver specimens of these HBc antibody positive patients had no histological features of chronic hepatitis B.

Although almost NASH-associated HCC was accompanied by liver cirrhosis according to previous reports^[1,17-19], the majority of our case series were accompanied by non-cirrhotic liver.

Furthermore, recent case reports^[2,30] about HCC arising from non-cirrhotic NASH have been accumulating. One possible explanation for this difference between our cases and other previous reports is that almost all patients with CC had clinical features consistent with NASH, but a diagnosis of NASH could not be confirmed by histology (likely “burnt out NASH”).

All cases of tumor recurrence in our series were observed within the first 2 years, no recurrence was observed after 2 years. These recurrence patterns of HCC suggested that the recurrence of HCC might be based on intrahepatic metastasis rather than multicentric carcinogenesis. NASH-associated HCC patients with non-curative treatments were not observed in our cases, because these patients did not receive liver biopsy or surgery. The existence of selection bias is unavoidable.

In conclusion, patients with non-cirrhotic NASH may be a high-risk group for HCC, and regular surveillance for HCC is necessary for non-cirrhotic NASH patients as well as cirrhotic patients.

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Role of diet in the management of inflammatory bowel disease

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Abstract

Many studies have looked at connections between diet, etiology, signs and symptoms associated with inflammatory bowel disease (IBD). Although these connections are apparent to clinicians, they are difficult to prove qualitatively or quantitatively. Enteral feeding and polymeric diets are equally effective at bringing about remission in Crohn's disease (CD). Parenteral feeding is also effective, although none of these methods is as effective as corticosteroid therapy. However, enteral feeding is preferred in the pediatric population because linear growth is more adequately maintained *via* this route. Exclusion diets in patients brought into remission using an elemental diet have been shown to maintain remission for longer periods. Studies that aim to isolate culpable food groups have shown that individuals react differently on exposure to or exclusion of various foods. The commonly identified food sensitivities are cereals, milk, eggs, vegetables and citrus fruits. Studies that have looked at gut mucosal antigen behavior have shown higher rectal blood flow, in response to specific food antigens, in those with CD over healthy subjects. Exclusion of sugar shows little evidence of amelioration in CD. Omega 3 fatty acids show promise in the treatment of IBD but await larger randomized controlled trials. Patients frequently notice that specific foods cause

aggravation of their symptoms. Whilst it has been difficult to pinpoint specific foods, with advances in the laboratory tests and food supplements available, the aim is to prolong remission in these patients using dietary measures, and reduce the need for pharmacotherapy and surgical intervention.

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Key words: Crohn's disease; Ulcerative colitis; Exclusion diet; Elimination diet

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INTRODUCTION

The etiology of inflammatory bowel disease (IBD) is considered multifactorial. Genetic, infective and environmental theories exist, as well as those centered around host immunity, intraluminal gut flora, food allergies and hypersensitivity^[1].

Whilst pharmacological therapy plays a major role, many patients prefer to control their symptoms by the most conservative means possible. Our aims in IBD therapy are to downregulate inflammation, and reduce the incidence of relapse and the healing time.

Dietary therapy encompasses dietary modifications suggested by physicians and those that patients make autonomously. The putative mechanisms of action are due to bowel rest, provision of nutrients, alteration of bowel flora or alteration of antigenic stimuli. The gastrointesti-

nal flora and its interaction with nutritional factors has a huge impact on the environment, especially in genetically predisposed individuals. Nutrients as components of cell structure or antigens can induce inflammatory mediator expression and suboptimal levels of nutrients, which may have an impact on tissue repair and other cellular processes^[2]. Other reviews have concluded that nutrients that tend to affect the immune responses of the host (n-3 fatty acids, antioxidants) are likely to play a role in the treatment of IBD^[3].

In this review, we examine the literature for dietary interventions in IBD, such as exclusion/elimination diets, enteral nutrition and total parenteral nutrition (TPN), and review the evidence that they induce and/or maintain remission in patients with IBD.

METHODOLOGY

PubMed, Cochrane and MEDLINE were searched using the broad key words Crohn's disease (CD), ulcerative colitis (UC) and IBD cross-referenced with exclusion diet, elimination diet, diet therapy and nutritional therapy. The results were limited to human studies and those available in English. They were not limited by the type or size of the trial. The search identified 503 papers of interest. When the titles were reviewed, 315 were found to be of interest by two independent reviewers. If there was any doubt, the papers were included. The 315 abstracts were reviewed again by the same two reviewers, out of which 104 were found to be relevant. These 104 were scrutinized for dietary evidence but only 38 of these had a dietary focus or provided evidence for it. Eighty-five secondary references were reviewed by abstract, of which, 45 were obtained and studied. Of these, 10 provided relevant evidence which was included in the review.

INDUCTION OF REMISSION IN CD

Elemental/polymeric diets

Elemental diets were chanced upon as a therapeutic option for CD patients when they were used to bolster their nutritional status and reduce inflammation^[4]. Liquid feeds are thought to work by reducing mucosal antigen exposure, partly due to the nature of the feed and partly to faster transit times. They also alter the fecal flora, which causes local immunomodulation downscaling, which enhances nutritional status and allows relative bowel rest^[5].

Compliance may be poor as elemental diets are not known for their palatability and are often delivered *via* a nasogastric tube, whereas the polymeric drinks are far more palatable. Several trials and meta-analyses have shown no significant difference in the efficacy of elemental diets over polymeric diets^[6,7].

Elemental diets offer a cheaper way of bringing about remission and without the side effect profile of TPN. In both adult^[8] and pediatric populations, elemental^[9] and polymeric^[10] feeds have been shown to be as effective as corticosteroid therapy in treating active CD. However, a

Cochrane review by Zachos *et al*^[11] has shown, in a meta-analysis, that enteral nutrition is not as effective as steroid therapy for inducing remission.

However, enteral therapy for CD has its role in selected cases, in particular, in children in whom steroids may cause growth retardation^[12]. Food exclusion with liquid diet is very difficult to maintain, therefore, these are rarely long-term solutions. Unfortunately, a staged return to normal feeding often leads to relapse.

Exclusion diets

The East Anglia Multicentre Controlled Trial showed that various food intolerances were perceived in individual patients, and among the more common were cereals, dairy produce and yeast^[13]. This work looked at the use of exclusion diets as an intervention in active CD. The exclusion diet was based around daily reintroduction of a single food type. If it caused diarrhea or pain, it was eliminated. All patients were treated initially with an elemental diet, and those who attained remission followed a reduced prednisolone course or the exclusion diet pathway. Jones *et al*^[14] have shown that maintenance of remission, by identification and avoidance of food intolerances, is possible, often without pharmaceutical adjuncts. Testing for these sensitivities has proven difficult, because testing shows a large number of sensitivities in unselected populations, which are of doubtful clinical significance^[15]. Jones *et al*^[14] have tested a diet rich in unrefined carbohydrate against an exclusion diet. Seven out of 10 patients on the exclusion diet stayed in remission for 6 mo, while none of those on the carbohydrate-rich diet remained in remission. Pearson *et al*^[16] have conducted a study of 42 CD patients after induction of remission by elemental diet. Single foods were investigated using open and double blind rechallenge over 5 d. Fourteen patients dropped out due to flare-ups that were thought to be unrelated to food, and caused by inability to comply with the regimen. Twenty of the remaining patients identified food intolerances and eight did not. This research group has concluded that food intolerance is not as frequent as claimed in other studies, and that it is variable in its intensity and occurrence.

Parenteral feeding in CD

TPN allows bowel rest while supplying adequate caloric intake and essential nutrients, and removes antigenic mucosal stimuli. However, TPN is expensive, invasive and has a number of side effects. TPN has been shown to bring about remission in CD^[17,18]. Müller *et al*^[18] have found that, in 30 consecutive complicated CD patients, 3 wk of TPN as an inpatient followed by an additional 9 wk at home, during which time, no medication or oral intake was allowed, 25 patients avoided surgery. These patients returned to work and needed no further medication and ate normal meals subsequently. In a prospective randomized controlled trial (RCT), 51 patients with active CD refractory to medical treatment were treated with TPN and nil by mouth, defined formula diet *via* a nasogastric tube, or partial parenteral nutrition^[17]. Clinical remission was

obtained in 71% of the patients on TPN, 58% on enteral feeding, and 60% on partial parenteral feed.

Enteral vs parenteral feeding

There has been controversy regarding the enteral *vs* parenteral route for feeding in patients with IBD. Comparison of TPN against elemental diet in a group of 36 patients showed no significant difference in the number of days to remission, the drop in Crohn's disease activity index (CDAI) score, the erythrocyte sedimentation rate (ESR), or albumin^[19]. However, in other studies that have agreed with this finding, neither was proven to be as beneficial as corticosteroids, except one study in a pediatric population^[20]. In that study, Sanderson *et al*^[20] entered 17 children into an RCT, in which eight were given an elemental diet for 6 wk *via* a nasogastric tube, and seven were given adrenocorticotrophic hormone injections and oral prednisolone and sulfasalazine. The elemental diet was equally effective at improving the Lloyd-Still disease activity index scores, C-reactive protein (CRP), ESR and albumin. The elemental diet was markedly better at maintaining linear growth. Whilst strong evidence exists supporting the primary use of enteral feeding in children with CD^[21], it is not commonplace in the treatment of adults.

Omega-3 fatty acids

Shoda *et al*^[22] have noted that the gradual replacement of n-3 polyunsaturated fatty acids with n-6 polyunsaturated fatty acids results in an increased incidence of CD. This implies that there is the potential to modulate immune responses by altering the ratio of polyunsaturated fatty acids in favor of n-3 rather than n-6^[23]. Meister and Ghosh have shown that fish-oil-enriched enteral diet, when incubated with intestinal tissue from 11 subjects with IBD and four controls, reduced inflammation modestly in CD and significantly in UC^[24]. Inflammatory improvement was assessed by analyzing the interleukin (IL)-1 receptor antagonist/IL-1 β ratio. The greater the ratio, the less inflamed the tissue. A systematic review of the effects of n-3 fatty acids in IBD by MacLean *et al*^[25] has identified 13 controlled trials that investigated the effects of n-3 fatty acids. The results were mixed but in the three studies that looked at steroid requirements, this was found to be reduced. However, this was statistically significant in just one of these studies^[26].

MAINTENANCE OF REMISSION IN CD

Exclusion diets

Jones has looked at exclusion diets for the maintenance of remission of CD and has shown that, in personalized exclusion diets, 62% of the patients maintained remission at 2 years and 45% at 5 years, with no other medical intervention^[19]. This was compared to the European Cooperative Crohn's Disease Study in 1984 in which the placebo arm of the study had no patient who maintained remission after 2 years of follow-up^[27].

A Cochrane review of the maintenance of remission in CD has suggested that larger, high-powered controlled trials are required to confirm current hypotheses relating to diet and maintenance of remission^[28]. Trials of diet against azathioprine and infliximab also have been suggested to investigate quantitative effects of nutritional supplements and their impact on cost-effectiveness and quality of life.

Enteral feeding

Enteral feeding has been shown to have a role in preventing relapse in inactive CD patients (predominantly in children)^[29], but the effect has also been observed in a Japanese study of adult CD patients^[30]. Esaki *et al*^[31] have demonstrated in a trial of 145 patients with CD (mostly induced into remission with TPN) that, under maintenance with elemental/polymeric nutrition, the risk of recurrence was lower in those with small bowel rather than large bowel involvement.

DIETARY MANAGEMENT IN UC

Maintenance of remission in UC

UC does not seem to be ameliorated by bowel rest and elemental diets in the same way as CD is^[32-34]. However, patients still express concern about specific food types, and there does appear to be an association with a western diet^[35]. In a study that has investigated self-reported food intolerance in chronic IBD, patients with CD and UC have reported that they felt intolerant to specific dietary triggers and restricted their diet accordingly^[36]. The same study has shown that the pattern and frequency of food intolerance did not differ between CD and UC patients. This has been reinforced by work from our own group that has investigated food intolerances detected by measuring IgG4 antibodies to specific food antigens^[37,38]. There is no evidence to support the use of elemental/polymeric feeding in the treatment of UC^[12,23].

Omega-3 fatty acids

Omega-3 fatty acids derived from fish oils have been shown to be of benefit in a double-blind RCT that looked at patients with distal UC. That study found that the group treated with 3.2 g eicosapentaenoic acid or 2.4 g docosahexaenoic acid daily had significantly better clinical and sigmoidoscopic scores compared with the control group who took sunflower oil, after 3 and 6 mo. This supports the idea that omega-3 oils suppress natural cytotoxicity^[39].

ADDITIONAL DIETARY FACTORS

Fiber

Dietary fiber has been investigated as a means of increasing short-chain fatty acid (SCFA) production. IBD has been linked with impaired SCFA production. SCFAs are mainly produced by the anaerobic bacterial fermentation of undigested carbohydrates and fiber polysaccharides.

In 1995, Galvez *et al*^[40] reviewed a number of studies that concluded that dietary fiber confers clinical benefits in patients with IBD because it maintains remission and reduces colonic damage. This is thought to occur by increasing SCFA production and by altering the gut flora towards predominantly non-pathogenic bacteria.

Fats

The properties of omega-3 fatty acids have been discussed elsewhere in this review. Other studies have revealed an inverse correlation between the percentage of energy derived from long-chain triglycerides and the efficacy of enteral feeds in achieving remission^[41,42].

Sugars

A high intake of sugar has been shown to be linked to CD in a number of trials, hence its possible etiological role has led to therapeutic trials of sugar avoidance^[43]. Most of these trials also have promoted a high fiber intake. The only trial to look solely at sugar avoidance has shown no statistically significant benefit^[44].

ANTIGENIC RESPONSE TO FOOD

Van den Bogaerde *et al*^[45] have published a trial in which the reactivity of peripheral lymphocytes to food, yeast and bacterial antigens was studied. They found that 23 out of 31 patients with CD responded to one or more antigens, compared to five out of 22 in the control group. They also correlated *in vitro* sensitization and *in vivo* changes with histological and blood flow changes. Skin testing and rectal exposure to six food antigens and saline were tested in 10 patients and 10 controls. The results showed that CD patients demonstrated *in vitro* and *in vivo* sensitization to food antigens and this was gut specific^[46].

Levo *et al*^[47] have shown that patients with IBD have higher serum concentrations of IgE. They also have shown that the levels are higher still in those with active disease over those in remission. However, this difference is not statistically significant. In 1998, another study was performed to investigate food-specific IgE as well as IgG, and IgE anti-IgE autoantibodies using serum from normal subjects, patients with CD and those with food allergies. They found that food-specific IgE was not detected at all in the CD group but they did have higher levels of IgG and IgE anti-IgE autoantibodies. They concluded that, even if IgE is an autoantigen in CD, it is not thought to take part in the pathophysiology of the adverse food reactions commonly reported by the patients^[48].

Western diets are more strongly associated with CD^[35]. There are several theories as to whether this may relate to the increased intake of sucrose, refined carbohydrate, and omega-6 fatty acids, and reduced intake of fruit and vegetables. Urban diets contain large quantities of microparticles such as natural contaminants like dust, and food additives which may be antigenic. CD patients allocated to a low microparticle diet experienced a reduction in disease activity and in steroid requirement

compared to a control group on a normal diet^[49].

The Table 1 lists the reviewed studies that have investigated dietary exclusion and sensitivity to foods.

SUMMARY

Although many studies have looked at diet therapy and IBD, mixed opinions exist as to the importance that food intolerance plays in the pathophysiology of IBD. In those that have looked at food sensitivity, this was done using different methods. Riordan *et al*^[13] have observed sensitivity to corn in seven patients; wheat, milk and yeast in six; egg, potato, rye, tea and, coffee in four; and apples, mushrooms, oats and chocolate in three. Ballegaard *et al*^[36] have found sensitivity, using questionnaires, to vegetables (particularly onions and cabbage), fruits (apples, strawberries, and citrus fruits) and to meat (especially beef). Van den Bogaerde *et al*^[45] have shown in a case-control study using lymphocyte proliferation that, out of 31 CD patients, 16 reacted to cabbage and peanuts, 14 to cereals, 13 to milk, and nine to citrus fruits.

As observed by Hunter^[50], epidemiologists tend to look at statistical relationships that lead to studies of sugar, sweet, coke and chocolate intake because patients with CD eat and drink more of these substances than control subjects. Clinicians focus on the foods that patients associate with their symptoms and therefore avoid. As a result, exclusion diets have tended to concentrate more on dairy products, cereals and yeast. Other work is being carried out on polyunsaturated fatty acids, especially omega-3 oils, and their anti-inflammatory effects.

Current elemental and polymeric diets have a role to play in the management of CD, particularly in children. Exclusion diets are of use particularly for maintenance of remission. TPN is of value and has been shown to be as effective as elemental diets, but none have proven as effective as corticosteroid therapy. However, TPN remains a crucial method for administering nutrition in patients with severe disease, who are not able to tolerate enteral feeding.

Despite early ideas about the involvement of sugars in the etiology of CD, the omission of sugar has not been found to be of benefit. Omega oil has shown promising results, particularly in reducing inflammation in UC, and to a lesser degree, in CD.

CONCLUSION

IBD has a multifactorial etiology but food sensitivity/intolerance appears to play a role, and the culpable foods vary on an individual basis. Techniques to identify food intolerance require refining. Progress has been made by looking at factors such as IgG4 responses to food antigens, but a large expanse of work exists in trying to determine people's food sensitivities and the degree to which these affect disease activity. Without further research, it remains unclear whether dietary manipulation will continue to have a role solely in symptom control, or

Table 1 Reviewed studies looking at dietary exclusion and sensitivity to foods

Ref.	Trial type	No. of patients	Diets compared	Findings	Outcomes/measures	GRADE rating
Voitk <i>et al</i> ^[4]	Retrospective	13	Elemental diet	Patients with UC. Showed improved weight and positive nitrogen balance	Weight, nitrogen balance and nutritional state	1D
Borrelli <i>et al</i> ^[10]	Open RCT	38	Oral steroid <i>vs</i> polymeric feed only	Clinical remission comparable (67% steroid group, 79% polymeric group). Significantly improved histological and endoscopic scores in the polymeric group	Clinical remission, histological and endoscopic scores at 10 wk	1A
Riordan <i>et al</i> ^[13]	Multicentre double blinded RC	136	Exclusion diet <i>vs</i> steroid treatment	Patients were maintained in remission on exclusion diets	Hemoglobin, albumin, ESR, CRP, remission rates over 2 yr	1A
Jones <i>et al</i> ^[14]	Controlled trial	20	Unrefined carbohydrate rich diet <i>vs</i> exclusion diet	7 out of 10 on the exclusion diet remained in remission for 6 mo <i>vs</i> none of the other group	Clinical remission	
Pearson <i>et al</i> ^[16]	Prospective cohort	42	Food intolerances after remission induced with enteral feeding	20 patients identified food intolerances and eight did not. 14 did not complete the study	Food intolerances were found in CD but were variable and short-lived	2C
Greenberg <i>et al</i> ^[17]	RCT	51	TPN <i>vs</i> formula diet <i>via</i> NG <i>vs</i> partial parenteral and oral food	Clinical remission in 71% of parenteral group, 60% of partial parenteral group and 58% defined formula group	Relapse rates, weight, albumin, arm circumference, triceps skinfold thickness	1B
Jones <i>et al</i> ^[19]	Randomised	36	TPN <i>vs</i> elemental for induction of remission in CD	Both were successful with no significant differences. Elemental diet was cheaper, safer and simpler	CDAI, ESR and serum albumin	2B
Sanderson <i>et al</i> ^[20]	RCT	17 children	Elemental <i>via</i> NG for 6 wk <i>vs</i> high dose steroids	Elemental feed equally effective in children to high dose steroids. Linear growth better in elemental group over 6 mo	Lloyd-Still score, ESR, CRP, albumin, linear growth and body weight	2A
Wilschanski <i>et al</i> ^[29]	Retrospective	65	Nasogastric supplemental feeding	Continued nasogastric feeding post resumption of normal diet maintained remission for longer and showed improved linear growth	Relapse rate, linear growth	1D
Esaki <i>et al</i> ^[31]	Retrospective	145	Enteral <i>vs</i> non enteral (where enteral applies to elemental or polymeric feeds)	Enteral feeding showed a lower relapse rate than non-enteral	Rate of relapse based on CDAI scores	1D
Axelsson <i>et al</i> ^[33]	Cohort	34	Elemental diet for IBD refractory to improvement on high dose steroids	31 had been on high dose steroids prior to trial. 15 went into remission on elemental feed alone. 6 achieved remission with introduction or increase in prednisone dose	Serum iron, transferring, albumin, creatinine clearance, ESR, urea clearance, fecal volume, number of bowel movements	1B
Ballegaard <i>et al</i> ^[36]	Questionnaire	130		Sensitivity was commonly reported to vegetables (40%), fruit (28%), milk (27%), meat (25%) and bread (23%). No differences were found between the UC and CD groups		1C
Brandes <i>et al</i> ^[44]	RCT	20	Refined sugar rich <i>vs</i> refined sugar excluded	The sugar rich diet was stopped in 4 patients due to flare-ups. In those with mild disease there were no detrimental effects in either group	CDAI	2B
Van den Bogaerde <i>et al</i> ^[45]	Case-control study	31 CD, 22 controls	Peripheral lymphocytes were incubated with food and bacterial antigens	Lymphocyte proliferation to all food and bacterial antigens was higher in CD patients than controls	Lymphocyte proliferation	1C
Van den Bogaerde <i>et al</i> ^[46]	Case-control study	10 CD, 10 controls	Skin testing and rectal exposure to 6 food antigens	CD patients had significant in vivo and in vitro sensitization to food antigens which is gut specific	<i>In vivo</i> - rectal blood flow. <i>In vitro</i> - lymphocyte proliferation	1C

whether complete remission may be possible using these methods in combination with pharmacological agents.

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Celiac disease in Middle Eastern and North African countries: A new burden?

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festations are similar between both populations except for a high prevalence of short stature in some ME and NA countries. Few studies have addressed atypical or silent CD. As in the West, diagnosis is initially made by serological tests and is confirmed by small intestinal biopsies. Gluten-free diet is the main mode of treatment with a higher apparent adherence rate than in the West. Most disease complications result from malabsorption. The disease is strongly associated with HLA DQ2 and to a lesser extent with HLA DQ8 alleles. In conclusion, CD prevalence is underestimated, with little data available about its malignant complications. Disease parameters in the ME and NA are otherwise similar to those in Western countries.

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Key words: Celiac disease; Gluten-free diet; Insulin dependent diabetes mellitus; Iron deficiency anemia; Middle East

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Abstract

Celiac disease (CD) is now recognized as a common disorder among Middle Eastern (ME) and North African (NA) populations. The aim of this review is to assess the available data regarding CD in the ME and NA and to compare this information with that of Western countries. A literature review was performed using the electronic databases PubMed and Medline (1950-2008) as search engines, and "celiac disease" was used as a Mesh term. The search was limited to ME and NA countries. The prevalence of CD in ME and NA countries among low risk populations is similar to that of Western countries, but is higher in high risk populations such as those with type 1 diabetes. It is underestimated because of lack of clinical suspicion and lack of patient awareness. Clinical presentations in term of gastrointestinal, hematologic, skeletal, and liver mani-

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INTRODUCTION

Celiac disease (CD) is an autoimmune disorder which affects genetically predisposed individuals upon the ingestion of gluten. Its prevalence has been underestimated, but it is now considered one of the most common genetic disorders in the West with a prevalence of 1%-2.67%^[1-3].

In Middle Eastern (ME) and North African (NA) countries, the literature regarding CD has expanded signif-

icantly. The number of original articles published from the region during the last 30 years is about 120 with around 30, 29, 16, and 10 articles coming from Israel, Turkey, Tunisia, and Iran, respectively. Most studies are epidemiological. The true prevalence of the disease is underestimated and its clinical features have not been fully determined due to small sample size, selection bias, limited knowledge about CD and limited funds available for research.

The aims of this review of CD in ME and NA countries are to compare the disease parameters to those in Western countries, namely its epidemiology in low and high risk populations, its most common clinical presentations, the diagnostic tests used and their reported sensitivity and specificity. In addition, we determine the efficacy of the treatment modalities used, compliance with a gluten-free diet (GFD) and disease complications.

EPIDEMIOLOGY OF CD IN THE MIDDLE EAST AND NORTH AFRICA

Until the 1990s, the prevalence of CD in ME and NA countries was considered low. However, with the introduction of anti-endomysial antibodies (AEA) and anti-gliadin antibodies (AGA) testing, CD has been more readily reported from developing countries^[4], and its prevalence seems similar to that of Western countries^[4-7]. However, this prevalence varies from 0.14% to 1.17% in low risk, and from 2.4% to 44% in high risk populations (Tables 1 and 2). This difference is attributed to the heterogeneity of the studied populations, subject selection, diagnostic strategies used, and whether confirmatory biopsies were performed or not. For example, two studies from Tunisia reported a five-fold difference in CD prevalence, probably due to the use of different screening methods^[8,9].

CD in low risk populations

Prevalence of CD among low risk populations ranges from 0.14% to 1.3% as assessed by serological markers and from 0.033% to 1.17% assessed by biopsies (Table 1). The prevalence was found to be > 1% in two studies from Turkey^[5,6], 0.5%-1% in six reports from Turkey, Egypt, Iran, Tunisia and Israel^[7,8,10-13], and < 0.5% in five reports from Jordan, Lebanon, Tunisia, and Kuwait^[9,14-17].

Most studies screened healthy blood donors^[6,9-11,17], of whom young males represented > 70%. One study screening healthy individuals for CD in Iran, 50% of whom were females, reported a similar disease prevalence of 1% among both men and women^[7]. On the other hand, Green *et al*^[2] reported that the disease was 2 to 3 times more common in women. Another study from Tunisia on healthy blood donors, 30% of whom were females, reported a disease prevalence of 0.4% among women compared to 0.22% among men^[17]. Exclusion of subjects with iron deficiency anemia (IDA), abnormal liver function tests (LFTs) and the low percentage of participating females may have led to the underestimation of disease prevalence. In addition, low suspicion among physicians may have contributed to the low reported incidence of CD^[8,14]. Finally, studies restricted to patients pre-

sending with severe symptoms might have underestimated the true prevalence of the disease by missing asymptomatic and mildly symptomatic individuals^[15].

CD in high risk populations

High risk populations include patients with positive family history, insulin dependent diabetes mellitus (IDDM) and/or thyroiditis, and those with symptoms of malabsorption such as chronic diarrhea, refractory IDA and weight loss. Among these, the prevalence of CD ranges from 2.4% to 44% assessed by serological markers and biopsies (Table 2).

In Western countries, the prevalence of CD among patients with IDDM is 1%-12% assessed by serological markers and 1%-11% by biopsies^[1]. The disease is more common in children than adults with IDDM. In nine reports from ME and NA countries, the prevalence of CD among IDDM patients was 2.4%-20% assessed by serological markers and 2.4%-16.4% by biopsies (Table 2). Small sample size and a high rate of consanguinity may have contributed to the high prevalence. CD and IDDM share many genetic factors including HLA DR3-DQ2 and HLA DR4-DQ8 haplotype^[18,33].

CD is common in patients with autoimmune thyroid diseases. In Western countries a prevalence of 4%-5% is reported^[34,35], with an average of 1.5%-6.7% as assessed by serological markers and 3% by intestinal biopsies^[1]. The prevalence of CD among Tunisian patients with Grave's disease was 3.7% by serological markers and 2.5% by biopsies^[19]. An increased prevalence of autoimmune thyroid antibodies among CD patients on a GFD^[36] has also been reported. In Turkey, 5.9% of patients with autoimmune thyroiditis had positive CD serology^[20]. Autoimmune thyroid disease and CD share a common genetic background (HLA DQ2 and HLA DQ8)^[20].

Prevalence of CD in relatives

In the US, the prevalence of CD was found to be 4.5% and 2.5% in first and second-degree relatives of patients with the disease, respectively^[3]. The National Institutes of Health (NIH) estimates the prevalence of CD among first degree relatives to be 4%-12%, assessed by biopsy.

In two studies from Algeria and Turkey, the prevalence of CD in patients' first degree relatives was 3.4% and 1.7%, respectively^[21,33]. Among 381 first degree relatives, 26 had positive serology, and villous atrophy was present in 13 of 16 who had biopsies performed^[21]. Also, clustering of CD within families has been reported from Jordan and Algeria^[14,21]. There are no twin studies of CD in ME and NA countries. The high rate of consanguinity in these countries might contribute to a higher prevalence of CD and provides an opportunity for studying genotype-phenotype correlations.

CLINICAL PRESENTATION

The clinical presentation of CD varies from silent disease to full-fledged severe intestinal and extra-intestinal manifestations^[11,2,37]. A study comparing Turkish and US

Table 1 Prevalence and incidence of celiac disease in Middle Eastern and North African countries among low risk populations

Country	Population	Method	Confirmation by duodenal biopsy	Result
Central Anatolia, Turkey ^[5]	906 hospitalized adults	t-TG ¹	Yes	Prevalence 1%
Turkey ^[6]	2000 healthy blood donors	t-TG ^{1,2}	Yes (incomplete)	Prevalence by serology 1.3% and by histology 1.17%
Iran ^[7]	2799 healthy individuals	t-TG ¹ , AEA ¹	Yes	Prevalence 0.96%
Tunisia ^[8]	6286 school children	t-TG ¹ , AEA ¹	Yes (incomplete)	Prevalence 0.64%
Tunisia ^[9]	1418 healthy blood donors	AEA ¹	Yes	Prevalence 0.14%
Iran ^[10]	2000 healthy blood donors	AGA ¹ , AEA ¹	Yes	Prevalence 0.6%
Israel ^[11]	1571 healthy blood donors	AEA ¹ , t-TG	Yes (incomplete)	Prevalence 0.63%
Turkey ^[12]	1263 healthy school children	t-TG ¹	Yes (incomplete)	Prevalence by serology 0.87% and by histology 0.63%
Egypt ^[13]	1500 healthy children	t-TG ^{1,2} , AEA ¹	Yes	Prevalence 0.53%
Jordan ^[14]	494 000 children	No serology done	Yes	Incidence 1 in 2800 live births, point prevalence 7:100 000
Kuwait ^[15]	60 000 newborn over 5 years	NR	Yes	1 in 3000 births
Lebanon ^[16]	42 600 hospitalized children	AGA, AEA	Yes	5 per 1000 hospital admissions
Tunisia ^[17]	2500 healthy blood donors	AGA ^{1,2} , AEA ¹	No	Prevalence 0.3%

¹IgA; ²IgG. AEA: Anti-endomysial antibodies; AGA: Anti-gliadin antibodies; tTG: Tissue transglutaminase; NR: Not reported.

Table 2 Prevalence of celiac disease in Middle Eastern and North African countries among high risk populations

Country	Population	Method	Confirmation by duodenal biopsy	Prevalence (%)
Egypt ^[13]	150 children with diarrhea and failure to thrive	t-TG ^{1,2} , AEA ¹	Yes	4.7
	250 children with IDDM	t-TG ^{1,2} , AEA ¹	No	6.4
Saudi Arabia ^[18]	123 pts with IDDM	AGA ¹ , ARA ¹	Yes (incomplete)	4.9-8.1
Tunisia ^[19]	161 pts with Grave disease	AEA ¹ , t-TG ¹	Yes (incomplete)	3.7
Turkey ^[20]	136 pts with autoimmune thyroiditis	t-TG ¹	Yes (incomplete)	5.9
Algeria ^[21]	116 children with IDDM	AGA ^{1,2} , AEA ¹	Yes	16.4-20
Iran ^[22]	100 pts with chronic diarrhea	AGA ¹ , AEA ¹	Yes	19
Iran ^[23]	825 children with chronic diarrhea	AGA ¹ , AEA ¹	Yes	6.5
Iran ^[24]	250 pts with IDDM	Total IgA, AEA ¹	Yes	2.4
North-Eastern Libya ^[25]	243 pts with high clinical suspicion	NR	Yes	31.7
Eastern Saudi Arabia ^[26]	145 pts with high clinical suspicion	AEA ¹ , ARA ¹	Yes	4-11
Egypt ^[27]	25 pts with refractory iron deficiency anemia	AGA ² , AEA ¹ , t-TG ² , ARA	Yes	44
Turkey ^[28]	100 pts with IDDM	AEA ¹	Yes	6
Iraq ^[29]	40 pts with IDDM	NR	Yes	15
Libya ^[30]	234 pts with IDDM	AGA, t-TG, ARA, AEA	Yes	10.3
Tunisia ^[31]	348 pts with IDDM	AEA ¹ , t-TG	Yes	2.6-4
Turkey ^[32]	122 pts with IDDM	Total IgA, AEA ¹	Yes	2.45

¹IgA; ²IgG. IDDM: Insulin dependent diabetes mellitus.

patients found that the former presented mostly with diarrhea and anemia and the latter with atypical symptoms such as fatigue, abdominal pain and bloating^[38]. Whereas some reports from Turkey, Jordan and Iran address silent and atypical CD^[5,14,39], little is known about the prevalence and the clinical, serologic and histopathologic features of patients with atypical or silent CD in this region, the so-called celiac iceberg.

GI manifestations

GI complaints are the most common presenting symptoms. They include diarrhea, abdominal pain, constipation, bloating, flatulence, nausea and vomiting. Lo *et al*^[40] report a drop in the percentage of CD patients presenting initially with diarrhea to 43%, compared to 73% before 1993.

Among six studies assessing chronic diarrhea in ME and NA countries, CD prevalence was 6.5%-21%^[22,23,41-44]. In Iran, Lebanon, Iraq, Saudi Arabia and Kuwait, CD was one of the most common causes of chronic diar-

rhea^[22,41-44]. In Egypt, 4.7% of children presenting with diarrhea and failure to thrive had CD^[13].

The reported prevalence of GI manifestations has varied widely among different studies (Table 3). This may be due to the low number of patients evaluated or a delay in their presentation. For example, al-Hassany^[45] reported 10 cases with advanced CD who all had diarrhea, abdominal distension and weight loss. Diarrhea and abdominal distension were significantly more common in younger children, whereas abdominal pain, failure to thrive and growth retardation were more common among those who were older. This is attributed to the predominance of classical CD among younger children versus atypical CD seen among older children^[14,46].

About one third of children with CD in Western countries develop short stature^[50]. In ME and NA countries, short stature was discovered to be the presenting symptom in 7.7% to 53% of patients. The highest prevalence of short stature was reported from Jordan where 26% of children with CD had rickets. In Turkey, 51% of patients had a

Table 3 Clinical presentation of celiac disease in the Middle East and North Africa

Country	Population	Abdominal distention/ flatulence (%)	Diarrhea (%)	Short stature (%)	Weight loss/failure to grow (%)	Abdominal pain (%)
Iran ^[7]	29 adults with CD detected by screening	55.5	22.2	NR	NR	18.5
Jordan ^[14]	34 pts with CD	44.0	65.0	53.0	NR	44.0
Kuwait ^[15]	20 children with CD	80.0	100.0	25.0 (rickets)	100.0	NR
Lebanon ^[16]	65 children with CD	26.2	60.0	NR	49.2	NR
Turkey ^[33]	60 Adults with CD	48.3	66.7	NR	50.0	NR
Iran ^[39]	52 pts with CD	32.7	48.1	23.1	78.8	NR
Iraq ^[45]	10 children with CD	100.0	100.0	NR	100.0	NR
Turkey ^[46]	104 children with CD	60.6	81.7	45.2	44.0-50.0	23.0
Turkey ^[47]	45 children with CD	NR	40.0	51.0	NR	NR
Libya ^[25]	77 children with suspected CD	49.0	53.0	23.0	100.0	15.0
Saudi Arabia ^[26]	10 children with suspected CD	NR	50.0	10.0	30.0	30.0
Egypt ^[27]	11 pts with anemia and CD	18.2	45.5	45.5	57.1	63.6
Saudi Arabia ^[48]	16 adults with CD	NR	37.0	18.5	NR	18.5
Libya ^[49]	39 pts with with CD	61.5	59.0	7.7	82.0	20.5

CD: Celiac disease.

height < 2.5 standard deviations below the mean^[47].

Many CD patients are initially diagnosed as having irritable bowel syndrome (IBS). Green *et al*^[51] reported that up to 36% of American patients with CD were previously diagnosed with IBS. In Iran, 12% of IBS labeled patients turned out to have CD^[39].

Hematological manifestations

The prevalence of anemia at the time of CD diagnosis is 12%-69%^[52]. IDA is the most common form and may be the only finding in 45% of patients with sub-clinical CD^[53]. Worldwide prevalence of CD among patients with IDA is 2.8%-8.7% and may be as high as 15%^[54,55]. Folate and B12 deficiency may contribute to anemia in CD, and surprisingly, anemia of chronic disease is relatively common^[52]. In ME and NA countries, anemia occurs in 20%-80% of CD patients and the majority of cases are attributable to iron deficiency^[14,15,22,24-26,33,45-47]. An Egyptian study found that 4% of young IDDM patients with anemia had CD^[56]. Another paper reports a CD prevalence of 44% among 25 Egyptian patients evaluated for refractory IDA^[27].

Osteoporosis

The prevalence of osteopenia and osteoporosis in CD patients is 30%-50% and 3.4%-14%, respectively^[57,58]. In ME and NA countries, 5 studies of CD patients reported a prevalence of hypocalcemia of 3.3%-27.4%^[33,45,46] and of osteoporosis of 13.5%-16.7%^[33,39]. In Saudi Arabia, osteomalacia and IDA were the most common presenting symptoms, accounting for 43.5% of the clinical presentations^[48]. In Tunisian children with CD diagnosed by screening, osteopenia prevalence was 34.7%^[8]. Delay in the diagnosis of CD may account for the high prevalence of osteoporosis^[33].

Abnormal LFTs

Hypertransaminasemia is an early manifestation of liver involvement in CD. Five to 10% of patients with elevated serum aminotransferases end up being diagnosed with

CD^[59]. Liver biopsy may reveal lesions ranging from reactive hepatitis to cirrhosis, which may be partially or totally reversed with a GFD. In ME and NA countries, few studies have reported abnormal LFTs among patients with CD^[21,46]. A Turkish study reported increased transaminases and hypoproteinemia in 38.3% and 4% of CD patients, respectively^[46]. The latter may be attributed to protein-losing enteropathy and/or decreased hepatic synthetic activity^[46]. An Iranian group reported increased transaminases among 25% of CD patients^[22]. Transaminases levels normalized in all patients, except those with cirrhosis, on a GFD^[22,46].

Prevalence of autoimmune diseases among patients with CD

About 30% of patients with CD have other autoimmune disorders such as IDDM and autoimmune thyroiditis^[60,61]. In ME and NA countries, the prevalence of autoimmune diseases among CD patients was demonstrated to be as low as 1.9% in Turkish patients^[46] and as high as 33% in Iranian patients^[28]. Many of these patients were diagnosed with CD after substantial delays^[39]. The prevalence of IDDM in CD patients is 6.7%-18.5% and affected patients are typically older than those with IDDM alone^[33,39,48]. They are mostly females with longer duration of diabetes^[24]. Autoimmune diseases are more common in patients with IDDM and CD than in those with CD or IDDM alone^[28,33,62]. In a Turkish study, adult patients with IDDM and CD had a 33.33% prevalence of other autoimmune diseases such as autoimmune thyroiditis^[28]. This may be an overestimation due to small sample size. Importantly, a French study demonstrated a reduction in the development of autoimmune diseases among CD patients adherent to a GFD compared to those not adherent to such a diet^[63]. This highlights a major role of early diagnosis and therapy of CD in order to reduce the burden of autoimmunity.

DIAGNOSIS

The diagnosis of CD is challenging and requires a high

Table 4 Serological diagnosis of celiac disease in Middle Eastern and North African countries

Country	Population	AGA (%)		AEA (%)		t-TG Ab (%)	
		Sensitivity	Specificity	Sensitivity	Specificity	Sensitivity	Specificity
Turkey ^[6]	2000 healthy individuals	NR	NR	NR	NR	90.0	NR
Iran ^[7]	2799 healthy individuals	NR	NR	19.0	100.0	100.0	99.0
Israel ^[11]	1571 healthy blood donors	NR	NR	20.0	100.0	50.0-60.0	25.0-38.0
Tunisia ^[17]	2500 healthy blood donors	100.0	84.0	NR	NR	NR	NR
Algeria ^[39]	116 children with IDDM	IgA AGA, IgG AGA, and IgA AEA					
		Sensitivity 80.0		Specificity 100.0			
Turkey ^[46]	104 children with CD	76.0 (IgA), 94.0 (IgG)		90.0	NR	NR	NR
Saudi Arabia ^[26]	145 high risk patients	NR		NR	100.0	NR	NR
Egypt ^[27]	11 children with CD	100.0		81.8	21.4	72.7	85.7
Israel ^[68]	39 children with CD and 22 healthy children	NR		100.0	100.0	NR	NR

¹Sensitivity and specificity of all tests simultaneously.

level of suspicion. All screening algorithms start with serological tests and include testing for IgA and IgG anti-gliadin antibodies (AGA), IgA and IgG anti-endomysial antibodies (AEA), IgA and IgG tissue transglutaminase (tTG) and a new generation of anti-gliadin antibodies to deamidated synthetic gliadin peptides (DGP)^[64]. The AEA test is currently the gold standard because of its high sensitivity and almost 100% specificity^[1,65]. Furthermore, high levels of both AEA and tTG have very high sensitivity and specificity^[66]. Small intestinal biopsy remains the gold standard diagnostic test^[1,67].

Diagnosis of CD in ME and developing countries

In 24 out of 28 studies of prevalence of CD in ME and NA countries, patients were initially screened with serological tests. In those testing positive, confirmation of the diagnosis by duodenal biopsy was performed in the majority of cases. In 7 out of 24 studies, confirmation by biopsy was incomplete (Tables 1 and 2). In 4 studies, intestinal biopsies were the initial mode of diagnosis because of the unavailability of serological tests^[14,15,25,29]. Serological marker sensitivity and specificity varied widely because of difference in the choice of gold standard, bias in patient selection, population differences and methodology used, including number of biopsies (Table 4). AEA sensitivity ranges from 20%^[7,11] to > 90%^[17,46,68], with a specificity reaching 100%^[7,11,26,68]. The low AEA sensitivity reported from 2 studies conducted in Iran and Israel is attributed to improper intestinal mucosal sampling, the low power of AEA-immunofluorescence in detecting early CD, and the poor performance of the AEA in the presence of milder degrees of villous atrophy^[11,69]. These factors may have resulted in missing many cases of CD^[7,11]. tTG IgA sensitivity is 70%-100%^[6,7,57], and specificity is 99%^[7]. The sensitivity of IgA AGA is about 80%, while that of IgG AGA is 90%-100%^[17,27,46]. AEA testing was used in 19 out of 24 studies, while t-TGA and AGA testing were used in 12 and 9 studies, respectively (Tables 1 and 2). While the serological tests have shown high sensitivity and specificity for the diagnosis of CD, there are problems including

differences in test kit sensitivity and specificity^[70], as well as an apparent lower performance in the clinical setting compared to research laboratories^[71].

The Marsh classification is a histologic grading system that reflects the varying degrees of intestinal mucosal villous atrophy and inflammatory changes that occur in patients with CD^[1]. Marsh classification is used by almost all investigators in the histological diagnosis and classification of CD severity.

TREATMENT

A GFD remains the mainstay of CD treatment. Adherence to a GFD in Western countries is reported to be less than 50%^[72]. Treatment with a GFD results in improvement of many clinical and serological parameters^[1,73]. Also, adherence to a GFD for five consecutive years or more significantly reduces the incidence of malignancies such as cancer of the mouth, pharynx, esophagus and lymphoma^[74]. Moreover, adherence to a GFD has a protective effect on the development of many autoimmune diseases such as IDDM, inflammatory bowel diseases, hepatitis and hematologic disorders^[63].

Adherence to a GFD in ME and NA countries ranges from 50% to 100%^[10,14,15,18,22,23,39,59,62]. The vast majority of adherent patients have good response ranging from 60% to 100%^[10,14-16,18,22,23,28,39,48,49]. Moreover, patients' level of compliance and response were used interchangeably in some studies^[14,15,48]. The higher level of adherence to a GFD in comparison to Western countries may be due to the limited number of patients studied and the use of less rigorous parameters to assess adherence.

The reasons for poor compliance are not clear. Wheat and barley are major diet constituents with few acceptable alternatives^[22], rendering the convincing of parents that bread is the cause of diarrhea very hard^[15]. Also, convincing patients with atypical CD to adhere to a GFD is difficult^[4]. Finally, lack of information about CD manifestations, lack of benefit from a GFD and lack of encouragement to adhere to such a diet may contribute. More than 10% of adults with CD do not adhere strictly

to long term GFD and more than 30% who believe they are, are actually consuming grams of gluten daily^[4]. Diabetic patients adhering to a GFD will have fewer episodes of hypoglycemia and better diabetes control^[26]. Demir *et al.*^[46] report improvement in growth velocity and rapid catch up of height and weight in children with CD who adhere to a GFD. Moreover, adherence to a GFD resulted in improvement of hepatic histopathological changes. In addition to a GFD, supplementation of calcium, vitamin D and iron may accelerate normalization of serological markers and reduce the rate of fractures and complications resulting from anemia^[39].

There are no reports regarding refractory CD or its treatment from ME or NA countries. In addition, no trials of immunosuppressive or immune-modulator drugs have been described. Currently, both a permeability blocker and oral enzyme preparations are in clinical trials in the US and Europe^[75-77]. While the safety and effectiveness of these potential therapies need to be determined, the financial burden of the addition of drugs to a GFD may be prohibitive in developing countries.

COMPLICATIONS

Complications of CD range from malabsorption to cerebellar ataxia, dilated cardiomyopathy^[78], infertility, lymphoma, as well as oropharyngeal, gastrointestinal and thyroid malignancies^[45,79]. Metabolic bone disease, malignancies and autoimmune conditions may develop if treatment is delayed^[80].

In the ME and developing countries, most complications result from malabsorption. Mortality in hospitalized patients with CD is relatively increased in the first 3 years after diagnosis, particularly in patients with malabsorption, those with delayed diagnosis, and those poorly adherent to a GFD^[33]. Prolonged INR due to Vit K malabsorption is found in 25% of patients and improves on a GFD^[81]. However, low levels of serum cholesterol, HDL, LDL and phospholipids in 46 Algerian patients with CD did not improve on a GFD^[82]. Hypocalcemia and hypovitaminosis D contribute to osteoporosis which occurs in 16.7%^[43] of CD patients, similar to what is reported in Western countries^[57,83]. There have been case reports of hepatic vein thrombosis in North African subjects with CD^[84,85].

A mortality rate of 3.3% was reported in 60 Turkish patients with CD, mainly due to malignancies^[33]. In Turkey and Iran, non-Hodgkin lymphomas are the most common CD-associated malignancies^[33,39]. Similar to Western countries, there is an increased prevalence of gastrointestinal malignancies and of non-Hodgkin lymphoma in CD patients. Oropharyngeal malignancies, as well as neurological, cardiac and cutaneous complications of CD, have been described in Western countries^[1,2,37,78,79], but have not been well documented in ME countries. Left ventricular subclinical systolic dysfunction has been reported in Turkish children with CD, with a negative correlation between myocardial systolic wave

and serum IgA AEA level^[86].

Mortality rate among hospitalized patients with CD in Turkey was found to be increased 2-fold^[33], although it is not clear what the control group was in this study. While increased mortality in CD patients has been well documented in the West using standardized mortality ratio (SMR)^[1], which is the ratio of observed deaths in patients with celiac disease to expected deaths on the basis of age- and sex-specific rates in the region under study, no such data has been published from the Middle East.

GENETICS

Most CD patients have HLA DQ2 or HLA DQ8 alleles, which account for 40% of the total genetic predisposition to CD^[1,2]. Moreover, HLA DQ2 and DQ8 are expressed in 30% of the general population, suggesting the presence of additional factors for CD development. One such factor is *CTLA-4* gene polymorphism, a non HLA gene thought to regulate T-cell immune function^[87]. As in the West, CD is found in ME and NA countries to be strongly associated with HLA DQ2 (DQA1*0501 and DQB1*0201)^[88-93]. HLA DQ8 (DQA1*0301 and DQB1*0302) is less strongly associated with CD^[92]. *HLA B8*, a gene expressed in MHC I antigen presenting cells, is found to be associated with CD in Algeria^[89], Iraq^[94,95] and Turkey^[91]. Carriers of this gene are at increased risk of developing CD^[91]. In addition, Saharawi patients with atypical CD were found to over-express the MHC class I chain-related gene A (MICA) allele 5.1^[89]. These associations have been reported in Western countries^[96]. Increased prevalence of HLA-A25(10) in Turkish children with CD is reported, suggesting that this genotype is particularly encountered among this population^[91]. No such association has been described in Western countries.

CONCLUSION

The prevalence of CD disease in ME and NA countries may be underestimated due to lack of awareness and low suspicion of the disease. Whether mass screening for the disease should be done in high risk populations, such as patients with short stature, chronic diarrhea, IDA, IDDM, and those with a positive family history is not clear. The best screening modality is not yet determined, though tTG IgA is probably the most economic.

Large prospective studies are needed to assess the true incidence, the clinical course, the efficacy of treatment modalities employed, patient compliance, disease complications and response to treatment in ME and NA countries. The association of CD with other autoimmune diseases and the presence of specific genetic markers would be areas of interesting future research. The high rate of consanguinity among people of the same ethnic background in this part of the world might provide an opportunity for establishing genotype-phenotype correlations in CD.

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Effects of *Haobie Yangyin Ruanjian* Decoction on hepatic fibrosis induced by carbon tetrachloride in rats

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Abstract

AIM: To explore the anti-fibrotic effect of *Haobie Yangyin Ruanjian* Decoction (HYRD) on CCl₄-induced hepatic fibrosis in rats and its modulation on the transforming growth factor (TGF) β -Smad signaling pathway.

METHODS: Fifty-six healthy Wistar rats were randomly divided into five groups: normal control group ($n = 6$), CCl₄-induced hepatic fibrosis group ($n = 14$) and three treatment groups (the treated rats received HYRD *via* oral administration at daily dosages of 8.2, 2.5 and 0.82 g/kg, respectively) of HYRD ($n = 12$, respectively). Experimental hepatic fibrosis was induced by subcutaneous injection of carbon tetrachloride solution (CCl₄ dissolved in peanut oil, 4:6, V/V) with 0.5 mL/100 g body weight for the first time, and then 0.3 mL/100 g body weight twice a week for 8 wk. In the former 2 wk, rats were raised by feedstuff I (80% corn meal, 20% lard, 0.5% cholesterol). After 2 wk, they were raised by feedstuff II (corn meal and 0.5% cholesterol). Except for the control group, 30% alcohol solution was given orally to each rat every other day from the beginning, 1 mL for each rat. Liver function

parameters and hepatic hydroxyproline content were detected by chromatometry. Serum levels of hyaluronic acid (HA), type IV collagen (CIV), type III procollagen (PCIII) and laminin (LN) were assayed with radioimmunoassay. Deposition of collagen was observed with hematoxylin-eosin staining and collagen staining. Gene expression of *TGF β 1* and *Smad3* were detected with real-time reverse transcriptase-polymerase chain reaction and Western blotting, respectively.

RESULTS: The serum levels of alanine transaminase and aspartate transaminase were increased in the model group compared with the control group ($P < 0.01$), and they were decreased in the three treatment groups compared with the model group. The serum levels of total protein and albumin were decreased in the model group and increased in the three treatment groups. The hepatic hydroxyproline content and serum levels of PCIII, HA, LN and CIV were markedly increased in the model group compared with the control group, and decreased in the treatment groups. The gene expression of *TGF β 1* and *Smad3* was enhanced in the model group compared with the control group, and HYRD could down regulate their expression.

CONCLUSION: HYRD can inhibit hepatic fibrosis induced by CCl₄ in rats, which is probably associated with its down-regulation on fibrogenic signal transduction of TGF β -Smad pathway.

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Key words: *Haobie Yangyin Ruanjian* Decoction; Hepatic fibrosis; Transforming growth factor β -Smad signaling; Rat model; Carbon tetrachloride

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INTRODUCTION

A variety of pathological factors, including viral hepatitis (especially hepatitis B and C), alcohol and drug abuse, metabolic diseases due to overload of iron or copper, autoimmunity against hepatocytes or bile duct epithelium, and congenital abnormalities can cause hepatic injury. Lifestyle changes (mainly exercise withdrawal and weight gain) have probably raised the prevalence of non-alcoholic fatty liver disease (NAFLD), which is the first cause of chronic liver disease in Western world. All these chronic hepatic diseases can cause hepatic fibrosis (HF)^[1]. If hepatic fibrosis treatment was delayed, hepatic cirrhosis would be developed. Hepatic fibrosis, cirrhosis in particular, is associated with significant morbidity and mortality. Hepatic fibrosis is characterized by imbalance between extracellular matrix synthesis and degradation. Extracellular matrix mainly results from hepatic stellate cells (HSCs) which can be transformed into myofibroblast initially. Transforming growth factor (TGF) β -Smad signal pathway plays an important role in this process^[2], it can activate HSCs and promote collagen synthesis^[3]. Research has shown TGF β 1 is a key cytokine in determining fatty liver and non-alcoholic steatohepatitis (NASH)^[4]. Therefore, TGF β -Smad signal pathway has become a main target in hepatic fibrosis treatment^[5]. Although new therapeutic approaches have recently been proposed, there is no established therapy for hepatic fibrosis. *Haobie Yangyin Ruanjian* Decoction (HYRD) is a kind of traditional Chinese Medicine. The aim of the present study was to investigate its protective effects and mechanism in a rat model of CCl₄-induced hepatic fibrosis.

MATERIALS AND METHODS

Composition of HYRD

The composition of HYRD include *Herba Artemisiae Annuae*, *Carapax Trionycis*, *Salviae Miltiorrhizae*, *Rhizoma Polygoni Cuspidati*, *Radix Curcumae*.

Animals and experiment protocol

Fifty-six healthy Wistar rats, female and male, weighing 237.8 ± 8.5 g were obtained from the Experimental Animal Center of Academy of Medical Science of Chinese People's Liberation Army (Beijing, China). Animal certificate: SCXK-(Army)2007-004. The rats were randomly divided into normal control group ($n = 6$), model group ($n = 14$), and three treatment groups ($n = 12$, respectively). Except for the normal control group, all the rats were subcutaneously injected with solution of carbon tetrachloride dissolved in peanut oil (CCl₄: peanut oil = 4:6, V/V), 0.5 mL/100 g body weight for the first time, and then 0.3 mL/100 g body weight twice a week for

8 wk. In the former 2 wk, rats were raised with feedstuff I (80% corn meal, 20% lard, 0.5% cholesterol). After 2 wk, they were raised with feedstuff II (corn meal and 0.5% cholesterol). Except for the normal control group, 30% alcohol solution was given orally to each rat every other day from the beginning, 1 mL for each rat. For the normal control group, the peanut oil was injected to each rat subcutaneously.

The rats in the three treatment groups, including a high-dose group (8.2 g/kg per day, calculated as Decoction), a medium-dose group (2.5 g/kg per day), and a low-dose group (0.82 g/kg per day), were given HYRD daily *via* oral administration, 1 mL/100 g body weight. Except for the dead, all the rats were anesthetized with ether. Blood was taken from the abdominal vein, centrifuged at 4°C, 3000 r/min, for 20 min, and serum was kept at -20°C for assay.

Liver laboratory tests

Serum levels of alanine transaminase (ALT), aspartate transaminase (AST), albumin (Alb) and total protein (TP) were measured using commercially available kits (Jiancheng Inst. Biotechnology, Nanjing, China) according to the manufacturer's instructions.

Hepatic hydroxyproline content

Liver tissue (100 mg) was prepared for hydroxyproline (Hyp) determination according to a modified method by Jamall *et al.*^[6]. The Hyp content of the liver as an indirect measure of tissue collagen content was expressed as milligram per gram of dry weight (mg/g).

Serum levels of hyaluronic acid, type IV collagen, type III procollagen and laminin

Serum levels of hyaluronic acid (HA), type IV collagen (CIV), type III procollagen (PCIII) and laminin (LN) were determined by radioimmunoassay (RIA) using commercially available kits (Beifang Inst. Biotechnology, Beijing, China) according to the manufacturer's instructions.

Histological examination

Liver tissues were taken from the left lobe of the liver of each rat, and fixed in 15% buffered paraformaldehyde, and dehydrated in a graded alcohol series. Specimens were embedded in paraffin blocks, cut into 5 μ m-thick sections and placed on glass slides. The sections were stained with hematoxylin-eosin (HE) and ponceau's^[7], respectively. Fibrosis was graded according to the method of Scheuer^[8] as follows: stage 0: no fibrosis; stage 1: an increase of collagen without the formation of septa (small satellite expansion of the portal fields), expansion of portal tracts without linkage; stage 2: formation of incomplete septa not interconnecting with each other, from the portal tract to the central vein; stage 3: complete but thin septa interconnecting with each other, which divide the parenchyma into separate fragments; and stage 4: complete cirrhosis, similar to stage 3 with thicker septa. Pathological examination was performed by the same pathologist who was blinded to the treatment assignment for the rats.

Determination of TGF- β 1 mRNA level in liver tissue by real-time reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA was extracted from liver tissues of each group with the tissue/cell total RNA isolation kit (Trizol Reagent, Invitrogen, USA) according to the manufacturer's protocol. The quantity and purity of RNA were detected by determining absorbance at 260/280 nm using a spectrophotometer (BECKMAN COULTER Co., USA). Total RNA was reversibly transcribed into complementary DNA (cDNA) using the cDNA synthesis kit [TaKaRa RNA PCR Kit(AMV)Ver 3.0, Dalian, China] according to the manufacturer's protocol. The ABI PRISM 7900 HT Real Time PCR System (ABI Co., USA) and real-time PCR kit (2 \times SYBGreen RT-PCR Master Mix, UCL, USA) were employed based on the manufacturer's instructions. The specific primers for the target gene and β -actin were synthesized by Dalian TaKaRa Biotechnology Company. TGF- β 1: 5'-TGGCGTTACCTTGGTAACC-3' (forward); 5'-GGTGTGAGCCCTTTCCAG-3' (reverse). β -actin: 5'-ACCCCTTAAGGCCAACCCTGAAAAG-3' (forward); 5'-TCATGAGGTAGTCTGTCAGGT-3' (reverse).

Two-step PCR procedure was used as follows: pre-denaturation for 30 s at 95°C, 1 cycle; 94°C for 15 s and 56°C for 40 s, 40 cycles. The final products were identified by electrophoresis in 1.5% agarose gel and melt curve analysis. Melt curve detection: 95°C 15 s, 60°C 15 s and 95°C 15 s. The final results were described with the relative values ($2^{-\Delta\Delta C_t}$). The calculation and analysis were performed by the Sequence Detection Software version 2.1 in the ABI PRISM 7900 HT Real Time PCR System (ABI Co., USA).

Determination of Smad3 level in liver tissue by Western blotting

Total protein was extracted from liver tissue and analyzed with bicinchoninic acid (BCA) protein concentration assay kit (Beyotime Inst. Biotechnology, Jiangsu, China). Sample protein was separated by electrophoresis in 12% SDS-PAGE separating gel with Bio-Rad electrophoresis system (Bio Rad Laboratories, Hercules, CA, USA). The primary antibodies (rabbit anti Smad3 antibody, 1:1000 dilution, MILLIPORE Inc., USA) were incubated at 4°C overnight. The corresponding horseradish peroxidase-conjugated secondary antibodies (anti-rabbit IgG, 1:5000 dilution, Zhongshanjinqiao Biotechnology Inc., China) were incubated at room temperature. Immobilon™ Western Chemiluminescent HRP Substrate (MILLIPORE Inc., USA) and Quantity ONE (BIO-RAD) were employed for revealing and quantitative analysis of the blots. β -actin protein was used as the internal control.

Statistical analysis

All values were expressed as mean \pm SD. Comparisons were analyzed by one-way ANOVA using the SPSS 12.0 statistical package. Differences were considered statistically significant if the $P < 0.05$.

Table 1 Effect of HYRD on serum levels of ALT, AST, TP and Alb (mean \pm SD)

Groups	ALT (U/L)	AST (U/L)	TP (g/L)	Alb (g/L)
Control	20.94 \pm 8.76 ^b	26.11 \pm 11.81 ^b	76.9 \pm 13.1 ^a	36.3 \pm 5.2 ^b
Model	62.40 \pm 25.59	99.93 \pm 38.76	45.8 \pm 14.8	24.3 \pm 5.3
Low-dosage HYRD group	47.92 \pm 19.65	70.72 \pm 28.53 ^a	53.2 \pm 23.5	28.0 \pm 7.4
Medium-dosage HYRD group	44.36 \pm 20.50 ^a	74.06 \pm 25.14 ^a	58.8 \pm 24.9	30.6 \pm 7.5 ^a
High-dosage HYRD group	34.29 \pm 11.42 ^b	49.95 \pm 19.47 ^b	67.1 \pm 20.2 ^a	33.0 \pm 5.6 ^b

^a $P < 0.05$, ^b $P < 0.01$ vs model group. HYRD: Haobie Yangyin Ruanjian Decoction; ALT: Alanine transaminase; AST: Aspartate transaminase; TP: Total protein; Alb: Albumin.

Table 2 Effect of HYRD on hepatic hydroxyproline content (mean \pm SD)

Groups	Hydroxyproline(mg/g dry weight)
Control	0.18 \pm 0.13 ^b
Model	2.39 \pm 0.28
Low-dosage HYRD group	1.89 \pm 0.99
Medium-dosage HYRD group	1.29 \pm 0.56 ^b
High-dosage HYRD group	1.22 \pm 0.45 ^b

^b $P < 0.01$ vs model group.

RESULTS

Effect of HYRD on liver function

The serum levels of ALT and AST were increased in the model group compared with the control group ($P < 0.01$). Compared with the model group, the serum levels of ALT and AST were decreased in the three treatment groups. The serum levels of TP and Alb were decreased in the model group compared with the control group. Compared with the model group, the serum levels of TP and Alb were increased in the treatment groups (Table 1).

Effect of HYRD on hepatic hydroxyproline content

Hepatic hydroxyproline content was markedly increased in the model group compared with the control group ($P < 0.01$). Compared with the model group, the levels of hydroxyproline were significantly decreased in the treatment groups (Table 2).

Effect of HYRD on serum levels of CIV, PCIII, HA and LN

The serum levels of PCIII, HA, LN and CIV were increased in the model group compared with the control group. Compared with the model group, the serum levels of PCIII, HA, LN and CIV were decreased in the treatment groups (Table 3).

Effect of HYRD on hepatic histopathological changes

At the end of the study, normal hepatic lobules, without fibroplasia and inflammatory cell infiltration could be

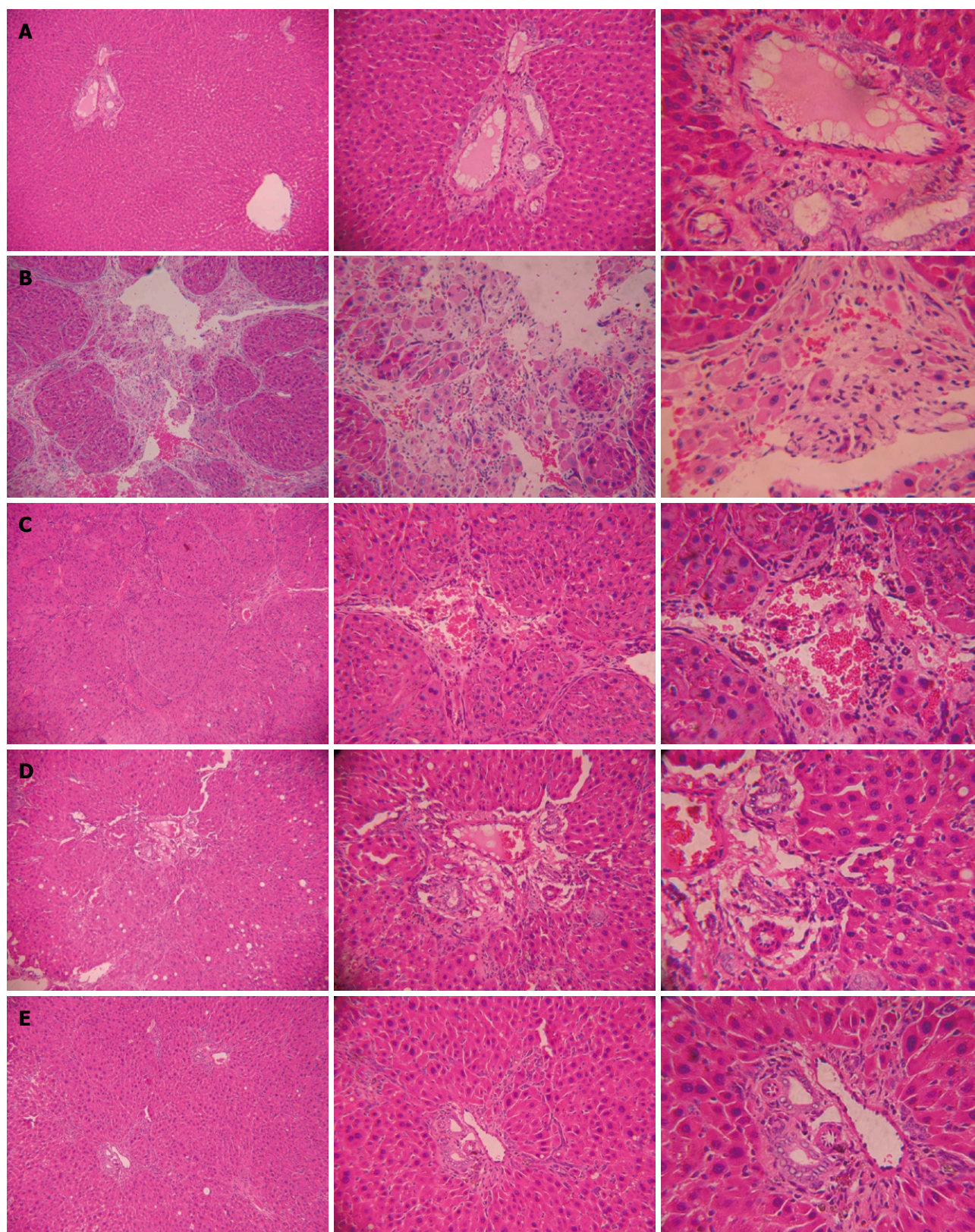


Figure 1 Histological profiles of liver tissues in rats. A: Normal rats; B: Rats with hepatic fibrosis; C: Low-dosage of *Haobie Yangyin Ruanjian* Decoction (HYRD)-treated rats; D: Medium-dosage of HYRD-treated rats; E: High-dosage of HYRD-treated rats. Left: Low-power magnification, $\times 40$; Middle: Middle-power magnification, $\times 100$; Right: High-power magnification, $\times 200$.

observed in normal rats (Figure 1A). Complete septa interconnecting with each other were formed, which divided the parenchyma into separate fragments and a great number of inflammatory cells were infiltrated

in intralobules and interlobules, cell degeneration and focal necrosis were found in rats with hepatic fibrosis (Figure 1B), which were improved after HYRD treatment (Figure 1C-E).

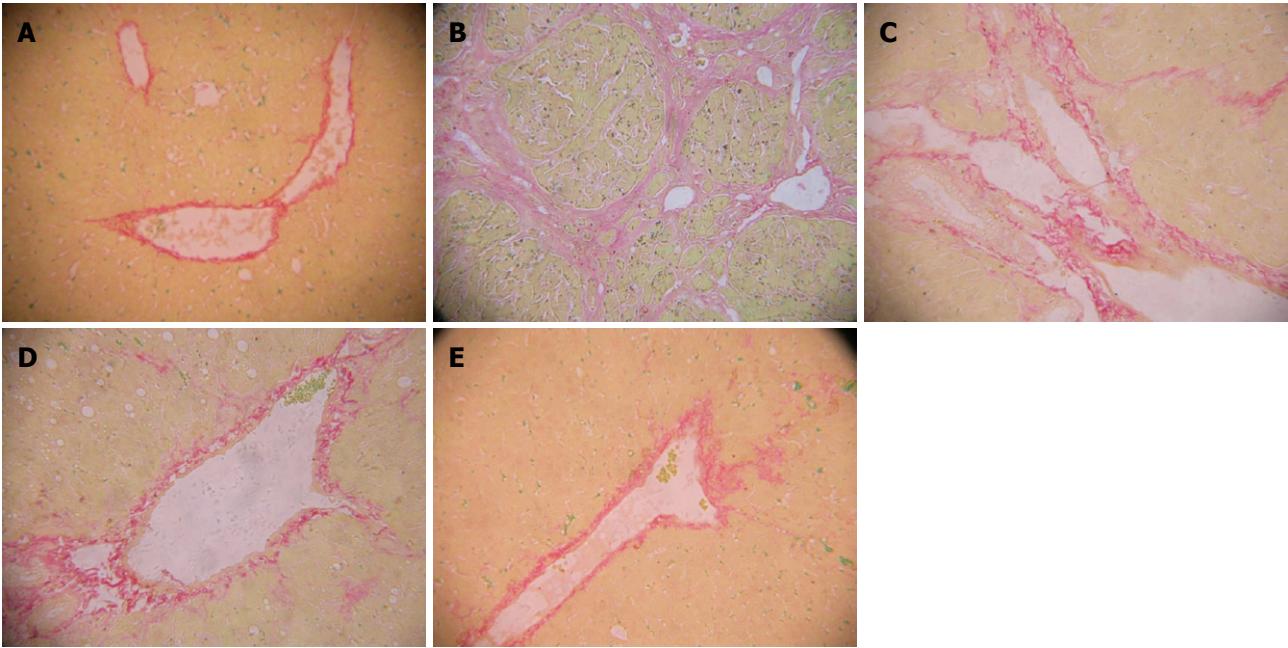


Figure 2 Profiles of liver tissues in rats. A: Normal rats; B: Rats with hepatic fibrosis; C: Low-dosage of HYRD-treated rats; D: Medium-dosage of HYRD-treated rats; E: High-dosage of HYRD-treated rats. Stained with ponceau's, $\times 100$.

Table 3 Effect of HYRD on serum levels of PCⅢ, HA, LN and CIV (mean \pm SD)				
Groups	PCⅢ (μ g/mL)	HA (ng/mL)	LN (ng/mL)	CIV (ng/mL)
Control	15.16 \pm 15.12 ^b	205.30 \pm 48.92 ^a	82.02 \pm 8.86	21.71 \pm 1.76
Model	35.73 \pm 17.90	563.82 \pm 335.54	89.57 \pm 7.59	29.20 \pm 6.17
Low-dosage HYRD group	27.87 \pm 10.13	464.19 \pm 283.41	79.86 \pm 9.52	26.38 \pm 8.61
Medium-dosage HYRD group	24.72 \pm 10.87 ^a	256.46 \pm 95.98 ^b	86.34 \pm 8.30	24.41 \pm 4.46
High-dosage HYRD group	20.34 \pm 13.92 ^b	161.51 \pm 48.29 ^b	83.26 \pm 13.20	26.22 \pm 7.97

^a $P < 0.05$, ^b $P < 0.01$ *vs* model group. PCⅢ: Type Ⅲ precollagen; HA: Hyaluronic acid; LN: Laminin; CIV: Type Ⅳ collagen.

Table 5 Expression of TGF- β 1 and Smad3 (mean \pm SD)		
Groups	TGF- β 1/ β -actin	Smad3/ β -actin
Control	1.00 \pm 0.00 ^b	0.62 \pm 0.08 ^b
Model	3.29 \pm 2.08	1.33 \pm 0.10
Low-dosage HYRD group	2.52 \pm 1.57	1.20 \pm 0.07 ^b
Medium-dosage HYRD group	2.14 \pm 1.42	1.16 \pm 0.05 ^b
High-dosage HYRD group	1.68 \pm 0.51 ^a	0.79 \pm 0.06 ^b

^a $P < 0.05$, ^b $P < 0.01$ *vs* model group. TGF: Transforming growth factor.

Effect of HYRD on hepatic collagen deposition

The rat liver was stained with ponceau's, the collagen fiber was shown red. Collagen deposition was markedly increased in the model group compared with the control group ($P < 0.01$). Compared with the model group, collagen deposition was significantly decreased in the treatment groups ($P < 0.01$) (Table 4, Figure 2).

Table 4 Liver histopathological semi-quantitative scores (mean \pm SD)						
Groups	<i>n</i>	Scores				
		0	I	II	III	IV
Control	6	6				
Model	13				2	11
Low-dosage HYRD group	9				4	5
Medium-dosage HYRD group	11		1	3	5	2
High-dosage HYRD group	11	1	3	5	2	

^b $P < 0.01$ *vs* model group.

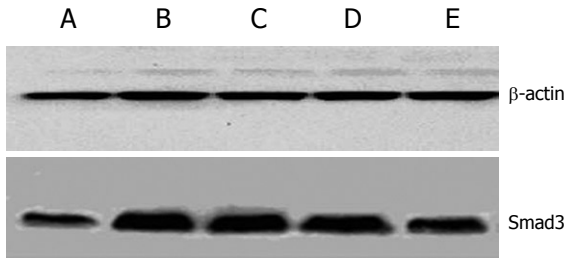


Figure 3 Western blotting for Smad3 expression in rats. A: Normal rats; B: Rats with hepatic fibrosis; C: Low-dosage of HYRD-treated rats; D: Medium-dosage of HYRD-treated rats; E: High-dosage of HYRD-treated rats.

Effect of HYRD on TGF- β 1 and Smad3 expression in liver

Expression of TGF- β 1 and Smad3 in liver was increased significantly in the model group ($P < 0.01$), and decreased obviously in the treatment groups (Table 5, Figure 3).

DISCUSSION

Hepatic fibrosis is thought to be a reversible disease, CCl₄-induced hepatic fibrosis in rats is a reproducible model for studying the pathogenesis of hepatic fibrosis and cirrhosis^[9]. CCl₄ can cause oxidative stress reaction *via* toxic free radicals. Lipid peroxidation of cytomembrane plays an important role in early stage of hepatic injury, which can activate lipocyte (HSCs). The activation of HSCs “induced by some critical cytokines” is considered to be of great importance during the long period of hepatic fibrosis^[10]. This activated HSC then becomes the main source of most cytokines and collagen proteins. Among the cytokines mediating factors, *TGF-β1* has been demonstrated in most researches to be an essential pro-fibrogenesis factor^[11-15]. In addition to that, *TGF-β-Smad* signaling pathway is the main pathway of *TGF-β*^[16-18], which transfers the stimulating signal from outside into the affected cells. The *Smad* proteins consist of a large family of transcription factors. *Smads* are *TGF-β* receptor substrates with a demonstrated ability to propagate signals. Briefly, two different transmembrane protein serine/threonine kinases, named *TGF-β* receptor type I and II, respectively, are brought together by the ligand, which acts as a receptor assembly factor^[19]. Before this occurs, receptor I is inactive. During the *TGF-β* signal transduction, receptor II is activated first. *TGF-β* and its receptor then form an activated complex. In the ligand-induced complex, active receptor II activates the receptor I kinase. The type I receptors specifically recognize the *Smad* subgroup known as receptor-activated *Smads* (R-*Smads*), which are *Smad 2* and *Smad 3*^[20]. Then R-*Smads* are activated and form a complex consisting of R-*Smads* and *Smad 4*, which belong to Co-*Smad*. The *Smads*-complex then is accumulated in the nucleus. This procedure leads to the formation of the functional transcriptional complexes. Both the R-*Smads* and the Co-*Smads* in this complex may participate in DNA-binding and recruitment of transcriptional cofactors^[21,22]. After transferring into the nucleus, the transcriptional complex binds to the certain domain of the target gene and cause the gene expression such as collagen production. The excess collagen production would lead to collagen deposition in liver tissue and hepatic fibrosis or cirrhosis at last. Thus, the down-regulation of *TGF-β* expression, modulation of *TGF-β-Smad* signaling and inhibition of the accumulation of activated HSCs by modulating either their activation and/or proliferation or promoting their apoptosis are important therapeutic strategy.

According to the Chinese Medicine theories, hepatic fibrosis is caused by internal damp (Shi), heat (Re), poison (Du), blood stasis (Yu), and both Qi and Yin asthenia. In this study, not only CCl₄ but also cholesterol, lard and alcohol were used to establish a hepatic fibrosis model. CCl₄ is poison, cholesterol, lard and alcohol produce damp and heat, which cause healthy energy asthenia, blood stasis exacerbation, unrelievable damp and heat, and induce hepatic fibrosis. Thus, the main Chinese Medicine approach to hepatic fibrosis is to eliminate heat, dispel damp, activate blood, promote Qi and cultivate

Yin^[23,24]. HYRD is composed of *Herba Artemisiae Annuae*, *Carapax Trionycis*, *Salviae Miltiorrhizae*, *Rhizoma Polygoni Cuspidati*, *Radix Curcumae*. The Decoction can activate blood and remove stasis, clear heat and eliminate damp, soften hard lumps and dispel nodes.

Herba Artemisiae Annuae can improve immunocyte activity, whose derivative artesunate can inhibit hepatic fibrosis^[25], and *Carapax Trionycis* can inhibit hepatic collagen deposition, promote collagen degradation^[26]. *Salviae Miltiorrhizae* and *Rhizoma Polygoni Cuspidati* can inhibit lipid peroxidation, reinforce organism immunity, improve hepatic microcirculation, inhibit adhesion of leucocyte and platelet to endotheliocyte, promote hepatocyte regeneration and collagen degradation^[27,28]. *Radix Curcumae* can activate blood and remove stasis, clear heat and eliminate damp^[29].

In conclusion, HYRD can inhibit lipid peroxidation, improve hepatic function, lessen collagen deposition, and prevent hepatic fibrosis *via* modulating *TGF-β-Smad* signaling pathway.

COMMENTS

Background

In China, the incidence of hepatic cirrhosis is still high. If treated properly at fibrosis stage, cirrhosis can be prevented. However, no effective antifibrotic drugs are available at present. According to the Chinese Medicine theories, hepatic fibrosis is caused by internal damp (Shi), heat (Re), poison (Du), blood stasis (Yu), and both Qi and Yin asthenia. *Haobie Yangyin Ruanjian* Decoction (HYRD) can activate blood and remove stasis, clear heat and eliminate damp, soften hard lumps and dispel nodes.

Research frontiers

Recent research showed hepatic fibrosis can be reversed by regulating collagen metabolism, inhibiting hepatic stellate cell (HSC) activation or by promoting HSC apoptosis. Hepatic extracellular matrix mainly results from HSC, which can be activated by fibrogenesis signal pathway.

Innovations and breakthroughs

This study has confirmed that HYRD can improve liver function, alleviate hepatic fibrosis, which is probably associated with its down-regulation on fibrogenic signal transduction of transforming growth factor (TGF) β -*Smad* pathway.

Applications

The HYRD can prevent hepatic fibrosis, which implies that it will be a good medicine for patients with chronic hepatic injury, this article can provide some scientific data for its application and development.

Peer review

This paper has reinforced my conviction that there is some good in this therapeutic approach. The study is statistically well-managed. Data are clear and convincing.

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Expression and functional characterization of platelet-derived growth factor receptor-like gene

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Abstract

AIM: To investigate the role of platelet-derived growth factor receptor-like gene (*PDGFRL*) in the anti-cancer therapy for colorectal cancers (CRC).

METHODS: *PDGFRL* mRNA and protein levels were measured by reverse transcription-polymerase chain reaction (RT-PCR) and immunohistochemistry in CRC and colorectal normal tissues. *PDGFRL* prokaryotic expression vector was carried out in *Escherichia coli* (*E. coli*), and purified by immobilized metal affinity chromatography. The effect of *PDGFRL* protein on CRC HCT-116 cells was detected by 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT), clone counting, cell cycle, and wound healing assay.

RESULTS: Both RT-PCR and immunohistochemistry showed that the expression of *PDGFRL* in colorectal normal tissues was higher than in cancer tissues. Recombinant *pET22b-PDGFRL* prokaryotic expression

vector was successfully expressed in *E. coli*, and the target protein was expressed in the form of inclusion bodies. After purification and refolding, recombinant human *PDGFRL* (*rhPDGFRL*) could efficiently inhibit the proliferation and invasion of CRC HCT-116 cells detected by MTT, clone counting and wound healing assay. Moreover, *rhPDGFRL* arrested HCT-116 cell cycling at the G0/G1 phase.

CONCLUSION: *PDGFRL* is a potential gene for application in the anti-cancer therapy for CRC.

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Key words: Platelet-derived growth factor receptor-like; Colorectal cancer; Prokaryotic expression; Reverse transcription-polymerase chain reaction; Immunohistochemistry

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INTRODUCTION

Colorectal cancer (CRC) is one of the most common malignant tumors in the world. It is estimated that 783 000 new cases are diagnosed each year, and the number has increased rapidly since 1975^[1]. CRC is the third most common cancer and the second leading cause

of cancer related mortality in the Western world. The incidence of CRC in China has increased rapidly over the past few decades^[2]. The molecular mechanism of carcinogenesis and development of CRC is still not fully understood.

Tumor suppressor genes are genes that can slow down cell division, repair DNA errors, and tell cells when to die (a process known as apoptosis or programmed cell death). When tumor suppressor genes do not work properly, cells can grow out of control, leading to cancer. About 30 tumor suppressor genes have been identified, including *p53*, *BRCA1*, *BRCA2*, *APC*, *RB1*, and platelet-derived growth factor receptor-like gene (*PDGFRL*). Tumor suppressor genes cause cancers when they are inactivated, and encode proteins that normally serve as a brake on cell growth. When such genes are mutated, the brake may be lifted, resulting in the runaway cell growth known as cancer. Gain of oncogene function associated with loss of tumor suppressors is now widely accepted as a hallmark of cancer initiation and progression^[3].

PDGFRL is located in chromosome 8p21.3-8p22, which is commonly deleted in sporadic hepatocellular carcinoma, CRC, breast cancer, and non-small cell lung cancers^[4,5]. While its precise biological function is not known, *PDGFRL* encodes a 375aa product with significant sequence similarity to the ligand-binding domain of platelet-derived growth factor receptor β . Mutations in *PDGFRL* have been found in individual cancer samples^[6-9]. An in-depth study using micro-cell-mediated chromosome transfer found that *PDGFRL* expression is decreased in the majority of breast cancer cells^[10]. Recently, *PDGFRL* is identified to play a central role in the tumor suppressor network by adopting a network perspective^[11]. Furthermore, *PDGFRL* was found to be involved in the suppression of the tumor metastatic phenotype as a strong candidate gene^[12].

To further identify new genes involved in the pathogenesis of CRC, we analyzed the expression of *PDGFRL* in CRC tissues and normal tissues by both reverse transcription-polymerase chain reaction (RT-PCR) and immunohistochemistry, and found that *PDGFRL* was expressed at higher levels in normal tissues than in CRC tissues. We constructed *pET22b-PDGFRL* recombinant prokaryotic expression vector before expression and purification of the *PDGFRL* recombinant protein were completed. The biological features of protein were identified to inhibit the proliferation and invasion of CRC HCT-116 cells by 3-(4,5-dimethylthiazolyl)-2,5-diphenyltetrazolium bromide (MTT), clone counting, and wound healing assay. In addition, *rhPDGFRL* arrested HCT-116 cell cycling at the G0/G1 phase.

MATERIALS AND METHODS

Cell culture

HCT-116 CRC cells and human umbilical vein endothelial cells (HUVEC) were maintained by our lab and cultured in Dulbecco-modified Eagle medium (DMEM; Gibco) supplemented with 10% bovine calf serum (BCS)

(Gibco). Cells were maintained at 37°C in an atmosphere of humidified air with 50 mL/L CO₂.

Collection of tissues

Fifteen samples of CRC tissues and paired non-cancerous tissues (5 cm away from tumor) were obtained from the First Hospital of Changsha. Written consent was obtained from the patients, who agreed with the collection of tissue samples. The resected tissue samples were immediately cut into small pieces, and snap frozen in liquid nitrogen until use. All tumor tissue and paired non-cancerous tissue samples were pathologically confirmed.

RT-PCR

RNA isolated from tissues and cells was reversibly transcribed and amplified using the RT-PCR System (Fermentas). Primer sequences used were sense 5'-CAAGAAGGTGAAGCCCCAAAT-3' and antisense 5'-ACAAGGAACCACAGCCTGTCT-3' for *PDGFRL*. A 587-bp Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) fragment was amplified as an internal control. For *GAPDH*, the forward primer 5'-AATCCCA TCACCATCTTCCA-3', and the reverse primer 5'-CC TGCTTCACCACCTTCTTG-3' were used. After heating at 95°C for 1 min, PCRs were exposed to 30 cycles (*GAPDH*, 25 cycles) of 95°C for 30 s, 60°C for 30 s, and 68°C for 1 min and 30 s with a final extension at 68°C for 10 min. The relative mRNA levels were normalized to that of *GAPDH* and the ratio of *PDGFRL* to *GAPDH* was calculated.

Immunohistochemistry

Paraffin sections were deparaffinized with xylene and rehydrated in graded alcohol. Endogenous peroxidase activity was blocked by incubation in 3% hydrogen peroxide at reverse transcription (RT) for 10 min. Non-specific binding was blocked with phosphate buffered saline Tween-20 (PBST) containing 10% goat serum for 2 h at RT. *PDGFRL* antibody (Abcam) was added to each slide and incubated at 4°C overnight. Following three washes, slides were incubated with Envision (DAKO) for 40 min at RT. Diaminobenzidine was used as a chromogen. Sections were counterstained with hematoxylin, dehydrated, and mounted. Evaluation of immunohistochemical slides was done with a Nikon Eclipse E800 microscope at $\times 100$ magnification. The intensity of the staining was scored on a scale of 0 to 3+ where 0, 1, 2 and 3 represented no staining and weak, moderate or strong staining, respectively. The mean staining scores for tissues and the mean fold change in protein expression was calculated.

Plasmid construction

A DNA fragment encoding the gene *PDGFRL* was amplified by PCR using a sense primer, 5'-TGAG CCATGGATCAACACCTTCC-3', and an anti-sense primer, 5'-AAGCTCGAGGGAAAACCTCAACAGT-3'. The primers were introduced to an *NcoI* site (sense) and an *XbaI* site (anti-sense), respectively. The amplification

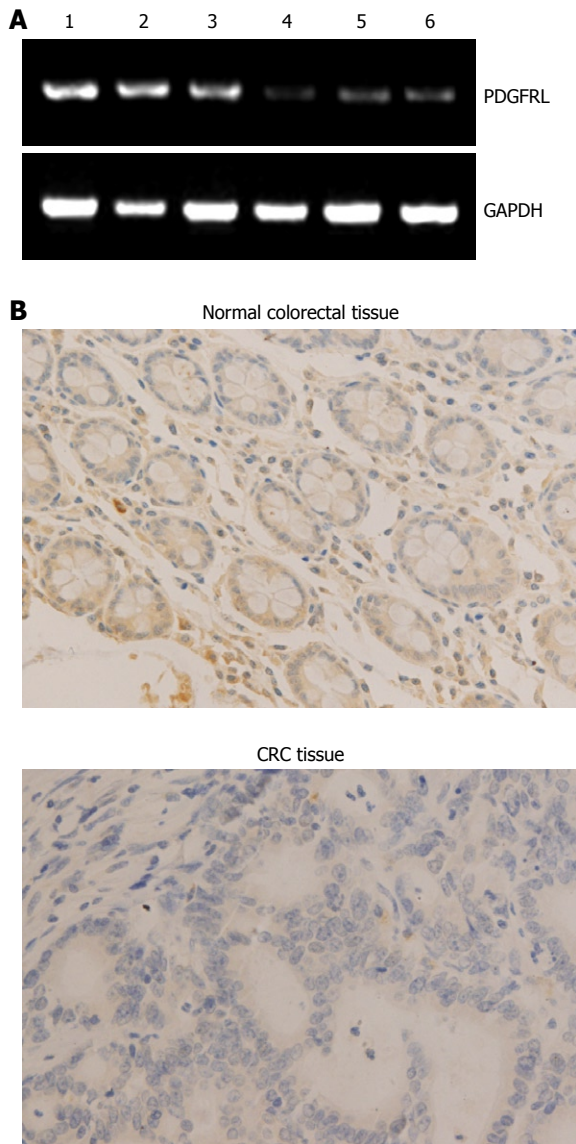


Figure 1 Expression of platelet-derived growth factor receptor-like gene (PDGFRL) in colorectal cancer and normal tissues. A: Reverse transcription-polymerase chain reaction (RT-PCR) analysis shows that mRNA levels in colorectal cancer tissues were lower than in normal tissues. 1-3: Normal colorectal tissues; 4-6: Colorectal cancer tissues; B: Immunohistochemical analysis illustrates that immunoreaction signal of PDGFRL in cancer tissues was weak compared with normal tissues. (Magnification, $\times 100$). GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; CRC: Colorectal cancers.

was performed using $2 \times$ pfu PCR MasterMix (MBI Fermentas) on an Eppendorf PCR instrument. The PCR products were purified by 1% agarose gel electrophoresis and double digested and ligated into the expression vector pET22b(+), resulting in a pET22b-PDGFRL plasmid with the sequence encoding the gene PDGFRL. The constructed plasmid was transformed into competent *Escherichia coli* (*E. coli*). DH5 α cells and the transformed strains were grown in Luria-Bertani (LB) broth supplemented with ampicillin (100 μ g/mL). All strains were incubated at 37°C with constant shaking (220 r/min). The recombinant was identified by PCR, double endonuclease digestion and DNA sequencing.

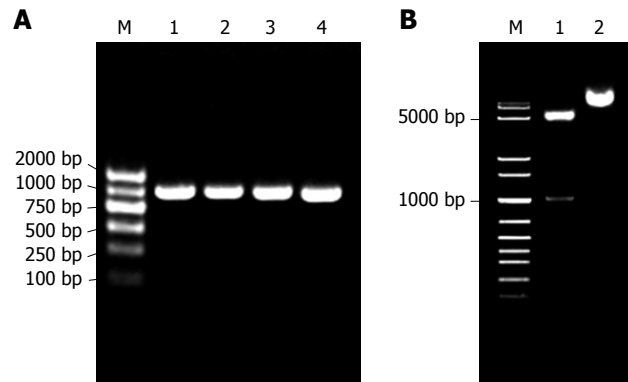


Figure 2 Construction of pET22b-PDGFRL. A: Bacterial colony PCR for detection of DH5 α clones with prokaryotic recombinant expression vector pET22b-PDGFRL. M: DNA ladder; 1-4: Positive bacterial colonies; B: Double endonuclease digestion of the recombinant vector pET22b-PDGFRL. M: DNA ladder; 1: Double digestion with NcoI/XhoI; 2: pET22b-PDGFRL without digestion.

Bacterial expression of recombinant human PDGFRL (rhPDGFRL)

The recombinant plasmid pET22b-PDGFRL was transformed into *E. coli* expression strain BL21 (DE3) cells. One colony was picked up and was grown in 3 mL LB rich medium containing 100 mg/L ampicillin. After 8 h, 1 mL of the BL21 (DE3) cells were introduced into 100 mL of LB medium containing 100 mg/L ampicillin. Bacteria were grown at 37°C until an A_{600} of 0.6 was reached. Then, isopropyl-b-D-thiogalactopyranoside (IPTG) was added to induce protein expression at 30°C. To check the expression of pET22b-PDGFRL, *E. coli* were induced at different final concentrations of IPTG such as 0.1, 0.5, 1.0, 1.5 and 2.0 mmol/L, and different time points such as 1, 2, 4 and 6 h, respectively. The cells were harvested by centrifugation at $4800 \times g$ for 30 min and the pellet was resuspended in 50 mmol/L sodium phosphate, 0.3 mol/L NaCl, pH 8.0. The resuspended cells were lysed by sonication. The cells after lysis were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Purification of rhPDGFRL

The protein was further purified by immobilized metal affinity chromatography (MagExtractor[®] His-tag protein purification kit, TOYOBO). The extracts were fractionated by gel filtration column chromatography (AKTA explorer 10S with HiLoad 16/60 Superdex 75 pg column, GE Healthcare) in 50 mmol/L Tris-HCl (pH 7.5) and 100 mmol/L NaCl. These fractionated extracts were desalinated using Slide-A-Lyzer[®] dialysis cassettes (Pierce Biotechnology), separated by denaturing SDS-PAGE, and stained with Coomassie brilliant blue (CBB). Western blotting was performed using an anti-His antibody (Abcam)^[13].

MTT assay

MTT assay was performed to measure cell viability and proliferation in external factors. The 3rd generation

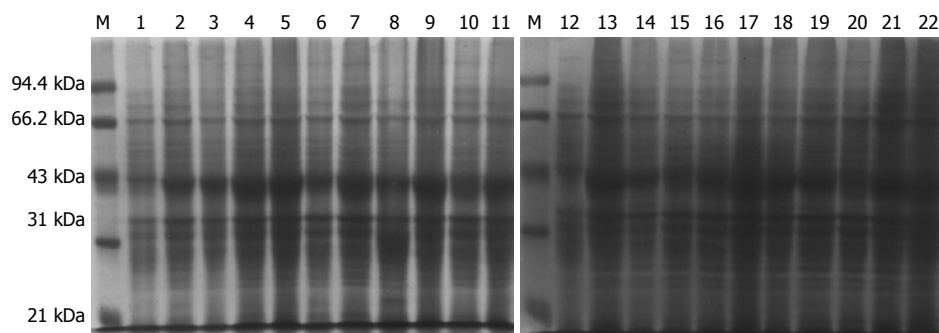


Figure 3 Protein expression in *Escherichia coli* containing *pET22b-PDGFRL*. Time course and different concentrations of isopropyl- β -D-thiogalactopyranoside (IPTG) analysis of *pET22b-PDGFRL* protein expression by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). M: Molecular weight standards; 1, 12: Uninduced bacterial lysate; 1-h (2-6), 2-h (7-11), 3-h (13-17), 4-h (18-22) induced samples at different concentrations of IPTG culture (0.1, 0.5, 1.0, 1.5, 2.0 μ g/mL).

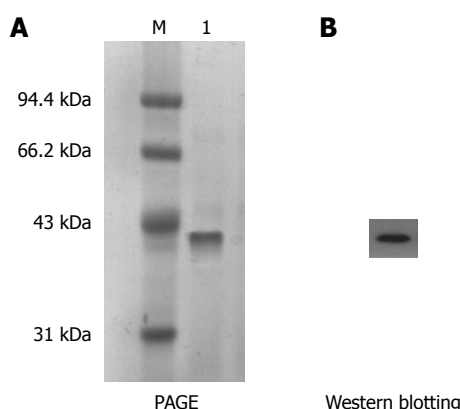


Figure 4 Purification of His-PDGFRL recombinant protein. A: Purification of His-tagged His-PDGFRL recombinant protein by immobilized metal affinity chromatography. M: Molecular weight standards; 1: The purified protein; B: Purification of recombinant protein confirmed by immunoblotting using anti-His-Tag antibody.

human HUVEC and HCT-116 cells were made into cell suspension with a density of 1×10^4 /mL, which was inoculated into 96-well plates separately. The purified *rhPDGFRL* with different plates concentrations (0, 0.5, 1.0, 1.5 and 2.0 μ g/mL, suspended in DMEM plus 1% BCS) was added to HUVEC and HCT-116 cells. After two days, 5 mg/mL MTT was added into the wells and incubated at 37°C for 3 h. The supernatant was blotted and added with DMSO (dimethyl sulfoxide). Absorbance of the dye was measured at a wavelength of 490 nm on a Microplate Reader. HCT-116 cells were then treated with *rhPDGFRL* or bovine serum albumin (BSA) (suspended in DMEM plus 1% BCS) and MTT assay was performed.

Colony formation assay

Crystal violet (CV) staining of cells and clone counting were used to measure cell proliferation. Dilute the HCT-116 cells into a 6-well plate separately and each pole contained 1000 cells. The *rhPDGFRL* or BSA (1.5 μ g/mL) was added to HCT-116 cells. After 2 wk, 0.05% CV was added into the plates. The cells were fixed for 10 min with 4% Paraformaldehyde (PFA) and stained for 30 min with 0.05% CV. The plates were carefully rinsed in ddH₂O until

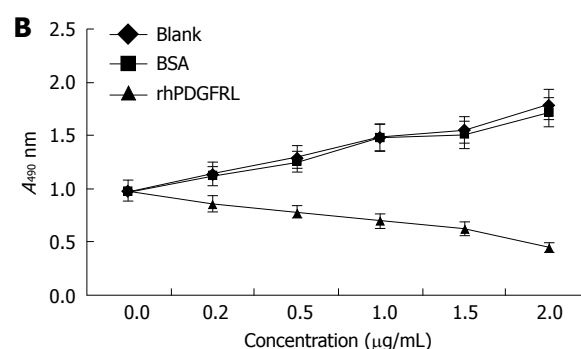
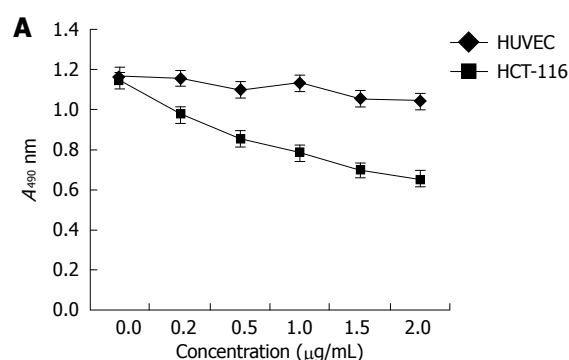


Figure 5 PDGFRL inhibits HCT-116 cell proliferation. A: MTT assay shows that HCT-116 cells grew slowly compared with human umbilical vein endothelial cells (HUVEC) cells after PDGFRL protein treatment, $P < 0.05$, HCT116 vs HUVEC; B: HCT-116 cells were treated with *rhPDGFRL* (HCT-116/*rhPDGFRL*) or bovine serum albumin (BSA) (HCT-116/BSA), $P < 0.05$, *rhPDGFRL* vs BSA. Data are expressed as mean \pm SD of three independent experiments.

no color appeared. Clone forming efficiency for individual type of cells was calculated according to the number of colonies/number of inoculated cells \times 100%.

Flow cytometry of cell cycle

The impact of *rhPDGFRL* on the HCT-116 cell cycling was examined by flow cytometry. HCT-116 cells indicated were seeded into a 6-well plate at a density of 3.5×10^5 cell/well. Once the cells were grown at 70%-80% confluence, HCT-116 cells were treated with the *rhPDGFRL* or BSA (1.5 μ g/mL). Cells were harvested at 48 h and resuspended in fixation fluid at a density of 10^6 /mL, 1500 μ L propidium iodide (PI) solution was added, and

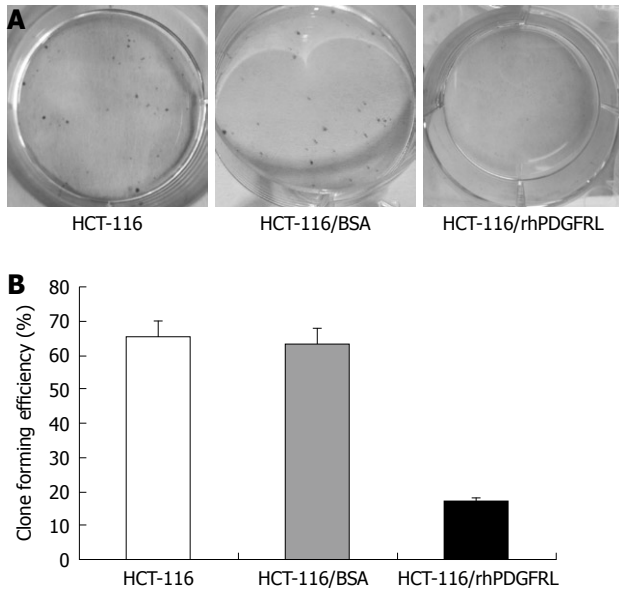


Figure 6 Crystal violet (CV) staining of cells and clone counting assay. A: CV staining of HCT-116 cells; B: Quantitative analysis of colony formation. Data are expressed as the efficiency of colony formation (%) and expressed as the mean \pm SD of three separated experiments, $P < 0.05$, HCT-116/rhPDGFRL vs HCT-116/BSA.

the cell cycle was detected by FACS Caliber (Becton Dickinson).

Monolayer wound healing assay

Wound healing assay was applied to measure cell invasion. HCT-116 cells indicated were seeded into a 6-well plate at a density of 3.5×10^5 cell/well. Once the cells were grown to a monolayer, a wound was made and the rhPDGFRL or BSA ($1.5 \mu\text{g/mL}$) was added to the HCT-116 cells. The distance of cell migration was calculated by subtracting the distance between the lesion edges at 48 h from the distance measured at 0 h. The relative migrating distance of cells was measured by the distance of cell migration/the distance measured at 0 h.

RESULTS

Expression of PDGFRL in CRC and normal tissues

To verify PDGFRL in CRC and normal tissues, we did RT-PCR and immunohistochemical analysis in 15 human CRC and adjacent normal tissue samples. In RT-PCR analysis, mRNA levels in CRC tissues were lower than in normal tissues (Figure 1A). By immunohistochemical analysis, immunoreaction signal of PDGFRL in cancer tissues was weak compared with normal tissues (Figure 1B). Semi-quantitative analysis of mRNA and protein expression for PDGFRL was performed in CRC and adjacent normal tissue samples. The relative mRNA expression of PDGFRL in CRC tissues was 0.11, but the one in the adjacent normal tissues was 0.78. Likewise, the relative protein level of PDGFRL in adjacent normal tissues was 8.2, but the one in CRC tissues was only 1.3. Both RT-PCR and immunohistochemistry showed that the expression of PDGFRL in colorec-

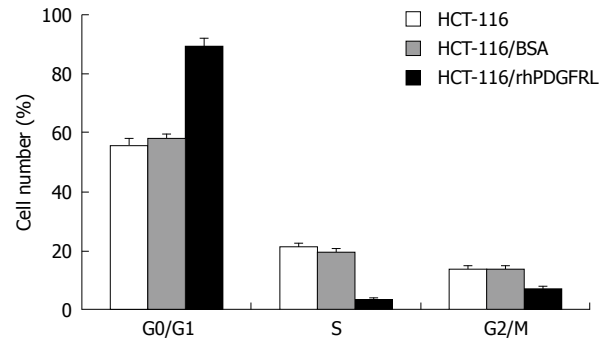


Figure 7 Analysis of cell cycle. HCT-116, HCT-116/BSA, and HCT-116/rhPDGFRL cells were fixed with 70% ethanol and stained with PI, followed by FACS analysis. Data are expressed as mean \pm SD of three independent experiments, $P < 0.05$, HCT-116/rhPDGFRL vs HCT-116/BSA.

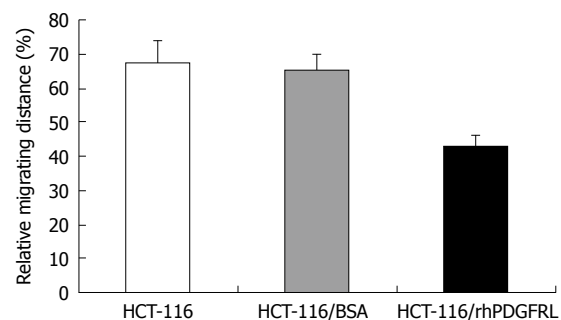


Figure 8 Measurement of migration distance in HCT-116, HCT-116/BSA, and HCT-116/rhPDGFRL cells. Data are expressed as the mean \pm SD of three independent experiments, $P < 0.05$, HCT-116/rhPDGFRL vs HCT-116/BSA.

tal normal tissues was higher than in cancer tissues based on the identification of PDGFRL as a tumor suppressor.

Construction of pET22b-PDGFRL

As shown in Figure 2, the prokaryotic expression recombinant pET22b-PDGFRL was successfully constructed using bacterial colony PCR (Figure 2A), restriction enzyme digestions (Figure 2B) and complete sequencing (data not shown).

Expression and purification of pET22b-PDGFRL recombinant protein

The prokaryotic expressive vector pET22b-PDGFRL was transformed into the *E. coli* BL21 (DE3) expression host strain for protein over-expression. Several potential clones were identified with DNA sequencing (data not shown). The expression and purification were identified by, respectively, running the crude lysate and the elution fractions on a 12% SDS-PAGE gel and subsequently staining with Coomassie brilliant blue. Experiments of IPTG concentration and time course were performed to determine the kinetics of protein expression in the bacterial culture (Figure 3). As a result, the cells should be harvested 4 h after 0.1 mmol/L IPTG induction, as the largest amount of the correct 42 kDa size pET22b-PDGFRL protein was produced at this time and concentration point.

The expressed protein at 0.1 mmol/L IPTG for 4 h

A

1	CCTGCGTCCC	CGCCCCGCGC	AGCCGCCGCG	CTCCTGCGCT	CCGAGGTCCG	AGGTTCCCGA
61	GATGAAGGTC	TGGTGCTGCT	TTGGTCTTCT	GCTGGTGAC	GAAGCGCTGG	AGGATGTTAC
121	TGGCCAACAC	CTTCCCAAGA	ACAAGCGTCC	AAAAGAACCA	GGAGAGAATA	GAATCAAACC
181	TACCAACAAG	AAGGTGAAGC	CCAAAATTCC	TAAATGAAG	GACAGGGACT	CAGCCAATTC
241	AGCACCAAAG	ACGCAGTCTA	TCATGATGCA	AGTGCTGGAT	AAAGGTCGCT	TCCAGAAACC
301	CGCCGCTACC	CTGAGTCTGC	TGGCGGGGCA	AACTGTAGAG	CTTCGATGTA	AAGGGAGTAG
361	AATTGGGTGG	AGTACCCCTG	CGTATCTGGA	CACCTTTAAG	GATTCTCGCC	TCAGCGTCAA
421	GCAGAATGAG	CGCTACGGCC	AGTTGACTCT	GGTCAACTCC	ACCTCGGCAG	ACACAGGTGA
481	ATTCAGCTGC	TGGGTGCAGC	TCTGCAGCGG	CTACATCTGC	AGGAAGGACG	AGGCCAAAAC
541	GGGCTCCACC	TACATCTTTT	TTACAGAGAA	AGGAGAACTC	TTTGACCTT	CTCCCAGCTA
601	CTTCGATGTT	GTCTACTTGA	ACCCGGACAG	ACAGGCTGTG	GTTCTTGTC	GGGTGACCGT
661	GCTGTCGGCC	AAAGTCACGC	TCCACAGGGA	ATTCAGGCC	AAGGAGATCC	CAGCCAATGG
721	AACGGACATT	GTTTATGACA	TGAAGCGGGG	CTTTGTGTAT	CTGCAACCTC	ATTCCGAGCA
781	CCAGGGGTGTG	GTTTACTGCA	GGGCGGAGGC	CGGGGCGAGA	TCTCAGATCT	CCGTCAAGTA
841	CCAGCTGCTC	TACGTGGCGG	TTCCAGTGG	CCCTCCCTCA	ACAACCATCT	TGGCTTCTTC
901	AAACAAAGTG	AAAAGTGGGG	ACGACATCAG	TGTGCTCTGC	ACTGTCCTGG	GGGAGCCCGA
961	TGTGGAGGTG	GAGTTCACCT	GGATCTTCCC	AGGGCAGAAG	GATGAAAGGC	CTGTGACGAT
1021	CCAAGACACT	TGGAGGTTGA	TCCACAGAGG	ACTGGGACAC	ACCACGAGAA	TCTCCCAGAG
1081	TGTCATTACA	GTGGAAGACT	TCGAGACGAT	TGATGCAGGA	TATTACATT	GCACTGCTCA
1141	GAATCTTCAA	GGACAGACCA	CAGTAGCTAC	CACTGTTGAG	TTTTCTGAC	TTGGAAAAGG
1201	AAATGTAATG	AACTTATGGA	AAGCCCATT	GTGTACACAG	TCAGCTTTGG	GGTTCCTTTT
1261	ATTAGTGCTT	TGCCAGAGGC	TGATGTCAAG	CACCACACCC	CAACCCAGC	GTCTCGTGAG
1321	TCCGACCCAG	ACATCCAAAC	TAAAAGGAAG	TCATCCAGTC	TATTACAGA	AGTGTTAACT
1381	TTTCTAACAG	AAAGCATGAT	TTTGATTGCT	TACCTACATA	CGTGTTCTTA	GTTTTTATAC
1441	ATGTGTAAAC	AATTTTATAT	AATCAATCAT	TTCTATTAAA	TGAGCACGTT	TTTGTAAGAA
1501	AT					

B

Met	Lys	Val	Trp	Leu	Leu	Leu	Gly	Leu	Leu	Leu	Val	His	Glu	Gla	Leu	Glu	Asp	Val	Thr	Gly
ATG	AAG	GTC	TGG	CTG	CTG	CTT	GGT	CTT	CTG	CTG	GTG	CAC	GAA	GCG	CTG	GAG	GAT	GTT	ACT	GGC

Figure 9 PDGFRL protein contains a putative signal peptide of 21aa. A: *PDGFRL* cDNA sequences contain an open reading frame (ORF) of 1128-base pairs that is matched in bold; B: ORF encodes a protein of 375 amino acids (aa) with a putative signal peptide of 21aa.

was further purified by immobilized metal affinity chromatography. The electrophoretic analysis revealed that the His-tagged His-*PDGFRL* recombinant protein was purified to near homogeneity and migrated as a 42 kDa band (Figure 4A). Moreover, the purification of the recombinant protein was confirmed by immunoblotting using anti-His-Tag antibody (Figure 4B).

***PDGFRL* inhibits HCT-116 cell proliferation**

MTT assay was performed to measure the effect on cell viability. HCT-116 cells were found to grow slowly compared with HUVEC cells after *PDGFRL* protein was added (Figure 5A). The proliferating ability of HCT-116 cells decreased gradually with an increasing concentration of *PDGFRL*, but normal cell line HUVEC had no evident change in proliferation. Next, we treated HCT-116 cells with *rhPDGFRL* or BSA, and found that the cell viability of HCT-116 treated with *rhPDGFRL* (HCT-116/*rhPDGFRL*) decreased compared with that treated with BSA (HCT-116/BSA), which was not distinguishable from blank group (Figure 5B). A similar pattern of inhibitory effect of *rhPDGFRL* in HCT-116 cells was achieved in colony formation assay (Figure 6). Following incubation for 2 wk, a few colonies from HCT-116/*rhPDGFRL* cells were generated compared with HCT-116 or HCT-116/BSA. Therefore, the low MTT activity and a small number of cell colonies from HCT-116/*rhPDGFRL* cells demon-

strated that *rhPDGFRL* inhibited the growth of HCT-116 cells *in vitro*.

Impact of *PDGFRL* on HCT-116 cell cycle

To further explore the cause of the decrease in cell viability, we examined the effects of *PDGFRL* on cell cycle. As illustrated in Figure 7, HCT-116 cells treated with *PDGFRL* blocked the cell cycle in G1 phase. The G0/G1-phase fraction increased from 57.8% (HCT-116/BSA) to 89.4% (HCT-116/*rhPDGFRL*). These data indicated that *PDGFRL* arrested HCT-116 cell cycling at the G0/G1 phase, which may inhibit the growth of HCT-116 cells.

Effects of *PDGFRL* on the migration of HCT-116 cells

We examined the impact of *PDGFRL* on the migration of HCT-116 cells by the wound healing assay as shown in Figure 8. Following incubation of physically wounded cells for 48 h, the mobile distance of HCT-116/*rhPDGFRL* cells was found significantly shorter than that of controls.

DISCUSSION

Despite curative surgery, nearly 4 out of 10 patients with CRC experience disease relapse, 1 of 5 will develop liver metastases, and 1 of 12 will develop pulmonary

metastases^[14]. The survival of CRC patients is still a key issue to address and the need for drugs curing CRC is urgent.

The tools and concept of gene therapy are being applied to the development of new effective treatment strategies for human cancer^[15]. Most human cancers are associated with multiple interacting and cooperating mutations in protooncogenes and tumor suppressor genes. Cancer therapies that target oncogenes usually seek to block or reduce their action, while those aimed at tumor suppressor genes seek to restore or increase their action. In several model systems, some features of the tumor phenotype can be suppressed *in vitro* through the restoration of expression of tumor suppressor genes such as *Rb* and *p53*^[16,17]. It is interesting that most investigators have found that pVHL suppresses tumorigenicity in a nude mouse assay but not *in vitro*^[18,19]. Protein phosphatase-2A (PP2A) has progressively been considered as a potential tumor suppressor. PP2A activation by forskolin, 1,9-di-deoxy-forskolin and FTY720 effectively antagonize leukemogenesis in both *in vitro* and *in vivo* models of these cancers^[20-22]. PP2A is now a highly promising target for developing a new series of anticancer agents potentially capable of overcoming the drug-resistance^[23]. It is necessary to identify new genes applied in the treatment for CRC.

In this study, both RT-PCR and immunohistochemistry showed that the expression of *PDGFRL* in colorectal normal tissues was higher than in cancer tissues based on the identification of *PDGFRL* as a tumor suppressor. To investigate the role of *PDGFRL* in the anti-cancer therapy for CRC, pET-22b (+) prokaryotic expression vector was used to construct *rhPDGFRL*.

The pET-22b (+) vector carries an N-terminal pelB signal sequence for potential periplasmic localization, plus optional C-terminal His-tag sequence. However, in our study, periplasmic secretion of *PDGFRL* protein was too small to collect the purified protein, and most of expressive proteins were produced in an insoluble form in *E. coli* (data not shown).

PDGFRL cDNA sequences contain an open reading frame (ORF) of 1128-base pairs encoding a protein of 375 amino acids (aa) with a putative signal peptide of 21aa (Figure 9). The signal peptide could not be expressed stably in prokaryotic expression vector in high yield, and therefore the signal peptide was deleted in the construction of the recombinant *pET22b-PDGFRL*^[24,25].

In this report, *in vitro* bioactivity of *PDGFRL* was determined by MTT, clone counting, cell cycle and wound healing assay. When *PDGFRL* protein was added to HCT-116 cells, MTT, clone counting and wound healing assay showed that proliferation and invasion of HCT-116 cells decreased. This result indicated that *PDGFRL* as a tumor suppressor inhibited the growth of CRC cells. In addition, *PDGFRL* arrested HCT-116 cell cycling at the G0/G1 phase. The results of this study extended our previous knowledge of *PDGFRL* as a tumor suppressor in CRC. Further characterization of *PDGFRL* will provide new insights into the role of *PDGFRL* in the molecular pathogenesis and therapy of CRC.

ACKNOWLEDGMENTS

We thank Dr. Di Wang, Department of Pathology, The First Hospital of Changsha, for supplying tumor and normal adjacent tissue sections.

COMMENTS

Background

Colorectal cancer (CRC) is the third most common cancer and the second leading cause of cancer related mortality in the Western world. The incidence of CRC in China has increased rapidly over the past few decades. The molecular mechanism of human carcinogenesis and development of CRC is still not clear.

Research frontiers

Mutations in platelet-derived growth factor receptor-like gene (*PDGFRL*) as a tumor suppressor have been found in individual cancer samples and *PDGFRL* expression is decreased in the majority of breast cancer cells. Recently *PDGFRL* is identified to play a central role in the tumor suppressor network by adopting a network perspective. Furthermore, *PDGFRL* was found to be involved in the suppression of the tumor metastatic phenotype as a strong candidate gene.

Innovations and breakthroughs

This study extended the previous knowledge of *PDGFRL* as a tumor suppressor in CRC.

Applications

Further characterization of *PDGFRL* will provide new insights into the role of *PDGFRL* in the molecular pathogenesis and therapy of CRC.

Terminology

PDGFRL is located in chromosome 8p21.3-8p22, which is commonly deleted in sporadic hepatocellular carcinoma, CRC, breast cancer, and non-small cell lung cancer.

Peer review

By different methods, the authors analyzed the role of platelet-derived growth factor receptor-like gene in CRC and its possible application in anti-cancer therapy. The manuscript is well-written and the study is conducted appropriately.

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Melatonin and Doxorubicin synergistically induce cell apoptosis in human hepatoma cell lines

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RESULTS: Treatment with Melatonin (10^{-8} - 10^{-5} mol/L) alone had a dose-related inhibitory effect on cell proliferation but no cytotoxic effect on hepatoma cell lines HepG2 and Bel-7402. Interestingly, when combined with Doxorubicin, Melatonin significantly increased the effects of cell growth inhibition and cell apoptosis. Furthermore, TUNEL staining and flow cytometry revealed that cooperative apoptosis induction was associated with decreased expression of Bcl-2 as well as increased expression of Bax and Caspase3.

CONCLUSION: The synergism of Melatonin and Doxorubicin inhibits hepatoma cell growth and induces cell apoptosis.

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Key words: Melatonin; Doxorubicin; Human hepatoma cell line; Apoptosis

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Abstract

AIM: To investigate whether Melatonin has synergistic effects with Doxorubicin in the growth-inhibition and apoptosis-induction of human hepatoma cell lines HepG2 and Bel-7402.

METHODS: The synergism of Melatonin and Doxorubicin inhibited the cell growth and induced cell apoptosis in human hepatoma cell lines HepG2 and Bel-7402. Cell viability was analyzed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay. Cell apoptosis was evaluated using TUNEL method and flow cytometry. Apoptosis-related protein Bax, Bcl-2 and caspase-3 expressions were measured by immunohistochemical staining.

Fan LL, Sun GP, Wei W, Wang ZG, Ge L, Fu WZ, Wang H. Melatonin and Doxorubicin synergistically induce cell apoptosis in human hepatoma cell lines. *World J Gastroenterol* 2010; 16(12): 1473-1481 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v16/i12/1473.htm> DOI: <http://dx.doi.org/10.3748/wjg.v16.i12.1473>

INTRODUCTION

Melatonin, a chief secretory product of the pineal gland, has diverse physiological functions. It plays a crucial role in regulating circadian rhythm, and is involved in immunomodulation, hematopoiesis, and antioxidative

processes^[1-5]. A large number of studies have demonstrated that Melatonin has important oncostatic properties^[6,7]. It inhibits cell proliferation in several cancer cell lines including human B-lymphoma cells^[8], human myeloid leukemia cells HL-60^[9] and human neuroblastoma cancer cells^[10]. Melatonin decreased the growth rates of tumors *in vivo* both in transplantable animal model and the animal model induced by the administration of carcinogens^[11,12].

Hepatocellular carcinoma (HCC) is the fifth most common cancer in the worldwide, and more than half a million new cases occur annually^[13]. In some areas of Asia and the Middle East, HCC ranks as the first cause of death from cancer. The incidence of HCC is also increasing in Europe and the United States^[14]. Chemotherapy is one of the common strategies in HCC treatment, especially for unresectable tumors. Conventional chemotherapeutic drugs such as Doxorubicin often have severe side effects that limit their efficacy. Combined therapy with multiple drugs or modalities is a common practice in the treatment of cancer, which can achieve better therapeutic effects than a single drug or modality, and can reduce the side effects and resistance to drugs as well. Thus it is imperative to develop new agents which can help enhance the drug effectiveness as well as reduce the toxicity.

The present study was designed to investigate the growth-inhibiting and apoptosis-inducing effects of Melatonin alone or combined with Doxorubicin in order to develop a new adjuvant therapy for HCC.

MATERIALS AND METHODS

Cell culture

Human hepatoma cells HepG2 and Bel-7402 were purchased from Shanghai cell bank, Chinese Academy of Sciences, cultured in Dulbecco's modified Eagles's medium and RPMI-1640 medium respectively. Each was supplemented with 10% (v/v) heat-inactivated fetal bovine serum and antibiotics and incubated at 37°C in a humid atmosphere with 50 mL/L CO₂.

Drugs and chemicals

The Doxorubicin injection was purchased from Shenzhen Main Luck Pharmaceuticals Inc. (Shenzhen, China, Cat-NO0407E1, 10 mg/ampoule); Melatonin, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetra-zolium bromide (MTT) and Rnase A was from Sigma (St Louis, MO, USA). DMEM and RPMI-1640 medium was from Gibco BRL Life Technologies Inc. (Grand Island, New York, USA); Rabbit polyclonal antibodies against human Bcl-2, Bax and caspase-3 were all purchased from Lab Vision Corporation (Fremont, California, USA) and the streptavidin-biotin-peroxidase (S-P) reagents kit was obtained from Fuzhou Maxim Biotech, Ltd. (Fuzhou, China). Cell Apoptosis PI Detection Kit was provided by Nanjing KeyGen Biotech Co. Ltd. (Nanjing, China). DeadEnd™ Colorimetric TUNEL System was from Promega Biotech Co. Ltd. (Madison, WI, USA).

In vitro cytotoxicity examination

Cytotoxicity was measured using MTT assay. HepG2 and

Bel-7402 cells in exponentially growth phase were cultured at a density of 1×10^4 cells/well in a 96-well plate. After treatment with various concentrations of drug for 48 h, MTT solution (5.0 mg/mL in phosphate-buffered saline) was added (20.0 µL/well), and the plates were incubated for another 4 h at 37°C. The purple formazan crystals were dissolved in 150.0 µL Dimethyl Sulfoxide DMSO per well. After 10 min, the plates were read on microplate reader (American Bio-Tek) at 490 nm. The cells without drugs were used as control. Assays were performed in three independent experiments. The percentage of cytotoxicity was calculated as follows: Cytotoxicity (%) = $(1 - A_{490} \text{ of experimental well}) / A_{490} \text{ of control well}$. The median inhibitory concentration (IC₅₀) (defined as the drug concentration at which cell growth was inhibited by 50%) was assessed from the dose-response curves.

Analysis of in vitro drug interaction

The coefficient of drug interaction (CDI) was used to analyze the synergistical inhibitory effect of drug combination. CDI is calculated as follows: $CDI = AB / (A \times B)$. According to the absorbance of each group, AB is the ratio of the combination groups to control group in A_{490} ; A or B is the ratio of the single agent groups to control group in A_{490} . Thus CDI value less than, equal to or greater than 1 indicates that the drugs are synergistic, additive or antagonistic, respectively. CDI less than 0.7 indicates that the drugs are significantly synergistic.

TUNEL assay

The cells were cultured in 6-well plates containing cover slips overnight. After treatment with various concentrations of Melatonin or Doxorubicin or in combination for 48 h, the cover slips were washed twice with PBS and fixed in 4% paraformaldehyde solution for 25 min at room temperature. Apoptotic cells were detected by terminal deoxynucleotidyl-transferase-mediated dUTP nick end-labeling (TUNEL) assay using DeadEnd™ Colorimetric TUNEL System Kit from Promega (Madison, WI), following the manufacturer's instructions. The TUNEL assay results were quantitatively analyzed by biological image analysis system which consists of Nikon ECLIPSE 80i biology microscope, Nikon Digital Camera DXM 1200F, ACT-1 version 2.63 software (Japan).

Immunocytochemistry

Cells were seeded onto glass coverslips overnight. After incubation with various concentrations of the drugs for 48 h, the cover slips were washed twice with PBS and added 4% paraformaldehyde solution for 30 min at room temperature. Immunohistochemical staining for Bcl-2, Bax and caspase-3 was measured using the standard S-P method. The detailed manipulation was conducted according to the manufacturer's instructions. As negative control, PBS was used instead of primary antibody, and other steps were followed in the same way. The immunocytochemical results were quantitatively analyzed by biological image analysis system which consists of Nikon

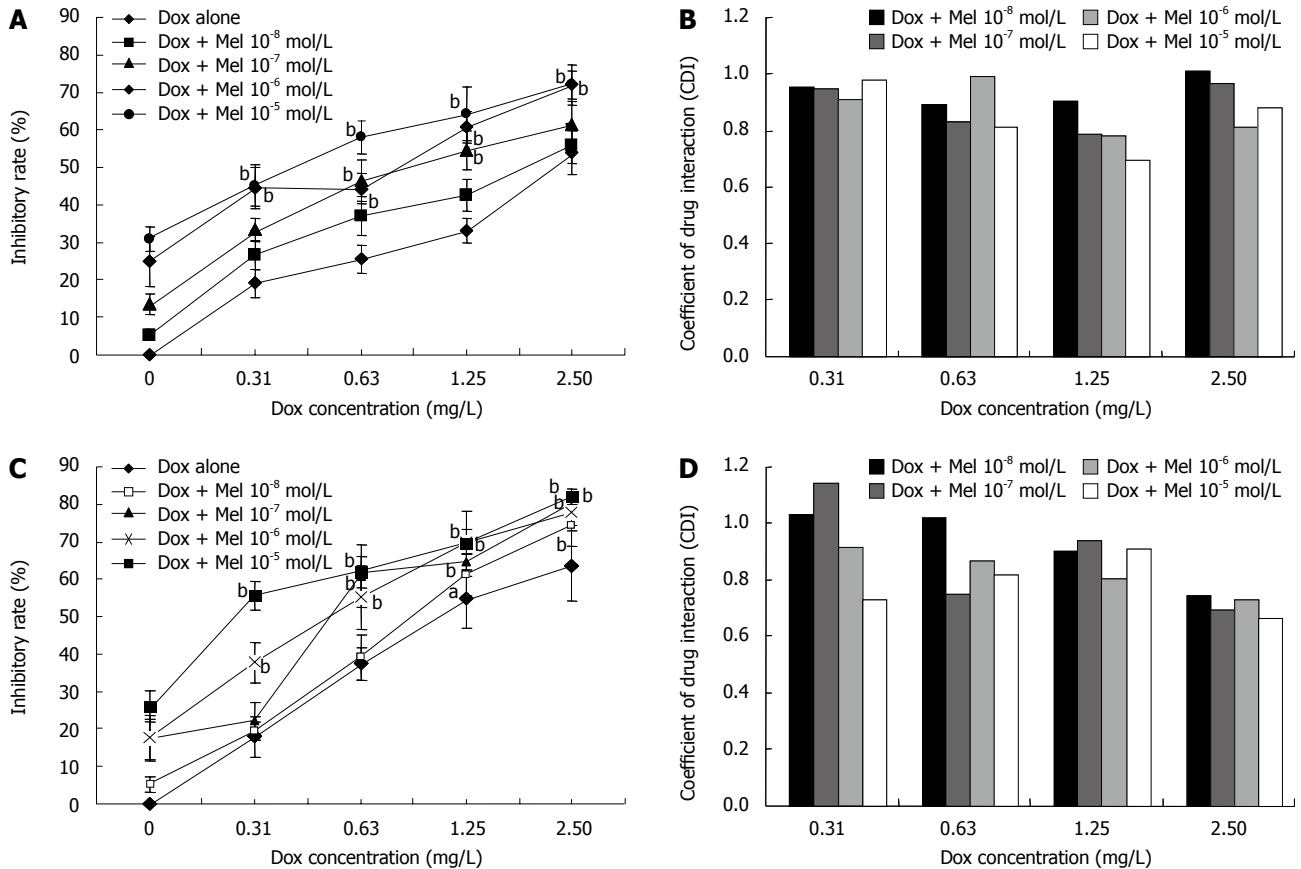


Figure 1 Synergistic effects and CDI of Melatonin and Doxorubicin on HepG2 and Bel-7402 cells. HepG2 cells (A) and Bel-7402 cells (C) were treated with 10^{-8} , 10^{-7} , 10^{-6} and 10^{-5} mol/L Melatonin and 0.31, 0.63, 1.25 and 2.50 g/L Doxorubicin for 48 h; CDI values of the combination group in HepG2 cells (B) and Bel-7402 cells (D). Data are presented as mean \pm SE (error bar) of triplicate cultures. ^a $P < 0.05$, ^b $P < 0.01$ vs Dox treated alone.

ECLIPSE 80i biology microscope, Nikon Digital Camera DXM 1200F, ACT-1 version 2.63 software (Japan), and JEOA 801D morphological biological image analysis software version 6.0 (Jie Da Technologies, Inc., China). The average absorbance value was analyzed by 5 randomly selected optical fields under microscopy ($\times 400$).

Flow cytometry

The cells were grown in 6-well plates and then treated with Melatonin and/or Doxorubicin at the desired concentrations. After exposure to drugs for 48 h, cells were trypsinized, washed twice with cold PBS and centrifuged. The cell pellet was resuspended in 1 mL cold PBS and fixed in 9 mL of 70% ethanol at -20°C for at least 12 h. Then cells were centrifuged and resuspended in 500 μL PBS, RNase A was added and the cells were incubated at 37°C for 30 min. Propidium iodide (PI) staining buffer was added in the dark at room temperature for 30 min (according to the procedure program of the Cell Apoptosis PI Detection Kit). A minimum of $1 \times 10^6/\text{mL}$ cells for each group was analyzed using an EPICS XL-MCL model counter (Beckman Coulter, Fullerton, CA, USA).

Statistical analysis

Biostatistical analyses were done using the SPSS 11.5 software package. All experiments were repeated at least three times. The results of multiple experiments are

given as the mean \pm SE. Non-parametric Kruskal-Wallis test was used to detect differences among the different experimental groups. The Mann-Whitney U -test was subsequently used for statistical evaluation in two-group comparisons. Pearson correlation coefficient was used for continuous independent and dependent variables. A level of $P < 0.05$ was accepted as statistically significant.

RESULTS

Inhibitory effect of Melatonin on hepatoma cell proliferation

HepG2 and Bel-7402 cells incubated with various concentrations of Melatonin alone for 48 h showed a dose-dependent reduction of cell proliferation. The inhibitory rate of Melatonin (10^{-8} , 10^{-7} , 10^{-6} and 10^{-5} mol/L) on HepG2 cells was $5.27\% \pm 1.53\%$, $13.53\% \pm 2.73\%$, $24.91\% \pm 6.60\%$, and $30.78\% \pm 3.28\%$, ($r = 0.993$, $P < 0.01$) and that on Bel-7402 cells was $5.35\% \pm 2.05\%$, $17.06\% \pm 5.51\%$, $17.73\% \pm 5.91\%$, and $26.09\% \pm 4.23\%$, respectively ($r = 0.952$, $P < 0.05$). The IC_{50} of Melatonin on HepG2 and Bel-7402 cells were 7.623×10^{-5} and 4.693×10^{-4} , respectively.

Synergistic cytotoxicity of combined Melatonin and Doxorubicin

To investigate the synergistic inhibitory effects of Mela-

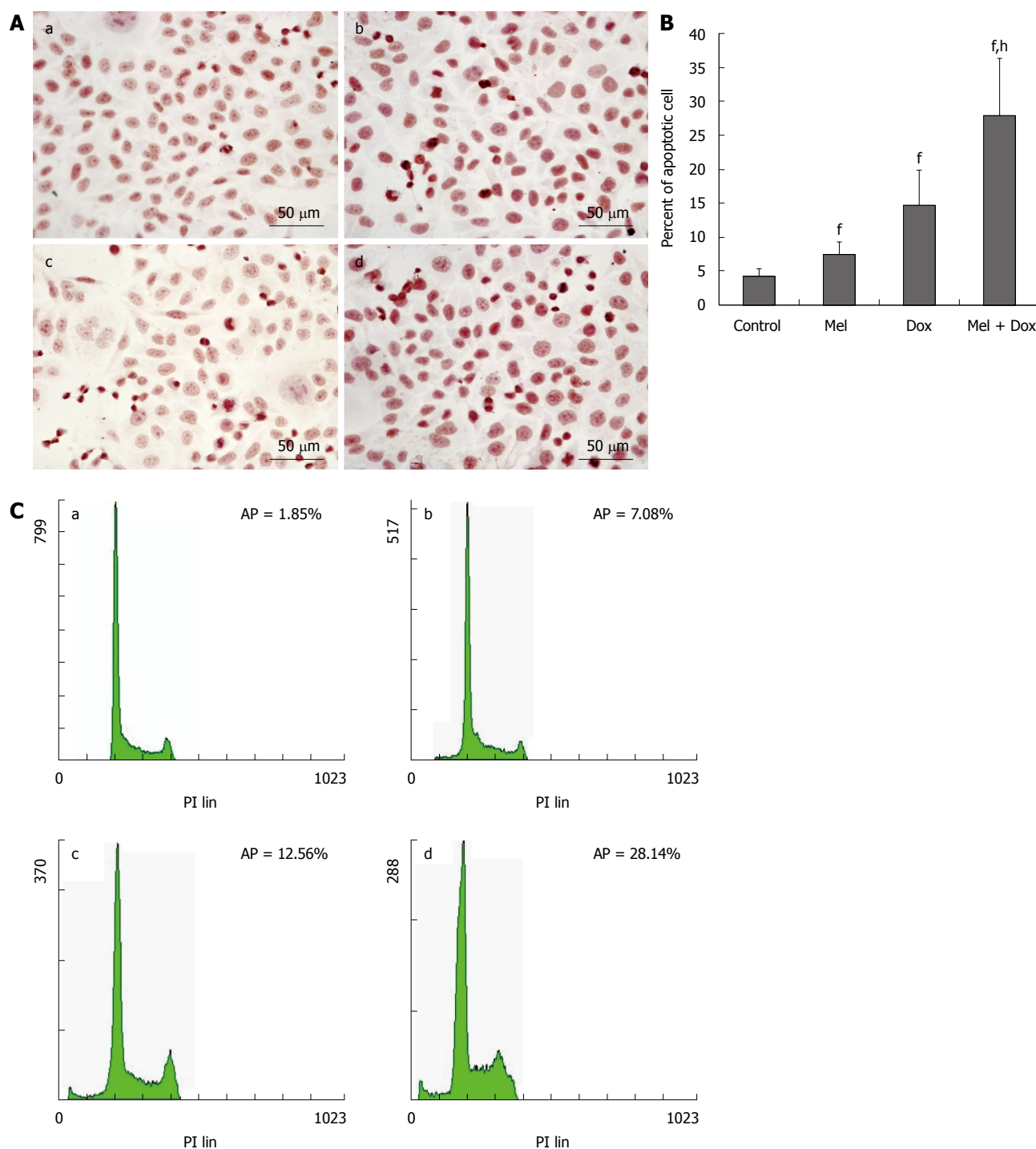


Figure 2 Effects of Melatonin and/or Doxorubicin on apoptosis of HepG2. A: Morphological changes of HepG2 cells treated with Mel and Dox. a: Untreated cells; b: 10^{-5} mol/L Mel; c: 1.25 mg/L Dox; d: 10^{-5} mol/L Mel plus 1.25 mg/L Dox; B: Percentage of apoptotic cells by TUNEL assay. Data are presented as mean \pm SD (error bar) of triplicate cultures. ^f $P < 0.01$ vs control, ^h $P < 0.01$ vs Dox treated alone; C: Apoptotic cells determined by FCM assay. a: Untreated cells; b: 10^{-5} mol/L Mel; c: 1.25 mg/L Dox; d: 10^{-5} mol/L Mel plus 1.25 mg/L Dox.

tonin and Doxorubicin, four different doses of Melatonin (10^{-8} , 10^{-7} , 10^{-6} and 10^{-5} mol/L) and four different concentrations of Doxorubicin (0.31, 0.63, 1.25 and 2.5 mg/L) were chosen and assessed. As depicted in Figure 1, Melatonin increased the cytotoxicity of Doxorubicin on HepG2 (Figure 1A and B) and Bel-7402 cells (Figure 1C and D). CDI was used to evaluate the nature of the interaction. When 10^{-5} mol/L Melatonin was combined with 1.25 mg/L Doxorubicin, the synergistic inhibitory effects

is the strongest on HepG2 (Figure 1B). Doxorubicin and Melatonin had the strongest synergism on Bel-7402 when 10^{-5} mol/L Melatonin was combined with 2.5 mg/L Doxorubicin (Figure 1D).

Proapoptotic effect of combined Melatonin and Doxorubicin

HepG2 and Bel-7402 cells were treated with different concentrations of Melatonin, Doxorubicin or a combination

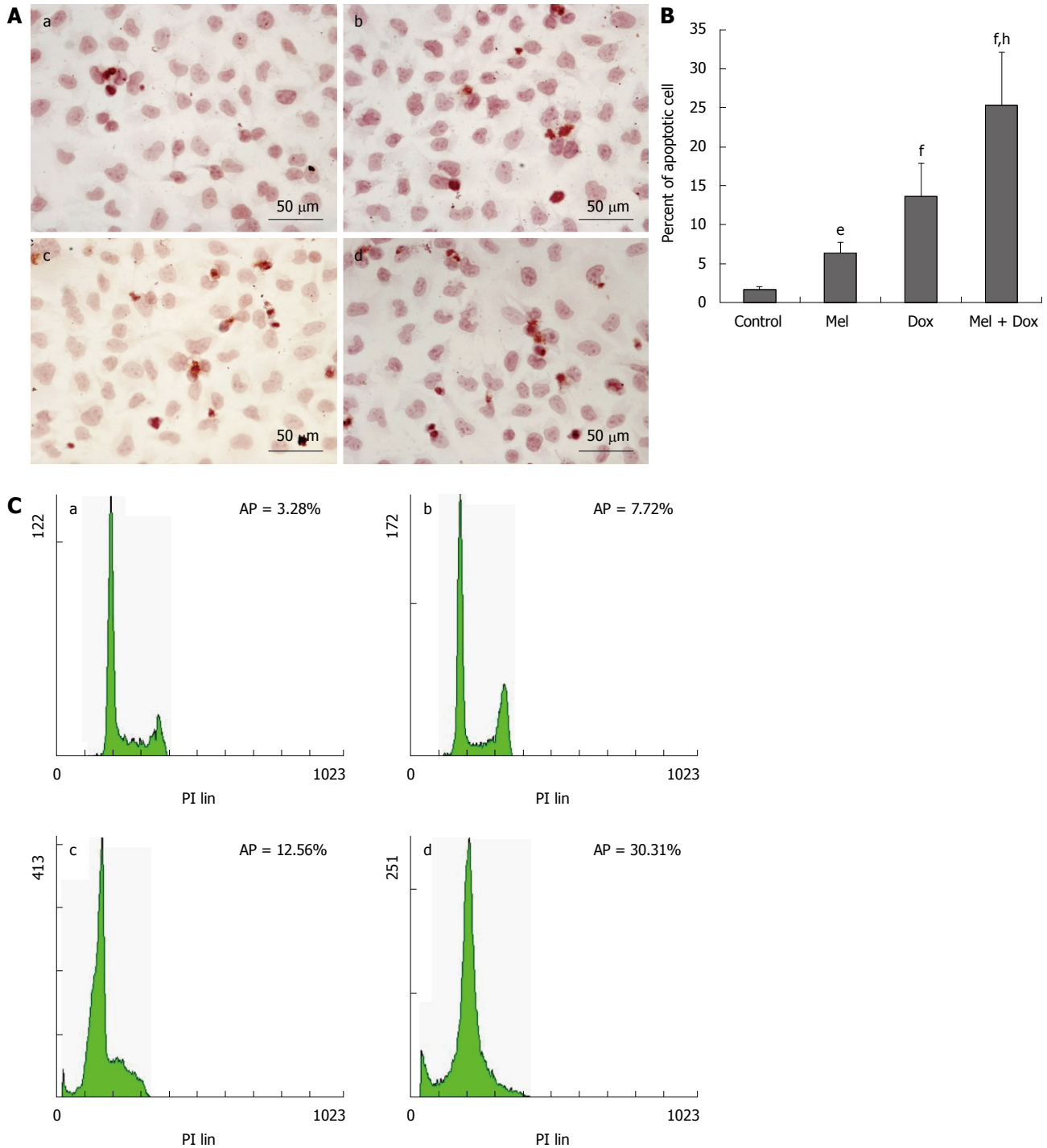


Figure 3 Effects of Melatonin and/or Doxorubicin on apoptosis of Bel-7402. A: Morphological changes of Bel-7402 cells treated with Mel and Dox. a: Untreated cells; b: 10⁻⁵ mol/L Mel; c: 2.5 mg/L Dox; d: 10⁻⁵ mol/L Mel plus 2.5 mg/L Dox; B: Percentage of apoptotic cells by TUNEL test. Data are presented as mean \pm SD (error bar) of triplicate cultures. ^a*P* < 0.05, ^b*P* < 0.01 vs control, ^c*P* < 0.01 vs Dox treated alone; C: Apoptotic cells determined by FCM assay. a: Untreated cells; b: 10⁻⁵ mol/L Mel; c: 2.5 mg/L Dox; d: 10⁻⁵ mol/L Mel plus 2.5 mg/L Dox.

of both for 48 h, respectively, and apoptosis was assessed by TUNEL and FACS methods. We found that in the combination treatment group, there was a greater degree of induction of apoptosis than that caused by either agent alone. In HepG2 cells, the number of apoptosis cells in Melatonin (10⁻⁵ mol/L) or Doxorubicin (1.25 mg/L) alone treatment group was only slightly increased when compared with the cell control group. However, the apoptotic rate

increased greatly when the cells were treated with combined Doxorubicin and Melatonin (Figure 2A and B). Similar results were found in Bel-7402 cells (Figure 3A and B). These results were confirmed by FCM assay. As shown in Figure 2C and Figure 3C, the sub-G1 peak, which appeared before the G1 phase that represents apoptotic cell population, was slightly increased in these two cell lines treated with Doxorubicin or Melatonin alone. The

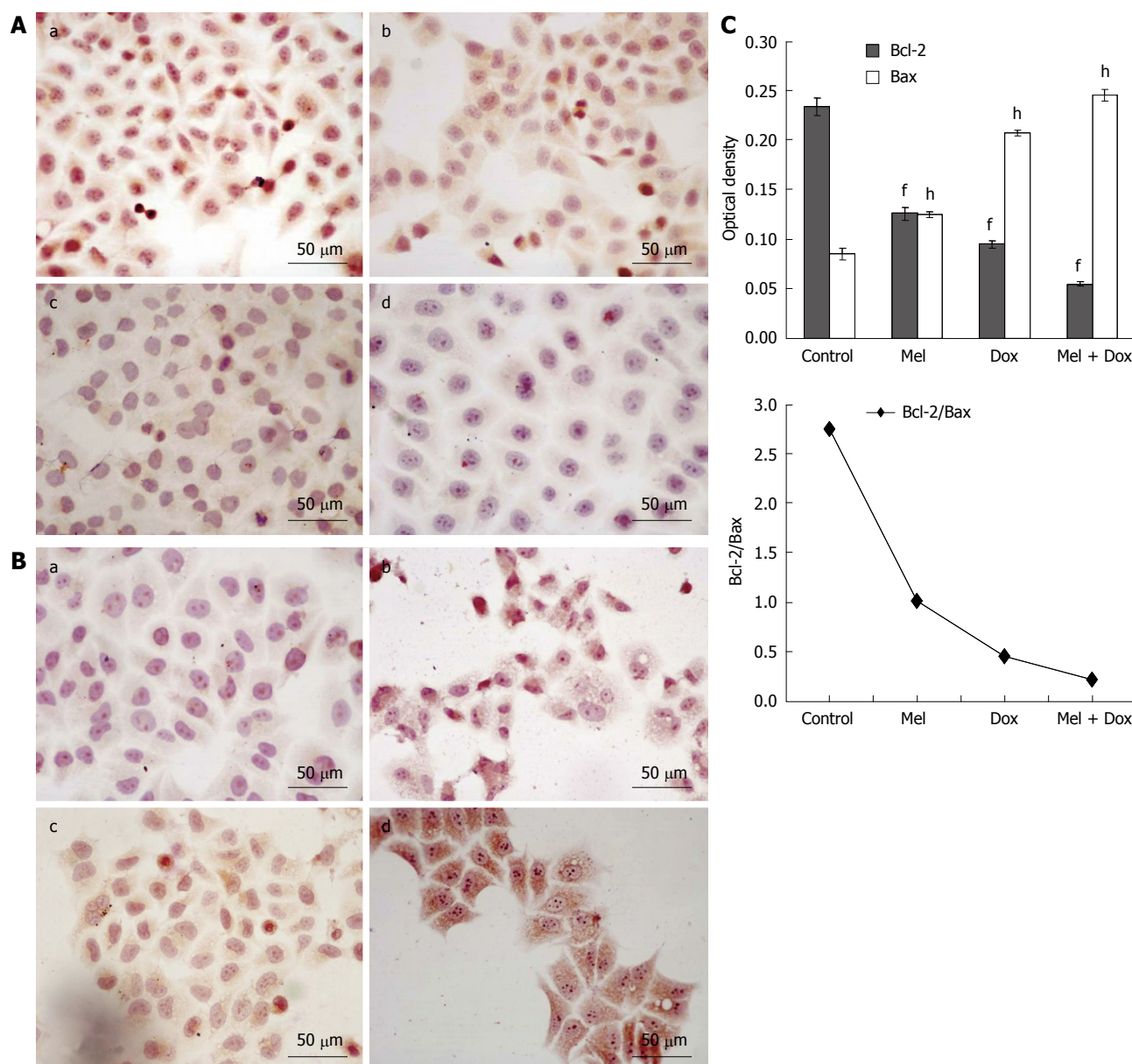


Figure 4 Effect of Melatonin and/or Doxorubicin on the expression of Bcl-2 (A) and Bax (B) in HepG2 cells, the quantitative analysis (C) of Bcl-2 and Bax. Quantitative analysis of Bcl-2 and Bax, expression by Biological Image Analysis System. Data are presented as mean \pm SD (error bar). a: Untreated cells; b: 10^{-5} mol/L Mel; c: 1.25 mg/L Dox; d: 10^{-5} mol/L Mel plus 1.25 mg/L Dox. S-P ($\times 400$). ^f $P < 0.01$ vs control of Bcl-2, ^h $P < 0.01$ vs control of Bax.

apoptotic peak was dramatically increased when the cells were exposed to combined Melatonin and Doxorubicin.

The standard positive Bcl-2 and Bax expressions were stained brown or yellow mainly in cytoplasm or membrane. After treatment for 48 h, the expression of Bcl-2 decreased (Figure 4A and Figure 5A) and in contrast, there was a significant increase of Bax expression, especially in the combination groups (Figure 4B and Figure 5B). The results were quantitatively analyzed by biological image analysis system and the ratio of Bcl-2/Bax decreased correspondingly (Figure 4C and Figure 5C).

To determine whether caspase-3 plays a role in Melatonin and/or Doxorubicin mediated apoptosis of HepG2 and Bel-7402 cells, we assessed the activated caspase-3 protein level of the two cell lines before and after treatment with Melatonin and/or Doxorubicin using

S-P method. The standard positive caspase-3 expressions were stained brown or yellow mainly in cytoplasm or nucleus. As shown in Figure 6, the expression of activated caspase-3 increased especially in the combination group after the two cell lines were exposed to drugs for 48 h.

DISCUSSION

Our results in the present study indicate that Melatonin inhibits growth of HepG2 and Bel-7402 cells in a dose-dependent manner. Meanwhile, combinations of Melatonin and Doxorubicin results in enhanced growth inhibitory effect and induction of cell apoptosis when compared with Melatonin or Doxorubicin used alone. These results suggest that chemotherapy combined with Melatonin may increase the therapeutic effect of anti-

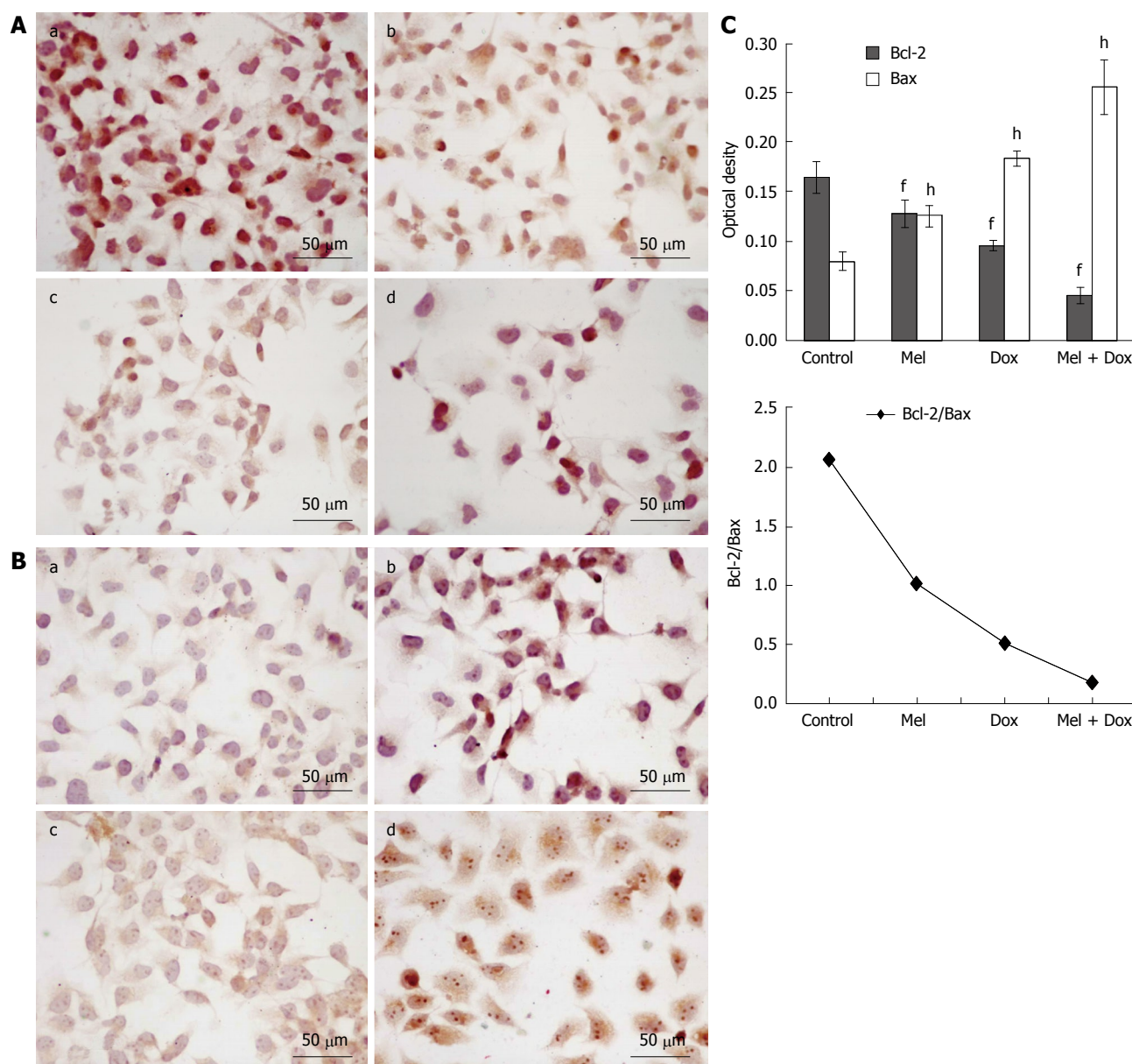


Figure 5 Effect of Melatonin and/or Doxorubicin on the expression of Bcl-2 (A) and Bax (B) in Bel-7402 cells, the quantitative analysis (C) of Bcl-2 and Bax. Quantitative analysis of Bcl-2 and Bax, expression by Biological Image Analysis System. Data are presented as mean \pm SD (error bar). a: Untreated cells; b: 10^{-5} mol/L Mel; c: 2.5 mg/L Dox; d: 10^{-5} mol/L Mel plus 2.5 mg/L Dox. S-P ($\times 400$). ^f $P < 0.01$ vs control of Bcl-2, ^h $P < 0.01$ vs control of Bax.

cancer drugs. Our results demonstrated that Melatonin had a synergetic effect with Doxorubicin in inhibiting the proliferation of non-small cell lung cancer (A-549)^[15]. Moreover, combining with the study of Martin-Renedo *et al.*^[16], our observation extend the possibility that Melatonin may provide a potential approach for the development of agents for the treatment and prevention of HCC.

Although the exact mechanism of the cytotoxicity of Melatonin against tumor cells is not very clear, many potential mechanisms have been proposed for the growth inhibition by Melatonin on cultured cells and animal models. These mechanisms include the induction of apoptosis^[17], the direct augmentation of natural killer (NK) cell activity^[17], which increases immunosurveillance, as well as the stimulation of cytokine production, e.g. interleukin IL-2, IL-6, IL-12 and interferon IFN γ ^[18]. Apoptosis or

programmed cell death is an essential physiological process that plays a critical role in development and tissue homeostasis. However, apoptosis is also involved in a wide range of pathological conditions^[19]. Apoptotic defects are a common event in oncogenesis and contribute to drug resistance^[20,21]. It is therefore interesting to search for ways to facilitate this apoptotic process after the use of chemotherapeutic drugs. Many studies have demonstrated that Melatonin could induce apoptosis *in vitro* and *in vivo*. To investigate the apoptosis-inducing effect of Melatonin as a single agent and combined Melatonin and Doxorubicin in hepatoma cells, the morphological changes and apoptotic rate were detected. The cells treated with the drugs showed the typical characteristics of apoptosis, which were more prominent in the combination group. Similarly, an apoptotic peak appeared before G1 phase when treated

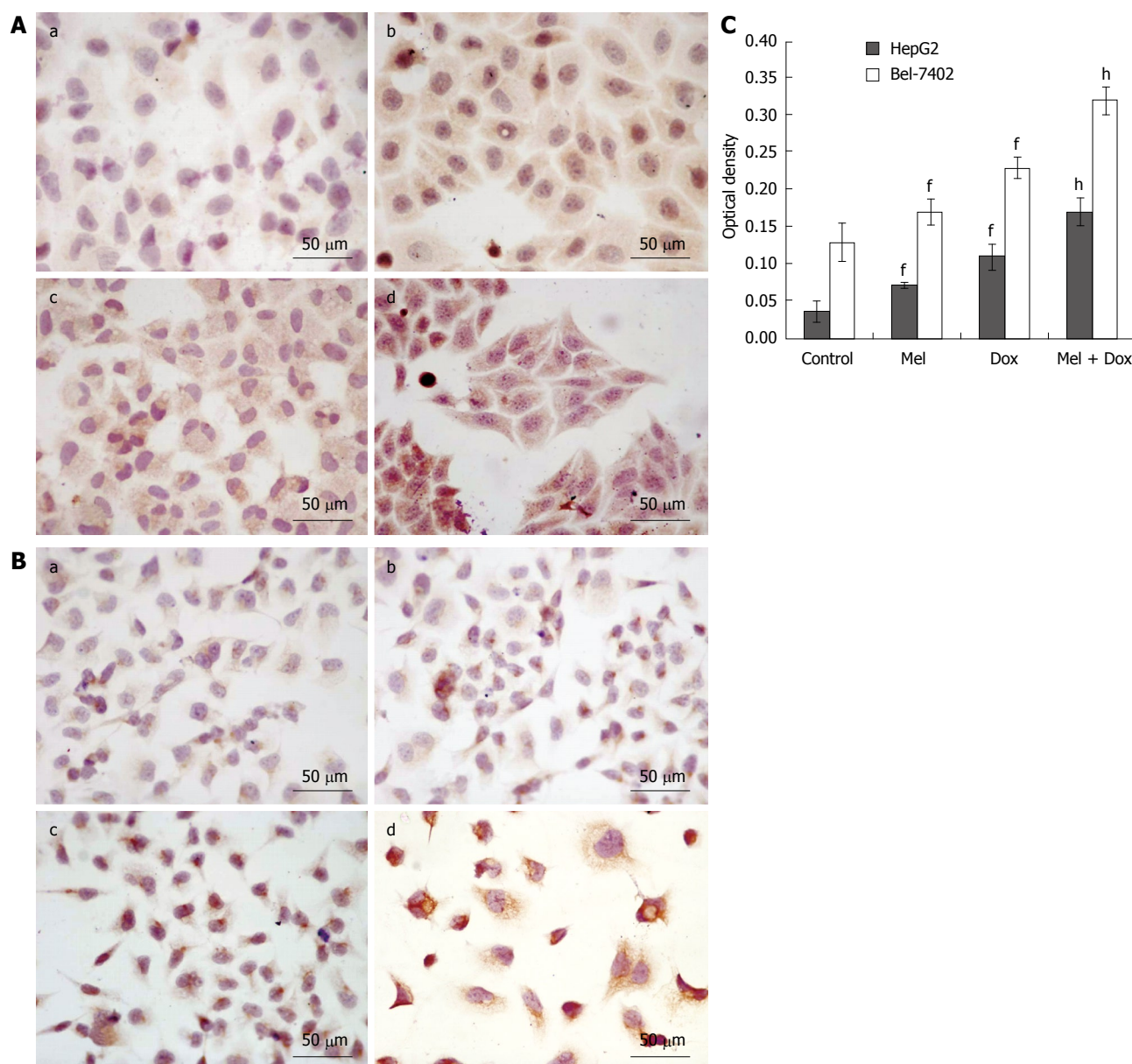


Figure 6 Effect of Melatonin and/or Doxorubicin on the expression of Caspase-3 in HepG2 (A) and Bel-7402 cells (B), and the quantitative analysis (C) of Caspase-3 expression. A: (a) Untreated cells; (b) 10^{-5} mol/L Mel; (c) 1.25 mg/L Dox; (d) 10^{-5} mol/L Mel plus 1.25 mg/L Dox; B: (a) Untreated cells; (b) 10^{-5} mol/L Mel; (c) 2.5 mg/L Dox; (d) 10^{-5} mol/L Mel plus 2.5 mg/L Dox. S-P ($\times 400$); C: Quantitative analysis of Caspase-3 expression by Biological Image Analysis System. Data are presented as mean \pm SD (error bar). ^f $P < 0.01$ vs control of Caspase-3, ^h $P < 0.01$ vs Dox alone.

with Melatonin or Doxorubicin alone, and a significant synergistic effect on the induction of apoptosis was observed in the combination group.

Several factors contribute to apoptosis, but the key elements are categorized into two main families of proteins including caspase enzymes and Bcl-2 family^[22]. Bcl-2 family is a set of cytoplasmic protein members that regulate apoptosis. The two main groups of this family, Bcl-2 and Bax proteins, are functionally opposed: Bcl-2 acts to inhibit apoptosis, whereas Bax counteracts this effect^[23,24]. Caspases are crucial mediators of programmed cell death (apoptosis). Among them, caspase-3 is a frequently activated death protease, catalyzing the specific cleavage of many key cellular proteins^[23,25]. Thus, we studied bcl-2

and bax and caspase-3 protein in hepatoma cell lines. The current findings show that Melatonin and/or Doxorubicin increased the expression of Bax and caspase-3, decreased the expression of Bcl-2. Furthermore, a significant decrease in the ratio of Bcl-2/Bax was observed when Melatonin was treated in combination with Doxorubicin, which was correlated with the incidence of apoptosis.

In conclusion, these results indicate that Melatonin in combination with Doxorubicin has significantly synergistic growth-inhibitory and apoptosis-including effects on the human hepatoma cell lines HepG2 and Bel-7402, which may be related to down-regulation of Bcl-2, up-regulation of Bax, and activation of caspase-3. Melatonin is expected to be an adjuvant drug in HCC treatment in the future.

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COMMENTS

Background

Hepatocellular carcinoma (HCC) is a major contributor to cancer incidence and mortality in the world. Currently, No effective treatment is available. Therefore, there is a critical need to develop effective chemotherapeutic strategies for hepatomas.

Research frontiers

Melatonin, the chief secretary product of the pineal gland has shown anti-neoplastic activities in both cell lines and animal models. However, studies on the oncostatic effects of Melatonin in the treatment of hepatocarcinoma are limited. In this study, the authors demonstrate that Melatonin has synergistic effects with Doxorubicin on the growth-inhibition and apoptosis-induction of human hepatoma cell lines.

Innovations and breakthroughs

This is the first report on the anti-proliferation, induction of apoptosis by Melatonin and Doxorubicin synergistically in human hepatoma cell lines.

Applications

Melatonin might be useful as an adjuvant drug in hepatocarcinoma treatment.

Terminology

Melatonin is a small lipophile molecule that is essentially secreted by the pineal gland and its synthesis shows a circadian pattern. Melatonin plays an important role in biological rhythm resynchronization, sleep induction, immunomodulation and tumor inhibition.

Peer review

The study puts forward an interesting point in Melatonin-related chemotherapy and provides some experimental evidence that Melatonin can be of interest in treatment of hepatocellular carcinoma. This cell culture-based study focused on the molecular synergism of Melatonin and Doxorubicin in apoptosis of hepatoma cells. The main limit of the study is that cultured cell lines were exclusively used.

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Effect of Recql5 deficiency on the intestinal tumor susceptibility of *Apc^{min}* mice

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CONCLUSION: Recql5 has a tumor suppression role in the mouse gastrointestinal tract.

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Key words: Recql5; *Apc*; Tumor suppressor; Genome instability; Colon cancer; *Apc^{min/+}* mice

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Abstract

AIM: To investigate whether Recql5, a DNA helicase that plays an important role in the maintenance of genome integrity, is a tumor suppressor in the gastrointestinal tract in mice.

METHODS: We generated cohorts of both *Recql5*-proficient and *Recql5*-deficient *Apc^{min/+}* mice and compared the tumor susceptibility in their gastrointestinal tracts.

RESULTS: Recql5 deficiency in *Apc^{min/+}* mice resulted in a significant increase in the tumor incidence in both the colon ($P = 0.0162$) and the small intestine ($P < 0.01$). These findings have provided the first genetic evidence for a tumor suppression role of Recql5 in the gastrointestinal tract of mice. Importantly, since mouse Recql5 and human RECQL5 are highly conserved, these findings also suggest that RECQL5 may be a tumor suppressor for human colon cancer.

INTRODUCTION

Cancer is a complicated genetic disorder, which may result from a myriad of deleterious oncogenic events induced by both endogenous and environmental insults, which perturb the normal growth control and physiological functions of cells^[1,2]. Most tumors are found to harbor genetic changes of either activation of proto-oncogenes, or inactivation of tumor suppressor genes (TSGs), or both^[3]. In particular, inactivation of TSGs represents an important early event of carcinogenesis in colorectal cancer. Generally, TSGs can be categorized into two major types, so-called “gatekeeper” and “caretaker” genes^[4,5]. Gatekeepers, such as the Retinoblastoma gene and the Adenomatous polyposis coli (*APC*) gene, have pivotal roles in cell proliferation by regulating cell cycle checkpoints, apoptosis and signaling transduction^[6,7]. It has been hypothesized that the loss of caretakers

provides the initial changes for the initiation of carcinogenesis, whereas mutations in gatekeepers provide the necessary “promotion” effect for the fully fledged development of cancer.

Chromosome instability (CIN) is one of the hallmarks of many cancer cells, and it has been suggested that CIN, both structural and numerical, contributes to the development of malignancies, and in particular, colorectal cancer^[8,9]. CIN may occur through many different mechanisms, such as DNA breaks, centrosome amplification, chromatid cohesion instability and cell cycle checkpoint defects^[9,10]. We have reported recently that deletion of Recql5, a member of the RecQ DNA helicase family, in mice resulted in a rearrangement type of CIN and an increased susceptibility to cancer in a number of organs and tissues, but not in the intestinal tract^[11]. Nonetheless, given that CIN is known to play an important role in the development of colorectal cancer, we suspected that Recql5 might have a role in tumor suppression in the gastrointestinal (GI) tract but that such an effect could not be readily detected in our previous study using straight *Recql5* knockout mice. *Apc^{Min}* mice have been widely used as a sensitizing background for assessing the potential oncogenic effect in the GI tract of specific genetic alterations^[12]. *Apc^{Min}* mice carry a spontaneous point mutation in one of the two copies of the *Apc*, the mouse homologue of the human *APC* TSG. In humans, mutations in this *APC* TSG give rise to familial adenomatous polyposis syndrome^[13]. In adult *Apc^{Min}* mice, the loss of the remaining wild-type copy of the *Apc* gene in the colonic epithelium predisposes these cells to tumorigenesis, leading to development of adenomas both in the small intestine and colon. The number of tumors developed per mouse as well as the size of these tumors can be affected by the genetic background of the animals. Thus, they have been used extensively for assessing the potential oncogenic effects of specific genetic alterations^[12].

In the current study, we have examined the potential effect of Recql5 deficiency on tumorigenesis within the gastrointestinal tract in *Apc^{Min/+}* mice, taking advantage of the sensitized genetic background for analyzing tumorigenesis in the GI tract provided by this well established model^[14].

MATERIALS AND METHODS

Mouse work

Recql5^{+/-} and *Recql5^{-/-}* mice were generated from crossings between *Recql5^{+/-}* mice, which were maintained in a mixed genetic background (87.5% C57BL/6 and 12.5% 129sv) as described previously^[15]. C57BL/6J (B6) and C57BL/6J-*Apc^{Min}*/J (*Apc^{Min/+}*) mice were purchased from the Jackson Laboratory (Bar Harbor, ME). All mice were propagated in the Case Western Reserve University American Association of Laboratory Animals accredited barrier-free facility. Mice were fed a commercially available rodent breeder diet, 5010 (PMI LabDiet). All cages, food, bedding, and water were autoclaved before

use. All procedures were approved by the Case Western Reserve University Institutional Animal Care and Use Committee.

Analysis of intestinal adenomas in *Apc^{Min/+}* mice

Recql5^{+/-} female mice were mated with B6-*Apc^{Min/+}* male mice. The resulting *Recql5^{+/-}Apc^{Min/+}* progeny were intercrossed to obtain both *Recql5^{+/+}Apc^{Min/+}* and *Recql5^{-/-}Apc^{Min/+}* mice. Genotyping was carried out by standard PCR methods^[16]. At 90 d of age, *Recql5^{+/+}Apc^{Min/+}* and *Recql5^{-/-}Apc^{Min/+}* mice were euthanized by CO₂ asphyxiation for quantitative analysis of intestinal adenomas. The entire intestine tract from duodenum to anus was removed, washed in phosphate buffered saline (PBS), opened longitudinally and pinned luminal side up on a wax dissection plate. Intestinal adenomas (macroadenomas with maximal diameters ≥ 1 mm, microadenomas < 1 mm) along the entire intestine were counted by microscopic examination at 10 \times magnification followed by fixation with 10% formalin in PBS (Fisher Scientific). Digital images of polyps and a metric ruler were captured using SPOT software 3.2.5 for Macintosh and an RT color SPOT camera mounted on a Leica MZFLIII workstation (Diagnostic Instruments). The percentages were calculated by dividing numbers of mice with more than 100 macroadenomas against total numbers of mice.

Statistical analysis

Statistical analyses were performed with the two-sample student's *t*-test using the Prism software package (Graph-Pad Software).

RESULTS

To investigate whether Recql5 deficiency may affect the adenoma multiplicity by either accelerating the loss of wild type *Apc* or by promoting the progression of tumors, we introduced the *Recql5* knockout allele into the *Apc^{Min/+}* background and analyzed the phenotype of intestinal adenomas at 90 d of age when mice are not severely affected by other complications, such as anemia.

We found that under our specific experimental conditions and a C57BL/6J \times 129Sv mixed genetic background, *Recql5^{+/+}Apc^{Min/+}* mice developed 72.9 ± 12.7 (mean \pm SE) macroadenomas (diameter ≥ 1 mm) along the entire intestinal tract at 90 d (Figure 1A and C). Remarkably, *Recql5^{-/-}Apc^{Min/+}* littermates developed 142.5 ± 9.4 macroadenomas at the same age, which is significantly higher than that observed in their *Recql5^{+/+}Apc^{Min/+}* littermates ($P = 0.0032$) (Figure 1A and C, Table 1). In particular, 15 out of 19 *Recql5^{-/-}Apc^{Min/+}* mice (78.9%) had more than 100 macroadenomas (Figure 1B and C) compared with only 1 out of 7 (14.2%) of such individuals in the *Recql5^{+/+}Apc^{Min/+}* cohort. Interestingly, however, although the average number of microadenomas (diameter < 1 mm) was higher in the *Recql5^{-/-}Apc^{Min/+}* cohort than in mice of the corresponding *Recql5^{+/+}Apc^{Min/+}* cohort, the difference did not reach a signifi-

Table 1 Effect of *Recql5* status on the intestine polyp multiplicity (\pm SE) of *Apc^{min/+}* mice at age 90 d

Genotype	Size	Upper small intestine (duodenum and jejunum)	Lower small intestine (ileum)	Large intestine (colon)
<i>Recql5^{+/+}Apc^{min/+}</i>	Macroadenoma (≥ 1 mm)	23.70 \pm 4.04	52.20 \pm 10.90	1.20 \pm 0.65
	Microadenoma (< 1 mm)	10.80 \pm 2.57	25.20 \pm 7.77	0.80 \pm 0.65
<i>Recql5^{-/-}Apc^{min/+}</i>	Macroadenoma (≥ 1 mm)	28.20 \pm 3.38 ($P = 0.270$)	108.00 \pm 11.50 ($P = 0.006$)	2.80 \pm 0.47 ($P = 0.041$)
	Microadenoma (< 1 mm)	13.10 \pm 2.04 ($P = 0.360$)	41.40 \pm 4.82 ($P = 0.070$)	0.90 \pm 0.33 ($P = 0.450$)

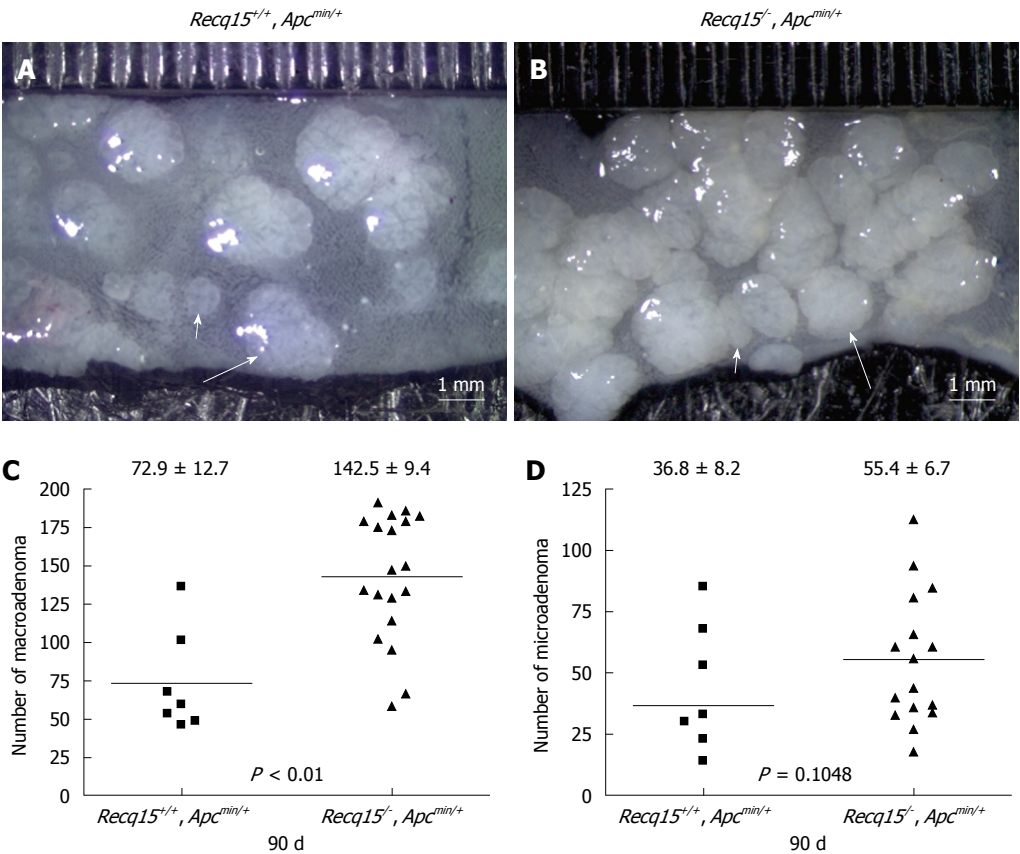


Figure 1 Effects of *Recql5* deficiency in the development of intestinal adenomas in the *Apc^{Min/+}* mice. *Recql5^{+/+}Apc^{Min/+}* and *Recql5^{-/-}Apc^{Min/+}* mice were monitored and sacrificed at 90 d of age. A, B: Representative microscopic images of sections from the ileum of a *Recql5^{+/+}Apc^{Min/+}* (A) and a *Recql5^{-/-}Apc^{Min/+}* (B) mouse. A microadenoma (diameter < 1 mm, short arrows) and a macroadenoma (diameter ≥ 1 mm, long arrows) are indicated in (A, B); C, D: Multiplicity of macroadenomas (C) and microadenomas (D) in the intestine of individual mice at 90 d of age. Mean and SE are also shown at the top of each column. Each data point represents the total number of macroadenomas (C) or microadenomas (D) in a single mouse.

cant level ($P = 0.1048$, Figure 1D, Table 1).

Previous studies have shown that although *Apc^{Min/+}* mice are highly prone to intestinal adenomas, colonic tumors were relatively infrequent (< 50% penetrance) in these mice^[16]. We found, however, that *Recql5* deficiency had a significant impact on the incidence of colonic tumors in these mice. It resulted in an increase of the colonic tumor incidence to 94.1% (16 out of 17 mice) (Figure 2). Moreover, microscopic examination revealed that some *Recql5^{+/+}Apc^{Min/+}* mice developed only small polyp-like nodules in their colons (Figure 2A, Table 1), whereas most *Recql5^{-/-}Apc^{Min/+}* mice had multiple macroadenomas in each colon (Figure 2B, Table 1). Together, this data clearly indicate that *Recql5* deficiency could play an important role in the development of intestinal adenomas in *Apc^{Min/+}* mice.

DISCUSSION

Previous studies have shown that *Apc^{Min/+}* mice on a congenic C57BL/6 background develop 30 to 50 macroadenomas at 90 to 120 d of age, respectively^[16]. We found that under the mixed genetic background resulting from intercrossing between B6/129.*Recql5^{-/-}* and B6.*Apc^{Min/+}* mice, *Recql5^{+/+}Apc^{Min/+}* mice developed 72.9 \pm 12.7 macroadenomas (diameter > 1 mm) along the entire intestinal tract at 90 d. This elevated adenoma development in *Recql5^{+/+}Apc^{Min/+}* mice suggests the existence of possible modifier(s) in this particular genetic background or a difference in environmental factors, such as diet. Importantly, comparing with cohorts of mice that were selected based on sibling pairs allows us to clearly show that the loss of *Recql5* in *Apc^{Min/+}* mice has a great impact

COMMENTS

Background

Colorectal cancer is a major type of human cancer. Knowledge regarding the molecular basis for the etiology of this disease can help in identifying novel biomarkers for its early diagnosis or in improving the efficacy of intervention regimens.

Research frontiers

This is the first report about the role of Recql5, a DNA helicase that plays an important role in the maintenance of genome integrity, in suppressing tumorigenesis in the gastrointestinal (GI) tract in mice.

Innovations and breakthroughs

In this article, the authors reported that Recql5 has a role in suppressing tumorigenesis in the GI tract in mice. Since mouse Recql5 and its human homologue (RECQL5) are highly conserved, these new findings have implicated RECQL5 as a suppressor for colorectal cancer in humans. Thus, RECQL5 may be used as a biomarker for this disease. Moreover, they have recently shown that mutations in *Recql5* resulted in a significantly enhanced sensitivity to anticancer drug camptothecin, the prototype of irinotecan that is currently used to treat colorectal cancer patients. Thus, the expression of RECQL5 could be used as a criterion for selecting patients for irinotecan-based chemotherapies.

Applications

No direct application may be derived based on the findings from this study. However, these findings should justify further investigation of the potential role of RECQL5 mutations in human GI cancers and the potential use of RECQL5 as a colorectal cancer biomarker or drug target.

Peer review

This is a well conceived, concisely written article, which certainly deserves publication.

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Wilson disease: Histopathological correlations with treatment on follow-up liver biopsies

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METHODS: We report a group of 12 WD patients treated with zinc and/or penicillamine who underwent multiple follow-up liver biopsies. Demographic, clinical and laboratory data were gathered and all patients underwent an initial biopsy and at least one repeat biopsy.

RESULTS: Time to repeat biopsy ranged from 2 to 12 years. Six patients (non-progressors) showed stable hepatic histology or improvement. In one case, we observed improvement of fibrosis from stage 2 to 0. Six patients (progressors) had worsening of fibrosis. There was no significant correlation between the histological findings and serum aminotransferases or copper metabolism parameters. The hepatic copper concentration reached normal levels in only two patients: one from the non-progressors and one from the progressors group. The estimated rate of progression of hepatic fibrosis in the entire group was 0 units per year in the time frame between the first and the second liver biopsy (4 years), and 0.25 between the second and the third (3 years). In the progressors group, the rate of progression of liver fibrosis was estimated at 0.11 fibrosis units per year between the first and second biopsy and, 0.6 fibrosis units between the second and third biopsy.

CONCLUSION: The inability of clinical tools to detect fibrosis progression in WD suggests that a liver biopsy with hepatic copper quantification every 3 years should be considered.

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Key words: Wilson disease; Copper; Liver biopsy; Histopathology

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Abstract

AIM: To investigate the progression of hepatic histopathology in serial liver biopsies from Wilson disease (WD) patients.

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Cope-Yokoyama S, Finegold MJ, Sturniolo GC, Kim K, Mescoli C, Rugge M, Medici V. Wilson disease: Histopathological correlations with treatment on follow-up liver biopsies. *World J Gastroenterol* 2010; 16(12): 1487-1494 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v16/i12/1487.htm> DOI: <http://dx.doi.org/10.3748/wjg.v16.i12.1487>

INTRODUCTION

Wilson disease (WD) is an inherited, autosomal recessive disorder of copper accumulation that affects about one individual per 30 000 population^[1]. It is due to the dysfunction of a copper-transporting P-type ATPase that has a crucial role in copper excretion into the bile. The gene that encodes this P-type ATPase, ATP7B, is located on chromosome 13q14.3, and numerous gene mutations can impair the protein's function^[2,3], which leads to copper accumulation mainly in the liver, but also in the brain, cornea, and kidney. The most frequent clinical presentation of WD is liver involvement^[4]. Hepatic manifestations may vary from hepatomegaly and fatty liver, to acute hepatitis, with high serum aminotransferases, liver failure, jaundice, and cirrhosis. The earliest morphological features of WD are represented by micro- and macrovesicular hepatic steatosis, glycogenated nuclei in the periportal hepatocytes, and focal hepatocellular necrosis. With the progression of parenchymal damage and inflammation, fibrosis, and subsequently cirrhosis, invariably develop. Cirrhosis can have either a micronodular or a mixed macro-micronodular pattern, and it is rarely complicated by hepatocellular carcinoma or cholangiocarcinoma^[5]. Regarding the timing of the disease progression, cirrhosis is often diagnosed by the second decade, but there are some individuals who do not develop cirrhosis even after the fourth decade of life^[6,7]. The ultrastructural analysis is characterized typically by mitochondrial abnormalities, including variability in size and shape, increased density of the matrix material, and numerous inclusions of lipid and fine granular material, which may be copper^[8]. With adequate treatment, these changes may not occur. WD is a treatable disorder and early diagnosis is essential: the goal of therapy is to reduce copper accumulation by enhancing its urinary excretion (with chelating agents) and by decreasing its intestinal absorption (with zinc salts)^[9-11]. As a result of the rarity of the disease and the fact that the liver biopsy is not performed routinely during the follow-up of WD, unless clinically indicated, the progression and timing of the liver pathology and its correlation with different anti-copper treatments or with aminotransferase levels are poorly characterized. Previous studies have shown the possibility of improvement of the steatosis and inflammation grade^[12], and the fibrosis stage^[13,14] during long-term follow-up. However, studies on serial liver biopsies, as well as studies on the correlation between hepatic histology and clinical parameters, are lacking.

The overall objective of this study was to describe the evolution of liver histology in WD patients during penicillamine (PCA) and zinc treatment, to define the rate of progression of the liver damage, and to correlate the clinical and biochemical parameters of liver injury with hepatic copper concentration.

MATERIALS AND METHODS

We included 12 patients with WD from the Division of Gastroenterology and Hepatology, Padua University Hospital (Italy), who were followed from 1981 to 2006 and who underwent serial liver biopsies. The mean follow-up was 5 ± 3 years (range: 1-12 years). Patients with history of alcohol abuse, positive serology for hepatitis B and C, and features of the metabolic syndrome were excluded. The study was conducted according to the principles of the Declaration of Helsinki, and all patients gave informed consent before undergoing liver biopsy. WD was diagnosed when 24-h urine copper excretion was $> 100 \mu\text{g}/24 \text{ h}$, hepatic copper concentration was $> 250 \mu\text{g/g}$ dry weight, and serum ceruloplasmin was $< 20 \text{ mg/dL}$. All patients were treated with either PCA or zinc sulfate following the initial biopsy. Liver biopsy samples were obtained by the percutaneous route, using the Menghini method. Liver copper concentrations in dried liver tissue were measured by flame atomic absorption spectrophotometry. Demographic, clinical, and laboratory data were gathered, and all patients underwent an initial and at least one repeat biopsy. Selected laboratory values were recorded at the time of initial and repeat biopsies: serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST), 24-h urinary copper excretion, and hepatic copper concentration. Slides of formalin-fixed, paraffin-embedded tissue were stained with hematoxylin and eosin (HE), and with the histochemical stains Perls for hemosiderin and Van Gieson for fibrosis. Two experienced pathologists who scored the biopsies were blinded to all clinical and biochemical information, and to the order of execution of the biopsies. The biopsies were semi-quantitatively graded on three main parameters (inflammation, steatosis, and fibrosis). The grading of portal and lobular inflammation was 0, none; 1, mild; 2, moderate; and 3, severe. The grading of steatosis was 0, none; 1, up to 25%; 2, 25%-50%; 3, 50%-75%; and 4, 75%-100%. The staging of fibrosis was 0, none; 1, expansion of portal fibrous tissue; 2, early bridging, no nodules; 3, bridging fibrosis, early nodule formation; and 4, cirrhosis. Iron deposits were graded by a standard method, from 0 to 4, where grade 1 represents minimal iron deposition and grade 4 represents iron deposition throughout all zones^[15]. Patients were separated into two groups. We considered as progressors the patients who presented worsening of at least one unit of fibrosis. Inflammation and steatosis were described separately. Non-progressors presented stable or improved fibrosis scores. The rate of the fibrosis progression was calculated as the result of the mean difference in fibrosis scores divided by the mean interval in years between the first and second liver biopsies.

Statistical analysis

Data are expressed as mean \pm SD or as proportions. The goodness-of-fit test was used to determine whether the distributions of continuous variables were normal prior to analysis^[16]. Measurements were log-transformed as necessary to improve the normality of residuals and homoscedasticity (or homogeneity of variance) of errors before analysis. Paired *t* test was used to compare the underlying mean differences in response between follow-up and baseline. Separate analyses were performed for each variable and each follow-up time. Analysis of variance (ANOVA) was performed to assess: (1) whether there was a significant mean difference in each response between two independent samples (i.e. treatment groups); and (2) whether there was a significant mean difference between progressors and non-progressors, after controlling the potential effects of other variables. Pearson (Spearman) correlation coefficients were estimated to assess the magnitude and direction of a linear association between two given continuous (ranked) variables. Individual trajectories of serum measurement changes in response level over the follow-up time were estimated from linear random-effect models. Each response level was entered as the dependent variable and treatment, follow-up time, and treatment \times follow-up time interaction were entered as the independent variables. To account for inter-subject heterogeneity in the change of response level, intercept and time were modeled as random effects. A two-side *P* value of 0.05 was considered significant. All statistical analyses were performed using SAS, Version 9.1 (SAS Institute, Cary, NC, USA).

RESULTS

Clinical features

Table 1 summarizes the clinical features for progressors *vs* non-progressors. The mean age of the 12 patients at diagnosis was 17.3 years (range: 6-35 years), and three (25%) were female. Ten patients presented with different degrees of hepatic symptoms and two with mixed hepatic and neurological disease, mainly characterized by rigidity, tremors, and dystonia. At baseline, before anti-copper treatment, 24-h urinary copper concentration was 994 ± 1293 $\mu\text{g}/24$ h, and mean hepatic copper concentration was 491 ± 260 $\mu\text{g}/\text{g}$ dry liver. Five patients were started on PCA after the diagnosis, and the remaining seven on zinc salts. Most patients remained on the same drug during follow-up. One patient began zinc treatment that was later changed to PCA, and the therapy switch was indicated by the lack of improvement of liver enzymes. At diagnosis, the mean ALT was 84.1 ± 50.9 U/L, and AST was 62.8 ± 50.5 U/L. During follow-up, we observed a significant improvement of aminotransferase levels, with mean ALT 37.3 ± 20.6 U/L and AST 35.3 ± 34.8 U/L (*P* = 0.01 and 0.03, compared to baseline) at the time of the second liver biopsy and mean ALT 38.3 ± 17 U/L and AST 27.9 ± 9.9 U/L at the time of the third liver biopsy. There was no significant change in hepatic copper concentration over time (Figure 1), and

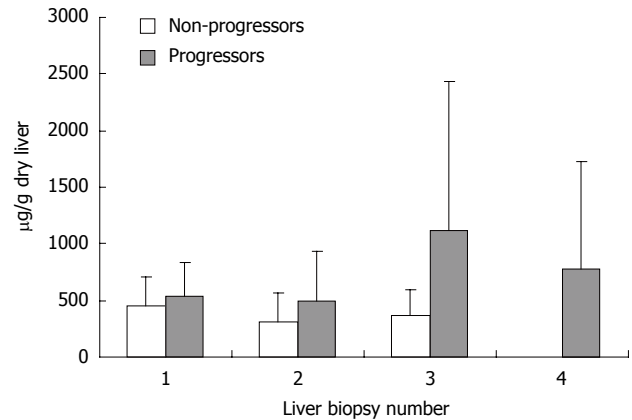


Figure 1 Evolution of the hepatic copper concentration in patients who presented with progression and without progression of the total histological score. Progressors had a higher hepatic copper concentration at all time points, but the difference between the two groups was not significant.

there was no significant difference in terms of laboratory data improvement between progressors and non-progressors (Table 1).

Hepatic histology

All patients underwent at least two liver biopsies. The average number of portal tracts was 14.9 ± 7.5 (range: 4-34). The mean interval between the first and the second liver biopsy was 4 years (range: 2-12 years) (12 patients). The mean interval between the second and third biopsy was 3 years (range: 2-4 years) (four patients). Two patients underwent a fourth liver biopsy after 2 and 3 years, respectively. None of the patients was on anti-copper treatment at the time of the first liver biopsy. The follow-up liver biopsies were indicated by an increase in the aminotransferase levels, or change of therapy, or were performed to monitor the potential disease progression. At baseline, five patients had grade 0 steatosis, three had grade 1, three had grade 2, and one presented with grade 3. Regarding the stage of fibrosis, seven had stage 0, three had stage 1, and two had stage 2. There were no cirrhotic patients. Three patients (two on zinc and one on PCA) showed overall improvement of the histological severity (patients 2, 3 and 6), with decreased steatosis (Figure 2A and B), and in one case, significant improvement of fibrosis, from stage 2 to 0 over 6 years of follow-up (Figure 2C and D). Three patients (one on zinc and two on PCA) showed no significant change in histology, although initial biopsies in this group showed mild lesions (patients 1, 4 and 5; Table 2). The patients with mixed neurological and hepatic phenotype were both included in the non-progressors group (patients 1 and 2). The six patients who manifested histological progression (patients 7-12) demonstrated worsening of inflammation and/or fibrosis (Figure 2E and F). Of these six patients, four had been started on zinc and two on PCA, and one was switched from zinc to PCA. One patient (#10) who showed an overall progression underwent the second liver biopsy 12 years after the first one, and over this time, the grade of steatosis and inflammation and the stage of fibrosis increased (Table 2).

Table 1 Baseline characteristics of patients who showed progression of the histologic score (progressors) *vs* those who showed overall improvement or no progression in histology (non-progressors)

	Progressors	Non-progressors
Age at diagnosis (mean \pm SD) (yr)	18.0 \pm 8.8	16.6 \pm 9.8
Phenotype	All hepatic phenotype	4 with hepatic phenotype; 2 with mixed hepatic and neurological phenotype
AST U/L (baseline) (normal range 15-43)	60.0 \pm 56.8 (range 18-44) (6)	64.6 \pm 51.3 (range 15-129) (6)
AST U/L (time of 2nd biopsy)	45.1 \pm 48.2 (range 20-143) (6)	25.5 \pm 9.7 (range 13-39) (6)
AST U/L (time of 3rd biopsy)	27.3 \pm 10.2 (range 13-44) (6)	28.7 \pm 10.8 (range 15-41) (3)
AST U/L (time of 4th biopsy)	24 and 28 (2)	
ALT U/L (baseline) (normal range 6-43)	83.5 \pm 40.2 (range 45-137) (6)	84.5 \pm 60.7 (range 15-164) (6)
ALT U/L (time of 2nd biopsy)	48.5 \pm 21.8 (range 24-80) (6)	26.1 \pm 12.3 (range 10-46) (6)
ALT U/L (time of 3rd biopsy)	42.0 \pm 17.2 (22 and 68) (2)	32.7 \pm 17.2 (range 8-48) (3)
ALT U/L (time of 4th biopsy)	47.0 \pm 7.0 (42 and 52) (2)	
24 h urinary Cu μ g/24 h (baseline)	679.7 \pm 504.0 (6)	1245.0 \pm 1649.0 (6)
Hepatic Cu mg/g dry liver (baseline)	534.4 \pm 298.7 (6)	455.0 \pm 245.3 (6)
Type of treatment	2 = penicillamine; 4 = zinc One patient switched to penicillamine during follow up	3 = penicillamine; 3 = zinc No change of treatment during follow up

The numbers in brackets represent the number of patients included in the analysis.

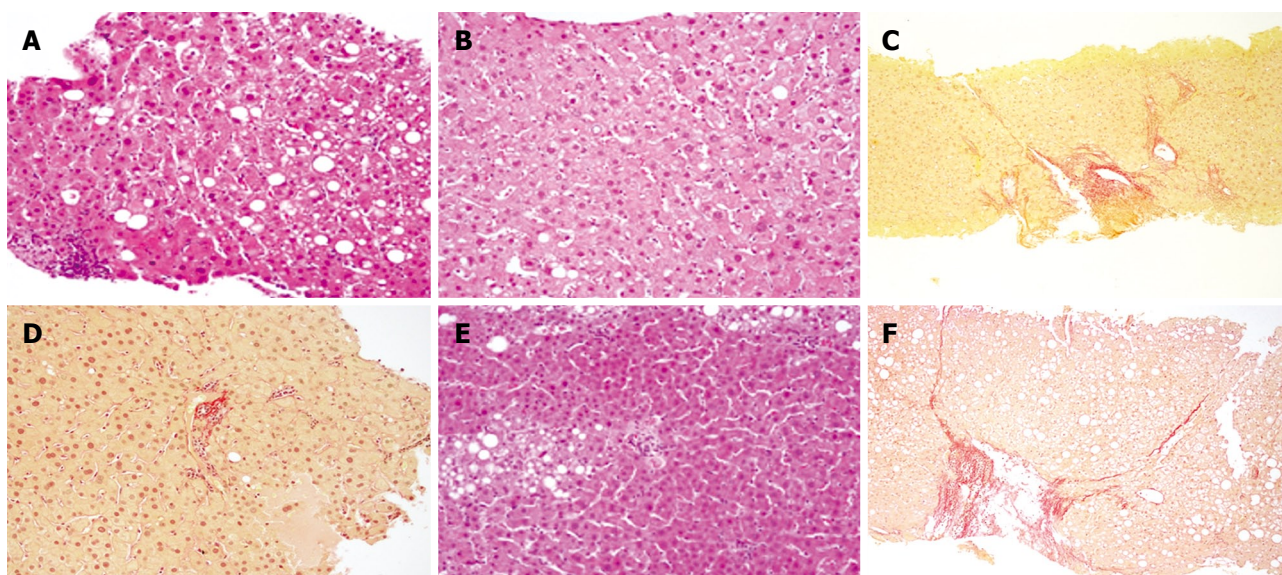


Figure 2 Biopsy of patients 3 and 9. A: Patient 3, initial biopsy, showing steatosis and focal inflammation (HE stain, 200 \times); B: Patient 3, third biopsy, 6 years after diagnosis, showing only rare steatosis and no inflammation (HE stain, 200 \times); C: Patient 3, initial biopsy, with bridging fibrosis (HE stain, 100 \times); D: Patient 3, second biopsy, without portal fibrosis (HE stain, 100 \times). Patient 3 was treated with zinc; E: Patient 9, initial biopsy, showing focal steatosis (HE stain, 200 \times); F: Patient 9, fourth biopsy, 8 years after diagnosis, showing bridging fibrosis and mild inflammation (Van Gieson stain, 100 \times). Patient 9 was treated with zinc.

Hepatic copper concentration was variable in the two groups, and did not correlate with histological findings. Progressors showed a mean hepatic copper concentration higher than non-progressors at all time points, but the result was not significant due to the small sample and high variability of the hepatic copper content (Figure 1). In our study, the hepatic copper concentration reached normal levels ($< 50 \mu\text{g/g}$ dry weight liver) in only two patients: one from the non-progressors group and one from the progressors group. The patients who did not show any change in histology also had consistent decreases in hepatic copper concentration, although levels were still elevated above normal. Iron staining was positive at baseline in two progressors and in one non-progressor. In one non-progressor the iron staining was positive after

4 years of anti-copper treatment (Table 2). There was no correlation between iron staining results and the severity of inflammation and fibrosis.

The histological progression did not correlate with subsequent aminotransferase levels or with the type of therapy. The estimated rate of progression of hepatic fibrosis (as result of the mean difference in fibrosis scores divided by the mean interval in years between the first and second liver biopsies) in the entire group was 0 units per year in the time frame between the first and the second liver biopsy, and 0.25 between the second and the third. However, among progressors, the rate of progression of fibrosis was estimated at 0.23 fibrosis units per year between the first and the second biopsy, and 0.6 units between the second and the third.

Table 2 Histopathology score of serial liver biopsies in WD patients who showed improved or stable hepatic fibrosis

Patient	Biopsy interval (yr)	Biopsy data			Iron staining	Treatment type
		Inflammation	Steatosis	Fibrosis		
Group NP						
1 ¹		0	0	0	None	-
	2	0	0	0	None	Zinc
2 ¹		0	2	0	None	-
	5	0	1	0	None	Zinc
3		2	1	2	None	-
	3	0	0	0	None	Zinc
	3	0	0	0	None	Zinc
		0	1	1	3+, diffuse	-
4		0	1	0	3+, diffuse	PCA
	2	0	0	0	None	-
5		0	0	0	None	-
	4	0	0	0	None	PCA
6		0	2	0	Not done	-
	2	1	1	0	None	PCA
	4	1	1	0	2+, focal	PCA
Group P						
7		0	2	0	None	-
	3	1	1	2	None	Zinc
8		1	1	0	Not done	-
	2	1	1	0	None	Zinc
	3	1	1	2	None	Zinc
	2	2	1	2	None	Zinc
9		0	3	1	1+, Kupffer	-
	3	0	3	1	None	Zinc
	2	1	3	2	None	Zinc
	3	1	3	2	None	Zinc
10		1	0	2	None	Zinc
	12	2	1	3	None	PCA
11		0	0	0	2+	-
	5	1	0	2	None	PCA
12		0	0	1	None	-
	5	1	1	2	None	PCA

Grade of inflammation and steatosis are also described. ¹Indicates the two patients who presented with mixed hepatic and neurological phenotype. NP: Non-progressors; P: Progressors; Zinc: Zinc sulfate; PCA: Penicillamine.

DISCUSSION

While our study confirms that, in WD, the clinical laboratory parameters do not correlate with the progression of hepatic histopathology, our newest finding is the rate of progression of fibrosis of 0 fibrosis units per year over a mean follow-up of 4 years after the diagnosis, and of 0.25 over 3 years between the second and third liver biopsy. We also observed improvement of the stage of fibrosis in two patients. Although some patients have been followed for several years, the overall amount of fibrosis in our study was low, with no patients demonstrating cirrhosis, even after long-term follow-up. However, our study covered a maximum of 12 years of follow-up, which might not be a sufficient time to observe the development of advanced-stage fibrosis in WD. A large study from Germany on 163 patients, 78 of whom underwent liver biopsy, showed variable hepatic involvement, with 37% patients presenting with cirrhosis, 36% with unspecified stage of fibrosis, and 54% with steatosis. Similar to our data, the hepatic copper concentration was highly variable, with a range from 95 to 3776 µg/g dry weight^[17].

Our finding that anti-copper treatments, zinc and PCA, were equally distributed between progressors and non-progressors is in agreement with previous studies that have demonstrated various responses to different type of treatments, including PCA, trientine, and zinc. There are six main previous studies on follow-up liver biopsies including a total of 42 WD patients (Table 3). The effect of PCA in the long-term progression of liver damage in WD has been described in three small groups of patients: four pediatric patients showed improvement or stable hepatic fibrosis after 2-7 years of treatment^[18], and seven adult patients showed marked improvement or disappearance of steatosis and improvement of mitochondrial morphological abnormalities after 3-5 years of PCA^[19]. Shiono *et al*^[20] have described an improvement in chronic active hepatitis in one patient after 6 years of PCA treatment, while in two patients with cirrhosis, there was no significant change in histopathology after 3-8.5 years of follow-up. Marcellini *et al*^[12] have described a pediatric population of 22 subjects that underwent a follow-up liver biopsy 10 years after diagnosis, and all subjects were treated with zinc sulfate. The

Table 3 Review of the case series describing the evolution of hepatic histology in WD

Grand <i>et al</i> ^[18] , 1975							
Interbiopsy interval 2-7 yr		Hepatic copper (µg/g dry weight)		AST/ALT		Histopathology	
Age at diagnosis (yr)		Before	After	Before	After	Before	After
1	24	NA	400	NA	NA	Inflammation 3+; connective tissue 13%; fatty vacuolization 0.5%	Inflammation 1+; connective tissue 7%; fatty vacuolization 0.5%
2	18	NA	80	NA	NA	Inflammation 2+; connective tissue NA; fatty vacuolization NA	Inflammation 2+; connective tissue 3%; fatty vacuolization 2%
3	11	1360	757	NA	NA	Inflammation 3+; connective tissue 17.6%; fatty vacuolization 5%	Inflammation 0/1+; connective tissue 7.6%; fatty vacuolization 3%
4	13.5	1112	90	NA	NA	Inflammation 4+; connective tissue 16%; fatty vacuolization 13%	Inflammation 0; connective tissue 14%; fatty vacuolization 8%
Sternlieb <i>et al</i> ^[19] , 1976							
Interbiopsy interval 3-5 yr		Hepatic copper (µg/g dry weight)		AST/ALT		Histopathology	
Age at diagnosis (yr)		Before	After	Before	After	Before	After
1	15	821	109	52/59	39/20	Mild fibrosis; steatosis	Mild fibrosis; marked diminution of steatosis
2	9	1004	945	76/136	20/44	Steatosis	Marked diminution of steatosis
3	12	866	737	180/190	24/26	NA	NA
4	15	1123	239	22/94	16/22	Severe steatosis	Resolution of severe steatosis
5	10	832	453	57/88	53/48	Inflammation; mild fibrosis; steatosis	Resolution of inflammation; mild fibrosis; diminution of steatosis
6	12	1177	1050	65/85	45/43	Inflammation; severe fibrosis and steatosis	Resolution of inflammation; diminution of severe steatosis and fibrosis
7	14	NA	NA	75/82	50/35	Inflammation; steatosis; cirrhosis	Diminution of steatosis and inflammation; persistence of cirrhosis
Shiono <i>et al</i> ^[20] , 2001							
Interbiopsy interval 3-8.5 yr		Hepatic copper (mg/g dry weight)		ALT (IU/L)		Histopathology (only stage of fibrosis)	
Age at diagnosis (yr)		Before	After	Before	After	Before	After
1	16	990	319	121	65	Cirrhosis with chronic active hepatitis	Cirrhosis
2	17	1025	200	241	109	Chronic active hepatitis	Chronic inactive hepatitis
3	19	524	190	17	25	Cirrhosis	Cirrhosis
4	23	540	129	19	18	Cirrhosis	Cirrhosis
Marcellini <i>et al</i> ^[12] , 2005							
Interbiopsy interval 10 yr		Hepatic copper (median of 22 pts) (mg/g dry weight)		AST/ALT (mean of 22 pts)		Histopathology summary of 22 pts	
		Before	After	Before	After	Before	After
Mean age at diagnosis (yr) 6.1 ± 2.5		873 (670-982)	690 (600-890)	110/94	21.7/23.7	Inflammation grade 1 in 81% of pts, grade 0 in 19%; steatosis grade 1 in 50%, grade 2 in 22.7%, grade 3-4 in 27.3%; fibrosis stage 1 in 54%, stage 3 in 46%	Resolution of inflammation in all pts; steatosis grade 1 in 90%, grade 2 in 10%; fibrosis stage 1 in 81%, stage 3 in 19%
Askari <i>et al</i> ^[13] , 2003							
Interbiopsy interval 4.4-10 yr		Hepatic copper		Child Pugh score (AST/ALT not available)		Histopathology (only stage of fibrosis)	
		Before	After	Before	After	Before	After
Mean age at diagnosis (yr) 25.4 ± 3.9		NA	NA	9-13	5	Cirrhosis Cirrhosis Cirrhosis	Fibrosis stage 2-3 Fibrosis stage 3-4 Fibrosis stage 1
Linn <i>et al</i> ^[14] , 2009							
Interbiopsy interval 3-7 yr		Hepatic copper (mg/g dry weight)		ALT (U/L)		Histopathology	
Age at diagnosis (yr)		Before	After	Before	After	Before	After
1	21	NA	NA	59	57	Mild fibrosis	Normal
2	13	1100	270	31	19	Normal	Cirrhosis

authors observed an improvement in all parameters of histological damage (inflammation, steatosis, and fibrosis) and an overall decrease in hepatic copper concentration; however, the level remained higher than normal in all patients^[12]. Askari *et al*^[13] have shown various degrees of improvement of fibrosis in three WD patients with cirrhosis, who were treated first with zinc and trientine and later only with zinc as maintenance treatment. One patient showed persistent stage 3-4 fibrosis; a second patient showed stage 2-3 and only one showed stage 1. Linn *et al*^[14] have described 17 patients who were followed for a median of 14 years and treated with zinc. In two cases, a second liver biopsy was performed after the baseline, which showed resolution of the initial mild fibrosis in one case and development of cirrhosis in the other. Although the data are heterogeneous and the progression of histopathological features is described following different criteria, it seems that there was an improvement in histology in most of the described patients, while we observed improvement or no progression only in 50% of cases. The explanation of our findings may be that we performed the liver biopsies when clinically indicated by the failure to respond to anti-copper agents, which potentially selected worse cases.

In previous work on WD, there have been variable changes in serum aminotransferases upon initiation of either PCA or zinc therapy, as well as persistently high levels resistant to either PCA or zinc therapy^[10,21]. In none of these studies was there a significant correlation between aminotransferase level and histological progression, which confirms the observation made by us and others that, in WD, an elevation of aminotransferase level is common and does not correspond to clinical worsening^[22]. The discovery of a higher hepatic copper concentration in progressors, as compared to non-progressors, is certainly not surprising and it may underline the importance of measuring hepatic copper during follow-up. Only one post-treatment specimen became positive for iron staining, and there was no correlation with fibrosis progression, which prevented us making any comparison with the results of Shiono *et al*^[20], which showed hepatic iron accumulation after long-term anti-copper treatment. Our study was limited by the relatively small number of patients; however, this was still one of the largest studies conducted on serial biopsies of this rare condition. The limited statistical power did not allow us to find significant correlations or predictive factors of histological progression, but we were able to derive important observations that might contribute to the long-term management of WD, considering that the timing and the indication for follow-up liver biopsy in WD has not been established yet. Our patients were recruited in the hepatology setting and were selected for this study according to the availability of serial liver biopsies. Nevertheless these patients' varied histopathology appears representative of the WD hepatic presentation. Our study focused on the role of liver biopsy in WD follow-up. However, liver biopsy, even as a fundamental diagnostic tool in WD and in chronic liver diseases, is limited by sampling error, which can affect both histologi-

cal evaluation and hepatic copper concentration, which is known to be significantly variable over time and among regenerative nodules in WD cirrhosis^[23,24]. Despite these limitations, our data are particularly valuable because of the rarity of WD and the infrequency of serial liver biopsies in this disease. Our observation of the inability of clinical tools to detect the progression of fibrosis despite treatment suggests that a liver biopsy with hepatic copper quantification every 3 years should be considered.

COMMENTS

Background

The earliest morphological features of Wilson disease (WD) are represented by micro- and macrovesicular hepatic steatosis, glycogenated nuclei in the periportal hepatocytes, and focal hepatocellular necrosis. With the progression of parenchymal damage and inflammation, fibrosis, and subsequently, cirrhosis invariably develop. As a result of the rarity of WD and the fact that liver biopsy is not performed routinely during follow-up of WD, unless clinically indicated, the progression and timing of the liver pathology is characterized poorly. Previous studies have shown the possibility of improvement during long-term follow-up of the steatosis and inflammation grade, and of the fibrosis stage. Studies on serial liver biopsies, as well as studies on the correlation between hepatic histology and clinical parameters are lacking.

Research frontiers

The research hotspots are: (1) what is the rate of hepatic fibrosis progression in WD; and (2) when is the best time to perform follow-up liver biopsies in WD patients?

Innovations and breakthroughs

The results indicate that the estimated rate of progression of hepatic fibrosis (as result of the mean difference in fibrosis scores divided by the mean interval in years between the first and second liver biopsies) in the entire WD group was 0 units per year between the first and second liver biopsy (4 years), and 0.25 between the second and third (3 years). However, among progressors the rate of progression of liver fibrosis was estimated as 0.23 and 0.6 fibrosis units per year between the first and second biopsy and between the second and third, respectively.

Applications

The results suggest that liver biopsy with hepatic copper quantification every 3 years should be considered.

Peer review

This paper investigate the progression of hepatic histopathology in serial liver biopsies from WD patients. The manuscript is well written, and it can be published in current form.

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Biochemical determination of lipid content in hepatic steatosis by the Soxtec method

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tween both assays was assessed by calculating the κ coefficient.

RESULTS: According to the histological classification, 38% of rats presented grade 0, 21% grade 1, 22% grade 2 and 20% grade 3. The amount of fat per 100 g tissue was 2.60 ± 0.64 g for grade 0, 3.87 ± 1.59 g for grade 1, 5.82 ± 1.37 g for grade 2 and 8.68 ± 2.30 g for grade 3. Statistically significant differences were found between the mean values for each of the histological grades ($P < 0.05$). The correlation for the quantification of fat in the liver between both assays was moderate ($\kappa = 0.60$).

CONCLUSION: The biochemical quantification of fat in liver tissue by the Soxtec method was correlated with the histological classification, although the agreement between the two tests was only moderate.

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Key words: Liver steatosis; Soxtec method; Quantitative analysis; Histology; Biochemistry

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Abstract

AIM: To establish a quantitative method to measure the amount of lipids.

METHODS: The livers of 53 male Wistar rats (225 g) with different degrees of hepatic steatosis were studied. This model of hepatic steatosis was based on a high carbohydrate, fat-free modified diet. Biopsies were classified into four grades depending on fat accumulation, using the Kleiner and Brunt classification. Total fat was studied by the Soxtec method (Soxtec™ 2050 Auto Fat Extraction System), and agreement be-

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INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) covers a wide spectrum that ranges from fatty liver alone to steatohep-

atitis, fibrosis or cirrhosis. Fatty liver alone is the most common histological lesion in patients with NAFLD^[1], and liver biopsy is the only diagnostic test that can reliably identify and quantify the degree of steatosis^[2,3].

The gold standard for detecting steatosis is the histopathological analysis of a liver sample collected by biopsy. The biopsy procedure is invasive and painful and presents a risk for patients^[4]. Actually, most hospitals use the histological method to determine the degree of steatosis. However, this method is subjective as it can vary, depending on the criteria for each pathologist. It is therefore necessary to develop a new method that is capable of determining the exact amount of fat in the liver.

Our aim was to compare a simple method for the isolation and purification of total lipids with the histological classification for quantifying fat deposits in a simple steatosis model in rats.

MATERIALS AND METHODS

Animals and diets

Male Wistar [CRL:Wi (Han)] rats (Charles River Laboratories) weighing approximately 225 g were studied. All experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals published by the United States Public Health Service. Animals were kept in separate cages with a regular 12-h light regime at a controlled temperature ($25 \pm 2^\circ\text{C}$). The modified diet (high carbohydrate, fat-free) consisted of carbohydrates (80%, as starch), protein (16%, as casein), and vitamins and minerals (4%) (PANLAB, Barcelona, Spain). The standard diet consisted of a balanced diet that contained carbohydrates (51%), protein (16%), vitamins and minerals (4%), and lipids (3%). The standard diet contained 2.9 kcal/g, and the modified diet 3.58 kcal/g. This model was based on that reported by Delzenne *et al.*^[5] and Bujanda *et al.*^[6].

Experimental procedures

The rats were separated into two groups: control (20 rats) and steatosis (33 rats). The control group was given free access to food and drink and was fed a standard diet during 28 d. The steatosis group was given free access to food and water 4 d/wk and fasted for the remaining 3 d (only access to water was allowed). They were fed a modified diet during the dietary restriction cycles (Figure 1).

All rats were killed after completing four cycles of feeding and fasting, i.e. 28 d after study start. The amount of food taken by the rats and the weight of the animals were controlled. At the end of the study, all rats were anesthetized using isoflurane (Forane®). The animals were placed in an induction chamber with 5% isoflurane and administered 0.2 mL/200 g buprenorphine (Buprex®) as an analgesic. Once asleep, they were maintained with 3% isoflurane and 0.5–0.75 L oxygen.

Pathological evaluation

A histological study was performed following a midline laparotomy to remove the liver. The hepatic index was obtained by dividing the liver weight by rat weight and mul-

tiplying by 100. Liver tissue samples taken at the time the rat was killed were immediately placed in 10% buffered formalin and subsequently embedded in paraffin. Liver sections were stained with hematoxylin and eosin using standard techniques. The sections were viewed without prior knowledge of the treatment group to which each animal belonged. Biopsies were classified into four grades, depending on fat accumulation, using the classification proposed by Kleiner *et al.*^[2], whereby a patient is classified as grade 0 when $< 5\%$ of hepatocytes are affected by fat vacuoles, grade 1 when fat vacuoles are seen in 5%–33% of hepatocytes, grade 2 when 33%–66% of hepatocytes are affected by fat vacuoles, and grade 3 when fat vacuoles are found in $> 66\%$ of hepatocytes. Two experienced pathologists blinded to the experiment evaluated all samples, and agreement between both pathologists was determined. In case of discrepancy, the opinion of a third histopathologist reviewed the biopsies.

Quantitative determination of total fat in liver

The Soxtec™ 2050 Auto Fat Extraction (Foss® Analytical, Hilleroed, Denmark) apparatus consisted of an extraction unit, a control unit and a drive unit. One gram of liver tissue was inserted into the extraction unit, solvent was added to the extraction cups in a closed system, and the cups were heated with an electric heating plate. The four-step extraction consisted of boiling, rinsing, solvent recovery and pre-drying. The results were calculated as total amount of fat (g) per 100 g tissue.

Samples were classified into four grades, according to the amount of fat detected in the steatosis group, using the data obtained for the control group and the maximum amount of fat detected in the steatosis rats as a reference.

Statistical analysis

Quantitative data were expressed as the mean \pm SD for the different animals in each group, and comparisons were made using a two-sided Student's *t* test. $P < 0.05$ was considered statistically significant. The agreement between biochemical assessments (grades) and histological steatosis grades (gold standard) was assessed by calculating the κ coefficient, which assessed how much better the agreement was than it would have been by chance alone ($\kappa = 1$ indicates perfect agreement; $0.80 < \kappa < 1$, excellent; $0.60 < \kappa < 0.80$, good; $0.40 < \kappa < 0.60$, moderate; $0.20 < \kappa < 0.40$, fair; $0 < \kappa < 0.20$, poor agreement). Weighted κ coefficients were calculated for ordered categorical data, whereas simple κ values were determined for other data.

RESULTS

Rat weight increased significantly in the control group (221 ± 10 to 355 ± 16 g), but remained similar in the group with fatty liver disease (222 ± 12 to 226 ± 14 g). The rats in the control group ate 31.11 ± 10.1 g/d and rats in the steatosis group ate 14.78 ± 3.2 g/d. The hepatic index was 4.47 ± 0.63 and 3.9 ± 0.31 in the steatosis and control groups, respectively.

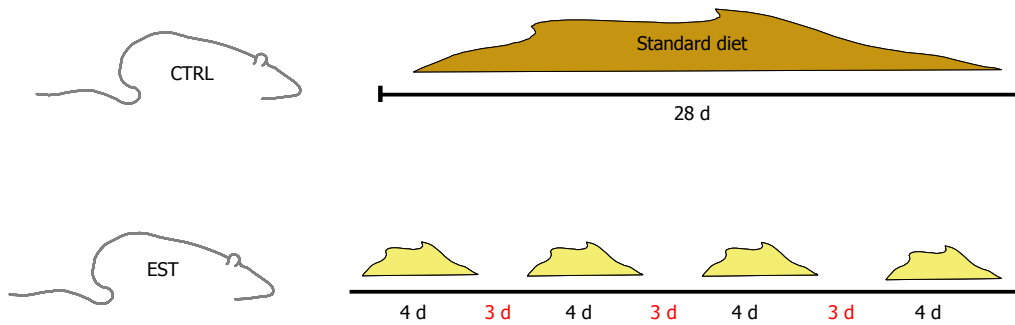


Figure 1 Summary of animal model.

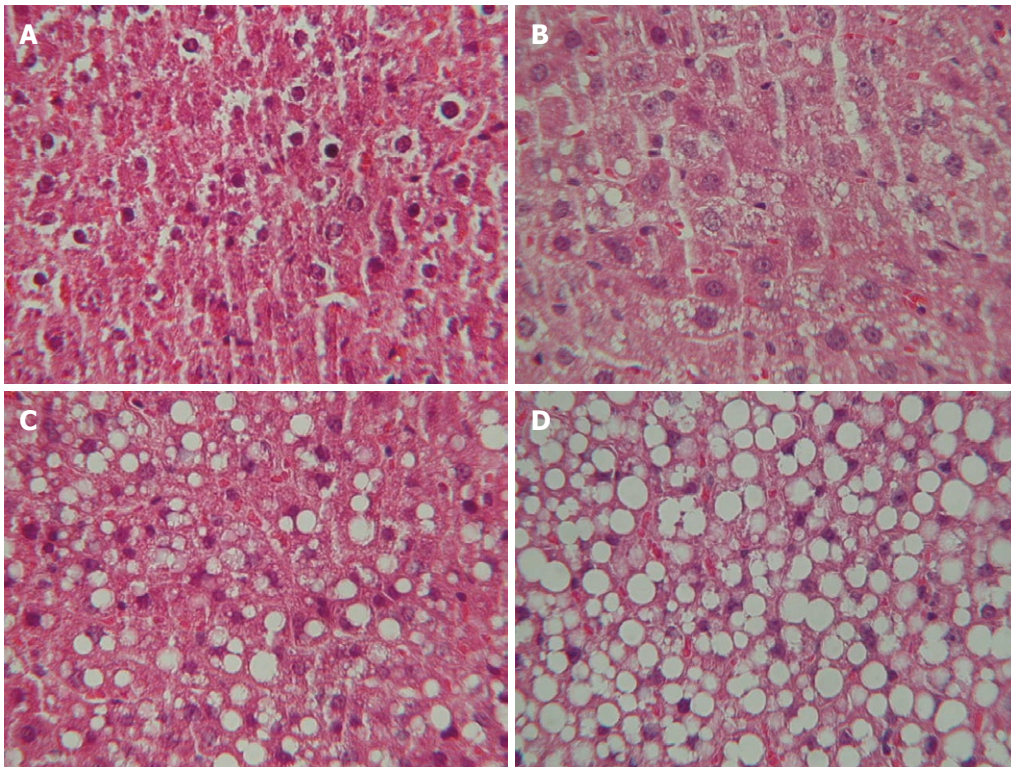


Figure 2 Different histological grades observed in the group of rats with steatosis. A: Grade 0; B: Grade 1; C: Grade 2; D: Grade 3.

Histological evaluation

No fatty infiltration was seen in the control group (Figure 1). Eleven rats (34%) in the steatosis group presented histological grade 1, 12 (36%) grade 2, and 10 (30%) grade 3. Inter-observer agreement was 0.89 and intra-observer agreement 0.92 (Figure 2).

Quantitative determination of total lipids

The amount of fat per 100 g tissue was 2.60 ± 0.64 g for histological grade 0, 3.87 ± 1.59 g for grade 1, 5.82 ± 1.37 g for grade 2, and 8.68 ± 2.30 g for grade 3. Statistically significant differences were found between the mean values for each of the histological grades ($P < 0.05$), except between grades 0 and 1 (Figure 2). The following biochemical classification was proposed on the basis of the amount of fat observed in the control group (2.6 g/100 g tissue) and the maximum amounts of fat observed in the steatosis group (11.16 g/100 g tissue). If we group

the rats for each histological grade and the average and SD we obtain the cut-off. The animals were classified as grade 0, when the amount of fat per 100 g tissue was < 2.6 g; grade 1, when the amount of fat was 2.6–5 g; grade 2, when the amount of fat was 5.1–8 g; and grade 3, when the amount of fat was > 8.1 g (Figure 3). The κ correlation between the histological and biochemical classifications was calculated to be 0.6 (moderate).

DISCUSSION

The amount of total fat determined by the Soxtec method correlates well with the Kleiner and Brunt histological classification^[2,3]. Quantifying fat biochemically is a more objective and accurate method of determining the fat content in liver tissue. In the Soxtec method, the crude fat is extracted from the liver by the Randall method^[7], a two-step extraction procedure that reduces fat-extraction

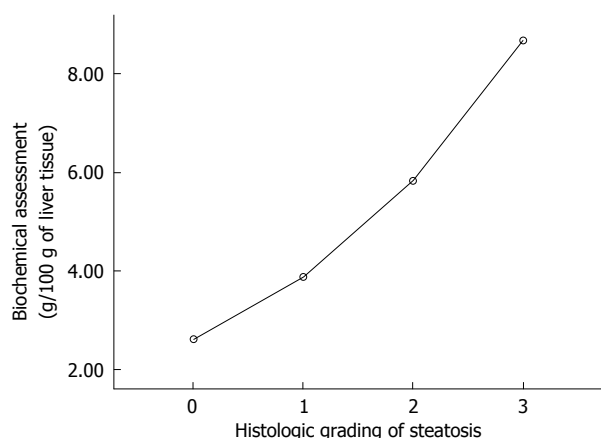


Figure 3 Biochemical determination of fat in liver tissue by the Soxtec method is correlated with the Kleiner and Brunt histological classification.

times to < 1 h per sample. This method for fat extraction has previously been used for meat and meat products and is accepted as an Association of Analytical Communities Official Method of Analysis (991.36)^[8]. It is recommended for Official First Action. This method is now automated and allows several samples to be analyzed simultaneously and rapidly (< 1 h). The approximate cost per sample is low, at around 22 euros. The Soxtec method is easier to perform than the classical biochemical method described by Folch in 1957 and is automated, which in our opinion, means it is the method of choice.

The diagnostic gold standard in another cumulative liver disease, namely hemochromatosis, is to determine the amount of iron present in a tissue sample obtained by liver biopsy. Other techniques, such as magnetic resonance imaging (MRI), which allow iron to be determined without the need for a liver biopsy, have been established based on this gold standard. In light of this, we are of the opinion that the best method for determining the amount of fat deposited in the liver is the biochemical method described herein, and that all other techniques, especially histological classification, are less accurate.

One of the main limitations of this biochemical technique, which is also inherent to histological classification, is that the amount of fat detected depends on the region where the biopsy is taken, which means that incorrect results can be obtained in cases of localized steatosis. Another limitation of biochemical determination is that it does not provide any information regarding other types of lesion that may be present, such as fibrosis or inflammation. The main limitations of histological classification include the subjective evaluation of the tissue sample by a pathologist, which can lead to incorrect results in the case of localized hepatic steatosis, and the higher cost with respect to biochemical analysis. The main advantage of histological classification with respect to biochemical analysis is that it provides information regarding other types of liver lesions. Despite its subjective nature, the majority of studies concerning the histological classification of fat in the liver have reported a good ($\kappa = 0.60$ -0.80) or excellent ($\kappa > 0.80$) inter- and intra-individual cor-

relation^[2,9]. Indeed, the correlation between the pathologists in our hospital was excellent ($\kappa = 0.89$). Another limitation for both tests is that both techniques (Soxtec method and histological) are invasive and therefore need a liver biopsy.

Several noninvasive imaging techniques, including ultrasonography, computed tomography, and MRI, can identify hepatic steatosis and have been advocated as diagnostic tests for NAFLD^[10-15] but sometimes cannot distinguish between simple steatosis and steatohepatitis, or stage degree of fibrosis accurately^[16]. However, the gold standard for diagnosing hepatic steatosis is still liver biopsy, and the most objective technique is biochemical determination.

In the not too distant future, we must be able to diagnose steatosis, without using invasive methods. Today, for the diagnosis, liver biopsy is used, but it would be very interesting to be able to perform diagnosis using noninvasive techniques. For example, determining the amount of liver fat by magnetic resonance techniques. It would be very useful to perform a predictive test, but for this, we need much more research about the interaction of different factors, molecules and genes^[17]. The identification of the molecular mechanism that leads to fat accumulation and oxidative imbalance in steatotic liver, as well as genome and proteome studies from patients at various stages of the disease, is expected to improve the diagnostic and therapeutic approaches. In this way, attractive pharmacological designs include new molecules that can decrease lipid levels in the liver and improve insulin sensitivity.

In summary, the biochemical determination of fat in liver tissue by the Soxtec method is the most objective and direct technique available and should therefore be considered the gold standard for this assay. The results obtained by this method complement those obtained from histological studies. Further studies are needed to confirm our findings and their application in humans.

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COMMENTS

Background

The prevalence of nonalcoholic fatty liver disease is high, and histological classification is currently considered to be the gold standard for quantifying fat deposits in the liver.

Research frontiers

Histological analysis is the gold standard to determine the amount of fat in the liver. However, this method is subjective because it can vary depending on the pathologist's criteria. We therefore propose a new biochemical method, the Soxtec method, to determine exactly the amount of fat in the liver.

Innovations and breakthroughs

This is the first study to report the comparison of histological and biochemical (Soxtec) methods in steatosis. This report highlights the importance of the new method to determine exactly the fat content.

Applications

Being able to determine the exact amount of fat is important because it can

be very useful in the treatment of these patients. This represents a future therapeutic intervention.

Peer review

The authors have proposed a novel biochemical method for evaluating hepatic steatosis. Its reliability was assessed compared with that of histopathological analysis. This study is interesting and significant, although more clinical and laboratory data are needed to determine whether the Soxtec method is practically available.

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Expression of matrix metalloproteinases 2 and 9 in human gastric cancer and superficial gastritis

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Abstract

AIM: To assess expression of matrix metalloproteinases 2 (*MMP2*) and *MMP9* in gastric cancer, superficial gastritis and normal mucosa, and to measure metalloproteinase activity.

METHODS: *MMP2* and *MMP9* mRNA expression was determined by quantitative real-time polymerase chain reaction. Normalization was carried out using three different factors. Proteins were analyzed by quantitative gelatin zymography (qGZ).

RESULTS: 18S ribosomal RNA (*18SRNA*) was very highly expressed, while hypoxanthine ribosyltransferase-1 (*HPRT-1*) was moderately expressed. *MMP2* was highly expressed, while *MMP9* was not detected or

lowly expressed in normal tissues, moderately or highly expressed in gastritis and highly expressed in cancer. Relative expression of *18SRNA* and *HPRT-1* showed no significant differences. Significant differences in *MMP2* and *MMP9* were found between cancer and normal tissue, but not between gastritis and normal tissue. Absolute quantification of *MMP9* echoed this pattern, but differential expression of *MMP2* proved conflictive. Analysis by qGZ indicated significant differences between cancer and normal tissue in MMP-2, total MMP-9, 250 and 110 kDa bands.

CONCLUSION: *MMP9* expression is enhanced in gastric cancer compared to normal mucosa; interpretation of differential expression of *MMP2* is difficult to establish.

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Key words: Gastric cancer; Superficial gastritis; Matrix metalloproteinases; Quantitative real-time polymerase chain reaction; Quantitative zymography

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INTRODUCTION

The matrix metalloproteinase (MMP) family is a group of 24 zinc-dependent endopeptidases in humans that degrade components of extracellular matrix (ECM), and are noteworthy due to their involvement in a great

number of physiological and pathological processes, including stomach diseases such as gastritis and gastric cancer^[1-3]. Hence MMPs, besides their ability to degrade ECM, participate in regulating growth, angiogenesis, invasion, immune response, survival and epithelial mesenchymal transition^[3]. As a result proteolytic parameters may be suitable as prognosis tools in gastric cancer.

The group of gelatinases comprises MMP-2 (gelatinase A) and MMP-9 (gelatinase B), both of which cleave proteins, solubilize pericellular matrix components such as chemokines, shed cellular ectodomains and have been implicated in angiogenesis stimulation by means of integrin- $\alpha v \beta^3$ ^[4]. Both MMP-2 and MMP-9 gelatinolytic activities can be studied by means of quantitative gelatin zymography (qGZ)^[5]. These proteases are synthesized predominantly by stromal cells rather than cancer cells, and it has been proposed that both contribute to cancer progression^[3]. Studies show that high levels of *MMP2* and/or *MMP9* have a significant correlation with gastric cancer invasion^[6,7] and moreover are associated with poor prognosis^[7,8]. This is important; hence both proteases could participate during invasion into the gastric wall and metastasis, which are key clinical parameters in defining patient treatment in gastric cancer.

Quantitative real-time polymerase chain reaction (qRT-PCR) is a highly specific and sensitive technique for gene expression analysis^[9]; it can provide the quantification of transcripts in many different tissues and cell lines for a limited number of genes, and is particularly suitable when availability of cells is limited, such as in microdissected or biopsy tissue studies^[10,11]. Gene expression analysis is increasingly important in the study of complex regulatory networks and in the understanding of disease pathogenesis. Multiple assays based on qRT-PCR have been developed for diagnosis, prognosis and monitoring of chronic and infectious diseases^[12,13]. In these assays, it is essential to relate gene expression to quantity of tissue analyzed. Ideally, the RNA target employed as an endogenous control gene should be expressed at a similar level between tissue samples at all stages of development and remain unaffected by experimental treatments^[14]. In general, housekeeping genes are selected to normalize for the variability between samples, which can occur due to constitutive expression. Unfortunately, there is no single RNA molecule for which expression is constant in all biological conditions and tissues samples^[14,15]. Therefore, the constant expression of an endogenous control gene, selected for each particular set of experimental samples, requires testing and validation before reliable data can be obtained. In this study we used qRT-PCR to assess genetic expression of two commonly used housekeeping genes: 18S ribosomal RNA (*18SRNA*) and hypoxanthine ribosyltransferase-1 (*HPRT-1*), and two members of the MMP family, *MMP2* and *MMP9*, in two related gastric diseases: gastritis and cancer^[16]. The variances of each gene and their correlation coefficients were analyzed. In addition, we used qGZ^[5] to determine the metalloproteinase activity of MMP-2 and MMP-9.

MATERIALS AND METHODS

Ethics

This study was given approval by the Hospital Ethics Committee which, from April 2007 to April 2008, permitted the recruitment of patients undergoing upper gastrointestinal endoscopy. This study complied with the code of ethics of the World Medical Association (Helsinki Declaration of 1964, as revised in 2002).

Clinical samples and histological analysis

Antral gastrointestinal biopsies from 28 patients were analyzed: 11 normal (7 females, 4 males: mean age 52 ± 14.86 years; range 28-72 years); 11 cases of superficial gastritis (9 females, 2 males: mean age 52 ± 12.56 years; range 29-72 years), and 6 cases of advanced gastric cancer (3 females, 3 males: mean age 58 ± 8.73 years; range 49-69 years). All gastrointestinal endoscopies were performed using an EVIS EXERA Video Gastroscope Olympus GIF-Q145 (Olympus, Wendenstr, HRB, DE). Biopsy specimens were collected from each patient for routine histological examination and for RNA/protein isolation.

RNA isolation

Antral gastric biopsy specimens were collected in phosphate buffered saline (PBS) solution and immediately immersed in a tissue stabilization solution (RNAlater®, Applied Biosystems, Foster City, CA, US). Tissues were homogenized in 1 mL of TRI reagent (Molecular Research Center, INC, Cincinnati, OH, US). RNA isolation was performed following the TRI reagent protocol. gDNA was digested using DNase I (Applied Biosystems). Quality and quantity of RNA were established by measuring the optical density of each sample at 260 and 280 nm.

Reverse transcription

RNA was pre-incubated with random primers and the reverse transcription reaction was performed at 42°C for 60 min. Expression levels of *18SRNA*, *HPRT-1*, *MMP2* and *MMP9* were assessed by qRT-PCR in 28 antral gastric mucosa tissue samples: 11 normal, 11 cases of superficial gastritis and 6 cases of advanced gastric cancer.

qRT-PCR

The qRT-PCR was performed using ABI PRISM 7500 Real-time PCR System as described by Nuttall *et al.*^[17]. All qRT-PCR reagents, plates, optical adhesives covers, primer and probe for *18SRNA*, *HPRT-1*, *MMP2* and *MMP9* were from Applied Biosystems (assay ID: 4308329, Hs99999909_m1, Hs00234579_m1 and Hs00234422_m1, respectively). Standard curves were prepared for each gene by serial dilution. The number of PCR cycles, termed cycle threshold (C_T), at which amplification entered the exponential phase was determined and this number was used as an indicator of the amount of target RNA in each sample. C_T values were used to classify genetic expression as very high ($C_T \leq 25$), high ($C_T = 26-30$), moderate ($C_T = 31-35$), low ($C_T = 36-39$) or not detected ($C_T = 40$) as

validated by Nuttall *et al.*¹⁷. The absolute quantity of the clinical samples was determined from comparison with the standard curve divided by three different factors: *18SRNA* C_T values; *HPRT-1* C_T values and geometric mean of *18SRNA/HPRT-1* C_T values.

Zymography

From the homogenized samples, the lower organic phase was transferred into fresh tubes and proteins were isolated following the protocol of the supplier (TRI reagent, Research Organic). Proteins were normalized and electrophoresed under non-reducing conditions. The gQZ was performed as described by Peake *et al.*⁵. Electrophoresis was performed using 5% polyacrylamide stacking gel and 10% resolving polyacrylamide co-polymerized with 1 mg/mL gelatin. MMP-2/MMP-9, human zymography standards (Millipore, Billerica, MA, US), were simultaneously loaded onto the gel. Gels were run in standard Tris-glycine-SDS running buffer. Gelatin gels were washed overnight by gentle shaking at room temperature in rinse buffer [50 mmol/L Tris-HCl pH 8.0, 5 mmol/L CaCl₂ and 2.5% (v/v) Triton X-100], incubated in 50 mmol/L Tris-HCl pH 7.5, 5 mmol/L CaCl₂ for 18 h. Parallel gels of gelatin zymography were incubated in buffers containing 10 mmol/L EDTA to inhibit metalloproteinase activity. Gelatinolytic activity appeared as a clear band over a blue background. Using a 170-8170 Molecular Imager Gel Doc XR System and Quantity one software (Bio-Rad) images taken at the same magnification were quantified by densitometry, on the basis of their contour quantity after background subtraction. The arbitrary densitometry units were correlated with a standard curve prepared by serial dilutions of human recombinant gelatinases across a linear range (0.039-1.25 ng/mL). A total of 19 samples were randomly selected for qGZ studies: 7 of 11 normal gastric mucosa; 7 of 11 gastritis cases, and 5 of 6 gastric cancer samples.

Statistical analysis

Statistical analyses were carried out using Sigma Stat (SPSS Inc.). C_T values were expressed as mean \pm SD and the variances of each gene were calculated. A Spearman correlation was used to compare *18SRNA* and *HPRT-1* expression patterns between normal, gastritis and cancer biopsies. A positive correlation coefficient $r > 0.5$ with $P < 0.05$ was considered significant. A Kruskal-Wallis test was used to examine differences observed, in both qRT-PCR and qGZ, between normal and gastritis tissue samples, and between normal and cancer tissue samples, where $P < 0.05$ was considered significant.

RESULTS

Relative expression of *18SRNA*, *HPRT-1*, *MMP2* and *MMP9*

The qRT-PCR analysis showed that in all samples the endogenous levels of *18SRNA* and *HPRT-1* were very high (C_T \leq 25) and moderate (C_T = 31-35), respectively. *MMP2* was highly expressed (C_T = 26-30) in all samples.

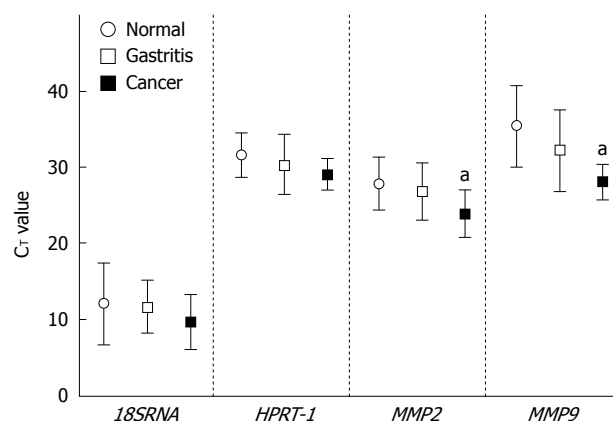


Figure 1 Relative mRNA expression of 18S ribosomal RNA (*18SRNA*), hypoxanthine ribosyltransferase-1 (*HPRT-1*), matrix metalloproteinases 2 (*MMP2*) and *MMP9* in three different groups of gastric mucosa tissues. Error bars represent the mean \pm SD. ^a $P < 0.05$ vs normal condition.

MMP9 was either not detected (C_T = 40, 3 samples) or lowly expressed in normal tissues (C_T = 36-39, 8 samples), while in gastritis it was not detected in 1 sample, moderately expressed in 5, and highly expressed in the remaining 5. In cancer samples, *MMP9* was moderately and highly expressed in 2 and 4 samples respectively. Using a Kruskal-Wallis test we found, as expected, for *18SRNA* and *HPRT-1* no significant differences between relative expression in gastric cancer and normal tissue, and between gastritis and normal tissue. For *MMP2* ($P = 0.039$) and *MMP9* ($P = 0.018$) there were significant differences between cancer and normal tissue samples (Figure 1).

The mean C_T value across all clinical samples of the endogenous control genes tested was 18.48 (SD 3.05) for *18SRNA* and 32.92 (SD 2.49) for *HPRT-1*. *HPRT-1* was the gene with the lowest C_T variance (5.98) in comparison with *18SRNA* (8.96). Analysis by Spearman rank correlation showed a high correlation between genetic expression of the *18SRNA* and *HPRT-1* genes in normal, gastritis and cancer clinical samples ($r = 0.800$, $P < 0.001$, data not shown).

Absolute quantification of *MMP2* and *MMP9* transcripts using different normalization factors

We subsequently determined the absolute mRNA levels of *MMP2* and *MMP9* using *18SRNA*, *HPRT-1* and the geometric mean of *18SRNA/HPRT-1* C_T values as normalization factors. We believed that the geometric mean of *18SRNA/HPRT-1* would indicate the central tendency of the expression of these commonly used endogenous genes in gastric mucosa tissue samples. In accordance with relative expression, we found significant differences ($P = 0.039$) for *MMP2* between gastric cancer and normal tissue, but not between gastritis and normal tissue, using *HPRT-1* as the normalization factor (data not shown). However, in the estimation of absolute expression of *MMP2* employing *18SRNA* and the geometric mean of *18SRNA/HPRT-1* as normalization factors, there were no significant differences found between gastric cancer and normal tissue, which is in

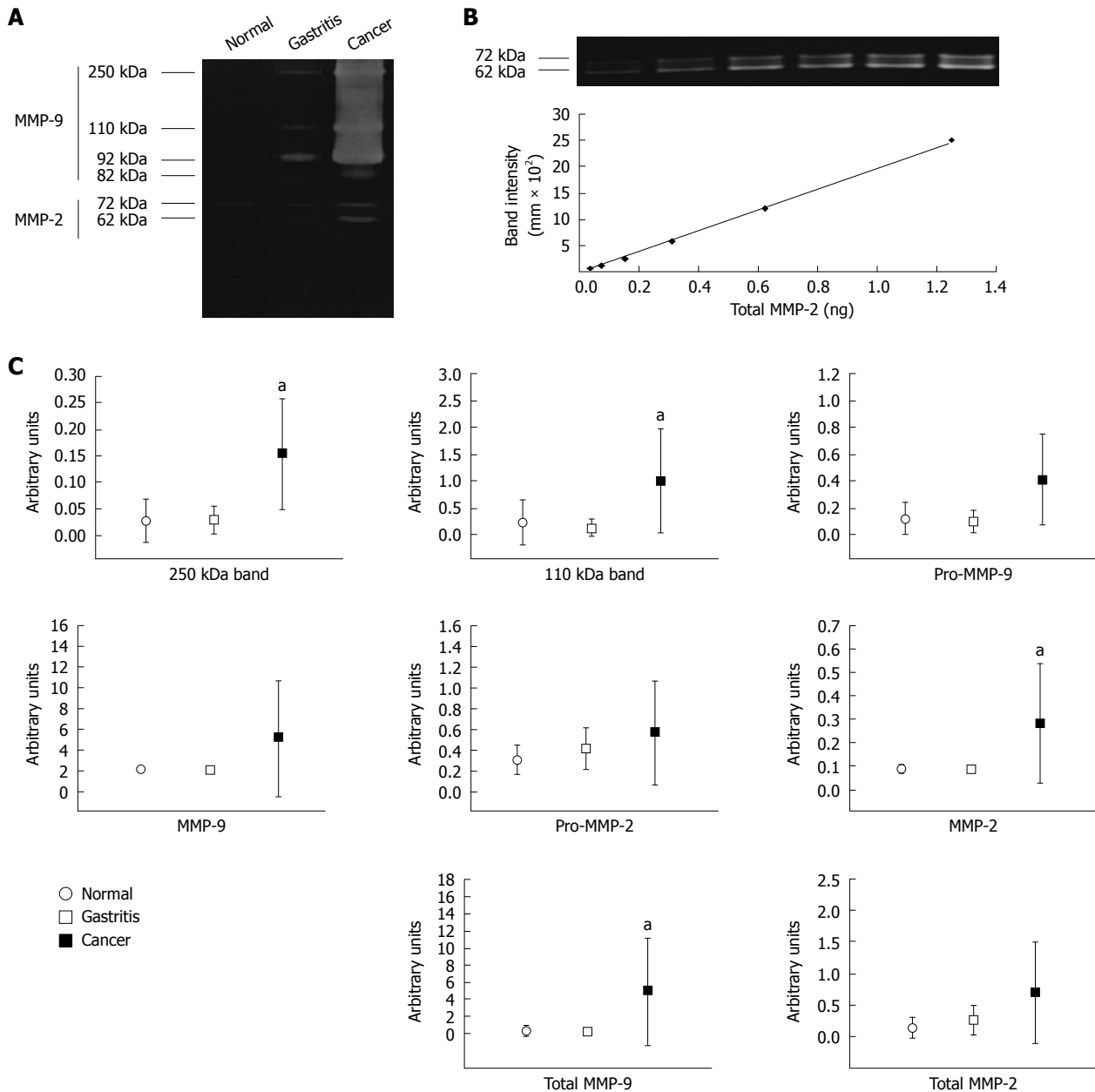


Figure 2 Validation of quantitative gelatin zymography (qGZ) of human mucosal biopsy homogenates. A: Representative zymogram of tested samples; B: Representative standard curve generated from human recombinant MMP-2 across linear range of 0.039-1.25 ng/mL, used to generate arbitrary units from densitometric data; C: Differential gelatinase activity of each of the six bands activity detected in normal (open circle), gastritis (open square) and gastric cancer (close square). Error bars represent the mean \pm SD. ^a $P < 0.05$ vs normal condition.

discordance with the relative expression data (data not shown). However, the P value for absolute expression of *MMP2* using geometric mean of *18SRNA/HPRT-1* was marginally significant ($P = 0.063$). In agreement with the relative expression data, there was a significant difference for *MMP9* between cancer and normal tissue, but not between gastritis and normal tissue, using all normalization factors (data not shown).

Gelatinase activity in gastritis, gastric cancer and gastric normal mucosa biopsies

Samples chosen for qGZ studies were analyzed by densitometry of each of the six bands activity: 250 kDa reported as MMP-9 homodimer^[18], 110 kDa reported

as MMP-9/lipocalin-2^[19], 92 kDa (Pro-MMP-9); 82 kDa (MMP-9); 72 kDa (Pro-MMP-2) and 62 kDa (MMP-2), see representative test samples in Figure 2A. Total MMP-9 was calculated by adding the 250, 110, 92 and 82 kDa band activities, and total MMP-2 by adding 82 and 72 kDa band activities. Arbitrary values for each individual band, total MMP-2 and total MMP-9 were determined from the standard curve; see representative standard curve generated from human recombinant MMP-2 on Figure 2B.

Using a Kruskal-Wallis test, we found no significant differences between gastritis and normal tissue in all bands. In addition, no significant differences were found for Pro-MMP-9, MMP-9, Pro-MMP-2 total MMP-2, between cancer and normal tissue (data not shown). However,

significant differences were found between cancer and normal tissue for 250 kDa ($P = 0.01$), 110 kDa ($P = 0.01$), MMP-2 ($P = 0.01$) and total MMP-9 ($P = 0.01$) (Figure 2C).

DISCUSSION

Various studies have reported high levels of MMP-2 and MMP-9^[6,20], and lipocalin-2^[6,21,22] in human gastrointestinal cancers, detected mainly by immune and zymography assays. In accordance with these, this study shows that the absolute quantity of *MMP9* mRNA is significantly enhanced in gastric cancer compared to normal mucosa, but interpretation of the differential mRNA expression among gastric cancer and normal mucosa of *MMP2* is conflictive, and appears to depend on the normalization factor employed. Our findings would suggest that the commonly used housekeeping genes *18S RNA* and *HPRT-1* are constitutively expressed at different levels in normal mucosa, gastritis and gastric cancer samples, although further studies with larger numbers of samples are required to confirm these findings. In addition, this study found that minor differences of the highly expressed gene *MMP2* between normal and cancer tissues could be obscured when using the equally highly expressed gene *18S RNA* as a normalization factor. Kubben and collaborators first reported that MMP-9 in complex with lipocalin-2 is increased in human gastric cancers compared to adjacent control tissue following detection by zymograms and immunoblotting^[23]. Our qGZ analysis reveals that MMP-9 homodimers, MMP-9/lipocalin-2 complexes, MMP-2 and total MMP-9 are significantly enhanced in gastric cancer compared to normal gastric mucosa. The formation of MMP-9/lipocalin-2 complexes has potential clinical value, as this action has been shown to protect MMP-9 from degradation *in vitro*^[24]. This may lead to the maintenance of an extracellular pool of latent MMP-9^[23]. Enhanced levels of these complexes are associated with poor prognosis in comparison to enhanced levels of MMP-9 alone. This study does not investigate the existence of possible ternary complexes of MMP-9/lipocalin-2/TIMP-1, which have been reported previously and show low gelatinase activity^[25]. Enhanced expression of MMP-2 and MMP-9 in human gastritis mucosal biopsy homogenates which are *Helicobacter pylori* (*H. pylori*)-positive has been reported using semiquantitative gelatin zymography^[19]; MMP-9 in serum samples from patients with gastritis *H. pylori*-positive^[26] and MMP-9 in infiltrative human gastric mucosal lymphocytes of *H. pylori*-associated gastritis are detected by flow cytometry^[27]. Interestingly, it has been shown by immunochemistry that Mmp-9 is increased in the murine *H. felis*-associated gastritis model and it has been associated with infection response and recruitment of immune cells^[28]. However, using qRT-PCR and qGZ technology we did not find significant differences in levels of *MMP2* and *MMP9* in gastritis biopsies compared to normal mucosa. There are several reasons which may account for this: (1) employment of different technology, qRT-PCR and qGZ, *vs* flow cytometry immunochemistry and semiquantitative gelatin zymography; (2) existence of

multiple levels of regulation of MMP expression and activity; and (3) our model was human superficial gastritis in which, at least initially, inflammation is confined to the portion of mucosa occupied by foveolae and the basal membrane is intact^[29]. In this regard it would be interesting to determine MMP expression and activity in gastritis characterized by strong tissue remodeling and degradation of the basal membrane, such as erosive gastritis^[29]. We would anticipate enhanced activity of MMPs in erosive gastritis compared to superficial gastritis.

In summary, we have shown that *MMP9* mRNA is significantly enhanced in gastric cancer compared to normal mucosa, while interpretation of the differential of *MMP2* transcripts among cancer and normal gastric mucosa is conflictive. The commonly used housekeeping genes *18S RNA* and *HPRT-1* appear to be constitutively expressed at different levels in normal mucosa, gastritis and gastric cancer tissues, although further studies are required. qGZ analysis reveals that MMP-9 homodimers, MMP-9/lipocalin-2 complexes, MMP-2 and total MMP-9 are significantly enhanced in gastric cancer compared to normal gastric mucosa. The potential clinical value of these findings should be fully explored in larger groups of gastritis and cancer patients.

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COMMENTS

Background

Gastric cancer in Mexico is the most frequent gastrointestinal malignant neoplasm and mortality due to this disease has been reported to be steady during the past three decades. Several precursor conditions, such as chronic gastritis, have been associated with the development of gastric cancer. Proteolysis represents an important mechanism for achieving precise cellular control of biological processes, through the highly specific hydrolysis of peptide bonds. Proteases such as matrix metalloproteinases 2 (MMP-2) and MMP-9 degrade basement membrane, cleave chemokines, shed cellular ectodomains and have been implicated in different gastrointestinal diseases, such as gastritis and cancer.

Research frontiers

MMP-2 and MMP-9 are fundamental enzymes in extracellular matrix (ECM) homeostasis. In this study the authors demonstrate differences in *MMP9* mRNA between gastric cancer and to normal mucosa; interpretation of differential expression of *MMP2* among cancer and normal gastric mucosa is conflictive. No differences for both genes were detected between gastritis and normal mucosa. Analysis of metalloproteinase activity has shown that MMP-9 homodimers, MMP-9/lipocalin-2 complexes, MMP-2 and total MMP-9 are significantly enhanced in gastric cancer compared to normal gastric mucosa but no differences were found among gastritis and normal condition.

Innovations and breakthroughs

Besides the ability of MMPs to degrade extracellular matrix components, they participate in regulating immune response, inflammation, invasion, angiogenesis, survival and epithelial mesenchymal transition; hence proteolytic parameters may be suitable as prognosis tools in gastrointestinal diseases. In the Mexican population, this is the first study to report *MMP2* and *MMP9* expression in two related gastric diseases: gastritis and cancer.

Applications

Knowledge about proteolytic profiles between normal gastric mucosa, precursor conditions and malignant mucosa is central to elucidation of regulatory pathways

involved in cancer development and progression. Combination of proteolytic gene expression profile and clinicopathological factors may provide insight into biology of these diseases.

Terminology

The MMPs are a family of zinc-dependent endopeptidases that consists of at least 24 members. These enzymes are able to degrade most components of ECM. The ECM is all secreted molecules that are outside cells. This network supports adhesion of cells and transmits signals through cell-surface adhesion receptors. The group of gelatinases comprises MMP-2 and MMP-9 both is noteworthy because they are involved in a great number of physiological and pathological processes including cancer.

Peer review

The authors investigated differential expression of MMP2 and MMP9 in gastric tumor and gastritis tissues using a population from Mexico, a high risk population of gastric cancer. They observed a significantly increased expression of MMP9 in tumor tissues. Results of this study were supported by previous studies. Strengths of this study are (1) biologically plausible mechanism, and (2) two different approaches for assessing expression of enzymes.

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Immediate virological response predicts the success of short-term peg-interferon monotherapy for chronic hepatitis C

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Abstract

AIM: To investigate the efficacy of short-term peg-interferon (PEG-IFN) monotherapy for chronic hepatitis C patients who achieved an immediate virological response.

METHODS: Defining an "immediate virological response (IVR)" as the loss of serum hepatitis C virus (HCV) RNA 7 d after the first administration of PEG-IFN α , we conducted a 12-wk course of PEG-IFN α 2a monotherapy without the addition of ribavirin for 38 patients who had low pretreatment HCV RNA load and exhibited IVR. The patients included 21 men and 17 women, whose ages ranged from 22 to 77 years (mean \pm SD: 52.0 \pm 17.8 years). There were 4 patients with HCV genotype 1b, 23 patients with genotype 2a and 4 patients with genotype 2b. HCV genotype was not determined for the remaining 7 patients. Patients were categorized into a sustained virological response (SVR) group, if serum HCV RNA remained negative for 24 wk after the end of treatment, or into a relapse group.

RESULTS: Based on the intention-to-treat analysis, 35 patients (92.1%) achieved SVR. One patient (2.6%)

relapsed with serum HCV RNA 12 wk after the end of treatment. Two patients (5.3%) withdrew from the study during the 24-wk follow-up period. With regard to the HCV RNA genotype, the SVR rates were 100% (4/4) for genotype 1b, 95.7% (22/23) for genotype 2a and 100% (4/4) for genotype 2b. The SVR rate in 7 patients, whose HCV RNA genotypes were not determined, was 71.4% (5/7).

CONCLUSION: Short-term PEG-IFN α 2a monotherapy is highly effective for chronic hepatitis C patients who have low pretreatment HCV RNA load and exhibit IVR.

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Key words: Chronic hepatitis C; Immediate virological response; Interferon therapy

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INTRODUCTION

Hepatitis C virus (HCV) infection is a leading cause of chronic hepatitis, liver cirrhosis and hepatocellular carcinoma. About 170 million patients are chronically infected with HCV worldwide. Interferon (IFN) therapy is important for suppressing the progression of chronic liver disease derived from HCV infection. Currently,

combination therapy with peg-interferon α (PEG-IFN α) and ribavirin (RBV) is the first-line therapy used to eliminate HCV in patients with chronic hepatitis C. The duration of treatment is determined based on the viral genotype, with treatment administered for 48 wk in patients with genotype 1 and 24 wk in patients with genotypes 2 or 3^[1,2]. However, long-term administration of IFN and RBV increases the cost of treatment and the risk of severe adverse events.

The efficacy of IFN therapy depends on the HCV genotype, pretreatment viral load and early viral kinetics^[3-7]. Thus, IFN therapy must be individualized and optimized according to the virological and clinical status of each patient. Several studies have shown that the duration of IFN therapy could be shortened in patients who achieve serum HCV RNA negativity during the early stages of treatment^[7-9]. Therefore, it is essential that we reduce the treatment duration for patients who exhibit a quick virological response to IFN therapy. In the present study, we focused on the initial response to PEG-IFN α administration and studied the efficacy of short-term PEG-IFN α 2a monotherapy for patients who had low pretreatment HCV RNA load.

MATERIALS AND METHODS

Definition of IVR and protocol for PEG-IFN α 2a therapy

We defined an “immediate virological response (IVR)” as the loss of serum HCV RNA 7 d after the first administration of PEG-IFN α . Patients with low pretreatment HCV RNA load ($< 1.0 \times 10^5$ IU/mL) were monitored for HCV RNA in the serum 7 d after the first administration of PEG-IFN α 2a. We scheduled a 12-wk treatment course of PEG-IFN α 2a without the addition of RBV for patients who achieved IVR. The patients received subcutaneous injection of 180 μ g of PEG-IFN α 2a (Pegasys, Roche) once per week. According to the circumstance of each patient, the dose was reduced to 90 μ g or the treatment course was terminated.

Patients

We administered PEG-IFN α 2a without the addition of RBV to a total of 59 patients with low HCV RNA load from December 2004 to November 2007. They were monitored for serum HCV RNA 7 d after the first administration of PEG-IFN α 2a. Among these, 38 patients who achieved IVR were enrolled in this study. The age, gender, HCV genotype, serum HCV RNA level, ALT level, hemoglobin (Hb), and the neutrophil and platelet (PLT) counts before treatment are shown in Table 1. The patients included 21 men and 17 women, whose ages ranged from 22 to 77 years (mean \pm SD: 52.0 ± 17.8 years). There were 4 patients with genotype 1b, 23 patients with genotype 2a and 4 patients with genotype 2b. HCV genotype was not determined for the remaining 7 patients. Among the 38 patients enrolled in this study, 37 received IFN therapy for the first time for HCV infection. Pt. 16 is the same patient as Pt. 3, who

received the same treatment course for HCV genotype 2a infection at 26 years of age. After the initial successful treatment, the patient was infected with genotype 2b HCV and re-treated at 28 years of age. Pt. 25 had been treated for hepatocellular carcinoma prior to this IFN treatment course.

Determination of serum HCV RNA load and the HCV genotype

Pretreatment serum HCV RNA levels were determined by RT-PCR using an Amplicor HCV monitor v2.0 series kit (Roche Diagnostics Co.), which had a detection limit of 5.0×10^3 IU/mL. When the serum HCV RNA level was below the detection limit, qualitative analysis of the HCV RNA was performed by RT-PCR using a COBAS Amplicor HCV test kit v2.0 (Roche Diagnostics Co.), which had a detection limit of 50 IU/mL. Qualitative analysis of HCV RNA was performed 7 d after the first administration for the evaluation of IVR, and at 24 wk after the end of treatment for the evaluation of the SVR. HCV genotype was determined using an HCV Genotyping SMITEST kit (Roche Diagnostics Co.).

Evaluation of the efficacy of PEG-IFN α 2a therapy

Patients were categorized into the sustained virological response (SVR) group, if serum HCV RNA remained negative for 24 wk after the end of PEG-IFN α 2a therapy. Patients were categorized into the relapse group if serum HCV RNA reappeared after the end of the treatment course.

Informed consent

All patients provided informed consent prior to their participation in this study. The study was conducted in accordance with the ethical guidelines of the Declaration of Helsinki and the International Conference on Harmonization Guidelines for Good Clinical Practice.

RESULTS

Completion of treatment

Figure 1 presents the dose and duration of PEG-IFN α 2a administration for each patient. Of 38 patients enrolled in this study, 26 (Pts. 1-26, 68.4%) completed a 12-wk treatment course. It was necessary to reduce the dose of PEG-IFN α 2a in 5 (Pts. 22-26) of these 26 patients. Twelve patients (Pts. 27-38, 31.6%) discontinued treatment prior to completion of the 12-wk course. In 6 (Pts. 30, 31 and 35-38, 15.8%) of these 12 patients, either self-withdrawal or financial problem was the cause for discontinuation. In 3 cases (Pts. 27, 28 and 32), the dose was reduced during the treatment, but the treatment was discontinued before completion of the 12-wk course.

Efficacy of the therapy

An intention-to-treat analysis was performed. Two patients (Pts. 27 and 35, 5.3%) withdrew from this study

Table 1 Patient characteristics

Pt. No.	Age (yr)	Gender	Genotype	Viral load (IU/mL)	ALT (IU/L)	Hb (g/dL)	Neutrophil (/mm ³)	PLT ($\times 10^3$ /mm ³)	Naive/re-treatment
1	51	M	2a	2.4×10^4	83	10.5	2729	254	Naive
2	56	M	1b	7.6×10^3	14	14.0	2565	268	Naive
3	26	F	2a	2.7×10^4	157	15.6	5217	284	Naive
4	67	M	2a	$< 5.0 \times 10^3$	63	15.2	3940	159	Naive
5	48	F	2a	1.1×10^4	121	12.1	1851	146	Naive
6	48	F	2a	7.0×10^4	48	12.7	3084	244	Naive
7	70	M	2a	5.3×10^4	119	13.7	2002	157	Naive
8	64	M	ND	$< 5.0 \times 10^3$	65	14.5	2200	148	Naive
9	22	M	ND	$< 5.0 \times 10^3$	51	15.3	5003	308	Naive
10	40	F	2a	1.1×10^4	198	14.2	2424	190	Naive
11	68	F	2a	9.3×10^3	31	11.8	3567	212	Naive
12	60	M	2a	$< 5.0 \times 10^3$	18	14.9	1520	265	Naive
13	68	F	2a	1.8×10^4	112	13.4	3338	190	Naive
14	76	M	2a	1.8×10^4	39	13.6	2540	272	Naive
15	25	F	2a	8.6×10^4	181	12.2	2649	260	Naive
16	28	F	2b	1.9×10^4	19	14.4	3522	324	Re-treatment
17	23	M	ND	$< 5.0 \times 10^3$	456	15.0	3483	257	Naive
18	57	F	2b	5.9×10^3	97	14.2	2080	306	Naive
19	48	M	2a	6.4×10^4	41	15.1	1688	235	Naive
20	56	M	2b	5.1×10^4	124	15.1	3810	82	Naive
21	34	F	2a	2.8×10^4	72	13.4	4004	98	Naive
22	32	F	2a	1.7×10^4	41	12.5	1853	259	Naive
23	74	M	ND	$< 5.0 \times 10^3$	37	10.6	1784	92	Naive
24	45	M	2a	1.0×10^4	41	16.3	4606	147	Naive
25	76	F	2a	$< 5.0 \times 10^3$	54	10.9	2948	192	Naive
26	53	F	1b	5.0×10^3	13	11.9	3438	204	Naive
27	27	F	ND	$< 5.0 \times 10^3$	46	15.1	2205	169	Naive
28	71	F	ND	5.0×10^3	40	14.1	2022	98	Naive
29	70	M	2a	5.1×10^4	81	14.5	2894	125	Naive
30	60	M	1b	5.7×10^3	40	15.1	1502	297	Naive
31	57	M	2a	2.8×10^4	61	15.5	3154	137	Naive
32	77	M	2a	$< 5.0 \times 10^3$	68	10.3	1430	125	Naive
33	69	F	1b	1.6×10^4	21	12.8	2772	186	Naive
34	37	F	2a	$< 5.0 \times 10^3$	42	13.1	2343	314	Naive
35	23	M	ND	$< 5.0 \times 10^3$	46	14.6	2772	238	Naive
36	36	M	2a	1.3×10^4	26	14.5	5126	242	Naive
37	57	F	2b	4.7×10^4	50	14.9	1628	130	Naive
38	71	M	2a	3.5×10^4	102	14.4	3852	109	Naive
mean \pm SD	52.0 \pm 17.8				76.8 \pm 77.6	13.7 \pm 1.5	2838.20 \pm 972.50	212.9 \pm 79.1	

ND: Not determined; ALT: Alanine transaminase; Hb: Hemoglobin; PLT: Platelet.

during the 24-wk follow-up period because they did not attend the hospital appointments. Thirty-five patients (92.1%) were negative for serum HCV RNA 24 wk after the end of treatment and were categorized into the SVR group (Figure 2). One patient (Pt. 6, 2.6%) was positive for serum HCV RNA 24 wk after the end of treatment and was categorized into the relapse group.

With regard to the HCV RNA genotype, the SVR rates were 100% (4/4) for genotype 1b, 95.7% (22/23) for genotype 2a and 100% (4/4) for genotype 2b (Figure 2). The SVR rate in 7 patients, whose HCV RNA genotypes were not determined, was 71.4% (5/7), as we could not evaluate the treatment effect for 2 patients because of self-withdrawal during the follow-up period.

Adverse events

The most frequent adverse events were flu-like symptoms, such as fever and pain. Fatigue appeared as the second most frequent. Other adverse events are sum-

marized in Table 2. Some patients who complained of insomnia were treated with hypnotics. Thrombocytopenia ($< 50\,000/\text{mm}^3$) occurred in 1 case (Pt. 23) and the dose was reduced for the 4th and 5th administration during the treatment course. Neutropenia ($< 750/\text{mm}^3$) appeared in 3 cases, and in 1 case (Pt. 32) it was necessary to reduce the dose of PEG-IFN $\alpha 2a$ from the 2nd to 5th administration. Anemia (Hb < 8.5 g/dL) did not occur in any of the patients. In 6 cases, it was necessary to reduce the dose of PEG-IFN $\alpha 2a$ because of fatigue (Pts. 22, 25 and 28), emotional lability (Pts. 26 and 27) or vertigo (Pt. 24). None of the patients discontinued the treatment course because of bone marrow suppression. In 5 cases it was necessary to discontinue the treatment course for the following reasons: 2 with fatigue (Pts. 28 and 34), 1 with stomatitis (Pt. 27), 1 with skin symptoms and diarrhea (Pt. 29) and the other with viral influenza infection (Pt. 32). These adverse events were not particularly severe. One case (Pt. 33) discontinued the treatment

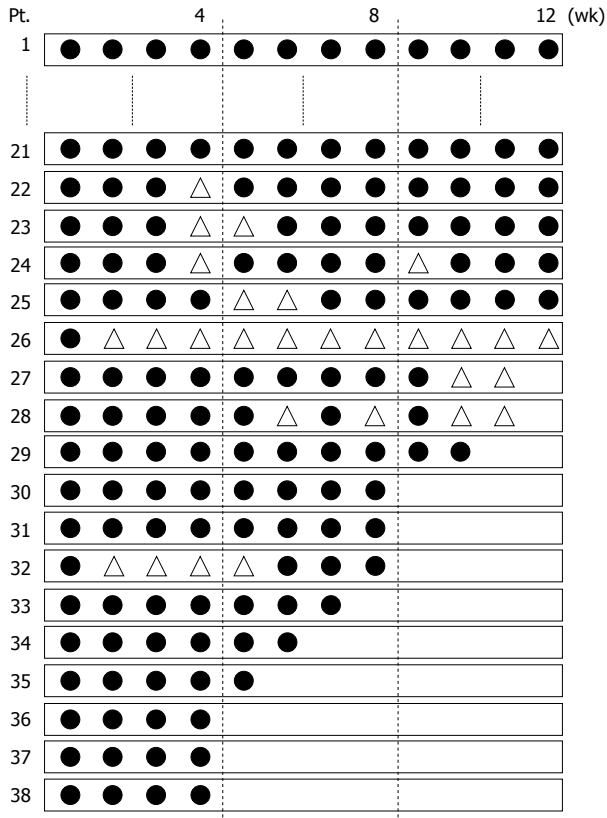


Figure 1 Administration of peg-interferon (PEG-IFN) α 2a. The patients received subcutaneous injections of 180 μ g of PEG-IFN α 2a (rotundity) once per week. Twenty-one patients (Pts. 1-21) completed a 12-wk treatment course without dose reduction. In 17 patients (Pts. 22-38), the dose was reduced to 90 μ g (triangle) or the treatment was terminated before completion of the 12-wk course.

course because of bacterial pneumonia, and recovered after antibiotic administration.

DISCUSSION

In the present study, we defined an “immediate virological response (IVR)” as the loss of serum HCV RNA 7 d after the first administration of PEG-IFN α . We then conducted a 12-wk treatment course of PEG-IFN α 2a in a population of patients who had low pretreatment HCV RNA load and who achieved IVR. This short-term PEG-IFN α 2a monotherapy without the addition of RBV exhibited an extremely high SVR rate.

The current standard therapy to eliminate HCV in patients with chronic hepatitis C is combination therapy with PEG-IFN α and RBV. While the SVR rate for 24 wk of PEG-IFN α /RBV therapy in patients with genotypes 2 and 3 is 78%-93%, the SVR rate for 48 wk of treatment for genotype 1 is 40%-51%^[1,2,10-13]. New methods of determining the adequate dose and duration of PEG-IFN α /RBV administration have been devised to increase the probability of SVR in the treatment for patients with genotype 1 and high pretreatment viral load^[9,14]. High dose and long-term IFN treatments, however, are expensive and contribute to additional risk for many adverse events.

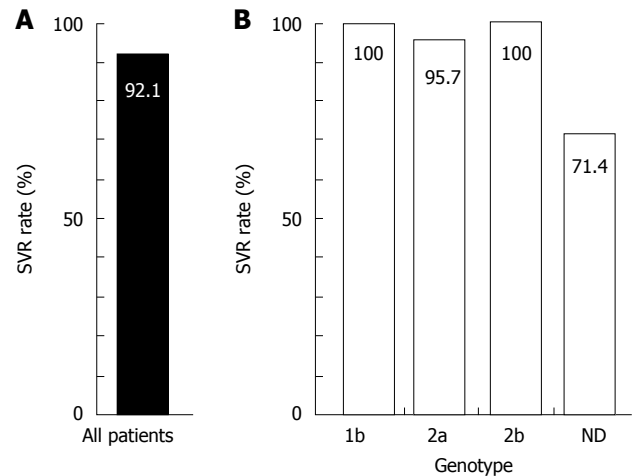


Figure 2 Efficacy of the therapy. A: The sustained virological response (SVR) rate in all patients; B: The efficacy of the therapy for each genotype.

Table 2 Adverse events

Adverse event	n (%)
Flu-like symptoms	
Fever	20 (52.6)
Pain	12 (31.5)
Fatigue	13 (34.2)
Insomnia	7 (18.4)
Neutropenia (< 750/mm ³)	3 (7.9)
Skin symptoms	3 (7.9)
Emotional lability	3 (7.9)
Gastrointestinal symptoms	2 (3.3)
Thrombocytopenia (< 50 000/mm ³)	1 (2.6)
Bacterial pneumonia	1 (2.6)
Influenza viral infection	1 (2.6)
Vertigo	1 (2.6)
Stomatitis	1 (2.6)

Major contributory factors for SVR are viral genotype (except genotype 1), low pretreatment viral load (< 1.0 \times 10⁵ IU/mL) and early loss of serum HCV RNA^[3-7]. Early virological response (EVR) and rapid virological response (RVR), which are indicated by loss of serum HCV RNA at weeks 12 and 4, respectively, are closely related to the SVR rate^[6,7,15,16]. Furthermore, Mangia *et al.*^[8] have reported that 12 wk of administration of PEG-IFN α /RBV to patients with HCV genotype 2 or 3 and who achieve RVR, results in a high probability of SVR. Tabaru *et al.*^[17] reported that the SVR rate was 100% after treatment with IFN α 2b for 6 wk for patients infected with HCV genotype 2a and low viral load. Establishing the minimum, and yet sufficient, IFN therapy period is important in terms of financial efficiency and for reduction of the risk of adverse events. Therefore, it is essential to establish a guideline to make the treatment period shorter than the standard length for patients who have a high probability of achieving SVR. For patients with low pretreatment HCV RNA load, the current standard IFN therapy which is allowed by Japanese National Medical Insurance, is 24-48 wk of PEG-IFN α 2a monotherapy without the addition of

RBV. RBV causes hemolytic anemia, and severe anemic symptoms sometimes appear in PEG-IFN α /RBV combination treatment^[18]. PEG-IFN α monotherapy can avoid these adverse events induced by RBV.

Thus, in the current study, we studied the efficacy of short-term PEG-IFN α 2a monotherapy for patients who had a low pretreatment HCV RNA load and exhibited IVR. Remarkably, 35 (97.2%) out of 36 cases that we were able to follow up to 24 wk after the last administration, were categorized into the SVR group. With regard to the HCV RNA genotype, SVR rates were 100% (4/4) for genotype 1b, 95.7% (22/23) for genotype 2a and 100% (4/4) for genotype 2b (Figure 2). These data might suggest that the efficacy of short-term PEG-IFN α 2a monotherapy for patients exhibiting IVR is independent of HCV genotype. Further analyses with a large population of patients should be conducted for each genotype of HCV, because the number of patients enrolled in this study, especially for genotype 1b, was small.

The patient (Pt. 6) who relapsed during this treatment course subsequently received 24 wk of PEG-IFN α 2a therapy and succeeded in achieving SVR. We consider that if short-term treatment failed to induce SVR, those non-SVR patients could be re-treated with long-term PEG-IFN α monotherapy (24-48 wk) or PEG-IFN α /RBV combination therapy. Thus, it is suggested that we should select short-term monotherapy at the first approach for patients with low pretreatment HCV RNA load and IVR.

Nine out of 35 cases who achieved SVR received less than 9 wk (4-8 wk) of drug administration. Therefore, there is the possibility that the treatment period could be shortened to less than 12 wk for a certain group of patients. Further analyses in randomized controlled trials with a large population of patients should be conducted to examine the efficacy of shorter treatment courses, such as 4 wk or 8 wk.

Frequent adverse events that generally appear during IFN therapy were seen in this study group of patients. However, no severe events were observed. None of the patients required discontinuation of therapy because of bone marrow suppression. While 1 case exhibited bacterial pneumonia as a severe complication, the patient recovered with antibiotic treatment. Thus, we consider that short-term monotherapy is safe.

PEG-IFN α monotherapy clearly has the advantage of avoiding adverse events induced by RBV. However, we have to consider the possibility that adding RBV to this short term PEG-IFN α therapy could offer shorter treatment duration or higher SVR rates. This option should be investigated in larger studies.

In conclusion, short-term PEG-IFN α 2a monotherapy is highly effective for chronic hepatitis C patients who have low pretreatment HCV RNA load and exhibit IVR. IVR is a simple and useful indicator of early viral kinetics to predict the high probability of SVR.

hepatitis C virus (HCV) genotype, pretreatment viral load and early viral kinetics. Therefore, IFN therapy must be individualized and optimized according to the virological and clinical status of each patient.

Research frontiers

Focusing on the initial response to PEG-IFN α administration, the authors proposed a new concept, the "immediate virological response (IVR)". Then the efficacy of short-term PEG-IFN α 2a monotherapy was investigated for patients who had low pretreatment HCV RNA load and exhibited IVR.

Innovations and breakthroughs

A 12-wk treatment course of PEG-IFN α 2a without the addition of RBV was highly effective for patients who achieved IVR. SVR rate for all patients in this study was 92.1% (35/38).

Applications

The present study has shown that short-term PEG-IFN α 2a monotherapy is an excellent treatment regimen for chronic hepatitis C patients who have low pretreatment HCV RNA load and exhibit IVR. It is also suggested that IVR is a simple and useful indicator of early viral kinetics to predict the high probability of SVR.

Terminology

An "IVR" was defined as the loss of serum HCV RNA 7 d after the first administration of PEG-IFN α .

Peer review

Yada *et al* reported that a 12-wk treatment course of PEG-IFN α 2a alone in a population of patients who had low pretreatment HCV RNA load and achieved immediate virological response results in extremely high SVR rates. The data are encouraging and important from a cost-effectiveness point of view.

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COMMENTS

Background

The efficacy of interferon (IFN) therapy for chronic hepatitis C depends on the

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Glycemic index and glycemic load of selected Chinese traditional foods

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Abstract

AIM: To determine the glycemic index (GI) and glycemic load (GL) values of Chinese traditional foods in Hong Kong.

METHODS: Fifteen healthy subjects (8 males and 7 females) volunteered to consume either glucose or one of 23 test foods after 10-14 h overnight fast. The blood glucose concentrations were analyzed immediately before, 15, 30, 45, 60, 90 and 120 min after food consumption using capillary blood samples. The GI value of each test food was calculated by expressing the incremental area under the blood glucose response curve (IAUC) value for the test food as a percentage of each subject's average IAUC value for the glucose. The GL value of each test food was calculated as the GI value of the food multiplied by the amount of the available carbohydrate in a usual portion size, divided by 100.

RESULTS: Among all the 23 Chinese traditional foods tested, 6 of them belonged to low GI foods (Tuna Fish

Bun, Egg Tart, Green Bean Dessert, Chinese Herbal Jelly, Fried Rice Vermicelli in Singapore-style, and Spring Roll), 10 of them belonged to moderate GI foods (Baked Barbecued Pork Puff, Fried Fritter, "Mai-Lai" Cake, "Pine-apple" Bun, Fried Rice Noodles with Sliced Beef, Barbecue Pork Bun, Moon Cakes, Glutinous Rice Ball, Instant Sweet Milky Bun, and Salted Meat Rice Dumpling), the others belonged to high GI foods (Fried Rice in Yangzhou-Style, Sticky Rice Wrapped in Lotus Leaf, Steamed Glutinous Rice Roll, Jam and Peanut Butter Toast, Plain Steamed Vermicelli Roll, Red Bean Dessert, and Frozen Sweet Milky Bun).

CONCLUSION: The GI and GL values for these Chinese traditional foods will provide some valuable information to both researchers and public on their food preference.

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Key words: Glycemic index; Glycemic load; Chinese traditional foods

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Chen YJ, Sun FH, Wong SH, Huang YJ. Glycemic index and glycemic load of selected Chinese traditional foods. *World J Gastroenterol* 2010; 16(12): 1512-1517 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v16/i12/1512.htm> DOI: <http://dx.doi.org/10.3748/wjg.v16.i12.1512>

INTRODUCTION

The glycemic index (GI) was firstly introduced by Jenkins *et al*^[1]. It was defined as the incremental area under the blood glucose response curve (IAUC) after a portion of food containing 50 g available carbohydrate expressed as a percentage of that after the same amount of carbohydrate

from a reference food, usually glucose or white bread, taken by the same subject. The glycemic load (GL) was more recently introduced to reflect overall glucose response^[2,3], and it was calculated as the GI value of the food multiplied by the amount of the available carbohydrate in a usual portion size, divided by 100. GI and GL of foods have been shown to related to some chronic diseases, such as diabetes^[2,3], metabolic syndrome^[4], cardiovascular disease^[5] and even some types of cancers^[6]. Low GI and GL diets could contribute to a reduction in body mass in overweight, obese adolescents^[7], or coronary heart disease (CHD)^[8] and have a small but clinically useful effect on medium-term glycemic control in patients with diabetes^[9].

The relevance of dietary GI and GL is still debated^[10] and the availability of a reliable table of GI is critical for continuing research and resolution of the controversy. The GI and GL values of over 2480 individual food items were listed in the more recent edition of the international GI and GL tables^[10], among which there were only about 50 Chinese foods. Chinese traditional foods, although some styles are very popular and well-known worldwide, are very different from western foods with recipes followed strictly as laboratory instructions.

In some studies investigating the effect of food intake on local people^[11,12], there were no precise GI and GL values for many local Chinese foods. They had to use international GI tables to find similar foods or seek for a “best estimate” from experts in this field. These studies indicated that there might be some differences between the estimated GI values and the real values. Furthermore, during recent years the GI and GL values of different local foods have been reported^[13-15]. Therefore, it was worthwhile to determine the GI and GL values of Chinese traditional foods so as to advise local individuals on their daily diets and provide tools to undertake related studies in this area. The purpose of this study was to determine GI and GL values of some Chinese traditional foods in Hong Kong, which would be preliminary information which may act as the basis for the development of a GI and GL database for Chinese traditional foods.

MATERIALS AND METHODS

Participants

Fifteen healthy adults (8 males and 7 females, mean \pm SE: age, 25.4 ± 1.2 years; BMI, 21.2 ± 0.6 kg/m²) volunteered to participate in the study. All subjects reported no history of diabetes and all female subjects were non-pregnant and non-lactating. The present study was approved by the University Clinical Research Ethical Committee and all subjects gave written informed consent.

Procedures

The GI values of 23 Chinese traditional foods were determined by using the Food and Agriculture Organization (FAO) recommended methods^[16]. All subjects were required to refrain from alcohol consumption and vigorous physical activities 24 h before test. They were

required not to consume unusually large meals and have balanced diets on the previous day. After 10-14 h overnight fast, the subjects were required to report to the lab between 8 am and 10 am. On arrival, the subjects rested for around 15 min and the baseline finger-prick capillary samples were collected. Then the subjects consumed either reference (50 g anhydrous glucose) or test foods containing 50 g of available carbohydrate, based on the information from the label of food or from the food nutrition content table provided by the Center of Food Safety, the Government of the Hong Kong Special Administrative Region^[17]. Each subject was given 50 g anhydrous glucose 3 times and 25 g anhydrous glucose twice as a reference. Among all the selected foods, 2 foods, which contained both 50 g and 25 g available carbohydrate, were tested twice to compare whether the different portion sizes gave the same result. Only one food contained 25 g available carbohydrate for size limitation.

The intervals between two tests were at least two days. Foods for testing were randomized in blocks of 4 foods^[18]. A drink of 250 mL water was served with test food in each test and all foods were required to be consumed within 10 min. Further blood samples were collected at 15, 30, 45, 60, 90, and 120 min after starting to eat. All the blood samples were analyzed with YSI glucose analyzer (YSI 1500, USA; YSI).

The food items included Baked Barbecued Pork Puff (BBPP), Fried Rice in Yangzhou-style (FRYS), Fried Fritter (FF), “Mai-Lai” Cake (MLC), Tuna Fish Bun (TFB), Sticky Rice Wrapped in Lotus Leaf (SRWLL), Steamed Glutinous Rice Roll (SGRR), “Pineapple” Bun (PAB), Jam and Peanut Butter Toast (JPBT), Fried Rice Noodles with Sliced Beef (FRNSB), Egg Tart (ET), Plain Steamed Vermicelli Roll (PSVR), Green Bean Dessert (GBD), Barbecue Pork Bun (BPB), Red Bean Dessert (RBD), Moon Cakes (MC), Glutinous Rice Ball (GRB), Chinese Herbal Jelly (CHJ), Instant Sweet Milky Bun (ISMB), Frozen Sweet Milky Bun (FSMB), Fried Rice Vermicelli in Singapore-style (FRVSS), Salted Meat Rice Dumpling (SMRD) and Spring Roll (SR). All foods were prepared on the test morning or the day before the test; when necessary the foods were steamed by hot water. Because of special cooking methods and habits, some Chinese traditional foods were actually mixed meals. However, they were very popular in China and all of them were prepared by fixed methods. Therefore, the GI value of the same kind of food produced in different places was expected to be similar. The detailed information of each food was listed in Table 1.

Statistical analysis

The individual GI value was calculated by expressing the IAUC for each test food as a percentage of each subject's average IAUC for the glucose. IAUC were calculated ignoring area beneath the fasting level^[16,19]. The mean of all the individual GI values for each test food calculated from all subjects was the GI value for that food. GL values were calculated by multiplying GI values of a food by the carbohydrate content of the usual portion

Table 1 Portion size, macronutrient composition and preparation of the test foods

Food Items	Serving (g)	Energy (kcal)	Protein (g)	Available CHO (g)	Fat (g)	Fiber (g)	Sugar (g)	Method of preparation
BBPP	161	708	14.3	50.0	50.0	2.1	9.2	Hoixe Cake Shop, H.K.; Instant
FRYS	217	412	15.2	50.0	17.1	4.8	1.3	Instant
FF	139	653	12.0	50.0	44.5	1.8	1.3	Ocean Empire International Ltd., H.K.; Instant
MLC	114	319	7.8	50.0	9.2	3.0	19.4	Instant
TFB	139	417	15.3	50.0	16.7	1.9	19.5	Maxim's MX, H.K.; Instant
SRWLL	167	351	12.7	50.0	11.2	2.0	3.0	Maxim's MX, H.K.; Instant
SGRR	109	283	5.6	50.0	7.1	1.9	6.4	Instant
PAB	91	319	7.8	50.0	10.0	1.5	9.1	Hoixe Cake Shop, H.K.; Instant
JPBT	106	350	11.7	50.0	11.7	5.5	12.7	Instant
FRNSB	250	350	10.0	50.0	12.8	5.5	1.8	Instant
ET	143	458	7.4	50.0	25.7	1.4	27.2	Maxim's MX, H.K.; Instant
PSVR	238	262	3.1	50.0	5.2	Trace	1.5	Instant
GBD	333	243	8.3	50.0	1.1	5.3	30.6	NISSIN brand, Nissin Foods Co., LTD.; Instant
BPB	119	309	8.7	50.0	8.7	1.9	16.7	Maxim's MX, H.K.; Instant
RBD	263	247	9.5	50.0	1.0	7.6	26.3	NISSIN Brand, Nissin Foods Co., LTD.; Instant
MC	80	324	6.6	50.0	10.9	2.5	NA	Kee Wah Bakery Shop, H.K.; Instant
GRB	115	424	5.8	50.0	23.1	Trace	30.6	Lee Chun Brand, Lee Chun Food Ltd., H.K.; Boiled
CHJ	333	212	0.0	50.0	0.0	0.0	36.4	Guang Jian Tang Brand, Kwong Tai Agency Co., Ltd.; Instant
ISMB	114	285	4.9	50.0	7.3	2.2	31.9	Instant
FSMB	114	285	4.9	50.0	7.3	2.2	31.9	AMOY Brand, Amoy Food Ltd., H.K.; Frozen, Steamed
FRVS (50 g)	333	533	21.0	50.0	28.0	12.0	3.2	Instant
FRVS (25 g)	167	267	10.5	25.0	14.0	6.0	1.6	Instant
SMRD (50 g)	200	360	11.4	50.0	13.4	3.4	0.7	Ocean Empire International Ltd., H.K.; Instant
SMRD (25 g)	100	180	5.7	25.0	6.7	1.7	0.3	Ocean Empire International Ltd., H.K.; Instant
SR (25 g)	114	388	10.4	25.0	27.4	2.2	2.5	Instant

BBPP: Baked barbecued pork puff; FRYS: Fried rice in Yangzhou-Style; FF: Fried fritter; MLC: "Mai-Lai" Cake; TFB: Tuna fish bun; SRWLL: Sticky rice wrapped in lotus leaf; SGRR: Steamed glutinous rice roll; PAB: "Pineapple" Bun; JPBT: Jam and peanut butter toast; FRNSB: Fried rice noodles with sliced beef; ET: Egg tart; PSVR: Plain steamed vermicelli roll; GBD: Green bean dessert; BPB: Barbecue pork bun; RBD: Red bean dessert; MC: Moon cakes; GRB: Glutinous rice ball; CHJ: Chinese herbal jelly; ISMB: Instant sweet milky bun; FSMB: Frozen sweet milky bun; FRVS: Fried rice vermicelli in singapore-style; SMRD: Salted meat rice dumpling; SR: Spring roll.

sizes of this food, divided by 100. The usual portion sizes of different foods were taken from manufacturers' information or from mean values of testing foods.

The differences in IAUC and GI values between male and female subjects were compared with independent samples *t*-test. The differences in IAUC and GI values between FRVS (50 g) and FRVS (25 g), SMRD (50 g) and SMRD (25 g) were compared with paired *t*-test. The differences in the mean IAUC value and within-subject coefficient of variation for repeated references (CVref) between Reference (50 g) and Reference (25 g) were also compared with paired *t*-test. The results were analyzed using the SPSS for Windows version 12.0 (SPSS, Chicago, IL) software package. The values were presented as mean \pm SE and the significance level was set at $P < 0.05$.

RESULTS

All subjects completed the experiment except one subject who did not consume the foods containing 25 g available carbohydrate for individual reasons. The determined GI and GL values of test foods were shown in Table 2.

The mean IAUC value of all the test foods calculated from male subjects (IAUCmale) was lower than that from female subjects (IAUCfemale) ($91.10 \pm 3.09 \text{ mmol min L}^{-1}$ vs $118.60 \pm 4.05 \text{ mmol min L}^{-1}$, $P < 0.001$), however there were no differences between the mean GI value of all the test foods determined from data of male subjects (GI_{male}) and that from female subjects (GI_{female}) (64 ± 2 vs 67 ± 2 , $P = 0.224$). When grouped by each test food, there were no differences either in the mean IAUC or GI value between male and female subjects.

The mean IAUC value calculated from the data elicited by 50 g anhydrous glucose (IAUCref50) was higher than that from the data elicited by 25 g anhydrous glucose (IAUCref25) ($167.54 \pm 14.54 \text{ mmol min L}^{-1}$ vs $108.40 \pm 8.86 \text{ mmol min L}^{-1}$, $P < 0.001$). However, there were no differences in the mean within-subject CVref (CVref = $100\% \times \text{SD}/\text{mean}$) ($28.00\% \pm 3.55\%$ vs $21.56\% \pm 3.74\%$, $P = 0.163$) for the 14 subjects between 50 g and 25 g anhydrous glucose.

The mean IAUC value calculated from the food containing 50 g carbohydrate was higher than that elicited by the same food containing 25 g carbohydrate (FRVS: 89.24

Table 2 Determined GI and GL values of test foods (mean \pm SE)

Food items	IAUC (mmol·min·L ⁻¹)	95% CI (mmol·min·L ⁻¹)	GI	95% CI	Available carbohydrate (g per serving)	GL (per serving)
Foods containing 50 g available carbohydrate						
BBPP	92.14 \pm 10.75	69.08-115.21	55 \pm 8	39-72	16.8	9
FRYS	133.70 \pm 13.85	103.99-163.40	80 \pm 6	67-92	133.6	107
FF	110.83 \pm 16.18	76.14-145.52	69 \pm 9	50-89	34.7	24
MLC	95.63 \pm 12.43	68.97-122.29	61 \pm 8	44-79	37.5	23
TFB	79.91 \pm 9.78	58.94-100.89	46 \pm 4	38-55	31.5	14
SRWLL	137.55 \pm 10.81	114.36-160.73	83 \pm 5	73-93	107.4	89
SGRR	143.03 \pm 12.96	115.24-170.83	89 \pm 8	71-107	43.3	39
PAB	106.39 \pm 11.69	81.32-131.46	65 \pm 8	48-83	33.9	22
JPBT	116.51 \pm 11.36	92.15-140.87	72 \pm 8	54-90	34.1	25
FRNSB	107.17 \pm 12.36	80.66-133.69	66 \pm 7	50-81	121	80
ET	74.70 \pm 7.82	57.93-91.46	45 \pm 3	38-53	22.1	10
PSVR	153.15 \pm 18.87	112.67-193.62	90 \pm 8	74-107	40.1	36
GBD	90.24 \pm 11.54	65.49-114.99	54 \pm 6	40-68	33.0	18
BPB	112.11 \pm 9.28	92.21-132.01	69 \pm 9	51-87	25.1	17
RBD	122.20 \pm 11.45	97.65-146.76	75 \pm 8	58-91	38.0	29
MC	90.96 \pm 9.65	70.26-111.66	56 \pm 7	42-70	52.2	29
GRB	98.76 \pm 13.04	70.78-126.74	61 \pm 10	37-82	95.2	58
CHJ	84.19 \pm 7.29	68.45-99.93	47 \pm 3	41-52	33.0	16
ISMB	115.44 \pm 12.42	88.80-142.07	67 \pm 5	57-78	20.8	14
FSMB	114.33 \pm 11.52	89.61-139.04	72 \pm 8	55-90	14.5	10
FRVS (50 g)	87.41 \pm 9.64	66.73-108.10	54 \pm 6	41-66	87.5	47
SMRD (50 g)	116.32 \pm 14.96	84.23-148.41	69 \pm 8	51-87	139.6	96
Foods containing 25 g carbohydrate						
FRVS (25 g)	64.26 \pm 11.09	40.30-88.22	58 \pm 9	39-78	87.5	51
SMRD (25 g)	85.99 \pm 9.15	66.24-105.75	81 \pm 7	65-97	139.6	113
SR (25 g)	53.05 \pm 7.27	37.34-68.75	50 \pm 5	39-60	20.5	10

± 10.17 mmol min⁻¹ L⁻¹ *vs* 64.26 ± 11.09 mmol min⁻¹ L⁻¹, SMRD: 117.23 ± 16.04 mmol min⁻¹ L⁻¹ *vs* 85.99 ± 9.15 mmol min⁻¹ L⁻¹, $P < 0.05$), however there were no differences between the two GI values (FRVS: 55 ± 6 *vs* 58 ± 9 , $P = 0.745$; SMRD: 70 ± 9 *vs* 81 ± 7 , $P = 0.319$).

DISCUSSION

The availability of reliable GI values of different foods is critical for not only researchers but also common people. The University of Sydney has determined the glycemic and insulin responses to more than 1750 foods and shown that the GI is a reproducible measure of day-long postprandial glycemia^[9]. In the more recent edition of international tables of GI and GL^[10], over 2480 GI values of individual food items were listed. Because of the close relationship between the food GI and human health, labeling of GI on foods has been proposed or is occurring in Australia, South Africa, Sweden, United Kingdom, and Germany, with several commercial laboratories measuring the GI of foods^[20].

During recent years, the GI values of some local foods have been measured in different countries prior to their utilization in research and clinical settings among the local population^[13-15]. Since there was little information about GI values of Chinese traditional foods in Hong Kong in the literature, and that had limited the related research in this area^[11,12], it was worthwhile to setup a GI and GL database for Chinese traditional foods. However, there are so many traditional and special Chinese foods, according to folk culture, district,

religion, and festival. For the famous classes divided by district, there are styles of Guangdong, Beijing, Shanghai, Sichuan, North-West, *etc.* These all above mentioned styles are well-known worldwide. Quite different from Western cooking whose recipes are followed strictly like laboratory instructions, Chinese cooking allows for a creative and stylistic touch to it and it is also one important reason why Chinese foods are always absent in the international GI and GL tables. In this study, by using a recommended standard method, GI and GL values of 23 Chinese traditional foods were determined.

Although GI was a classification of the blood glucose raising potential of carbohydrate foods, many other factors such as food form, particle size, cooking methods, presence of other macronutrients and starch structure, might affect the GI of foods^[14,21]. Fat and protein added to carbohydrate foods have been suggested to reduce the postprandial glycemic responses which occurred by different mechanisms, such as delaying gastric emptying^[22]; however, most of the studies found that the amount of protein or fat in commonly consumed foods did not affect the glycemic responses^[23,24]. It was also found in the present study that no relation existed between the amounts of fats or protein in foods and their GI values.

Though there was a recommended standard protocol for the determination of GI^[16,25], there were still some methodological factors which will influence the accuracy in GI determination. According to an inter-laboratory study^[21], the GI values of foods were more precisely determined using capillary than venous blood sampling. A recent study^[20] also found that the CV of the IAUC val-

ues was significantly lower for capillary than for venous blood. So in the present study, capillary blood samples were selected for determining the GI values of foods.

One study suggested that the composition and characteristics of the evening meal might influence glucose tolerance the next morning^[27]. However, no difference was found in another study on within-individual variation influenced by subject preparation between controlled trials and uncontrolled trials^[28]. Furthermore, a more recent report suggested that simply advising subjects to avoid certain types of foods was almost as good and might be more cost-effective^[20]. Thus, in the present study all the subjects were just advised to have a balanced dinner each night before the test. Furthermore, all subjects in the present study were also required to refrain from alcohol consumption and vigorous physical activities 24 h before test, foods for testing were randomized in blocks of 4 foods, and the intervals between two tests were at least two days^[16,18].

The FAO recommended the reference food test should be repeated at least three times in each subject^[16]. A recent study suggested that no evidence to justify doing 3 tests rather than 2 tests was found because the difference was small and not significant^[20]. In our study, the reference of 50 g anhydrous glucose was tested three times to determine the GI and GL values for 20 of the 23 foods.

In the present study, though the mean IAUC values calculated from female subjects were higher than that calculated from male subjects, there were no differences in the mean GI values between them, which is consistent with a previous study^[20].

One study showed that GI value was negatively related to the within-individual CVref, and low within-subject variation (CVref < 30%) was required for accuracy in GI determination^[20]. Another study also found that most of the variation of GI values was due to within-subject variation, and in normal subjects the mean CVref was about 25.0%^[22]. In the present study, both CVref of 50 g glucose and that of 25 g glucose were less than 30%, and there were no differences between the two values. This result might indicate that the determined GI values were accurate in some extent.

No differences were found between the two GI values determined for the same food containing different amounts of available carbohydrate (50 g and 25 g) in this study. It might suggest that when the portion of one food containing 50 g available carbohydrate was too large for subjects to consume, it was appropriate to select the portion of the food containing 25 g available carbohydrate to determine the GI values. The result was consistent with a previous study which showed that the relative glycemic responses to the foods containing different levels of available carbohydrate intake were the same, at least between 25 g and 100 g^[29].

In conclusion, the GI and GL values for these Chinese traditional foods in the present study provide some valuable information both to researchers and to common individuals on their food preference and they are

also preliminary references on the setup of a GI and GL database for Chinese traditional foods later.

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COMMENTS

Background

The glycemic index (GI) and glycemic load (GL) are closely related to some chronic diseases, however, little was known about the GI and GL values of Chinese traditional foods even in the latest international GI and GL tables.

Research frontiers

Previous studies have shown that GI is a reproducible measure of day-long postprandial glycemia and over 2480 GI values of individual food items were listed in the more recent edition of international tables of GI and GL. The relationship between food GI and human health has been largely investigated and is still one of the research hotspots in this research field. During recent years, the GI values of some local foods have been measured in different countries because most of the published GI data are based on analysis carried out in western countries, while this has limited the application of GI tables to local researchers or common people.

Innovations and breakthroughs

The present study determined the GI and GL values of some selected Chinese traditional foods which are very popular in Hong Kong. The results will provide some preliminary information which may act as the basis for the development of a GI and GL database for Chinese traditional foods.

Applications

The availability of reliable GI values of different foods is critical for not only researchers but also common people. Therefore, the results of this study will provide some valuable information both to researchers and to common individuals on their food preference.

Terminology

GI: GI was defined as the incremental area under the blood glucose response curve after a portion of food containing 50 g available carbohydrate expressed as a percentage of that after the same amount of carbohydrate from a reference food, usually glucose or white bread, taken by the same subject. The differences in the food GI values are mainly related to differences in the rate at which the carbohydrates are digested and absorbed. GL: GL was calculated as the GI value of the food multiplied by the amount of the available carbohydrate in a usual portion size, divided by 100.

Peer review

Blood glucose response to intake of traditional Chinese foods was investigated in this study. The aim of the study is interesting and the study design is good.

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Cigarette smoking and alcohol drinking and esophageal cancer risk in Taiwanese women

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METHODS: This is a multi-center, hospital-based, case-control study. Case patients consisted of women who were newly diagnosed and pathology-proven to have esophageal squamous cell carcinoma (ESCC) from three large medical centers (one from Northern and two from Southern Taiwan, respectively) between August 2000 and December 2008. Each ESCC patient was matched with 4 healthy women based on age (within 3 years) and hospital of origin, from the Department of Preventive Medicine in each hospital. A total of 51 case patients and 204 controls, all women, were studied.

RESULTS: Frequencies of smokers and drinkers among ESCC patients were 19.6% and 21.6%, respectively, which were significantly higher than smokers (4.4%) and drinkers (4.4%) among controls (OR = 4.07, 95% CI: 1.36-12.16, $P = 0.01$; OR = 3.55, 95% CI: 1.03-12.27, $P = 0.04$). Women who drank an amount of alcohol more than 158 g per week had a 20.58-fold greater risk (95% CI: 1.72-245.62, $P = 0.02$) of ESCC than those who never drank alcohol after adjusting for other covariates, although the sample size was small.

CONCLUSION: Cigarette smoking and alcohol drinking, especially heavy drinking, are the major risks for developing ESCC in Taiwanese women.

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Key words: Esophageal squamous cell carcinoma; Taiwanese women; Cigarette smoking; Alcohol drinking

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Abstract

AIM: To investigate the etiology of esophageal cancer among Taiwanese women.

Tai SY, Wu IC, Wu DC, Su HJ, Huang JL, Tsai HJ, Lu CY, Lee JM, Wu MT. Cigarette smoking and alcohol drinking and esophageal cancer risk in Taiwanese women. *World J Gastroenterol* 2010; 16(12): 1518-1521 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v16/i12/1518.htm> DOI: <http://dx.doi.org/10.3748/wjg.v16.i12.1518>

INTRODUCTION

Esophageal cancer, predominately esophageal squamous cell carcinoma (ESCC), was the 9th leading cause of cancer deaths in Taiwan in 2006^[1]. The age-adjusted mortality rate was 0.54 per 100 000 persons for females, much lower than for males (9.04 per 100 000 persons), due to low incidence rate of esophageal cancer in females (0.77 per 100 000 persons in females and 11.99 per 100 000 persons in males).

Most epidemiological studies, including ours, regarding the etiology of ESCC have been conducted in men or both combined genders^[2-6], but very few have only focused on the effect of risk/protective factors on esophageal cancer among women^[7,8]. Cheng *et al*^[7] conducted a population-based, case-control study in four regions in England and Scotland. They found that high body mass index in early adulthood and low consumption of fruit are the most important risk factors, but that breastfeeding may confer a protective effect, for esophageal adenocarcinoma^[7]. In the same group led by Sharp *et al*^[8], the researchers found that cigarette smoking and consumption of hot food and tea are significant risk factors for ESCC in the same four regions of the previous study^[7]. Since, in Taiwan, no study has examined the determinants of ESCC occurrence in women, we conducted a multicenter, hospital-based, case-control study to investigate this issue.

MATERIALS AND METHODS

Selection of cases and controls

Case patients were women who were newly diagnosed and pathology-proven to have ESCC from three large medical centers: National Taiwan University Hospital (NTUH) located in northern Taiwan, Kaohsiung Medical University Hospital (KMUH) and Kaohsiung Veterans General Hospital located in southern Taiwan. These three hospitals are the main medical centers in their geographic areas and are accessible to patients from all socioeconomic groups in Taiwan. We matched each case patient with 4 healthy women based on their age (within 3 years) and hospital of origin, from the Department of Preventive Medicine. In total, 51 cases and 204 controls, all women, were recruited for interview between August 2000 and December 2008. During the same period, we recruited 530 male ESCC patients from these three medical centers. The study women were interviewed to collect demographic and substance use information by trained interviewers using a standardized questionnaire which was used earlier in an esophageal cancer study of

Taiwanese men^[2,3]. This study was approved by the Human Subjects Committee of KMUH; informed consent was obtained from all subjects.

Questionnaire

The information collected regarding substance-use habits included whether the subject had been a habitual areca chewer, cigarette smoker or alcoholic beverage drinker in her lifetime. Subjects who had smoked more than 10 cigarettes per week for at least 6 mo were defined as cigarette smokers. Those who reported regularly chewing betel quid for at least 6 mo were defined as areca chewers. For those who were cigarette smokers or areca chewers, the amount of consumed tobacco or areca per week was also collected. For alcohol drinking, subjects who had drunk beer, wine or distilled spirits more than once a week for at least 6 mo were defined as alcoholic beverage drinkers. For those who had ever consumed alcohol, detailed information was collected as to percentage of alcohol content (categorized as < 10%, 10%-19%, 20%-49%, and \geq 50%) and number of alcohol drinks consumed per week. One drink was defined as a bottle or can of beer, a medium glass of wine, a small glass of port/sherry, or a nip of spirits/liqueur.

Statistical analysis

Descriptive analysis was applied to demographic data to determine respective distributions. The averaged alcohol intake (in grams) per week for each type of beverage was estimated by multiplying the midpoint value for each intake frequency by the standard drink volume per week and median percentage of alcohol content (categorized as < 10%, 10%-19%, 20%-49%, and \geq 50%). Unconditional logistic regression was used to assess the association between case/control status and use of substances (tobacco, alcohol, and areca) and other covariates. Data were analyzed using the SAS 9.1 statistical package; all *P*-values were two-sided.

RESULTS

The distributions of age, tea consumption, and educational level were comparable between case patients and controls (Table 1). The frequencies of smokers, drinkers, and areca chewers were higher in case patients than in controls. After adjusting for other covariates, the status of smoking and drinking remained significant. Compared to non-smokers, smokers had a 4.07-fold (95% CI: 1.36-12.16, *P* = 0.01) greater risk of developing ESCC in women. Compared to non-drinkers, drinkers had a 3.55-fold (95% CI: 1.03-12.27, *P* = 0.04) greater risk of developing ESCC (Table 1). Since smoking and drinking alcohol are the significant risk factors for ESCC, we further examined their dose-response effect by dichotomizing the average amount of alcohol intake per week by the median (cigarette smoking: 3.5 packs/wk; alcohol drinking: 158 g/wk). We found that women who drank an amount of alcohol more than 158 g per week had a 20.58-fold greater disease risk (95% CI: 1.72-245.62, *P* = 0.02) than those who

Table 1 Demographic characteristics and frequency of substance use in female esophageal squamous cell carcinoma patients and their controls (*n* = 255)

Variables	Cases (<i>n</i> = 51) <i>n</i> (%)	Controls (<i>n</i> = 204) <i>n</i> (%)	Crude OR (95% CI)	Adjusted OR ¹ (95% CI)	<i>P</i> -value ¹
Age (yr)					
≤ 65	17 (33.3)	74 (36.3)	1.00	1.00	0.2467
> 65	34 (66.7)	130 (63.7)	1.14 (0.60, 2.18)	1.56 (0.70, 3.50)	
Educational level (yr)					
≤ 9	36 (70.6) ²	179 (87.8)	1.00	1.00	0.0962
> 9	8 (15.7) ²	25 (12.3)	1.59 (0.67, 3.81)	1.78 (0.68, 4.64)	
Tea consumption					
No	13 (25.5) ²	37 (18.14)	1.00	1.00	0.1743
Yes	36 (70.6) ²	167 (81.86)	1.63 (0.79, 3.37)	1.82 (0.78, 4.24)	
Areca chewing					
Non-chewers	48 (94.1)	203 (99.5)	1.00	1.00	0.4016
Chewers	3 (5.9)	1 (0.5)	12.69 (1.29, 124.61)	3.41 (0.13, 88.80)	
Smoking status					
Non-smokers	41 (80.39)	195 (95.59)	1.00	1.00	0.0107
Smokers	10 (19.61)	9 (4.41)	5.28 (2.02, 13.82)	4.07 (1.36, 12.16)	
≤ 3.5 pack/wk	6 (11.76)	4 (1.96)		6.08 (1.43, 25.94)	
> 3.5 pack/wk	4 (7.84)	5 (2.45)		2.09 (0.39, 11.23)	
Alcohol consumption					
Non-drinkers	40 (78.4)	195 (95.6)	1.00	1.00	0.0378
Drinkers	11 (21.6)	9 (4.4)	5.96 (2.32, 15.32)	3.55 (1.03, 12.27)	
≤ 158 gm/wk	3 (5.9)	8 (3.9)		2.06 (0.44, 9.63)	
> 158 gm/wk	8 (15.7)	1 (0.5)		20.58 (1.72, 245.62)	

¹After adjusting for age (≤ 65 years *vs* > 65 years old), educational level (≤ 9 y *vs* > 9 yr), tea consumption (yes *vs* no), and status of smoking, areca chewing, and alcohol consumption (yes *vs* no); ²We got missing data with the educational level and tea consumption of cases.

never drank alcohol after adjusting for age, educational level, cigarette smoking, and areca chewing. Women who drank less than 158 g per week had a slightly elevated risk of ESCC, but not significantly, compared to non-drinkers (Adjusted OR = 2.06, 95% CI: 0.44-9.63). In contrast, we did not find any significant dose-response effect of smoking on the risk of developing ESCC (Table 1).

DISCUSSION

This multi-center hospital-based case-control study shows that cigarette smoking and alcohol drinking are the major risk factors for ESCC in Taiwanese women. The effect of alcohol drinking on ESCC risk is mainly confined to heavy drinkers (> 158 g/wk). Tobacco and alcohol are classified as class 1 carcinogens by the International Agency for Research on Cancer^[9]. Although cigarette smoking and alcohol drinking are well-known risk factors for ESCC^[10-15], the majority of findings have been from studies among men.

To our knowledge, only two papers have been published which solely investigate the risk/protective factors of esophageal cancer in women^[7,8]. Cheng *et al*^[7] first conducted a multi-center, population-based, case-control study in four regions in England and Scotland. They collected 74 incident cases of women with histologically confirmed diagnoses of esophageal adenocarcinoma, and 74 female controls matched by age. They found that high body mass index in early adulthood and low consumption of fruit are important risk factors for esophageal adenocarcinoma. In contrast, breastfeeding may confer a protective effect, but this needs further confirmation as suggested by the authors^[7]. In the same group

led by Sharp *et al*^[8], the researchers conducted another population-based, matched case-control study of histologically confirmed ESCC in women in the same four regions. There were 159 case-control pairs. They found that cigarette smoking and the consumption of hot food and tea are significant risk factors. However, they did not find that alcohol drinking was a significant risk factor for ESCC, a result which is different from our findings.

Tobacco contains numerous carcinogens and many studies have demonstrated its link to esophageal cancer^[8,12-14,16-19]. In point of fact, the likelihood that cigarette smoking is more of a promoter than a mutagenic initiator is seen epidemiologically, as when heavy smokers stop smoking their risk of developing lung cancer is greatly diminished. Moreover, the most predominant chemicals in cigarette smoke are known promoters as they share mechanistic properties of known tumor promoters (i.e. they have threshold levels of action; they are reversible in action; their biological effects can be overridden by anti-tumor promoters, anti-oxidants and chemopreventive agents in the diet, *etc.*)^[20-23]. However, for alcoholic beverages, it is not clear what exact mechanisms cause the increased risk of esophageal cancer. The commonest ingredient of all beverages is ethanol. Although ethanol has not been shown as carcinogenic in laboratory animals, it may act through its major metabolite, acetaldehyde, which is a carcinogen in animal models^[24,25]. Alternatively, ethanol could exert a promoting effect by either solubilizing tobacco-specific carcinogens or enhancing their penetration into the esophageal mucosa to cause direct toxicity or oxidative damage on the epithelial mucosa^[18,26]. In addition, alcoholic beverages may activate other carcinogenic compounds, such

as N-nitrosamines and urethane, to increase the risk of malignancies of the upper aerodigestive tract^[9,17,26].

A relatively small sample size was the major limitation in this study. Since the incidence rate of ESCC in Taiwanese women is extremely low (average 0.81 per 100 000 for the preceding 5 years), a large effort was made in this study to recruit all incident cases of ESCC from three medical centers in Taiwan. Because of small sample size, we are unable to investigate other factors, such as fruit and vegetable intake, which may have a modest protective effect on ESCC in women. In conclusion, our results suggest that cigarette smoking and alcohol drinking, especially heavy drinking, are the major risks affecting the development of ESCC in Taiwanese women.

COMMENTS

Background

Esophageal cancer, predominately esophageal squamous cell carcinoma (ESCC), was the 9th leading cause of cancer deaths in Taiwan in 2006. While most epidemiological studies regarding the etiology of ESCC have been conducted in men or both combined genders, very few have solely focused on the effect of risk/protective factors on esophageal cancer among women.

Research frontiers

Since, in Taiwan, no study has examined the determinants of ESCC occurrence in women, the authors conducted a multi-center, hospital-based, case-control study to investigate this issue.

Innovations and breakthroughs

The results of this study suggest that cigarette smoking and alcohol drinking, especially heavy drinking, are the major risk factors for developing ESCC in Taiwanese women.

Applications

This study suggests that to abstain from smoking and drinking can prevent the development of ESCC in women.

Peer review

This manuscript is a well-written article. It is a quite interesting study by mainly being held on women population. The authors investigated the etiology of esophageal cancer among women in Taiwan. It is a multi-center hospital-based case-control study which concluded that cigarette smoking and alcohol drinking, especially for heavy drinkers, are the major risk factors for developing ESCC in Taiwanese women.

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APC gene mutations in Chinese familial adenomatous polyposis patients

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(22.2%). Large fragment deletions were detected by MLPA in 2 families. The total mutation detection rate of micromutations and large fragment deletions was 78.6% (11/14).

CONCLUSION: The detection rate of *APC* gene germline mutation can be improved by direct sequencing combined with MLPA large fragment deletion detection.

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Key words: Adenomatous polyposis coli gene; Familial adenomatous polyposis; Large fragment deletion; Multiplex ligation-dependent probe amplification; Mutation

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Abstract

AIM: To study the characteristics of *APC* (adenomatous polyposis coli) gene germline mutation in Chinese patients with familial adenomatous polyposis (FAP).

METHODS: *APC* gene from 14 FAP families was amplified by polymerase chain reaction (PCR) and underwent direct sequencing to determine the micromutation type. For the samples without micromutation, the large fragment deletion of *APC* gene was examined by multiplex ligation-dependent probe amplification (MLPA).

RESULTS: There were gene micromutations in 9 families with a micromutation detection rate of 64.3% (9/14), including 6 frameshift mutations (66.7%), 1 nonsense mutation (11.1%) and 2 splicing mutations

INTRODUCTION

Familial adenomatous polyposis (FAP) is a rare autosomal dominant genetic disease, with an approximate incidence rate of 1/10000. Clinical manifestations are mainly multiple adenomatous polyps in the large intestine (more than 100 polyps in total), and most of these patients fall ill in adolescence. Adenomatous polyps are a type of precancerous lesions. Thereby, cancerization will occur before the age of 40 years in almost 100% of patients without treatment. It was shown that the occurrence of FAP was related to *APC* (adenomatous polyposis coli) gene

mutations located at 5q21-q22^[1]. *APC* gene micromutations were identified in about 60%-70% of FAP patients^[2], while large fragment deletion mutations of *APC* gene were identified in 10%-15%^[3,4]. *APC* gene mutation screening in FAP patients and their family members cannot only further explore the pathogenesis of FAP and understand the *APC* gene mutation spectrum of Chinese FAP, but also predict the risk of FAP in "healthy members" of their families. It is also helpful for monitoring and in the clinical treatment of high-risk individuals with mutant genes, and can effectively decrease the incidence and mortality of FAP^[5]. At present, only a few studies of Chinese FAP have been reported, and smaller sample size and lower detection rate (mostly at 50%) were the main problems in these studies. In order to further understand *APC* gene mutations in Chinese patients with FAP, a total of 14 FAP families were detected by direct sequencing combined with large fragment deletion detection in this study.

MATERIALS AND METHODS

Patients

From 2002 to 2008, 14 patients from FAP families diagnosed and treated in the General Hospital of Beijing Military Region were enrolled in this study. Diagnostic criteria were as follows: (1) more than 100 adenomatous polypi in total; (2) more than 20 adenomatous polypi in patients with a family history of FAP. The patients which included 7 males and 7 females were from Beijing, Hebei, Henan, Anhui, Inner Mongolia, Shan'xi, Fujian Provinces and other regions, and were aged 12-57 years (mean 35.21 years) with an onset age of 8-57 years (mean 28.14 years). All patients gave written informed consent.

Genomic DNA extraction

Ten milliliter peripheral venous blood was drawn from FAP patients and genomic DNA was extracted by the phenol/chloroform/isoamyl alcohol method.

Primer synthesis

Primer sequences for *APC* gene exons 1-15 were synthesized by Shanghai Sangon Biological Engineering Technology & Services Co. Ltd, as previously described^[6].

PCR

Twenty microlitre PCR amplification reaction system contained 100 ng template DNA, 0.2 mmol/L of dNTP, 1.5 mmol/L of Mg^{2+} , 0.1-0.2 μ mol/L of upstream and downstream exon primers and 1-1.5 U TaqDNA polymerase. PCR reaction conditions were as follows: initial denaturation at 94°C for 5 min, followed by 35-40 cycles of denaturation at 94°C for 30 s, annealing for 30 s and extension at 72°C for 30 s, and a final extension at 72°C for 5 min, preservation was carried out at 4°C. After 1.5% agarose gel electrophoresis (containing EB dye), PCR products were observed by a gel imaging instrument. Fragment sizes of PCR products were indicated with DL2000 DNA Marker.

DNA sequencing

PCR reaction products were purified, and sequencing was performed by a DNA automatic sequencer (ABI PRISM 3730XL, USA). Changes in base sequence were confirmed by reverse sequencing.

Biological analyses

Sequence analyses were performed by BioEdit software. For the changed base sequences, the mutational site and type were determined in NCBI, and then the mutations were identified as new by referring to the mutations reported in the Human Gene Mutation Database (<http://www.hgmd.cf.ac.uk/ac/gene.php?gene=APC>) and the UMD-APC mutations database (<http://www.umd.be/APC/>).

Detection of large fragment deletions

Large fragment deletions were detected by multiplex ligation-dependent probe amplification (MLPA) only in samples without micromutations, and the experimental procedures followed the instruction manual of the MLPA kit (SALSA MLPA kit P043 APC, MRC-Holland, Amsterdam, the Netherlands). One hundred nanogram template DNA was denatured at 98°C for 5 min and hybridized with the probe liquid at 60°C overnight, subsequently, with thermal stability enzyme ligase 65, and spliced with long and short probes with the same sequences at 54°C for 15 min. Finally, the spliced probes were amplified by PCR with universal primers. Electrophoresis and collection of PCR amplification products were finished in an ABI 3700 sequenator, and the results were analyzed by GeneMapper software to obtain a peak map and peak area.

RESULTS

A total of 9 micromutations were identified by direct sequencing among these 14 unrelated FAP families with a mutation rate of 64.3%, including 6 frameshift mutations (66.7%), 2 splicing mutations (22.2%) and 1 nonsense mutation (11.1%), of which 4 mutations including c.2336-2337insT, c.3923-3929delAAGAAAA, c.532-2A>T and c.4179-4180GAdelinsT have not previously been reported (Table 1 and Figure 1). Among the 5 patients without micromutations identified by direct sequencing, large fragment deletions were identified by the MLPA method in 2 patients, including large fragment deletions of exon 11 and 10A (Alternative exon) in one patient and a large fragment deletion of exon 15 start in another patient (three probes included start, middle and end in the MLPA detection of exon 15), and these two large fragment deletions have not previously been reported (Table 1 and Figure 2).

The detection rate of large fragment deletions in *APC* mutation-negative patients was 40%, while the total mutation rate of micromutations and large fragment deletions was 78.6%. Meanwhile, 7 Snp sites were detected in 14 families (Table 2). Missense mutations including c.2753C>A, c.4007G>C and c.3964C>T were found in

Table 1 Micromutations and large fragment deletions of APC gene germline mutation in Chinese patients with familial adenomatous polyposis detected in this study

Family No.	Exons/introns	Base changes	Protein changes	Mutation types
2	Exon15	c.3927-3931delAAAGA	p.Glu1309AspfsX4	Frameshift mutation
3	Exon15	c.4179-4180GAdelinsT ¹	p.Asp1394LeufsX21	Frameshift mutation
4	Exon15	c.2336-2337insT ¹	p.Leu779PhefsX9	Frameshift mutation
5	Exon10	c.1327G>T	p.Glu443X	Nonsense mutation
6	Exon15	c.3923-3929delAAGAAAA ¹	p.Lys1308ArgfsX11	Frameshift mutation
7	Intron7	c.657+1 G>A		Splicing mutation
8	Exon15	c.3183delACAAA	p.Gln1062X	Frameshift mutation
10	Exon15 start ¹			Large fragment deletion
11	10A (Alternative exon) and Exon11 ¹			Large fragment deletion
12	Exon15	c.3202_3205delTCAA	p.Ser1068GlyfsX57	Frameshift mutation
14	Intron4	c.532-2A>T ¹		Splicing mutation

¹Micromutations or large fragment deletions were not reported previously. Mutation sequencing was shown in Figure 1; Peak of large fragment deletion was shown in Figure 2.

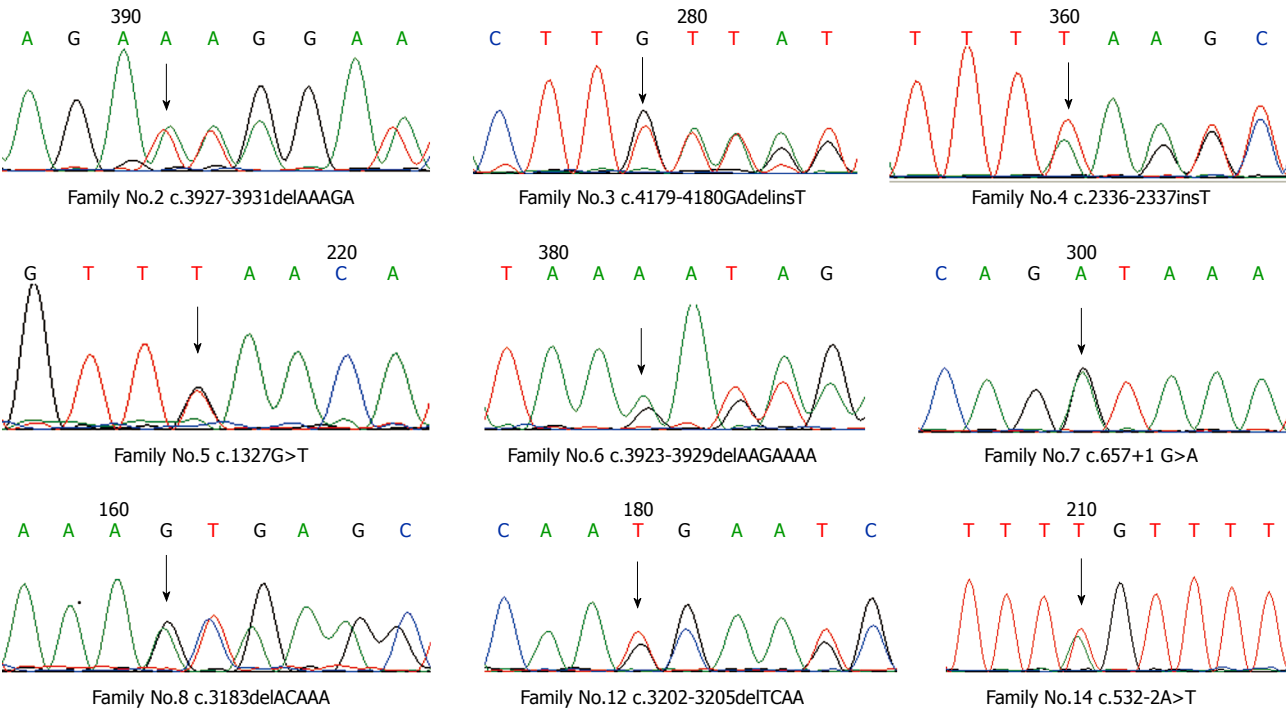


Figure 1 DNA sequencing of micromutations.

families 3, 11 and 14 besides frameshift mutations, large fragment deletions and splicing mutations, respectively. Due to difficulty in assessing pathopoiesis of missense mutations, these 3 missense mutations were excluded in the calculation of the mutation detection rate during analyses of the results. Moreover, one base substitution of intron 14 (c.653+8T>C) was found in family 13. Due to unknown effects of intron mutation, this was also excluded in the calculation of the mutation detection rate.

DISCUSSION

APC gene, a tumor suppressor gene, is the key gene in FAP. It is located at 5q21-q22 and contains an 8538 bp open reading-frame and a total of 15 exons. The APC gene product, APC protein, is a multi-regional binding

protein containing 2843 amino acids. The majority of APC gene mutations will result in the earlier formation of terminal codons in downstream. APC protein will lose biological activities due to its truncated change. Although there are numerous methods for detecting APC gene micromutations, direct sequencing is the most direct and accurate method. MLPA is a new method for detecting large fragment deletions which is rapid, sensitive, specific and reliable and has other advantages. In order to improve the detection rate of APC gene mutations, direct sequencing and the MLPA method for detecting large fragment deletions were combined in this study.

Direct sequencing showed that the APC gene mutation rate in Chinese FAP patients (Mainland) was 64.3%, which was significantly higher than that in Taiwan (50%)^[6], Hong Kong (50%)^[6] and other reports in the Mainland

Table 2 Snp sites of *APC* gene germline mutation in Chinese patients with familial adenomatous polyposis detected by DNA sequencing

Exons	APC gene loci	Base changes	Ncbi dbsnp ID	Positive families
Exon11	89271	C/T	refSNP ID: rs2229992	1-8; 10; 12-14
Exon13	90978	A/G	refSNP ID: rs351771	1-12
Exon15	102187	A/G	refSNP ID: rs41115	1-7; 9-14
Exon15	102742	A/G	refSNP ID: rs42427	1-14
Exon15	102976	G/T	refSNP ID: rs866006	1-13
Exon15	103173	A/T	refSNP ID: rs459552	1-14
Exon15	103588	A/G	refSNP ID: rs465899	1-14

(48.39%^[7] and 50%^[8]). This difference in Chinese *APC* gene mutation rates might result from different human subjects and detection methods and small sample sizes. The detection rate in this study was close to that in Japan (67%^[9] and 64.67%^[10]), lower than that in Greece (83%)^[6] and Chile (87.5%)^[11], and slightly higher than that in the Czech Republic (59.3%)^[12], Slovakia (61.5%)^[12] and South Korea (61%)^[13]. Combined with the MLPA method for detecting large fragment deletions, the total detection rate of *APC* gene mutations was up to 78.6%, which was higher than that reported in Czechoslovakia (67.6%)^[9].

Among the 9 micromutations detected, the majority were frameshift mutations (66.7%) located at exon 15 (66.7%). Eventually, 77.8% of the mutations including nonsense mutations and frameshift mutations resulted in the earlier formation of terminal codons, and truncated changes were found in APC proteins. Substitution of bases in site +1 of exon 7 splicing district in family 7 and in site -2 of exon 5 splicing district in family 14 might lead to abnormal mRNA splicing. Therefore, the synthesized proteins were different from the wild-type. Four new micromutations found in this study were located at codon 779, 1308, 1394 and c.532-2, respectively.

With the exception of the above-mentioned 9 micromutations, 7 Snp sites were identified in this study, which was consistent with NCBI reports. Base substitution located at c.653+8 in intron 14 was found in family 13. However, the mechanisms of intron mutation are not yet understood. Therefore, the relationship between the mutation at c.653+8 and the onset of FAP should be studied further.

We also found two mutations in one family, including: (1) a frameshift mutation c.4179-4180GAdelinsT and a missense mutation c.2753C>A in family 3; (2) large fragment deletions of exon 10A and exon 11 and a missense mutation c.4007G>C in family 11; and (3) splicing mutations c.532-2A>T and a missense mutation c.3964C>T in family 14. Because the frameshift mutation in family 3, large fragment deletion in family 11 and splicing mutation in family 14 were definite pathogenic mutations, it was difficult to identify the pathopoiesis of simultaneous missense mutations, indicating that undetected definite

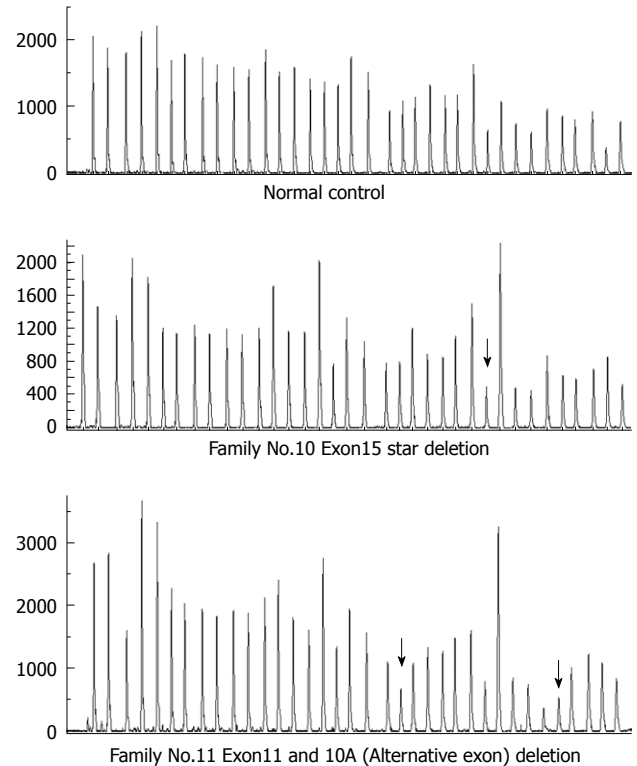


Figure 2 Peak of large fragment deletion detected by multiplex ligation-dependent probe amplification (MLPA). Arrows show the reduced relative peak area of the amplification product of that probe which means heterozygous deletions of corresponding exons.

pathogenic mutations would decrease after comprehensive screening for all exons of *APC* genes combined with large fragment deletion detection. Therefore, functional experiments should be carried out to identify the pathopoiesis of missense mutations and understand the causal relation with occurrence of diseases.

Two large fragment deletions were found by the MLPA method in patients without micromutations detected by direct sequencing. Deletions of 10A and exon 11 were found in one case. Because 10A was located at 1.6 kb in the downstream of exon 10, this indicated that deletions of exon 10A and exon 11 including some parts of intron 10 existed in this case. Deletion of exon 15 start was found in another case. These two large fragment deletions have not been reported in the Human Gene Mutation Database (<http://www.hgmd.cf.ac.uk/ac/gene.php?gene=APC>). MLPA showed that the detection rate of large fragment deletions in patients without *APC* gene mutations was 40% (2/5), which was higher than that in Belgium (15%)^[3] and the Netherlands (6.4%)^[14]. Because of the small sample size in the research on large fragment deletions (5 cases) and few reports on *APC* gene large fragment deletions in Chinese patients, studies on *APC* gene large fragment deletions in Chinese FAP need to be carried out with a larger sample size.

In addition, 10A, an additional *APC* gene exon, was found recently located at 1.6 kb in the downstream of exon 10. 10A consisted of 54 bases encoding 18 amino acids^[15]. One case of 10A deletion was detected in this

study. Therefore, *APC* gene screening should include exon 10A. Recently, the studies on *MYH* genes and *MYH* associated polyposis (MAP) revealed that MAP accounted for 5%-7.5 % of FAP. It was found by Sieber *et al*^[16] that diallelic *MYH* mutations accounted for 6.6 % of polyposis families without *APC* gene mutations, and accounted for almost one third of FAP in families with attenuation-type familial adenomatous polyposis. Detection of *MYH* gene mutations should be performed in patients who have no mutations shown by mutation detection and large fragment deletion detection of *APC* genes.

In conclusion, there were a variety of *APC* gene mutations in Chinese FAP, and mutation detection rates were relatively high. Four new micromutations and 2 new large fragment deletions were found in this study. *APC* gene mutation detection rates could be improved effectively by direct sequencing combined with the MLPA method for large fragment deletions. Therefore, it was very necessary to add large fragment deletion detection to conventional detection of molecular genetics.

COMMENTS

Background

Familial adenomatous polyposis (FAP) is a rare autosomal dominant genetic disease. Clinical manifestations are mainly multiple adenomatous polyps in large intestine, and most patients fall ill in adolescence. Cancerization will occur before the age of 40 years in almost 100% of patients without treatment. FAP is related to *APC* (adenomatous polyposis coli) gene mutations located at 5q21-q22.

Research frontiers

APC gene micromutations were identified in about 60%-70% of FAP patients, while large fragment deletions were identified in 10%-15%. Direct sequencing is the most accurate method for detecting *APC* gene micromutations. Multiplex ligation-dependent probe amplification (MLPA) is a new method for detecting large fragment deletions.

Innovations and breakthroughs

In order to improve the detection rate of *APC* gene mutations, direct sequencing and the MLPA method for large fragment deletion detection were combined in this study. The total detection rate of *APC* gene mutations was up to 78.6%. Moreover, 4 novel micromutations and 2 novel large fragment deletions were found in this study.

Applications

APC gene mutation detection rates could be improved effectively by direct sequencing combined with the MLPA method for large fragment deletions. Therefore, it was very necessary to add large fragment deletion detection to conventional detection of molecular genetics.

Terminology

Multiplex ligation-dependent probe amplification (MLPA) is a variation of PCR that permits multiple targets to be amplified with only a single primer pair. Each probe consists of a two oligonucleotides which recognise adjacent target sites on the DNA. One probe oligonucleotide contains the sequence recognised by the forward primer, the other sequence is recognised by the reverse primer. Only when both probe oligonucleotides are hybridized to their respective targets, can they be ligated into a complete probe.

Peer review

The work described in this report is a service to the community concerned with *APC* gene mutations. The discovery of new mutations in an important gene and disease sometimes can qualify for a publication, especially in a relevant journal. This article is well written and the work done properly.

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Surgically treated primary malignant tumor of small bowel: A clinical analysis

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Abstract

AIM: To evaluate the clinical presentation, treatment and survival of patients with primary malignant tumor of small bowel (PMTSB).

METHODS: Clinicopathologic data about 141 surgically treated PMTSB patients (91 males and 50 females) at the median age of 53.5 years (range 23-79 years) were retrospectively analyzed.

RESULTS: The most common initial clinical features of the patients were intermittent abdominal discomfort or vague abdominal pain (67.4%), abdominal mass (31.2%), bowel obstruction (24.1%), hematochezia (21.3%), jaundice (16.3%), fever (14.2%), coexistence of bowel perforation and peritonitis (5.7%), coexistence of gastrointestinal bleeding and shock (5.0%), and intraabdominal bleeding (1.4%). Ileum was the most common site of tumor (44.7%), followed by jejunum (30.5%) and duodenum (24.8%). PMTSB had a nonspecific clinical presentation. Segmental bowel resection ($n = 81$) was the most common surgical procedure, followed by right hemi-

colectomy ($n = 15$), pancreaticoduodenectomy ($n = 10$), and others ($n = 19$). Twenty-seven adenocarcinoma patients and 13 malignant lymphoma patients received adjuvant chemotherapy with 5-fluorouracil and cyclophosphamide, adriamycin, vincristine and prednisone, respectively. Information about 120 patients was obtained during the follow-up. The median survival time of PMTSB patients was 20.3 mo. The 1-, 3- and 5-year survival rate was 75.0% (90/120), 40.0% (48/120) and 20.8% (25/120), respectively. Adenocarcinoma was found in 73.7% (42/57), 21.1% (12/57) and 15.8% (9/57) of the patients, respectively. Gastrointestinal stromal tumor was observed in 80.0% (20/25), 72.0% (18/25) and 36.0% (9/25) of the patients, respectively. Carcinoid was detected in 100.0% (15/15), 80.0% (12/15) and 46.7% (7/15) of the patients, respectively. Malignant lymphoma was demonstrated in 69.2% (9/13), 30.8% (4/13) and 0% (0/13) of the patients, respectively.

CONCLUSION: *En bloc* resection is the principal therapy for most PMTSB and chemotherapy is the important treatment modality for malignant lymphoma and other malignant tumors of small bowel which cannot be radically removed.

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Key words: Small bowel; Malignant tumor; Diagnosis; Surgical treatment; Chemotherapy

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Han SL, Cheng J, Zhou HZ, Guo SC, Jia ZR, Wang PF. Surgically treated primary malignant tumor of small bowel: A clinical analysis. *World J Gastroenterol* 2010; 16(12): 1527-1532 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v16/i12/1527.htm> DOI: <http://dx.doi.org/10.3748/wjg.v16.i12.1527>

INTRODUCTION

Primary malignant tumor of the small bowel (PMTSB) accounts for 2% of all gastrointestinal (GI) tumors and 1% of GI tumor-related deaths^[1-5]. The small bowel is relatively resistant to carcinogenesis although it is considerably long (accounting for 70% of the whole digestive tract) and exposed to a wide variety of potentially noxious substances. In a review of over 11 000 primary GI malignant tumors, Martin^[6] found that only 2.4%, 10.8%, 16.4%, and 70.3% of primary malignancies are originated from the small bowel, esophagus, stomach, and colorectum, respectively. However, the diagnosis of PMTSB is difficult, because its symptoms and signs are nonspecific at presentation. Therefore, it is usually discovered at its advanced stage and often needs a surgical intervention due to acute complications. Sometimes, it is occasionally found during other surgical procedures^[6-11]. This retrospective study was to evaluate the clinical presentation, treatment and survival of PMTSB patients.

MATERIALS AND METHODS

Medical records of PMTSB patients at the tumor registry of our hospital between January 1988 and December 2007 were analyzed. Patients with tumor at the ampulla of Vater, pancreatic head, and ileocecal valve and metastatic cancer were excluded from the study. Only those with primary tumor arising from the duodenum, jejunum, or ileum were included.

One hundred and forty-one patients entered the study. Data on demography, clinical presentation, diagnosis, surgical treatment, histopathological findings, postoperative course, and survival time were collected from each patient. The TNM staging classification (AJCC system, 6th edition)^[12] was used to classify the extent of adenocarcinoma and carcinoid based on the histopathological and surgical reports. Stage I (T1-2, N0) was defined as tumor extending to muscularis propria, stage II (T3-4, N0) as tumor extending to subserosa, mesentery, and adjacent viscera, stage III (any T, N1) as tumor with regional lymph node metastasis, and stage IV (any T, any N, M1) as tumor with distant metastasis. Adenocarcinoma at stages I-IV was detected in 3, 14, 37 and 7 patients, respectively. Carcinoid at stages I-IV was found in 4, 8, 5 and 2 patients, respectively. Operation reports on the type, extent, and necessity of *en bloc* resection were reviewed. Operation was defined as radical if the tumor was completely removed both grossly and microscopically, and as palliative if the patients had distant metastasis at presentation, gross residual tumor at surgery, or positive margins microscopically. The World Health Organization standard grading system (well differentiated, moderately differentiated, poorly differentiated, and undifferentiated) was used to classify the histological types^[12]. In addition, Ann Arbor staging classification^[13] was used to classify the extent of malignant lymphoma. The diagnosis of gastrointestinal stromal tumor (GIST) was made as previously described^[14-16].

Statistical analysis

Data were analyzed using the SPSS software (version 13.0; SPSS, Inc., Chicago, IL). Patient records were reviewed during the follow-up or by direct contact with the patients, their relatives, or office visit. Survival rate was calculated on the day of histological diagnosis until death or last follow-up with the Kaplan-Meier method for analysis of censored data. Cox regression analysis was performed to assess the independent prognostic significance of parameters. $P < 0.05$ was considered statistically significant.

RESULTS

Clinical and diagnostic features

Of the 141 PMTSB patients, 91 were male and 50 were female. Their median age was 53.5 years (range 23-79 years). Ileum was the most common site of PMTSB (44.7%), followed by jejunum (30.5%) and duodenum (24.8%). The most common clinical features of PMTSB patients at initial presentation were intermittent abdominal discomfort or vague abdominal pain (67.4%), abdominal mass (31.2%), bowel obstruction (24.1%), hematochezia (21.3%), jaundice (16.3%), fever (14.2%), coexistence of bowel perforation and peritonitis (5.7%), coexistence of gastrointestinal bleeding and shock (5.0%), and intraabdominal bleeding (1.4%). Other symptoms were loss of appetite, diarrhea, anemia and loss of bodyweight (Table 1). The median time of symptoms was 2 mo (range 0-41 mo).

The preoperative diagnostic rate was decreased to 91.7% (11/12) at the duodenum, 70.6% (36/51) at the jejunum, and 60.3% (47/78) at the ileum, respectively. The most commonly used diagnostic techniques were ultrasonography (US) of the abdomen (90.1%), followed by computed tomography (CT) of the abdomen (80.1%), upper gastrointestinal radiography (31.9%) and upper endoscopy (25.5%). Additional techniques included ultrasonography, endoscopic retrograde cholangiopancreatography (ERCP), superior mesenteric arteriography, colonoscopy and bone scanning.

All the patients were diagnosed histopathologically after operation. Of the 141 patients, 61 (43.3%) were diagnosed as adenocarcinoma, 28 (19.8%) as GIST, 17 (12.1%) as carcinoid, 14 (9.9%) as malignant lymphoma, 10 (7.1%) as leiomyosarcoma, 6 (4.3%) as malignant melanoma, 3 (2.1%) as malignant neurilemmoma, and 2 (1.4%) as fibrosarcoma, respectively (Table 2). Twenty-three of the 61 patients (37.7%) who underwent curative resection were found to have lymph node metastases after surgery, which were not suspected before operation.

Surgical procedures

Of the 141 patients who underwent surgical intervention, 31 (22.0%) had emergency operation and 110 (78.0%) had selective operation. The emergency indications included bowel obstruction ($n = 24$), gastrointestinal bleeding ($n = 4$) and bowel perforation ($n = 3$). The most commonly used surgical procedure was segmental bowel resection

Table 1 Clinical symptoms and signs of PMTSB patients

Symptoms	n (%)
Abdominal pain	95 (67.4)
Abdominal mass	44 (31.2)
Bowel obstruction	34 (24.1)
Hematochezia	30 (21.3)
Jaundice	23 (16.3)
Fever	20 (14.2)
Bowel perforation coexistent peritonitis	8 (5.7)
Gastrointestinal bleeding and coexistent Shock	7 (5.0)
Intraabdominal bleeding	2 (1.4)

Others symptoms: Loss of appetite, diarrhea, anemia and loss of body-weight. PMTSB: Primary malignant tumor of small bowel.

Table 3 Surgical procedure for PMTSB patients

Procedure	n (%)
Operative intervention	
Emergent procedure	31 (22.0)
Elective procedure	110 (78.0)
Disease for emergent procedure	
Bowel obstruction	24 (77.4)
Gastrointestinal bleeding	4 (12.9)
Bowel perforation	3 (9.6)
Surgical procedure	
Segmental bowel resection	92 (65.3)
Right hemi-colectomy	15 (10.6)
Pancreaticoduodenectomy	10 (7.1)
Gastric bypass	13 (9.2)
Biopsy at laparotomy	5 (3.6)
Feeding jejunostomy	2 (1.4)
Biliary bypass	2 (1.4)
Enteric bypass	2 (1.4)
Radicality of procedure	
Radical ¹	104 (73.8)
Palliative ²	37 (26.2)

¹Radical resection: Negative margin, resection of all gross diseases, *en bloc* local resection when indicated; ²Palliative resection: Positive margin, gross residual disease.

(65.3%), followed by right hemicolectomy (10.6%), and pancreaticoduodenectomy (7.1%). Other procedures included gastric bypass ($n = 13$), biopsy only ($n = 5$), feeding jejunostomy ($n = 2$), biliary bypass ($n = 2$), and enteric bypass ($n = 2$).

Of the 141 PMTSB patients, 104 (73.8%) received a radical resection, 37 (26.2%) underwent diagnostic or palliative operation. Of the 32 patients who underwent a palliative resection, 15 had synchronous distant metastasis (liver metastasis in 10 and peritoneal dissemination in 5) in small bowel and its mesentery ($n = 11$), retroperitoneum ($n = 5$) and ovary ($n = 1$) (Table 3).

Postoperative complications

Postoperative complications occurred in 21 (14.9%) patients, including pancreatic anastomotic leak in 7 (5.0%), wound infection in 6 (4.3%), prolonged gastric emptying in 3 (2.1%), subphrenic abscess in 3 (2.1%), and gastrointestinal bleeding from gastrojejunostomy in 2 (1.4%) as shown in Table 4. The median hospital stay time of patients was 13.2 d (range 8-60 d).

Table 2 Histopathological type of PMTSB ($n = 141$)

Histopathological type	n (%)
Adenocarcinoma	61 (43.3)
GIST	28 (19.8)
Carcinoid	17 (12.1)
Malignant lymphoma	14 (9.9)
Leiomyosarcoma	10 (7.1)
Malignant melanoma	6 (4.3)
Malignant neurilemmoma	3 (2.1)
Fibrosarcoma	2 (1.4)

GIST: Gastrointestinal stromal tumor.

Table 4 Complications after operation

Complications	n (%)
Pancreatic anastomotic leak	7 (5.0)
Wound infection	6 (4.3)
Prolonged gastric emptying	3 (2.1)
Subphrenic abscess	3 (2.1)
Gastrointestinal bleeding	2 (1.4)
Total	21 (14.9)

Postoperative adjuvant therapy

Of the 141 PMTSB patients, 40 (28.4%) received adjuvant chemotherapy after operation. However, adenocarcinoma was treated with 5-fluorouracil (5-FU) and malignant lymphoma was treated with cyclophosphamide, adriamycin, vincristine and prednisone (CHOP).

Recurrence patterns

Recurrence of the tumor was found in 32 (22.7%) of the 104 patients after radical resection (at a single site in 13 and at multiple sites in 19). The most common sites of recurrence were liver and lung (65.6%), peritoneal carcinomatosis (21.9%) and intestinal mesentery (12.5%). Nine patients (28.1%) underwent further operative intervention, 8 (25.0%) received chemotherapy and/or radiotherapy, and 4 (12.5%) received no further treatment. Of the 9 patients who underwent a second operation, 7 received a palliative procedure and died of the disease progression at a median time of 10 mo (range 2-18 mo) after operation.

Survival rate of PMTSB patients according to histology

Information was obtained during the follow-up of 120 patients with PMTSB including adenocarcinoma ($n = 57$), GIST ($n = 25$), carcinoid ($n = 17$), malignant lymphoma ($n = 17$), leiomyosarcoma ($n = 3$) and malignant melanoma ($n = 1$). The median survival time of PMTSB patients was 20.3 mo. The 1-, 3- and 5-year survival rate was 75.0% (90/120), 40.0% (48/120) and 20.8% (25/120), respectively. Adenocarcinoma was detected in 73.7% (42/57), 21.1% (12/57), and 15.8% (9/57) of the patients, respectively. GIST was observed in 80.0% (20/25), 72.0% (18/25) and 36.0% (9/25) of the patients, respectively. Carcinoid was found in 100.0% (15/15), 80.0% (12/15) and 46.7% (7/15) of the patients, respectively. Malignant lymphoma was shown in 69.2% (9/13), 30.8% (4/13) and 0% (0/13) of the patients, respectively. In addition,

Table 5 Survival rate of PMTSB patients *n* (%)

Histopathological type	Loss of follow-up cases (<i>n</i>)	1-yr survival rate	3-yr survival rate	5-yr survival rate
Adenocarcinoma (<i>n</i> = 61)	4	42 (73.7)	12 (21.0)	9 (15.8)
GIST (<i>n</i> = 28)	3	20 (80.0)	18 (72.0)	9 (36.0)
Carcinoid (<i>n</i> = 17)	2	15 (100.0)	12 (80.0)	7 (46.7)
Lymphoma (<i>n</i> = 14)	1	9 (9.25)	4 (30.8)	0
Other tumors(<i>n</i> = 21)	11	3 cases of leiomyosarcoma survived (15, 39 and 71 mo, respectively) and 1 case of malignant melanoma survived 18 mo		

3 leiomyosarcoma patients had a survived time of 15, 39 and 71 mo, respectively. One malignant melanoma patient survived for 18 mo (Table 5).

DISCUSSION

Presentation and diagnosis of PMTSB

PMTSB is a rare malignancy. Most PMTSB patients have nonspecific clinical symptoms and signs^[1-6]. In this series, the most frequent symptoms were abdominal pain (67.4%), abdominal mass (31.2%) and bowel obstruction (24.1%), followed by hematochezia (21.3%), jaundice (16.3%), fever (14.2%), coexistence of bowel perforation and peritonitis (5.7%), coexistence of gastrointestinal bleeding and shock (5.0%), and intraabdominal bleeding (1.4%). The symptoms are similar to the reported findings^[1-3,10,11]. The median time of delayed diagnosis in our series was 2 mo.

Preoperative diagnosis of PMTSB is often difficult. In our series, the preoperative diagnostic rate was decreased to 91.7% at the duodenum, 70.6% at the jejunum, and 60.3% at the ileum, respectively. The most commonly used diagnostic techniques were US, CT, upper gastrointestinal radiography and upper endoscopy. Endoscopic biopsy proved that most duodenal tumors (91.7%) in our study were malignant before surgery, suggesting that the more distal the tumor is, the more difficult the preoperative diagnosis is^[16,17]. Since the accuracy of CT staging for small bowel adenocarcinoma is 47%-61%, it is only used in the detection of mesenteric infiltration and regional lymphadenopathy^[16-20].

Hatzaras *et al*^[1] showed that carcinoid tumor is the most common intestinal cancer, followed by adenocarcinoma. Our data demonstrate that ileum is the most common site (44.7%), followed by jejunum (30.5%), duodenum (24.8%), and that the most prevalent histological type is adenocarcinoma (43.3%), followed by GIST (19.8%), carcinoid (12.1%), malignant lymphoma (9.9%), leiomyosarcoma (7.1%), malignant melanoma (4.3%), malignant neurilemmoma (2.1%) and fibrosarcoma (1.4%), indicating that carcinoid tumor is more frequently found in ileum than adenocarcinoma in duodenum^[1,8,21-23].

Laparoscopy can help to stage small bowel malignant tumor; serosal infiltration, retroperitoneal fixation, lymph node metastasis and ascites^[23]. Laparoscopy can accurately assess and stage gastric adenocarcinoma^[24,25], thus avoiding unnecessary laparotomy for those with no indication for palliative surgery. However, laparoscopy is not appropriate for patients with obstruction or bleeding.

Treatment strategy and surgical procedure

Treatment of PMTSB is mainly based on its histopathological type, location and extent. If the tumor is located at jejunum or ileum, an aggressive segmental resection and primary anastomosis are indicated^[2]. If the tumor is located at ileum around the ileocolon junction, ileocolonic resection or right hemi-colectomy is indicated^[3]. The optimal resection extent of duodenal tumor has not been defined. Some authors advocate pancreaticoduodenectomy (PD) for all patients with malignant tumor of the duodenum, including those located at the third and fourth portions to ensure adequate *en bloc* resection^[6]. If the tumor is an adenocarcinoma or a carcinoid, *en bloc* resection and systemic lymph node dissection are indicated^[7]. If the tumor is a histopathologically proven malignant lymphoma before operation, systemic chemotherapy is the first choice of treatment and surgery selection is only indicated for those with bowel obstruction, perforation and bleeding^[26-29]. The value for routine extensive resection of adenocarcinoma or carcinoid has been recently challenged^[2,3,7,8,26,27]. Others support PD for proximal duodenal carcinoma, but segmental resection for tumor of the third and fourth portions^[27-29]. In our patients, the most commonly used surgical procedure was segmental bowel resection (65.3%), followed by right hemicolectomy (10.6%), PD (7.1%) and others.

Of the 61 patients (37.7%) who underwent curative resection, 23 were found to have lymph node metastasis after surgery, which was not suspected before operation. It has been shown that laparoscopy can help to stage GI cancer, thus avoiding unnecessary laparotomies. However, laparoscopy is not appropriate for patients with obstruction or bleeding or for those with no indication for palliative surgery^[24,25]. In this series, 31 (22.0%) patients required urgent laparotomy for intestinal obstruction, gastrointestinal bleeding, or perforation.

It has been reported that chemotherapy may be beneficial for PMTSB, but optimal chemotherapy and the degree of benefit remain to be defined^[27,30,31]. Bakaeen *et al*^[30] showed that chemotherapy with lomustine (CCNU), 5-FU, either alone or in combination with other therapies, can considerably improve symptoms and hormone level, and moderately inhibit tumor regression, particularly in patients with metastatic gastroenteropancreatic neuroendocrine tumors with minimal adverse effects^[30,31]. In our study, 40 patients (28.4%) received adjuvant chemotherapy after operation. However, adenocarcinoma and malignant lymphoma were treated with 5-FU and CHOP, respectively.

Surgical outcome

It has been reported that the 5-year survival rate of PMTSB patients after curative resection is 32%-47%^[1,20-22,32,33]. Howe *et al*^[31] have reported that the 5-year survival rate of patients after curative resection of localized, regional and distant metastatic PMTSB is 47.6%, 31% and 3.9%, respectively, which is similar to that of our patients. The median survival time of the 141 PMTSB patients was 20.3 mo. The 1-, 3- and 5-year survival rate was 75.0%, 40.0% and 20.8%, respectively. Adenocarcinoma was detected in 73.7%, 21.1% and 15.8% of the patients, respectively. GIST was found in 80.0%, 72.0% and 36.0% of the patients, respectively. Carcinoid was observed in 100.0%, 80.0% and 46.7% of the patients, respectively. Malignant lymphoma was demonstrated in 69.2%, 30.8% and 0% of the patients, respectively.

The site, clinical stage, and histological type do not influence the survival time of PMTSB patients. Howe *et al*^[31] reported that the median survival time of patients with tumors of duodenum, jejunum and ileum is 16.9 and 28-31 mo, respectively. In our study, 32 of 104 patients (22.7%) had distant metastasis or intra-abdominal carcinomatosis at presentation, which is consistent with the reported findings^[1,5,7,11,26]. Ito *et al*^[7] reported that the 5-year survival rate of T1/T2 and T3/T4 tumor patients is 82% and 58%, respectively ($P < 0.05$). In contrast, Bakaeen *et al*^[30] found that T stage can not predict the survival time of PMTSB patients. Curative resection, however, may not be possible owing to the late diagnosis of PMTSB. Dabaja *et al*^[29] also reported that the 5-year survival rate of patients with PMTSB at stage IV is 5%, which is much lower than that of those with PMTSB at stages I-III (36%). Although carcinoid is usually silent and diagnosed at its advanced stage, carcinoid patients have a good prognosis and a long survival time after effective treatment^[2,5,7,34,35].

In summary, *en bloc* resection is the principal procedure for most PMTSB patients and chemotherapy is the important treatment modality for malignant lymphoma and other small bowel malignant tumors with no indication for radical resection.

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COMMENTS

Background

Although small bowel is considerably long and exposed to a wide variety of potentially noxious substances, it is relatively resistant to carcinogenesis. Primary malignant tumor of the small bowel (PMTSB) accounts for 2% of gastrointestinal (GI) tumors and 1% of gastrointestinal tract cancer-related deaths. In a review of over 11 000 primary GI malignant tumors, Martin found that only 2.4%, 10.8%, 16.4% and 16.4% are originated from the small bowel, esophagus, stomach, and colorectum, respectively. However, the diagnosis of PMTSB is difficult, because its symptoms and signs are nonspecific at presentation. Therefore, PMTSB is usually discovered at its advanced stage and often needs surgical intervention due to acute complications. Sometimes, it is occasionally found during other

surgical procedures. The objective of this study was to evaluate the clinical presentation, treatment and survival of PMTSB patients.

Research frontiers

How to improve the early diagnosis and treatment of PMTSB is a hotspot in recent studies.

Innovations and breakthroughs

This study evaluated the clinical presentation, treatment and survival time of PMTSB patients.

Applications

The retrospective analysis of the clinical presentation, treatment and survival time of PMTSB patients showed that PMTSB can be made early diagnosed and can thus be rationally treated.

Peer review

This is a rather large series of PMTSB patients from a single center. The data provided in this study contribute to the early diagnosis and treatment of PMTSB.

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Successful endoscopic treatment of colonic gallstone ileus using electrohydraulic lithotripsy

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Abstract

The surgical management of gallstone ileus is complex and potentially highly morbid. Initial management requires enterolithotomy and is generally followed by fistula resection at a later date. There have been reports of gallstone extraction using various endoscopic modalities to relieve the obstruction, however, to date, there has never been a published case of endoscopic stone extraction from the colon using electrohydraulic lithotripsy. In this report, we present the technique employed to successfully perform an electrohydraulic lithotripsy for removal of a large gallstone impacted in the sigmoid colon. A cavity was excavated in an obstructing 4.1 cm lamellated stone in the sigmoid colon using electrohydraulic lithotripsy. A screw stent retractor and stent extractor bored a larger lumen which allowed for guidewire advancement and stone fracture *via* serial pneumatic balloon dilatation. The stone fragments were removed. Electrohydraulic lithotripsy is a safe and effective method to treat colonic obstruction in the setting of gallstone ileus.

INTRODUCTION

Gallstone obstruction of the colon is a rare event^[1]. The traditional approach has been an initial enterolithotomy followed by fistula resection as indicated^[2]. Although this has been shown to be successful, endoscopic therapies offer a less invasive approach. Non-surgical treatment of gallstone(s) impacted in the colon has been described using extracorporeal lithotripsy, dormia baskets and polypectomy snares^[3-6]. These techniques, while successful in situations where the gallstone is small enough to be endoscopically extracted or where it yields enough to be broken with less radical endoscopic methods, can be inadequate for management of large stones. We encountered a large calcified stone impacted in the sigmoid colon that required localized disintegration by use of intracolonic electrohydraulic lithotripsy (EHL).

CASE REPORT

A 92-year-old man presented with a 5-d history of obstruction followed by nausea and vomiting. His abdomen was mildly distended with left lower quadrant tenderness. His past medical history was significant for diverticulo-

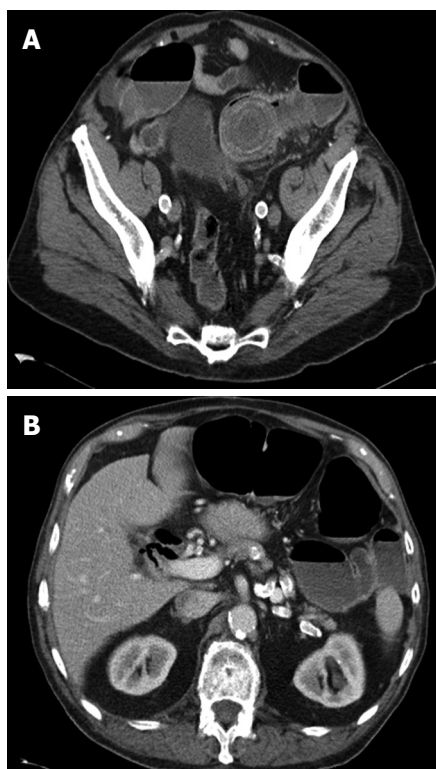


Figure 1 Computed tomography. A: Gallstone impacted in the sigmoid colon; B: Pneumobilia.

sis, coronary artery disease, severe aortic stenosis and an attack of right upper quadrant pain with nausea and vomiting 6 mo previously. Computed tomography demonstrated dilated loops of small and large intestine proximal to a 4.1 cm lamellated stone impacted in the sigmoid colon (Figure 1A), in addition to a separate 3.8 cm stone within the gallbladder lumen. There was fistulous communication between the gallbladder fundus and the transverse colon with resultant pneumobilia (Figure 1B). Due to his advanced age and extensive comorbidities, endoscopic intervention was recommended. In the endoscopy suite, the patient was placed in the left lateral decubitus position. An Olympus gastroscope (Olympus America Inc., Center Valley, PA) was advanced transanally to the impacted piston-shaped stone which was found to be lodged within the distal sigmoid colon at an area of narrowing, likely from prior diverticulitis. A 1.9 French EHL probe (Northgate Research Inc., Arlington Heights, IL) was passed through the biopsy channel of the gastroscope. Continuous normal saline instillation was used to provide a medium for EHL. EHL was performed by applying shocks to the center of the impacted stone using the Northgate SD-100 EHL generator with shock delivery at a setting of 70 to 100 watts (Figure 2A). A total of nine EHL probes were required to excavate a cavity through the core of the stone after 2200 cumulative shocks. The shocks did not produce fragmentation but rather a “tunnel” through the stone. Due to poor visualization, when a small amount of blood was seen exiting from the proximal end of the tunnel it was assumed

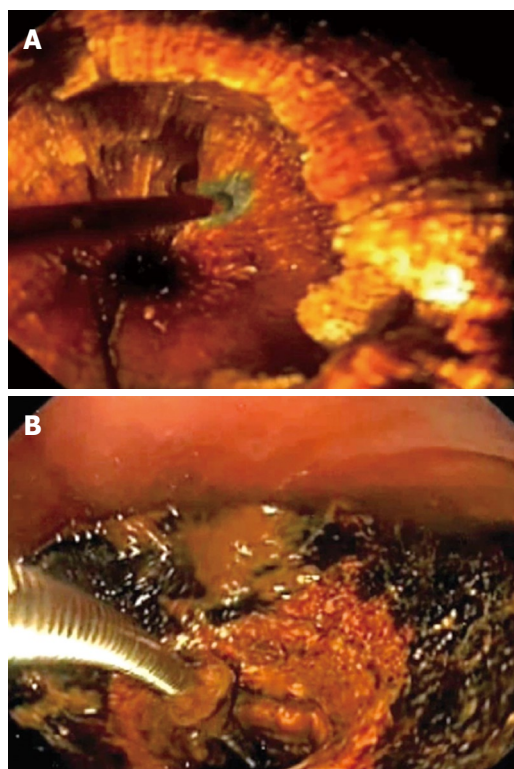


Figure 2 Endoscopy images. A: Electrohydraulic lithotripsy (EHL) shock to the center of the gallstone; B: Soehendra screw retractor in place for dilation of the gallstone lumen.



Figure 3 Four gallstone fragments after balloon fracture.

a complete lumen was created within the stone. A guidewire was advanced through the cavity into the proximal colon. A 7 French Soehendra screw stent retractor and a 10 French Soehendra screw stent extractor (Cook Medical, Winston-Salem, NC) were used to enlarge the proximal lumen (Figure 2B). This allowed passage of a dilating balloon to enable the stone to be fractured from within. Dilation using 18, 19 and 20 mm balloons inflated to maximum PSI was performed within the stone neolumen, causing it to fracture into four large pieces (Figure 3). These were retrieved using a Roth basket (US Endoscopy, Mentor, OH) and a large polypectomy snare. Concerns over the duration of the manipulation and resulting colonic wall edema necessitated termination of the procedure after a colonic decompression tube was placed over a guidewire that extended to the descending



Figure 4 Endoscopically assisted contrast evaluation of the cholecysto-colonic fistula with cholelithiasis.

colon. We were able to reconstruct the gallstone into its whole form, confirming that no potentially obstructing stone fragment was retained.

Due to concerns of biliary tract infection and recurrent gallstone ileus, an attempt at extraction of the existing gallstone within the gallbladder was attempted the following day. An Olympus IT gastroscope was advanced to the hepatic flexure where a large cholecystocolonic fistula was encountered. We advanced the endoscope through the fistula into the gallbladder fundus but no gallstone was encountered. Fluoroscopy and contrast injection through a biliary occlusion balloon (Olympus America, Inc.) demonstrated a stricture within the fundus and a large filling defect wedged within the gallbladder infundibulum consistent with the known retained gallstone (Figure 4). The stricture prevented further endoscopic manipulation. Therefore, the patient underwent resection of the fistulous tract with en bloc cholecystectomy and segmental colonic resection *via* a right subcostal incision. This confirmed a 4 cm long cholecystocolonic fistula from the fundus of the gallbladder to the hepatic flexure. The known retained stone plus an additional 1 cm stone were found within the infundibulum. This was walled off from the fundus due to the stricture. An intra-operative cholangiogram confirmed patency of the patient's biliary system without evidence of bile leak.

The patient recovered from a gastrointestinal standpoint without any complications related to the endoscopic procedures. Post-operatively, he developed decompensated congestive heart failure and pulmonary hypertension in addition to atrial fibrillation. He was discharged on post-operative day 25 after optimization of his cardiac function. The patient died at home 12 mo later secondary to aspiration pneumonia.

DISCUSSION

Gallstone ileus is a rare clinical entity accounting for 1% to 3% of intestinal obstruction and is more common in the elderly population^[1]. Gallstones most commonly impact in the terminal ileum (61%) followed by the jejunum (16%), stomach (14%), and large intestine (4%) at the rectosigmoid junction. The classic signs on imaging studies include di-

lated bowel and pneumobilia. Aberrantly located gallstones are present in about 50% of patients^[7]. Initial surgical enterolithotomy is the traditional treatment and this allows for relief of the obstruction in the short term. Fistula resection can be safely performed during a second surgical procedure if indicated^[2]. As this condition is predominantly encountered in an elderly population with a high incidence of comorbid conditions, the complication and mortality rates of surgical treatment are substantial^[1,2]. Endoscopically accessible impacted gallstones are amenable to less invasive alternative therapeutic options including EHL, extracorporeal shock wave lithotripsy, intracorporeal laser lithotripsy and endoscopic mechanical lithotripsy for fragmentation. To date, there have been a few case reports of the successful use of EHL in patients with gallstones impacted within the stomach and small intestine, but to our knowledge, this is the first report of the successful and safe use of EHL to remove an impacted gallstone within the sigmoid colon^[8-12]. The EHL technique allows for fragmentation of large, calcified stones that would not otherwise be amenable to other endoscopic modalities. Advanced endoscopic skills, in addition to the proper equipment, are necessary as the technique has the potential to be long and complicated, but can ultimately be successful.

The patient's retained gallstone posed a high risk for biliary infection and recurrent gallstone ileus and therefore the patient underwent an attempt at endoscopic retrieval of the retained gallstone within the gallbladder. Due to the stricture at the infundibulum, we were unable to manage this without operative intervention. However, the endoscopic extraction of the obstructing gallstone was able to eliminate an enterolithotomy procedure. This simplified his initial management and allowed him to undergo fistula resection under optimal conditions.

In summary, we present our technique for the first reported use of EHL to endoscopically extract an impacted gallstone within the lumen of the colon and believe that this is a safe and effective method to treat colonic obstruction in the setting of gallstone ileus.

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Ischemic colitis after mesotherapy combined with anti-obesity medications

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INTRODUCTION

Mesotherapy and anti-obesity medications are gradually gaining in popularity for purposes of body contouring and weight loss. However, there is a tendency to disregard their various adverse effects. Adverse effects of mesotherapy include bruising, edema, skin necrosis, abdominal hematoma at the site of injection, liver toxicity, demyelination of nerves, and atypical mycobacterial infection^[1]. Adverse effects of anti-obesity medications include increase of blood pressure, headache, constipation, and insomnia^[2-5].

Until now, there have been no case reports on the intestinal ischemia resulting from mesotherapy only or in combination with anti-obesity medications. We report on an interesting case of ischemic colitis after mesotherapy combined with anti-obesity medications in a 39-year-old female who had no risk factors.

CASE REPORT

A 39-year-old female was admitted to our hospital due to hematochezia persisting for 12 h. She had no previous history of disease. Eight days before admission, she visited an outside anti-obesity clinic where she underwent two days of injection therapy once per day into the sub-

Abstract

Mesotherapy and anti-obesity medications are gradually gaining worldwide popularity for purposes of body contouring and weight loss. Their adverse effects are various, but there is a tendency to disregard them. Ischemic colitis is one of the most common diseases associated with non-obstructive blood vessel disorders. However, there have been no case reports about the adverse effects resulting from mesotherapy only or in combination with anti-obesity medications. We report on an interesting case of ischemic colitis after mesotherapy combined with anti-obesity medications in a 39-year-old female who had no risk factors.

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Key words: Mesotherapy; Anti-obesity medications; Adverse effects; Ischemic colitis

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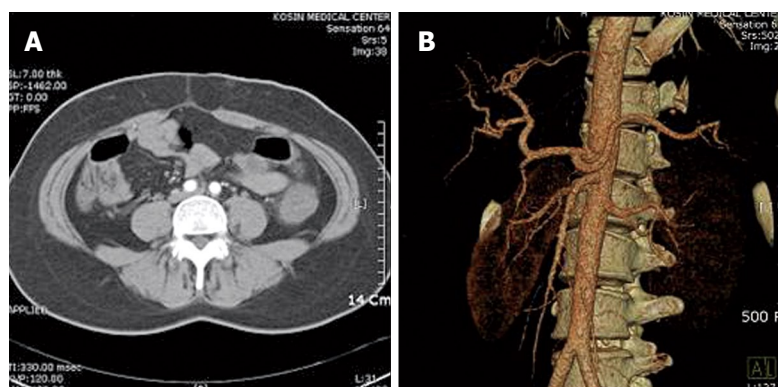


Figure 1 Abdominal computed tomography and angiography. A: Computed tomography showed diffuse bowel wall edema in the descending colon; B: Angiography showed no evidence of obstruction or stenosis in mesenteric vessels.

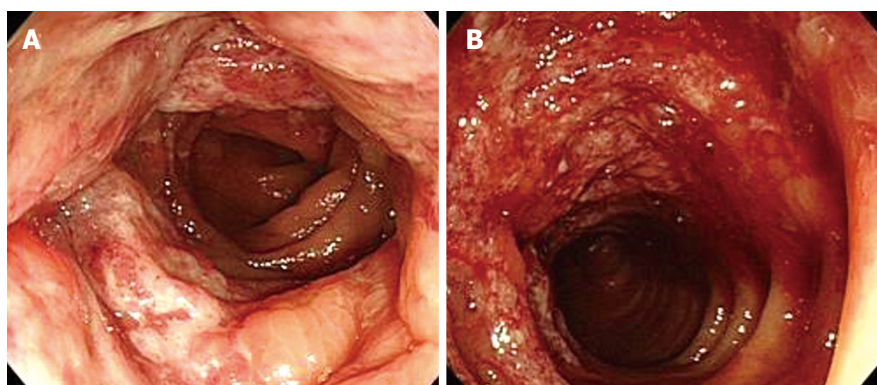


Figure 2 Sigmoidoscopic findings. Diffuse mucosal edema, erythema (A), friability and submucosal hemorrhage (B) from the splenic flexure to the sigmoid colon are shown.

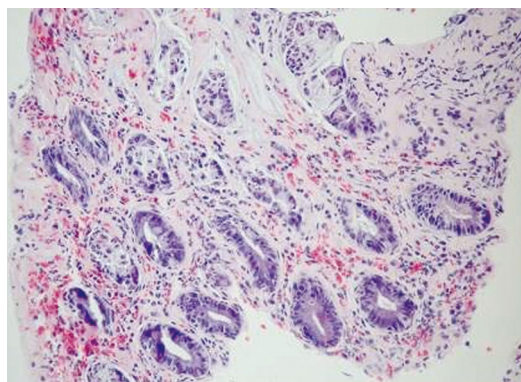


Figure 3 Microscopic findings of colonoscopic biopsy. Sections showed portions of colonic mucosa revealing sloughing of the surface epithelium and infiltration of mononuclear inflammatory cells in hyalinized lamina propria. (HE, $\times 200$).

cutaneous fat of the lower abdomen as mesotherapy, and took oral anti-obesity medications twice per day for two days. Medications for mesotherapy were aminophylline, epinephrine, and lidocaine at doses that could not be obtained outside the anti-obesity clinic. Oral anti-obesity medications were fluoxetine 10 mg, ephedrine hydrochloride 10 mg, anhydrous caffeine 50 mg, and green tea powder 250 mg per dose. Six days before admission, the patient stopped taking the mesotherapy and anti-obesity medications due to lower abdominal discomfort and nausea and vomiting. She nearly fasted, except for a small amount of liquid diet, until her admission to our hospital. A sudden onset of hematochezia and lower abdominal pain developed about 12 h prior to admission. She had no

history of any medication other than recent mesotherapy and anti-obesity medications. Her vital signs were within normal limits, including normal systolic and diastolic blood pressure. Physical examinations were normal, except for mild tenderness of the left lower abdomen, and fresh bloody stool was revealed with digital rectal examination. Height and body weight were 162 cm and 76.7 kg, respectively, with BMI 29.2 kg/m². Laboratory test results were within normal limits, including a hemoglobin 14 g/dL, platelet 351 000/uL, prothrombin time 11.3 s, protein C antigen 96% (normal range: 72%-160%), protein S antigen 81% (normal range: 60%-150%), B-type natriuretic peptide 35 pg/mL (normal range: less than 100 pg/mL), except for mildly elevated white blood cells 15 400/mm³. Electrocardiogram, 24-h Holter monitoring, and transthoracic echocardiography all had normal findings. Abdominal CT and CT angiography showed a markedly edematous wall of the entire descending colon and intact abdominal vasculature, including the superior and inferior mesenteric arteries (Figure 1). Colonoscopy showed diffuse edema, erythema, friability, and intramural hemorrhage with multiple active ulcers from the splenic flexure to the sigmoid colon, which were clearly demarcated with adjacent normal segments of colon (Figure 2). Microscopic findings showed portions of colonic mucosa revealing sloughing of the surface epithelium and infiltration of mononuclear inflammatory cells in hyalinized lamina propria (Figure 3). Two days after conservative management with intravenous fluids, electrolytes, and antibiotics composed of ciprofloxacin and metronidazole, bloody stool and abdominal pain markedly decreased and disappeared 6 d later, with complete normalization of

leukocytosis. The patient has been followed for 2 years on an outpatient basis without recurrence of specific symptoms.

DISCUSSION

Ischemic colitis is one of the most common diseases associated with non-obstructive blood vessel disorders. Ischemic colitis is common in people over 50 years old. However, it can develop rarely in women younger than 40 years old through use of oral contraceptives, vasoconstrictors, diuretics, cocaine and anti-depressants, or as a result of irritable bowel syndrome^[6-9].

Potential cardiac sources of embolism (CSE) (arrhythmia, structural cardiac abnormality such as valvular heart disease and structural aortic abnormality) were relatively common in patients with ischemic colitis^[10,11]. This suggests that when ambulatory ischemic colitis occurs, it is necessary to perform an exhaustive cardiovascular evaluation similar to those performed in other ischemic diseases^[11]. The optimal way to detect potential CSE was the combination of electrocardiogram, 24-h Holter monitoring, and transthoracic echocardiography^[11,12]. Anticoagulation therapy should also be considered for patients who have CSE^[10].

Cases of ischemic colitis in young patients have been reported^[12,13] to be associated with hereditary factors such as deficiencies of protein C, protein S, and antithrombin, factor V Leiden mutation, and prothrombin 20210G/A mutation, as well as acquired factors such as antiphospholipid antibodies^[13]. The mechanism of ischemic colitis is more often multifactorial^[11,12]. Therefore, an interaction between hereditary and acquired factors including the drugs which are used in mesotherapy and anti-obesity medication, is possibly the key to understanding why certain persons develop colonic ischemia at a specific time point especially at a young age^[12]. Accordingly, when ischemic colitis develops in young people, we should examine whether such hereditary and acquired factors as causes of it exist or not.

Mesotherapy is a recently introduced treatment that involves delivery of pharmacologic drugs into the mesoderm, which is the layer of fat or connective tissue under the skin. It is commonly used for purposes of body contouring and weight loss. Aminophylline is a common ingredient in mesotherapy that is used for lipolysis. It inhibits phosphodiesterase and activates hydrolysis of adipose tissue^[14]. Systemically, it stimulates the medullary respiratory center to induce diuresis and induces catecholamine release from the adrenal medulla^[15]. Epinephrine and lidocaine are not commonly used ingredients in mesotherapy. However, we think that they were administered for the purpose of causing local vasoconstriction and for pain relief at the subcutaneous injection site of the lower abdomen, respectively. Although the mesotherapy solution was injected locally into the subcutaneous fat, we think that its systemic effect could not be completely ignored.

Some oral agents are approved by the U.S. FDA as

anti-obesity medications for the purpose of long-term use: sibutramine, orlistat, and rimonabant. In addition, anti-obesity medications for short-term use include sympathomimetic amines, selective serotonin reuptake inhibitors (SSRI), ephedrine, and caffeine^[2,10,16]. Fluoxetine is an anti-depressant belonging to the SSRIs. Blockage of serotonin uptake in the nerve terminal and amplification of signals sent by serotonin neurons result in decreased food intake^[5]. There have been no reported cases of intestinal ischemia induced by use of fluoxetine as a single agent; however, three cases of intestinal ischemia induced by use of fluoxetine in combination with sumatriptan have been reported^[3]. Pseudoephedrine is an α -adrenergic agonist and potent vasoconstrictor. It inhibits appetite through release of noradrenaline in sympathetic nerve, and exerts a synergistic action when administered with caffeine^[2,10]. Dowd *et al*^[4] reported on 4 female patients aged 37 to 50 who developed ischemic colitis after taking pseudoephedrine. Caffeine promotes secretion of catecholamine and inhibits appetite. Green tea contains caffeine, and therefore exerts effects similar to those of caffeine^[17].

In this case, a second colonoscopy during the follow-up period could not be performed due to the patient's pregnancy and this is a weakness since we don't know if the findings of the first colonoscopy were resolved or not, although symptoms have completely disappeared.

We think that the potential important environmental factors for ischemic colitis in our patient were aminophylline (by increasing noradrenaline activity and inducing catecholamine release from the adrenal medulla), epinephrine (by acting as a direct vasoconstrictor), fluoxetine (by blocking serotonin uptake and so decreasing food intake and causing dehydration), ephedrine (by releasing noradrenaline in sympathetic nerves), caffeine (by exerting a synergistic action when administered with ephedrine), green tea (by caffeine effects and increasing the action and release of catecholamines), and unidentified hereditary and/or acquired factors might have contributed to the development of ischemic colitis. In addition, reduced body fluid caused by relative fasting and vomiting can be considered as aggravating factors in the development of ischemic colitis. Although two cases of ischemic colitis associated with anti-obesity medications with fenfluramine and phentermine have been reported^[18,19], they were not related to the agents prescribed for our patient. Therefore, to the best of our knowledge, our case is the first report in the world on ischemic colitis after mesotherapy combined with anti-obesity medications.

In conclusion, mesotherapy should be taken with great care by both the young and the old, particularly when combined with anti-obesity medications, because it can cause systemic vasoconstriction and result in various side effects, such as ischemic colitis. In addition, dehydration by relative fasting should be avoided during therapy because it can aggravate development of ischemic colitis.

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Transpapillary incision of refractory circumscript pancreatic duct stricture using wire-guided snare forceps

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Abstract

Endoscopic therapy of pancreatic duct (PD) strictures using balloon dilation and pancreatic duct stent (PS) placement has been reported to improve the severity of abdominal pain in selected patients with chronic pancreatitis (CP). However, some strictures are refractory and require frequent PS exchange to control symptoms. We describe two cases of successful endoscopic PD incision for difficult PD stricture using a wire-guided snare. The snare is partially opened within the strictured pancreatic duct while applying current, thus incising the duct. Although both cases were successful without complications we do not advocate that this method be used routinely because of the potential for severe complications, e.g. bleeding, ductal perforation or pancreatic parenchymal damage. In order to prevent these complications, we developed a wire-guided technique under fluoroscopic control. We think this procedure may be useful in patients with short, straight PD strictures. Although further study is required, this approach may have potential for selected patients with refractory PD strictures due to CP.

INTRODUCTION

Endoscopic therapy of pancreatic duct (PD) strictures using balloon dilation and pancreatic duct stent placement has been reported to improve the severity of abdominal pain in selected patients with chronic pancreatitis (CP)^[1-6]. However, some strictures are refractory and require frequent stent exchange to control symptoms. We describe two cases of successful endoscopic PD incisions for difficult PD strictures using a wire-guided snare. The snare is partially opened within the strictured pancreatic duct while applying electrical current, to incise the duct.

CASE REPORT

Case 1

A 35-year-old man with a 2-year history of acute chronic pancreatitis required repeated initial placement of a 7-Fr stent, followed by 10-Fr pancreatic duct stent exchange

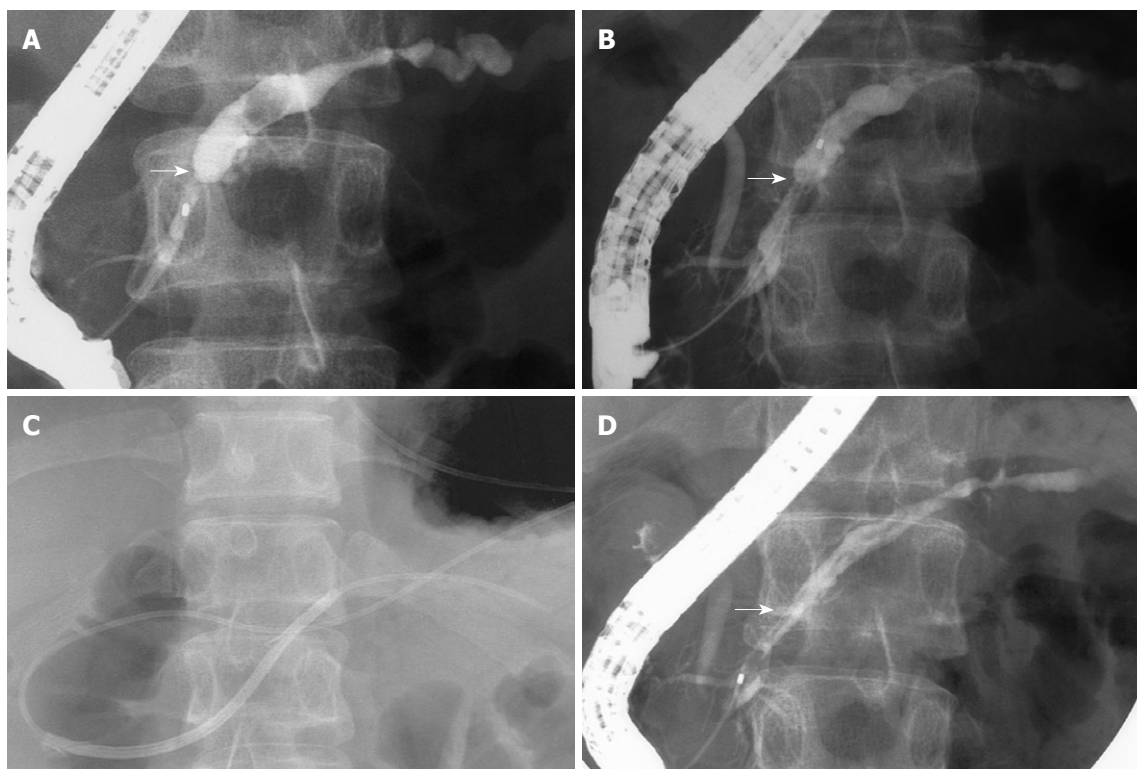


Figure 1 Pancreatic duct incision of a refractory pancreatic duct stricture. A: Pancreatography reveals a short pancreatic duct stricture (arrow) and upstream ductal dilation; B: Pancreatography after removal of a 10-Fr stent shows a stricture (arrow) still remaining; C: Wire-guided snare forceps are advanced into the pancreatic duct through the major papilla; D: Pancreatography reveals resolution of pancreatic duct stricture (arrow).

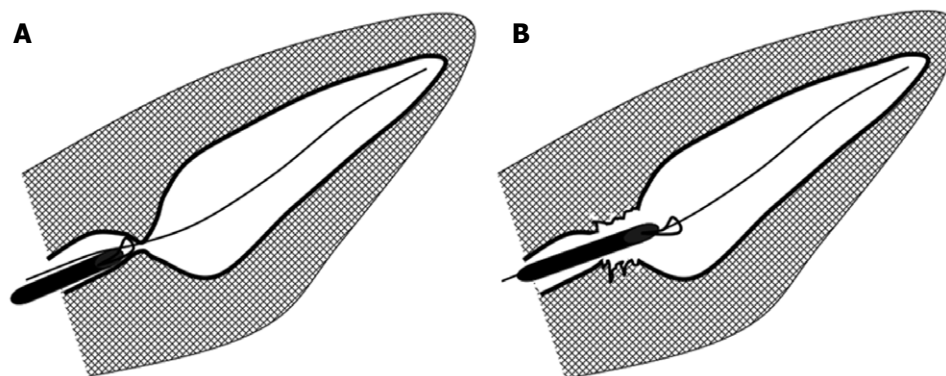


Figure 2 Diagram of pancreatic duct incision using snare forceps. A: Snare forceps are advanced into the pancreatic duct stricture; B: Pancreatic duct incision is performed with an electrocautery snare.

after a major papillotomy for management of a tight, short PD stricture (Figure 1A). Pancreatography after removal of the 10-Fr stent still showed a PD stricture (Figure 1B); the PD stent removal had been performed 6 mo previously at the patient's request. He was subsequently admitted with severe epigastric pain and recurrent pancreatitis. Pancreatography revealed obstruction of the PD and a 7-Fr stent was placed. We again recommended regular stent exchange or surgical therapy, but he refused. One month later, a transpapillary PD incision was performed in an attempt to produce stricture resolution without a stent. A guidewire was inserted across the stricture, and a standard snare (SD-5U-1, 6U-1, Olympus Medical Systems, Tokyo, Japan) was advanced over the

wire to the level of the stricture (Figure 1C). A PD incision was performed using an electrosurgical generator (ICC200; ERBE Elektromedizin GmbH, Tübingen, Germany) (Figure 2A and B). The patient experienced pain during the incision. A 10-Fr stent was placed without any serious complications such as bleeding or pancreatitis. Two weeks later the stent was removed (Figure 1D). The patient remains asymptomatic 3 years later.

Case 2

A 37-year-old woman had undergone regular replacement of 7-Fr and 10-Fr stents with a minor papillotomy across the minor papilla for management of a PD stricture (Figure 3A) due to pancreas divisum for the past

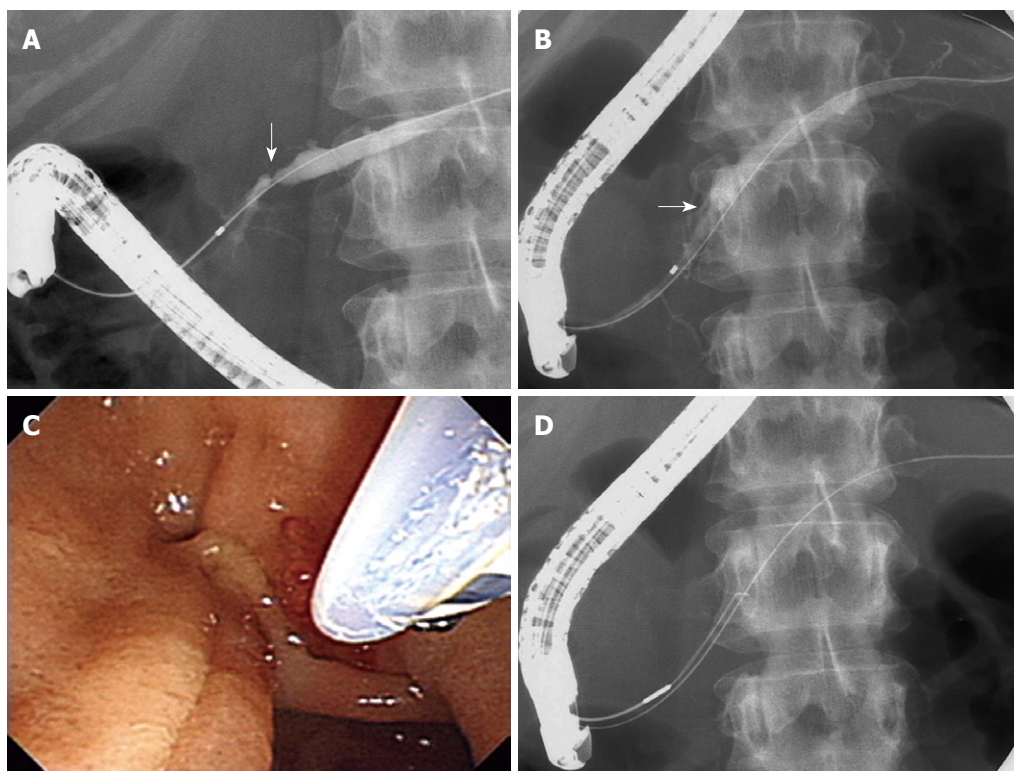


Figure 3 Pancreatic duct incision of a refractory pancreatic duct stricture. A: Pancreatography reveals a short pancreatic duct stricture (arrow) and upstream ductal dilation; B: Pancreatography after removal of a 10-Fr stent shows a stricture (arrow) still remaining; C: Pancreatic duct incision of the stricture using electrocautery snare; D: Wire-guided snare forceps advanced into the pancreatic duct through the major papilla.

3 years. Although pancreatography after removal of the 10-Fr stent still showed a PD stricture (Figure 3B), the stent was removed at the patient's request. However, 8 mo later, she developed severe epigastric pain due to recurrent pancreatitis. Since she refused regular stent exchange and surgical therapy, a PD incision was performed as described in the previous case. A 10-Fr stent was placed (Figure 3C and D). No complications occurred. The stent was removed one month later. She remains asymptomatic 18 mo later.

DISCUSSION

Placement of a pancreatic duct stent can often result in symptomatic improvement in patients with PD strictures caused by CP. However, long-term outcomes are not necessarily satisfactory. In patients with refractory PD strictures, regular, single or multiple stent replacements may be required for sustained symptom improvement^[4-6]. Such refractory strictures can be dilated using a Soehendra stent extractor^[7] or by self-expandable metal stent placement^[8]. Interestingly, Kawamoto *et al*^[9] reported the dissection of a difficult PD stricture using a needle-knife. Although the aim of their technique was recanalization of the pancreatic duct showing disconnection using a needle, our aim was persistent canalization without any stent.

Until now, endoscopic therapy using stents has been a well-established procedure. Nevertheless, several investigators have advocated that the technique of regular stent replacement is not recommended for CP with

circumscribed PD stenosis because outcomes of a surgical approach in these cases were superior to those of an endoscopic approach^[10-12].

In the present cases, the patients strongly refused surgical therapy. We therefore performed PD incision in anticipation that mechanical manipulation around the stricture might allow stricture resolution. We hypothesized that the snare could obtain a wider aperture compared to a sharp dissection using a needle knife. Although both cases were successful without complications we do not advocate that this method should be used routinely because of the potential for severe complications, e.g. bleeding, ductal perforation, or pancreatic parenchymal damage. In order to prevent these complications, we developed a wire-guided technique under fluoroscopic control. Furthermore, we advocate that the technique described here is rather experimental and should only be used in experienced hands due to the possibility of severe complications.

With regard to necessity and duration of a temporary stent placement after PD incision, this is a controversial issue because a relatively large PD incision can be expected. However, we used a stent to avoid acute obstructive pancreatitis due to edematous change for a period of 1 mo. We think this procedure may be useful in patients with circumscribed, straight PD strictures.

Limitations of this study include a small sample size, lack of a control group and no long-term outcomes.

In conclusion, although we do not think that this procedure can replace conventional endoscopic therapy

as the first-line treatment, this approach may have potential for selected patients with refractory circumscribed PD strictures due to CP.

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Early colon cancer within a diverticulum treated by magnifying chromoendoscopy and laparoscopy

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Abstract

We report a unique case of intramucosal carcinoma in a tubulovillous adenoma arising from a single diverticulum. Endoscopic mucosal resection (EMR) was carried out successfully and completely with the assistance of laparoscopy. A 71-year-old man was admitted to our hospital because of melena and anemia. Emergent colonoscopy showed diverticulosis in the right-sided colon. However, endoscopy could not exactly detect the bleeding site. A flat elevated polyp was found within a single diverticulum located in the descending colon and diagnosed as an intramucosal carcinoma, as magnifying chromoendoscopy revealed a type IV pit pattern. As his diverticular bleeding repeated, a right-sided hemicolectomy was decided for treatment, the polyp within the diverticulum was also completely removed by EMR with the assistance of laparoscopy. Although a colonic perforation was detected immediately after EMR, the perforation was closed with endoclips intraluminally and also repaired laparoscopically from the serosal side. Histologically, the resected lesion was an intramucosal well-differentiated adenocarcinoma and the surgical margin was free of tumor.

INTRODUCTION

Colonic neoplasia can arise from the normal mucosa near or within a diverticulum. Adenoma or adenocarcinoma located within a diverticulum is, however, a very rare phenomenon and only sporadic cases have been reported to date^[1-8]. The reported cases are often cases of advanced cancers and could only be diagnosed postoperatively. Therefore, the association of adenoma or early cancer with a diverticulum is exceptionally rare^[3,4,6-8]. We herein describe a case of intramucosal carcinoma in a tubulovillous adenoma developed in a colonic diverticulum, which could be correctly diagnosed by magnifying chromoendoscopy and was subsequently treated by endoscopic mucosal resection (EMR) with the assistance of laparoscopy.

CASE REPORT

A 71-year-old man was admitted to our hospital because of melena and anemia. Emergent colonoscopy showed diverticulosis in the right-sided colon. However, endoscopy could not definitely detect the bleeding source. Additionally, colonoscopy showed a single diverticulum in the descending colon, and a flat elevated polyp, 15 mm in

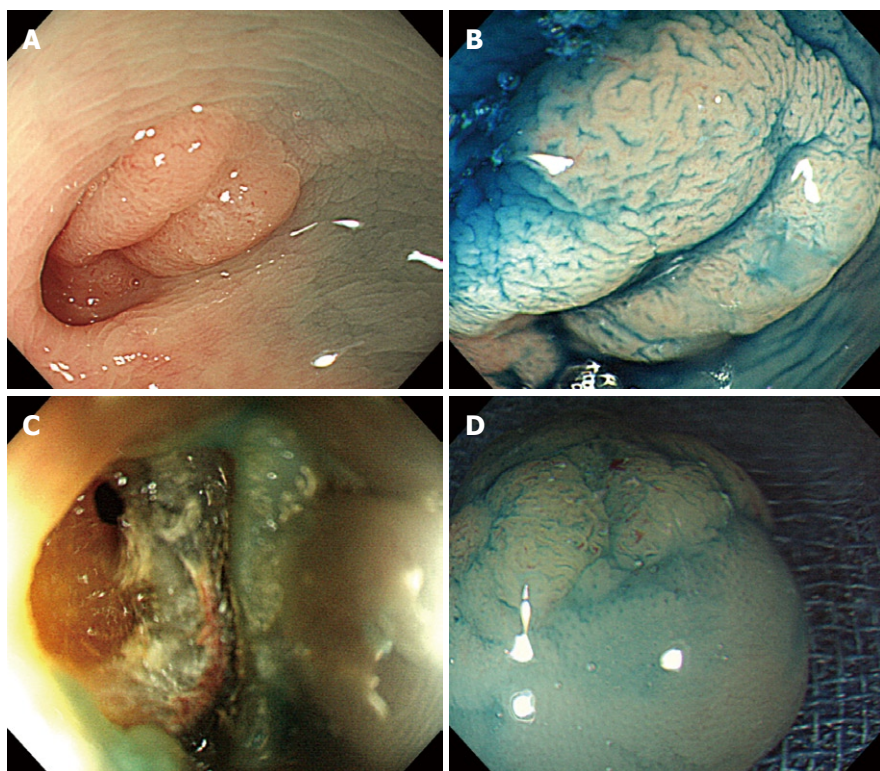


Figure 1 Colonoscopy and magnifying chromoendoscopy. A: Colonoscopy showing a single diverticulum in the descending colon and a flat elevated polyp (15 mm in diameter) within the diverticulum; B: Magnifying chromoendoscopy using 0.4% indigo-carmin dye spraying revealing a type IV pit pattern according to Kudo's classification; C: Endoscopy showing a pin-hole perforation immediately after endoscopic mucosal resection (EMR); D: Magnifying chromoendoscopy displaying negative neoplastic changes in the removed lesion.

diameter, within the diverticulum (Figure 1A). Magnifying chromoendoscopy using 0.4% indigo-carmin dye spraying revealed a type IV pit pattern according to Kudo's classification (non-invasive pattern according to Fujii's classification)^[9,10] (Figure 1B). Therefore, an endoscopic diagnosis of intramucosal carcinoma was established, and endoscopic resection was thus considered as the first line treatment. As his diverticular bleeding repeated, which could not be managed endoscopically, multiple blood transfusions were thus needed. Right hemicolectomy was decided for treatment. During laparoscopically assisted right hemicolectomy, EMR was also performed to remove the polyp within the diverticulum located in the descending colon. Glycerol was first injected into the submucosal layer to lift the polyp well (non-lifting sign negative), and subsequently EMR was successfully carried out. After EMR, a pin-hole perforation was detected endoscopically and laparoscopically (Figure 1C). Metal clips were used endoscopically to close the perforation which was also repaired laparoscopically. Magnifying chromoendoscopy of the removed lesion disclosed no neoplastic lesions at the surgical lateral margin (Figure 1D). The patient developed anal bleeding 6 d after surgery, and an urgent colonoscopy revealed bleeding at the anastomotic site of right hemicolectomy but not at the EMR site, and the bleeding was successfully stopped with metal clips. Histologically, the removed polyp was an intramucosal well-differentiated adenocarcinoma in a tubulovillous adenoma. No adenoma and adenocarcinoma were found

at the lateral and vertical margin of the removed lesion. No muscle layer was identified in the removed lesions on histology.

DISCUSSION

Cancer arising from diverticula may be diagnosed at the advanced stage despite its small size. As colonic diverticula lack of muscular coats, and thus cancer can more easily extend into the serosal surface than that arising from the normal mucosa apart from the diverticulum^[7]. We performed EMR to remove this lesion, as magnifying chromoendoscopy provided an endoscopic diagnosis of an intramucosal lesion. It is commonly accepted that colorectal cancers limited within the mucosal layer do not carry risks of lymph node metastasis and therefore are good candidates for endoscopic resection. Therefore, to avoid unnecessary surgery or endoscopic treatment, it is important to endoscopically evaluate the invasion depth of colorectal cancers before removal. The mucosal crypt patterns have been reported to be closely related to the histology and are helpful in endoscopic diagnosis by magnifying chromoendoscopy^[9,10].

Histologically, diverticula have a characteristically thin wall without muscular layer. Surgical treatment is, therefore, recommended even for mucosal cancer or adenomatous polyp near or within a diverticulum, as colonic perforation may easily occur after endoscopic resection for such lesions. As anticipated, our case developed

a colonic perforation immediately after EMR. Iatrogenic perforation is one of the most serious potential complications of colonoscopy^[11]. Treatment strategies for colonic perforation include non-operative management with or without endoscopic closure, laparoscopic repair, and open surgery. EMR was performed for this case in our department with the assistance of laparoscopy to treat the diverticular bleeding in the right-sided colon^[11,12]. Therefore, the perforation site of this case was first closed with endoclips intraluminally and thereafter repaired laparoscopically from the serosal side. In our opinion, this kind of pin-hole perforation can be managed only endoscopically with endoclips, and we would have tried EMR without laparoscopic assistance if there was no uncontrolled diverticular bleeding.

In conclusion, intramucosal cancer in a tubulovillous adenoma developed within a diverticulum can be correctly diagnosed by magnifying chromoendoscopy and successfully treated with minimally invasive surgery including EMR with the assistance of laparoscopy.

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Klippel-Trenaunay syndrome with gastrointestinal bleeding, splenic hemangiomas and left inferior vena cava

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Abstract

Klippel-Trenaunay syndrome is a congenital vascular anomaly characterized by a triad of varicose veins, cutaneous capillary malformation, and hypertrophy of bone and (or) soft tissue. Gastrointestinal vascular malformations in Klippel-Trenaunay syndrome may present with gastrointestinal bleeding. The majority of patients with splenic hemangiomatosis and/or left inferior vena cava are asymptomatic. We herein report a case admitted to the gastroenterology clinic with life-threatening hematochezia and symptomatic iron deficiency anemia. Due to the asymptomatic mild intermittent hematochezia, splenic hemangiomas and left inferior vena cava, the patient did not seek any help for gastrointestinal bleeding until his admittance to our department for evaluation of massive gastrointestinal bleeding. He was referred to angiography because of his serious pathogenetic condition and inefficiency of medical therapy. The method showed that hemostasis was successfully achieved in the hemorrhage site by embolism of corresponding vessels. Further endoscopy revealed vascular malformations starting from the stomach to the descending colon. On the other hand, computed tomography revealed splenic hemangiomas and left inferior vena cava. To the

best of our knowledge, this is the first Klippel-Trenaunay syndrome case presenting with gastrointestinal bleeding, splenic hemangiomas and left inferior vena cava. The literature on the evaluation and management of this case is reviewed.

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Key words: Klippel-Trenaunay syndrome; Gastrointestinal bleeding; Splenic hemangiomas; Left inferior vena cava

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INTRODUCTION

Klippel-Trenaunay syndrome (KTS) is a rare congenital disorder of the vascular system and is characterized by the following triad of clinical signs. (1) Haemangiomas due to cutaneous capillary dysplasias; (2) Soft tissue and/or bone hypertrophy; and (3) Venous and lymphatic anomalies^[1]. At least two signs are present to establish the diagnosis^[2,3].

Although seemingly uncommon, vascular malformations involving the gastrointestinal (GI) tract have been reported and can be a source of significant morbidity and even mortality^[1]. Clinical manifestations range from occult to massive, life-threatening hemorrhage. KTS patients with clinically significant hemorrhage usually require resection of the involved bowel segment^[1]. Visceral

vascular malformations in KTS have been described involving organs such as the GI tract, liver, spleen, bladder, kidney, lung and heart^[4]. Computed tomography (CT) of the abdomen and pelvis provides a simple, noninvasive means of assessing visceral hemangiomatous masses^[5].

The left inferior vena cava (IVC) in KTS has not been reported. The left inferior vena cava does not affect the blood circulation, so these patients have no symptoms. They are accidentally discovered by abdominal CT imaging.

We herein report the clinical presentation and management of a young male patient with the diagnosis of KTS.

CASE REPORT

A 19-year-old male patient was admitted to the Gastroenterology Clinic for management of life-threatening hematochezia and symptomatic iron deficiency anemia. When he was born, there was a few vascular malformations in his body and right leg. The lesion on body surface was enlarged along with his age. As the lesion limits the right leg, exeresis was performed. The pathological section revealed well-marked capillary and arteriolar proliferation in the epidermal layer. The patient had been frequently admitted to the emergency unit due to transfusion-dependent anemia caused by mild intermittent hematochezia since July 2003. But he did not seek any help for gastrointestinal bleeding. Afterwards, the mild intermittent hematochezia continued and the amount of gastrointestinal bleeding increased over time. He was admitted to our hospital for evaluation after a severe and debilitating attack of hematochezia.

Physical examination revealed that his skin was pale, and there were several vascular malformation lesions on the surface of his body (Figure 1A) and a giant scar at the lateral right knee joint, and the right leg was obviously thicker than the left (Figure 1B). Laboratory examination showed significant anemia with hemoglobin of 2.2 g/dL, hematocrit 8.9%, iron 2.8 $\mu\text{mol/L}$, and total iron binding capacity 98 $\mu\text{mol/L}$. The platelet count and coagulation parameters were normal.

We could not obtain hemostasis by medical treatment. During hospitalization, the patient had an attack of massive hematochezia with loss of consciousness. Due to the severe symptomatic anemia and increased amount of GI bleeding over time, interventional examination and therapy were considered. The digital subtraction angiography revealed the bleeding point was caused by vascular malformation at terminal ileum. Superselective vessel embolism was performed (Figure 2). The patient had never hemorrhaged since the treatment. Further endoscopic investigation was conducted (Figure 3). Gastrosocopy revealed several vascular malformation lesions at gastric antrum and gastric corpus. Colonoscopy revealed that there were several vascular malformation lesions from terminal ileum to the descending colon. Several vascular malformation lesions



Figure 1 Cutaneous vascular malformations and abnormal right leg in the patient. A: Cutaneous vascular malformations of the gluteal region and body; B: The enlarged right leg.

were found distributed from jejunum to ileum on the capsule endoscopy. Abdominal CT imaging showed left IVC and multiple hemangiomas in the spleen (Figure 4). Based on the vascular malformations in GI, splenic hemangiomas, cutaneous vascular malformation and the enlarged right leg, the patient was diagnosed as having KTS.

DISCUSSION

Klippel-Trenaunay syndrome is a term used to describe the combination of a cutaneous capillary malformation, varicose veins, and hypertrophy of bone and/or soft tissue. Most cases of KTS are sporadic, the syndrome affects males and females equally, has no racial predilection, and manifests at birth or during childhood^[4].

Involvement of the GI tract may be more common in KTS than previously believed (occurring in perhaps as many as 20% of patients) and may be unrecognized in patients without symptoms^[6]. The most common bleeding sites in the GI system are the distal colon and rectum. Jejunal vascular malformation and esophageal varices as bleeding sources caused by prehepatic portal hypertension were reported in the literature^[1]. It is rare that the vascular malformation distributes in the whole GI. The spectrum of the GI bleeding may vary from asymptomatic occult bleeding to life-threatening massive bleeding. GI hemorrhage usually begins in the first decade of life and tends to be intermittent. Investigation of GI bleed-

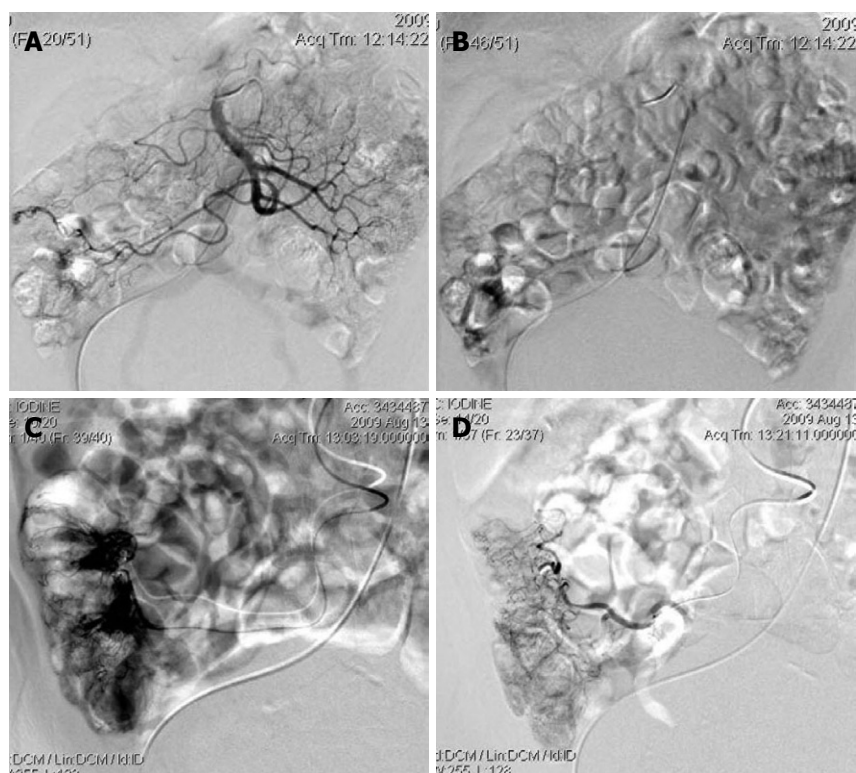


Figure 2 Angiograms demonstrate contrast opacification of the superior mesenteric artery and its branches. A: Some abnormal small veins that were filled earlier in the arterial phase; B: Manipulus contrast extravasation into the terminal ileum; C: Abnormal ectatic slow-emptying veins and extravasation of contrast material after superselective catheterization; D: No active bleeding after superselective vessel embolism with gelfoam.

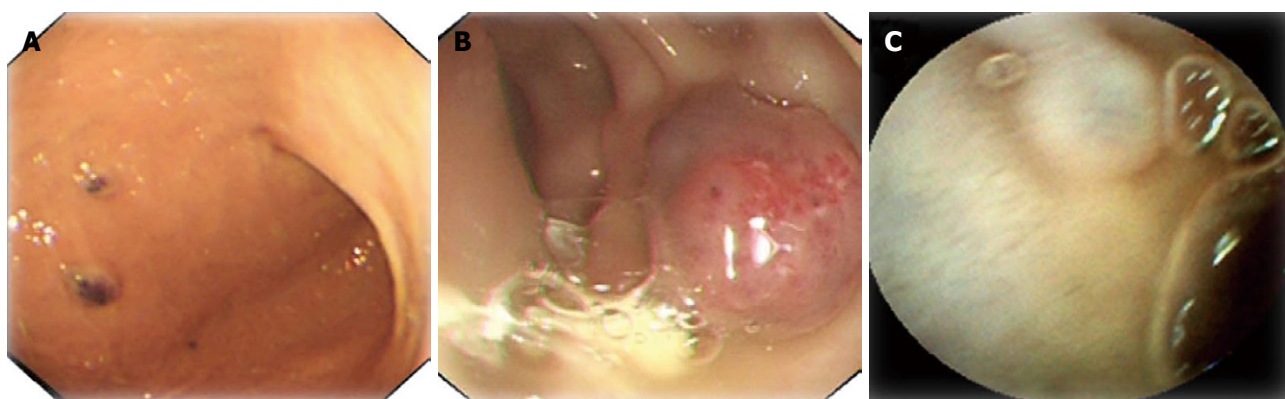


Figure 3 Lesions of gastrointestinal tract on endoscopy. A: Gastroscopy shows multiple polypoid mucosal nodules with abundant vasculature which had a purplish red chrysanthemum-like surface, which are centrally located at the greater curvature of the gastric antrum and gastric corpus; B: Colonoscopy shows several polypoid mass lesions with abundant vasculature, which are centrally located from the terminal ileum to the descending colon; C: Capsule endoscopy revealing several vascular malformation lesions distributing over the jejunum and ileum.

ing should begin with endoscopic examination in a patient with suspected KTS. Although endoscopy has the advantage of showing the localization and extension of vascular malformations, it might be misleading to accuse the vascular malformations observed during the endoscopy for bleeding due to possible metachromic localizations of vascular malformations in the different parts of the GI tract. With this point of view, endoscopic investigation of the entire GI tract should be made as the routine clinical practice for the exact localization and effective management of GI bleeding in patients with KTS. With the unawareness of this rare diagnosis, biopsies over the vascular malformations may be taken, which might be fatal in a patient with hematochezia^[7]. On the other hand, the patient could not tolerate the endoscopy during the

onset of serious hematochezia. So the interventional examination and therapy are ideal method at this time. First, advantages of angiography include the lack of requirement for bowel preparation. Secondly, angiography remains the gold standard for the diagnosis of vascular malformation. Following injection of contrast, vascular malformation can be recognized by ectatic slow-emptying veins, vascular tufts, or small veins that were filled earlier. Angiography is able to localize the bleeding source (when one is identified). Third, there is possibility of therapeutic intervention in some cases. Haemostasis can be achieved by intra-arterial infusion of vasopressin or arterial embolization *via* the angiographic catheter^[8].

Hemangioma is the most common benign primary tumor of the spleen. Splenic hemangiomas may occur

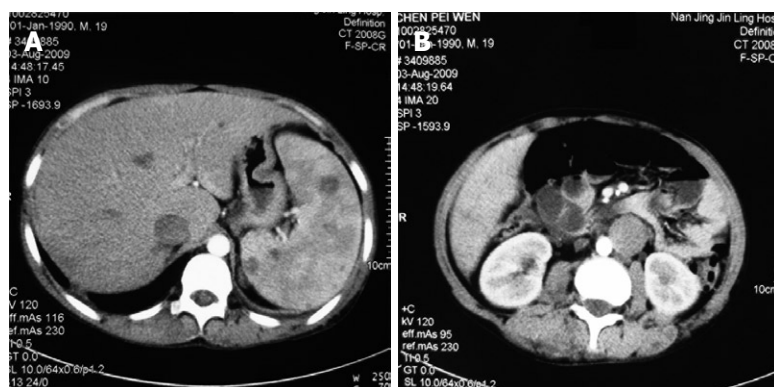


Figure 4 Contrast-enhanced abdominal computed tomography (CT) showing splenic hemangiomas (A) and left inferior vena cava (IVC) (B).

in a part of generalized angiomatosis as KTS. Complications of the splenic hemangiomas include rupture, hypersplenism, and malignant degeneration. The exact course for a given hemangioma is difficult to predict. Larger tumors (> 4 cm) are likely to be more prone to rupture than smaller ones, either spontaneously or from minor trauma, and may result in fatal hemorrhage. Spontaneous rupture has been reported as the most common complication, occurring in 25% of patients having large (> 4 cm) hemangiomas^[9]. Recent reviews in adult patients reported that asymptomatic patients with small splenic hemangioma (< 4 cm) have been managed conservatively with observation, and no rupture or other complications occurred^[10]. Kasabach-Merritt syndrome has been reported in patients with large hemangiomas^[11]. As our patient was asymptomatic with splenic hemangiomas < 4 cm, a conservative approach with observation was preferred and splenectomy was not performed.

A left IVC results from regression of the right supracardinal vein with persistence of the left supracardinal vein. The prevalence is 0.2%-0.5%^[12]. Typically, the left IVC joins the left renal vein, which crosses anteriorly to the aorta in the normal fashion, uniting with the right renal vein to form a normal right-sided prerenal IVC. The major clinical significance of this anomaly is the potential for misdiagnosis as left-sided para-aortic adenopathy^[13]. In addition, spontaneous rupture of an abdominal aortic aneurysm into a left IVC has been reported^[14]. The presence of vascular and renal anatomical anomalies may induce technical problems during abdominal aortic surgery^[15-17] and may give rise to serious intraoperative complications. Therefore, prior to aortic surgery, preoperative knowledge of the presence of such anomalies helps with operative planning and may reduce the associated risk of major venous hemorrhage^[15]. Abdominal CT is the most accurate investigation to discover such anomalies^[16].

The spectrum of clinical manifestations of KTS is wide and can include additional arterial and lymphatic system abnormalities beyond the classical manifestation^[18]. The potential of KTS to have widespread venous malformations in any part of the body implies that heterogeneous genetic mutations affecting mesodermal development may be present in patients with KTS^[19]. At present, molecular diagnosis for KTS is not available. In conclusion, KTS is a rare condition with protean manifestations. Hematochezia

should alert the physicians about the possibility of associated vascular malformations in the GI systems. Due to the progressive nature and wide extension of KTS lesions, endoscopic therapies have limited value in the management. Angiographic interventions should be used for visualizing the vascular anatomy and determining the disease extent. It is efficient to embolize the bleeding spots based on the examinations.

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Gastric cardia adenocarcinoma in Taiwanese men: Positive associations due to selection bias

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Abstract

The factors associated with an increase in gastric cardia adenocarcinoma are poorly understood. Environmental factors such as *Helicobacter pylori* (*H. pylori*) infection and diet have been hypothesized to play a role in the recently increased risk of this disease, but additional studies are needed. In conducting studies to establish the relationship between potential risk factors and gastric cardia adenocarcinoma, it is necessary to carefully consider the role of bias. In a recently published study, the reported associations between *H. pylori* as well as post-meal physical exertion and gastric cardia adenocarcinoma may have been greatly influenced by selection bias.

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Key words: *Helicobacter pylori*; Gastric cardia adenocarcinoma; Gastric cancer; Selection bias

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TO THE EDITOR

The positive association between *Helicobacter pylori* (*H. pylori*) infection and development of non-cardia gastric cancers is well established^[1,2]. Although various observational studies have been conducted to elucidate the roles of *H. pylori* and other environmental factors in the development of cancer in the gastric cardia^[3], these roles are still not well defined and therefore additional studies are needed.

A recent hospital based case-control study conducted by Chen *et al*^[4] attempted to address these roles, reporting a positive association between gastric cardia adenocarcinoma and *H. pylori* infection. Specifically, the study found that men with gastric cardia adenocarcinoma are almost three times more likely to have antibodies against *H. pylori* and also three times more likely to have conducted work or exercise after meals^[4]. While the authors did recognize that their conclusion may have been distorted by differential misclassification due to recall bias, the reported associations could also be explained by selection bias called exclusion bias.

Controls were cancer-free, inpatients from family medicine departments including inpatients reporting to the hospital for a general check-up^[4]. Furthermore, controls with "stomach-related" diseases were excluded from participating in the study^[4]. In order to prevent selection bias, controls should be selected so that they have a distribution of exposures similar to the population from which

cases arise^[5]. Rothman *et al*^[5] indicate that the exclusion of controls presenting with illness relating to and after the exposure “reduces the prevalence of the exposure in the controls ... hence biases the effect estimates upward”. Although the authors did not specify which stomach-related diseases were excluded, *H. pylori* infection has been identified as a major risk factor in the development of many stomach-related issues such as chronic gastritis, peptic ulcer disease and gastric adenocarcinoma^[6]. Furthermore, the development of precancerous lesions is preceded by a variety of stomach-related symptoms and factors including *H. pylori* infection possibly 20 years prior to the event^[1]. Hence, if control subjects with any of the aforementioned stomach-related diseases were excluded from the present study, fewer controls with antibodies against *H. pylori* would be identified compared to the study cases (men with gastric cardia adenocarcinoma). By excluding control subjects with stomach-related diseases, the authors inadvertently created an under representation of *H. pylori* in the control series compared to the population from which cases arose, which could explain the observed positive association between *H. pylori* infection and gastric cardia adenocarcinoma^[5].

Case-control studies have the potential to be internally valid and provide an efficient and cost effective means to estimate exposure-outcome associations, especially

for rare outcomes such as those in the present study. Of utmost importance in this and all studies, utilizing the case-control design, however, is the requirement to carefully select control subjects to avoid distorted estimates of effect for which post-study statistical analyses cannot ameliorate.

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Meetings

Events Calendar 2010

January 25-26
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International Conference on Medical
Negligence and Litigation in Medical
Practice

January 25-29
Waikoloa, HI, United States
Selected Topics in Internal Medicine

January 26-27
Dubai, United Arab Emirates
2nd Middle East Gastroenterology
Conference

January 28-30
Hong Kong, China
The 1st International Congress on
Abdominal Obesity

February 11-13
Fort Lauderdale, FL, United States
21th Annual International Colorectal
Disease Symposium

February 26-28
Carolina, United States
First Symposium of GI Oncology at
The Caribbean

March 04-06
Bethesda, MD, United States
8th International Symposium on
Targeted Anticancer Therapies

March 05-07
Peshawar, Pakistan
26th Pakistan Society of
Gastroenterology & Endoscopy
Meeting

March 09-12
Brussels, Belgium
30th International Symposium on
Intensive Care and Emergency
Medicine

March 12-14
Bhubaneswar, India
18th Annual Meeting of Indian
National Association for Study of
the Liver

March 23-26
Cairo, Egypt
14th Pan Arab Conference on
Diabetes PACD14

March 25-28
Beijing, China
The 20th Conference of the Asian

Pacific Association for the Study of
the Liver

March 27-28
San Diego, California, United States
25th Annual New Treatments in
Chronic Liver Disease

April 07-09
Dubai, United Arab Emirates
The 6th Emirates Gastroenterology
and Hepatology Conference, EGHG
2010

April 14-17
Landover, Maryland, United States
12th World Congress of Endoscopic
Surgery

April 14-18
Vienna, Austria
The International Liver Congress™
2010

April 28-May 01
Dubrovnik, Croatia
3rd Central European Congress
of surgery and the 5th Croatian
Congress of Surgery

May 01-05
New Orleans, LA, United States
Digestive Disease Week Annual
Meeting

May 06-08
Munich, Germany
The Power of Programming:
International Conference on
Developmental Origins of Health
and Disease

May 15-19
Minneapolis, MN, United States
American Society of Colon and
Rectal Surgeons Annual Meeting

June 04-06
Chicago, IL, United States
American Society of Clinical
Oncologists Annual Meeting

June 09-12
Singapore, Singapore
13th International Conference on
Emergency Medicine

June 14
Kosice, Slovakia
Gastro-intestinal Models in
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June 16-19
Hong Kong, China
ILTS: International Liver
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June 20-23
Mannheim, Germany
16th World Congress for
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Orlando, FL, United States
70th ADA Diabetes Scientific
Sessions

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Boston, Massachusetts, United States
10th OESO World Congress on
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September 10-12
Montreal, Canada
International Liver Association's
Fourth Annual Conference

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La Jolla, CA, United States
New Advances in Inflammatory
Bowel Disease

September 12-15
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Prague Hepatology Meeting 2010

September 23-26
Prague, Czech Republic
The 1st World Congress on
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October 07-09
Belgrade, Serbia
The 7th Biannual International
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October 15-20
San Antonio, TX, United States
ACG 2010: American College of
Gastroenterology Annual Scientific
Meeting

October 23-27
Barcelona, Spain
18th United European
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October 29-November 02
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Case-Based Approach to the
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Chinese journal article (list all authors and include the PMID where applicable)

- 2 **Lin GZ**, Wang XZ, Wang P, Lin J, Yang FD. Immunologic effect of Jianpi Yishen decoction in treatment of Pixu-diarrhoea. *Shijie Huaren Xiaohua Zazhi* 1999; **7**: 285-287

In press

- 3 **Tian D**, Araki H, Stahl E, Bergelson J, Kreitman M. Signature of balancing selection in Arabidopsis. *Proc Natl Acad Sci USA* 2006; In press

Organization as author

- 4 **Diabetes Prevention Program Research Group**. Hypertension, insulin, and proinsulin in participants with impaired glucose tolerance. *Hypertension* 2002; **40**: 679-686 [PMID: 12411462 PMID:2516377 DOI:10.1161/01.HYP.0000035706.28494.09]

Both personal authors and an organization as author

- 5 **Vallancien G**, Emberton M, Harving N, van Moorselaar RJ; Alf-One Study Group. Sexual dysfunction in 1, 274 European men suffering from lower urinary tract symptoms. *J Urol* 2003; **169**: 2257-2261 [PMID: 12771764 DOI:10.1097/01.ju.0000067940.76090.73]

No author given

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

Volume with supplement

- 7 **Geraud G**, Spierings EL, Keywood C. Tolerability and safety of frovatriptan with short- and long-term use for treatment of migraine and in comparison with sumatriptan. *Headache* 2002; **42** Suppl 2: S93-99 [PMID: 12028325 DOI:10.1046/j.1526-4610.42.s2.7.x]

Issue with no volume

- 8 **Banit DM**, Kaufer H, Hartford JM. Intraoperative frozen section analysis in revision total joint arthroplasty. *Clin Orthop Relat Res* 2002; **(401)**: 230-238 [PMID: 12151900 DOI:10.1097/00003086-200208000-00026]

Instructions to authors

No volume or issue

- 9 Outreach: Bringing HIV-positive individuals into care. *HRSA Careaction* 2002; 1-6 [PMID: 12154804]

Books

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- 10 **Sherlock S**, Dooley J. Diseases of the liver and biliary system. 9th ed. Oxford: Blackwell Sci Pub, 1993: 258-296

Chapter in a book (list all authors)

- 11 **Lam SK**. Academic investigator's perspectives of medical treatment for peptic ulcer. In: Swabb EA, Azabo S. Ulcer disease: investigation and basis for therapy. New York: Marcel Dekker, 1991: 431-450

Author(s) and editor(s)

- 12 **Breedlove GK**, Schorfheide AM. Adolescent pregnancy. 2nd ed. Wiczorek RR, editor. White Plains (NY): March of Dimes Education Services, 2001: 20-34

Conference proceedings

- 13 **Harnden P**, Joffe JK, Jones WG, editors. Germ cell tumours V. Proceedings of the 5th Germ cell tumours Conference; 2001 Sep 13-15; Leeds, UK. New York: Springer, 2002: 30-56

Conference paper

- 14 **Christensen S**, Oppacher F. An analysis of Koza's computational effort statistic for genetic programming. In: Foster JA, Lutton E, Miller J, Ryan C, Tettamanzi AG, editors. Genetic programming. EuroGP 2002: Proceedings of the 5th European Conference on Genetic Programming; 2002 Apr 3-5; Kinsdale, Ireland. Berlin: Springer, 2002: 182-191

Electronic journal (list all authors)

- 15 Morse SS. Factors in the emergence of infectious diseases. Emerg Infect Dis serial online, 1995-01-03, cited 1996-06-05; 1(1): 24 screens. Available from: URL: <http://www.cdc.gov/ncidod/EID/eid.htm>

Patent (list all authors)

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

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Write as mean \pm SD or mean \pm SE.

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Ezetimibe as potential treatment for cholesterol gallstones: The need for clinical trials

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Abstract

Obesity is associated with insulin resistance, non-alcoholic fatty liver disease (NAFLD) and gallstones. High fat diets (unsaturated fats) rich in cholesterol have been demonstrated to produce not only gallstones but also NAFLD and insulin resistance. Interestingly, a high incidence of gallstones is being reported in association with insulin resistance and NAFLD. Laparoscopic cholecystectomy is the best definitive therapy for symptomatic gallbladder disease. Ezetimibe is a drug that inhibits the absorption of both dietary and biliary cholesterol in the small intestine. Importantly, ezetimibe showed potential benefit not only in treating and preventing gallstones but also in insulin resistance and NAFLD. Further studies are required before the use of ezetimibe for the treatment of gallstones can be advocated.

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Key words: Ezetimibe; Gallstones; Insulin resistance; Non-alcoholic fatty liver disease

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INTRODUCTION

Gallstones are a common problem and is likely to have existed since the appearance of mankind on Earth. Interestingly, gallstones have been identified in autopsy studies of Egyptian mummies^[1]. Gallstones and associated complications cost approximately 6.5 billion dollars annually in the USA. The incidence of gallstones is more frequent in females than males and increase with age. The role of ethnicity and genetics is known in gallstone formation. Importantly, the distribution of gallstones in different populations appears to be related to high dietary intake of cholesterol and fats (Western diets). Furthermore, gallstones are primarily cholesterol or black pigment stones (bilirubin polymers) or brown pigment stones (calcium bilirubinate). The only established dietary risk is a high caloric intake. Other modifiable risk factors for gallstones are obesity, the metabolic syndrome, rapid weight loss, certain diseases (cirrhosis, Crohn's disease) and gallbladder stasis (from spinal cord injury or drugs such as somatostatin). Therefore, it is anticipated that diets containing fibre, vegetable protein, nuts, calcium, vitamin C, coffee, plus physical activity are all regarded as protective factors. In developed countries, cholesterol gallstones predominate; 15% are black pigment stones. It is thought that gallstones affect around 15% of white adults in developed countries. East Asians develop brown pigment stones in bile ducts, associated with biliary infection or parasites, or in intrahepatic ducts (hepatolithiasis). In view of the high prevalence of obesity and metabolic

syndrome, the burden of disease is epidemic in American Indians at around 60%-70%. The frequency of gallstones is reduced in Black Americans, Hispanics of mixed Indian origin, East Asians and sub-Saharan Africans^[2,3]. Most asymptomatic gallstone carriers require no therapy. Laparoscopic cholecystectomy is the best definitive therapy for symptomatic gallstone disease. Primary prevention is unproven but focuses on early identification and risk alteration to decrease the possibility of developing gallstones. Ursodeoxycholic acid has a limited role in stone dissolution but can prevent stone development in severe obesity during rapid weight reduction with diet therapy or after bariatric surgery^[4].

EZETIMIBE AND CHOLESTEROL GALLSTONES

Recently, it was shown both in animal models and humans that ezetimibe may prevent and treat cholesterol gallstones^[5]. Ezetimibe inhibits intestinal uptake of cholesterol with a half life of approximately of 22 h. The major metabolic pathway for ezetimibe consists of glucuronidation of the 4-hydroxyphenyl group by uridine 5'-diphosphate-glucuronosyltransferase isoenzymes to form ezetimibe-glucuronide in the intestine and liver. Approximately 78% of the dose is excreted in the faeces predominantly as ezetimibe, with the balance found in the urine mainly as ezetimibe-glucuronide^[6]. Niemann-Pick C1 like1 (NPC1L1), highly expressed in the jejunum of different species and only in human liver, is the main transporter of intestinal cholesterol. Mice deficient in NPC1L1 showed a significant > 70% reduction in cholesterol absorption, and a further reduction in cholesterol level with ezetimibe administration was not achievable. It was concluded that ezetimibe reduces intestinal absorption by inhibiting the action of NPC1L1. Ezetimibe significantly reduces low-density lipoprotein (LDL) cholesterol and is used as monotherapy or in combination with statins to treat hyperlipidaemia^[7].

Wang *et al*^[5] showed that ezetimibe treatment for 8 to 12 wk in male gallstone-susceptible C57L mice and in 7 patients (treated with 20 mg ezetimibe for 30 d) resulted in a decrease in intestinal cholesterol absorption and biliary cholesterol secretion. Ezetimibe also protected gallbladder motility function by desaturating bile. Furthermore, ezetimibe treatment promoted the dissolution of gallstones by forming an abundance of unsaturated micelles and reduced biliary cholesterol saturation. However, Tamel *et al*^[8] found that mice transgenic for *NPC1L1* gene, displayed an increase in biliary cholesterol concentration, suggesting that ezetimibe treatment may reduce biliary cholesterol secretion and increase the cholesterol saturation index. This is not in agreement with previous studies which showed the potential benefit of ezetimibe in treating gallstones, and may lead to speculation that it is actually intestinal cholesterol absorption which is largely responsible for the formation of cholesterol gallstones.

Furthermore, administration of ezetimibe in Golden Syrian hamsters fed a diet high in cholesterol and sun-

flower oil resulted in a significant reduction in absolute and relative cholesterol levels in bile^[9]. Ezetimibe treatment in C57BL female mice, prevented biliary crystals and normalized gallbladder wall fat and function^[10]. Importantly, gallstone-susceptible C57BL/6 inbred mice were fed control and lithogenic diets with or without simultaneous ezetimibe administration. Lithogenic diets increased biliary cholesterol content and secretion, and induced sludge or gallstone formation in 100% of the animals. Ezetimibe administration reduced intestinal cholesterol absorption by 90% in control animals and by 35% in mice receiving the lithogenic diets. Ezetimibe prevented the appearance of cholesterol crystals and gallstones. In addition, mice fed the lithogenic diets plus ezetimibe exhibited a 60% reduction in the biliary cholesterol saturation index. Of note, ezetimibe treatment caused a significant increase in bile flow (+50%, $P < 0.01$) as well as bile salt, phospholipid and glutathione secretion rates (+60%, +44% and +100%, respectively, $P < 0.01$), which was associated with a moderately increased expression of hepatic bile salt transporters^[11]. From the above discussion it is possible to suggest that ezetimibe acts by decreasing intestinal cholesterol absorption and biliary cholesterol secretion, preserving gallbladder motility function by de-saturating bile in mice, promoting the dissolution of gallstones by forming an abundance of unsaturated micelles and significantly reducing biliary cholesterol saturation and retarding cholesterol crystallization in the bile of patients with gallstones.

Another important precipitating factor for gallstones and a therapeutic target for ezetimibe is insulin resistance. Interestingly, Chang *et al*^[12] showed in 19 503 Korean men, that the prevalence of obesity, abdominal obesity, and metabolic syndromes in the subjects with gallstones were higher than in those without gallstones. The prevalence of elevated homeostatic model assessment (HOMA) (> 75 percentile) in subjects with gallstones was significantly higher than in those without gallstones, and this association remained even after the obesity stratification was applied. In multiple logistic regression analyses, only age and HOMA proved to be independent predictors of gallstones. Insulin resistance was positively associated with gallstones in non-diabetic Korean men, and this occurred regardless of obesity. Importantly, gallstones appear to be a marker for insulin resistance, even in non-diabetic, non-obese men. Furthermore, hepatic insulin resistance directly promotes the formation of cholesterol gallstones in mice^[13]. Nakeeb *et al*^[14] showed that in lean, non-diabetic volunteers without gallstones, gallbladder dysmotility is associated with an elevated fasting glucose as well as a high index of insulin resistance, and their conclusion was that insulin resistance alone may be responsible for gallbladder dysmotility which may result in acalculous cholecystitis or gallstone formation. Insulin resistance is also associated with non-alcoholic fatty liver disease (NAFLD)^[15]. Loria *et al*^[16] showed a higher prevalence of gallstones in association with NAFLD compared with a normal population. In liver biopsy screening for NAFLD, it was documented that 55% of subjects have gallstones^[17]. Recent studies showed the potential benefit of ezetimibe as treat-

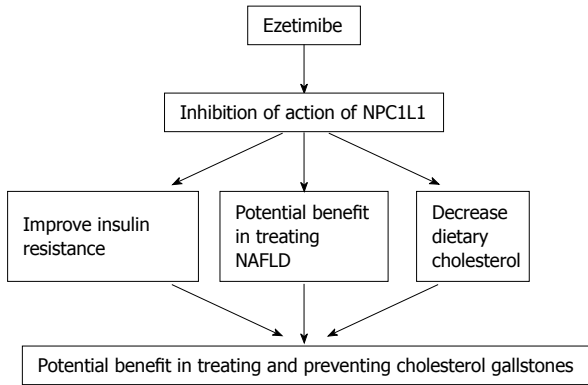


Figure 1 Mechanism of action of ezetimibe and ezetimibe may not only be of potential benefit in the treatment of cholesterol gallstones but also insulin resistance and NAFLD.

ment for NAFLD and associated hyperlipidaemia and insulin resistance. Interestingly, Zheng *et al*^[18] showed that ezetimibe treatment for 4 wk reduced alanine transaminase (ALT), hepatic triglyceride, hepatomegaly, cholesterol ester and free cholesterol in diet-induced obese mice fed a high fat/cholesterol diet for 7 mo with proof of NAFLD. Importantly, administration of ezetimibe in obese Zucker rats (a model of NAFLD and metabolic syndrome) resulted in a significant improvement in both cholesterol and triglyceride levels, hepatic steatosis and improved insulin resistance^[19]. This is in accordance with a recent study by Nomura *et al*^[20] who showed that ezetimibe improved hepatic insulin sensitivity.

CONCLUSION

In summary, Ezetimibe is a unique medication with potential for treating not only gallbladder stones but also insulin resistance and NAFLD (Figure 1). Taking all these factors into consideration, it is possible to suggest that a clinical trial designed to investigate the potential of ezetimibe for reducing biliary cholesterol saturation and insulin resistance in populations with a predisposition to cholelithiasis is now warranted.

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Liver magnetic resonance imaging: State of the art

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Abstract

Magnetic resonance imaging (MRI) has now been used for about three decades to characterize the human liver in a non-invasive way, that is without the need of using ionizing radiation or removing tissue samples. During the past few years, technical progress has been considerable and novel applications of MRI have been implemented in the clinic. The beginning of a new decade offers an excellent opportunity for having five experts to present their view on the current status of MRI (and magnetic resonance spectroscopy) in the study of perfusion, fat and iron contents, diffusion and the metabolism of diffuse liver diseases. This topic highlight series thus provides an update of current knowledge in the field of liver MRI.

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Key words: Liver; Magnetic resonance imaging

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focus on five magnetic resonance imaging (MRI) methods offering opportunities for parametric exploration of the liver^[1]. The first contribution focuses on the microcirculatory status in liver parenchyma and liver lesions as studied by perfusion MRI, i.e. MRI with the use of (contrast) agents to improve the contrast between the features on images^[2]. Great progress has been made in turning perfusion weighted imaging into a quantitative method for detecting tumor, evaluating tumor viability after therapy and, for instance, the diagnosis of liver cirrhosis and its severity. Another important development is that nowadays, the sampling of liver tissue for the assessment of hepatic steatosis, related to alcoholic and non-alcoholic liver disease, metabolic syndrome, obesity and insuline resistance, can be replaced by the use of MRI or magnetic resonance spectroscopy (MRS) for determining liver fat content^[3]. The next review is a thorough assessment of the present status in another novel application of MRI presenting an alternative to biopsy: liver iron content determination^[4]. Accurate evaluation of iron overload is necessary to establish the diagnosis of hemochromatosis and guide chelation treatment in transfusion-dependent anemia. Diffusion weighted imaging (DWI), best used in combination with conventional unenhanced MRI and perfusion weighted MRI, is a promising tool used in predicting tumor responsiveness and following up on-cological treatment since DWI might be capable of detecting recurrent disease earlier than conventional imaging^[5]. The final contribution features the use of MRS to study metabolism in diffuse liver diseases, diabetes and cancer^[6]. Although this method still is in the preclinical stage, it is anticipated that future developments such as clinical magnets with a higher field strength (3 T, 7 T) and improved delineation of multi-component signals, will lead to intensified research in metabolic syndrome, cardiovascular disease, hepato-biliary diseases, *etc.* We believe that this issue will be of interest not only to gastroenterologists, but also to those involved in metabolic studies, cell physiology and pathology.

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Liver fat content determined by magnetic resonance imaging and spectroscopy

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Abstract

Hepatic steatosis as the most prevalent liver disorder can either be related to alcoholic liver disease (ALD) or non-alcoholic fatty liver disease (NAFLD). In both conditions, hepatocytes excessively accumulate fat-containing vacuoles within their cytoplasm, which is the key histological feature. In contrast to ALD, NAFLD is commonly associated with metabolic syndrome, obesity and insulin resistance. To determine increased liver fat content, liver biopsy is currently considered the gold standard. Besides the invasive technique, various other non-invasive techniques have been developed, such as ultrasound, computed tomography (CT), magnetic resonance spectroscopy (MRS) and magnetic resonance imaging (MRI) based methods. Among these techniques, ultrasound and CT provide only qualitative information about hepatic steatosis, whereas MRS- or MRI-based

methods are able to determine even small amounts of fat accurately. These non-invasive magnetic resonance techniques have already proven their great potential, especially in longitudinal and cross-sectional studies regarding various metabolic conditions and medical treatment regimens. In this review, the most common, non-invasive MRS/MRI techniques for assessment of intrahepatic lipid content are described with their inherent advantages and limitations.

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Key words: Hepatic steatosis; Magnetic resonance imaging; Proton magnetic resonance spectroscopy; Lipids

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Springer F, Machann J, Claussen CD, Schick F, Schwenzer NF. Liver fat content determined by magnetic resonance imaging and spectroscopy. *World J Gastroenterol* 2010; 16(13): 1560-1566 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v16/i13/1560.htm> DOI: <http://dx.doi.org/10.3748/wjg.v16.i13.1560>

INTRODUCTION

Hepatic steatosis is a common finding during liver examination and is found in a broad spectrum of diseases. It is related to an increased deposition of triglycerides within the cytoplasm of hepatocytes. Besides alcoholic liver disease (ALD), intrahepatic accumulation of lipids can also be associated with obesity, insulin resistance and metabolic syndrome, and is then termed non-alcoholic fatty liver disease (NAFLD). NAFLD is constantly gaining prevalence throughout the western world and is related to obesity as an increasing problem in recent decades^[1,2]. Nevertheless, NAFLD can also be found in non-obese subjects with a body mass index within the normal range. Those patients

often suffer from insulin resistance. Thus, intrahepatic fat fraction denotes an interesting metabolic parameter for longitudinal or cross-sectional studies regarding various metabolic conditions. Moreover, it is considered an independent risk factor for insulin resistance and atherosclerosis^[3-7]. The current gold standard for quantification of intrahepatic lipid content is based on invasive liver biopsies and subsequent histological analysis. However, due to its invasive character, it is not useful for longitudinal studies or metabolic studies on otherwise healthy subjects.

Magnetic resonance spectroscopy (MRS) and magnetic resonance imaging (MRI) provide non-invasive means to accurately quantify intrahepatic lipid content^[8-10]. In contrast to other modalities such as ultrasound and computed tomography (CT), MRI/MRS are capable of detecting even small amounts of intrahepatic lipid accumulation^[10]. Therefore, MRI/MRS are especially useful to measure changes in hepatic steatosis during various treatment regimens. During recent years, clinical and research investigations have been performed on this subject.

This review gives an overview of various magnetic-resonance-based methods that are capable of quantifying intrahepatic lipid content non-invasively. Different strategies of ¹H-MRS, as well as phase-sensitive and frequency-selective MRI methods are described.

¹H-MRS

In 1993, Longo *et al.*^[11,12] first published their results of ¹H-MRS of liver parenchyma and correlated the data with CT studies and biopsies. In these studies, they found an excellent agreement between the different investigated methods. Since then, several studies have been performed that have further verified these results by means of whole-body MR scanning^[13-15].

However, various strategies have been developed to obtain volume-selective ¹H-MR spectra from liver parenchyma *in vivo*. Spectra are usually recorded from volumes ranging from 1 to 27 cm³, which are small enough to be positioned well in the liver parenchyma. To record reliable spectra from pure liver parenchyma, voxels have to be carefully placed in order to avoid artificial signal contributions from surrounding adipose tissue or intrahepatic blood vessels.

Two main strategies are used for single-voxel spectroscopy (SVS): point resolved spectroscopy (PRESS) or stimulated-echo acquisition mode (STEAM)^[16,17]. The PRESS acquisition scheme (multi-echo single-shot technique) uses a 90°-180°-180° pulse sequence with long echo time (TE) and allows for better visualization of metabolites with long T₁ relaxation times. In contrast, the STEAM sequence applies a 90°-90°-90° pulse sequence and is less sensitive to J-coupling effects. The STEAM sequence provides shorter TE and lower signal yield compared to PRESS, which is usually not a limitation for fat quantification in the liver. However, both techniques can be applied for intrahepatic fat quantification in clinical examinations.

Since both techniques only provide spectra of a small sub-region of the liver parenchyma, so-called spectroscopic imaging techniques with 2D or even 3D matrices of spectra have been developed to obtain detailed information on lipid distribution^[18,19]. Compared to SVS, these techniques are rarely used clinically for routine investigation of liver parenchyma, due to their rather long acquisition and post-processing times^[20,21]. In most cases of NAFLD, hepatic lipid distribution has been shown to be relatively homogeneous, which allows one to quantify intrahepatic fat fraction by only one single representative voxel^[22-24]. However, it should be noted that significant differences in sub-regions of both liver lobes have also been reported^[13].

The above-mentioned ¹H-MRS techniques have been applied in studies investigating NAFLD in the general adult population^[25]. Moreover, an increasing number of longitudinal clinical studies have been performed evaluating intrahepatic fat fraction in the obese population or patients at risk for developing type 2 diabetes^[26-31]. Intrahepatic fat fraction has also been evaluated in morbidly obese patients undergoing bariatric surgery^[32-35]. Moreover, additional cross-sectional studies have revealed different intrahepatic fat fractions depending on genetic background or hormonal status of the examined subjects^[36-42].

All of these MRS fat quantification techniques have been shown to be safe and non-invasive alternatives to the current invasive gold standard (liver biopsy). They have been tested regarding their accuracy and have shown high intra-individual reproducibility in repeated measurements^[13,23,25]. However, one has to consider that MR spectroscopic fat quantification relies on determination of overall volume fraction of lipids in the liver parenchyma. In contrast, in histological examinations, the percentage of hepatocytes that show distinct fat droplets is used for quantification. Thus, the reported percentage values that characterize steatosis from MR examinations might differ from those in histological analysis. On the other hand, data from MRI and histology correlate with each other and both techniques allow, nevertheless, for reliable quantification of intrahepatic lipid content.

It should be also mentioned that spectroscopic examinations are especially recommended for assessment of small lipid fractions in the liver, because sensitivity to low signal intensities from fat is higher than for imaging-based strategies. Furthermore, water and fat signals can be well distinguished.

FAT-SENSITIVE IMAGING METHODS

¹H-MRS capabilities are still not available on all standard clinical scanners and require dedicated prerequisites including spectroscopic sequences and post-processing software. Therefore, ¹H-MRS still remains a research tool for clinical studies and is usually not used in daily routine liver examinations. There are, nevertheless, MRI sequences that allow for reliable and accurate quantification of intrahepatic lipid content.

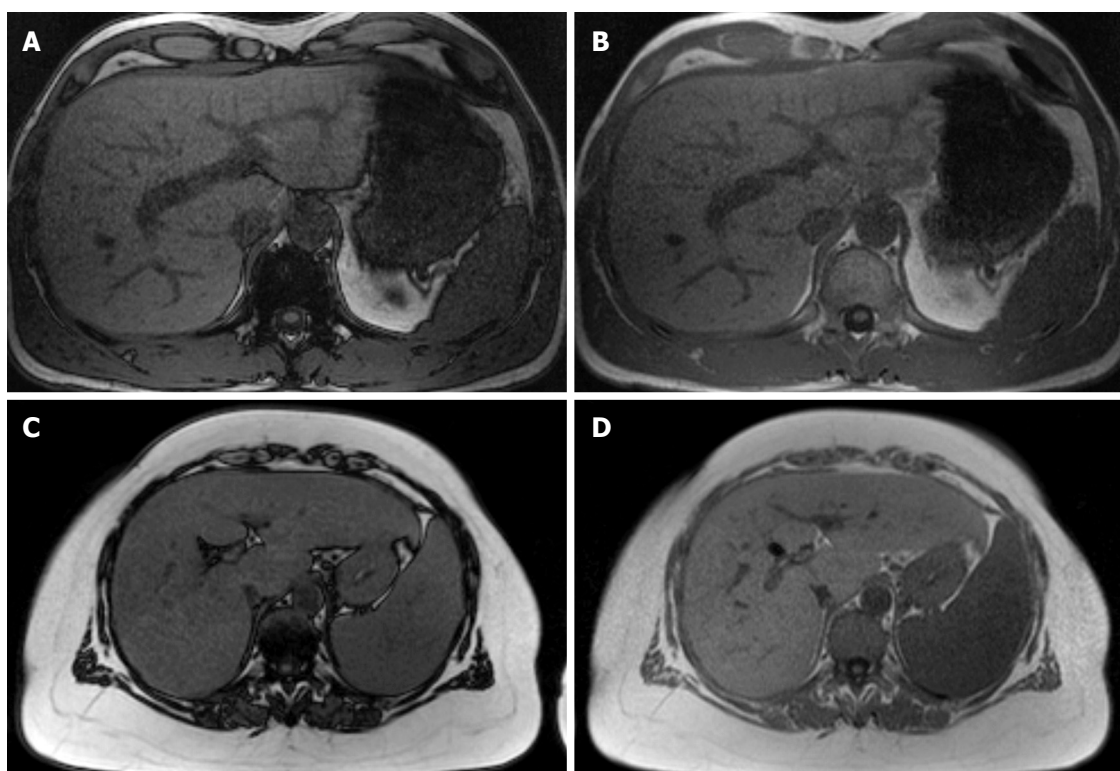


Figure 1 T1-weighted gradient echo images recorded with OP (parts A and C) and IP (B and D) conditions. A and B show a lean subject with almost equal signal intensity of the liver under OP (A) and IP (B) conditions, since no intrahepatic lipid storage is present. In contrast, C and D show an obese subject with lower signal intensity under OP conditions compared to IP conditions, which indicated relevant intrahepatic lipid storage.

Techniques based on differences in signal phase of water and fat

So-called in-phase/opposed-phase (IP/OP) techniques are available on most MR units and can be performed easily in routine examinations. Using this technique, T1-weighted images can be acquired extremely fast, with the use of multi-segment phased array coils and parallel imaging techniques. Moreover, T1-weighted gradient echo sequences can cover most of the liver parenchyma within a single breath-hold^[43-46]. The IP/OP technique is based upon the fact that, during TE, transverse magnetization vectors of fat and water develop a phase difference that results in decreased overall length of the magnetization vector under OP conditions. At a main magnetic field strength of 1.5 T, the frequency shift between fat and water is approximately 220 Hz, which results in OP conditions at a TE of about 2.4 ms and in-phase conditions at a TE of about 4.8 ms^[47-49]. The hepatic fat fraction can then be quantified by calculating the loss of signal intensity in OP images compared to IP images^[50-52], as shown in Figure 1. From congruent sets of IP and OP images, acquired within the same breath-hold, the fat fraction can be calculated pixel-wise and misregistration errors can be avoided. Thus, maps of intrahepatic fat fraction can be obtained to estimate liver fat content and show differences in regional fat distribution.

However, not only the phase difference between water and fat protons contribute to the observed signal loss in OP images, but also additional transverse and longitudinal relaxation effects may play a major role. Recent studies

have shown that especially transverse relaxation time can vary largely between different individuals, as well as intra-individually in the time-course of longitudinal studies^[46,53,54]. These changes in transverse relaxation time are mainly due to increased iron deposition in the liver parenchyma; either artificially acquired or, for example, hemochromatosis-associated^[55]. It has been shown that transverse relaxivity correlates well with serum ferritin levels^[53,56]. Thus, transverse relaxation time of liver parenchyma has to be measured additionally using a multi-echo gradient echo sequence. The data necessary for estimation of T2* can then be obtained within a single additional breath-hold. Integration of individual T2* values in the calculation of the fat fraction requires a somewhat more sophisticated approach^[57].

In contrast, longitudinal relaxation times are relatively stable throughout the population and individual calculation requires additional time-consuming sequences. Therefore, it seems legitimate for the general population to account for longitudinal relaxivity using constant values for longitudinal relaxation time of liver parenchyma.

Compared to the above-described gradient-echo-based IP/OP technique, Dixon *et al.*^[47] described in 1984 the use of a spin-echo technique with a small timing-offset of the 180° refocusing pulse, which is used to create a so-called OP image. The IP image is then acquired using a conventional spin-echo sequence. From these two images, fat- and water-selective images can be subsequently obtained. However, sensitivity to magnetic field inhomogeneity cannot be neglected and has prevented the wides-

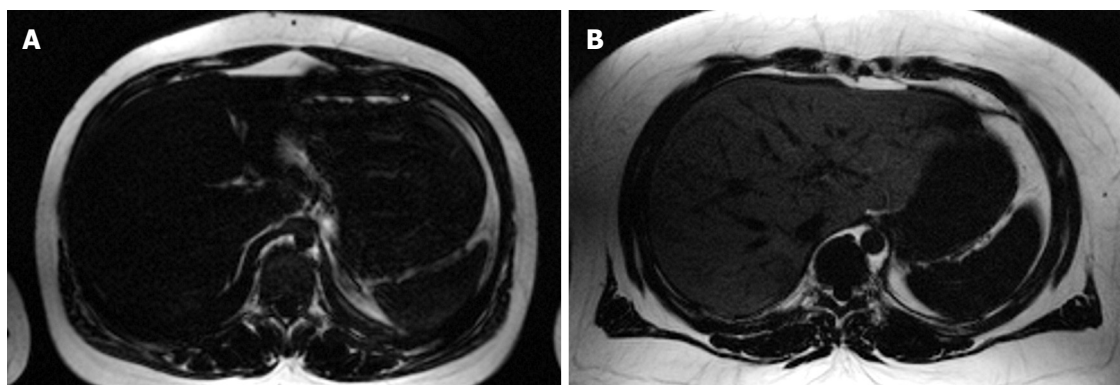


Figure 2 A and B show fat-selective spectral-spatial imaging of the body trunk. A lean subject with almost no intrahepatic lipid storage (A) and an obese subject with markedly increased lipid storage in the liver parenchyma is shown (B).

pread routine clinical usage of the Dixon technique. Since its introduction more than 20 years ago, several modifications have been reported that have aimed at overcoming its inherent limitations^[45,48,58,59]. Three-point Dixon methods have been developed that additionally acquire a third image with a phase shift of -180° or 360° . Then, using three different images and sophisticated phase-correction algorithms, true fat- and water-selective images are derived from the recorded data. This technique allows one to distinguish which constituent (water or fat) is predominant in each voxel^[60-66]. Acquiring all three images in a single breath-hold is often not possible, whereas recording in multiple breath-holds poses the problem of misregistration artefacts due to variable positions of the liver parenchyma.

Another approach was first described by Reeder *et al.*^[67-69] and is termed the IDEAL technique (iterative decomposition of water and fat with echo asymmetry and least squares estimation). Using optimized echo shifts and gradient echo imaging, it provides robust quantification of the intrahepatic fat fraction. This technique allows for fat quantification even in the presence of moderate inhomogeneities of the static magnetic field, which are often encountered in examinations of extremely obese patients on wide-bore MRI scanners. However, it is not free of limitations. Liu *et al.*^[70] have reported techniques for reduction of noise bias and longitudinal relaxation effects that affect quantification of the hepatic fat fraction in the IDEAL technique. These drawbacks can be partially overcome by small- or dual-flip angle approaches, magnitude discrimination and phase-constrained methods. Besides its capabilities in measuring parenchymal fat content, the IDEAL method has also been used for fat suppression in clinical studies of various body regions^[71-75].

Techniques based on frequency selective excitation

Previous studies have described a so-called spectral-spatial excitation technique to quantify fat content accurately in parenchymal organs and muscles^[57,76]. A combination of chemical shift selectivity and slice-selective excitation in gradient echo or spin echo imaging sequences provides a high sensitivity to detect even small amounts of fat^[23,49,77,78].

Furthermore, spatial information about parenchymal lipid distribution is also obtained. Slice-selectivity is implemented using six equidistant radio frequency pulses (time increment between pulses, 2.38 ms at 1.5 T) with nearly binomial amplitude ratios. These radio frequency pulses excite the methylene and methyl signal of fatty acids (0.8-2.0 ppm) selectively, as shown in Figure 2. Thus, signal contributions from water protons are below the noise level. To achieve this optimal spectral-spatial excitation, relatively homogeneous static magnetic fields are required, which makes adequate shimming procedures necessary. However, especially in wide-bore MR scanners that are designed to examine extremely obese patients, the inhomogeneity of the static magnetic field is often problematic. Even time-consuming shimming procedures might fail. For quantitative assessment of intrahepatic fat, adjacent subcutaneous or visceral fat is used as an internal reference because it contains almost 100% fat. The spectral-spatial excitation method is capable of detecting even small amounts of lipids (starting at 1%-2% volume fraction of fat in the liver), with additional spatial information about its distribution^[23]. However, some advantages and disadvantages of this technique should be noted. As a result of highly selective visualization of fat, the technique offers relatively low soft tissue contrast compared to conventional gradient echo sequences (Figure 3). Moreover, only a small number of representative slices can be acquired during a single breath-hold. Since only a reference region-of-interest in subcutaneous adipose tissue adjacent to liver parenchyma is needed for quantification of fat fraction, the calculation of intrahepatic lipid content can easily be done. Furthermore, there is no need for additional time-consuming sequences that are necessary to correct for transverse and longitudinal relaxation effects.

CONCLUSION

Several non-invasive methods have been developed for quantification of intrahepatic fat content using whole-body MRI scanners. Being aware of the inherent advantages and disadvantages of each technique, one has to choose carefully the appropriate method for specific examination

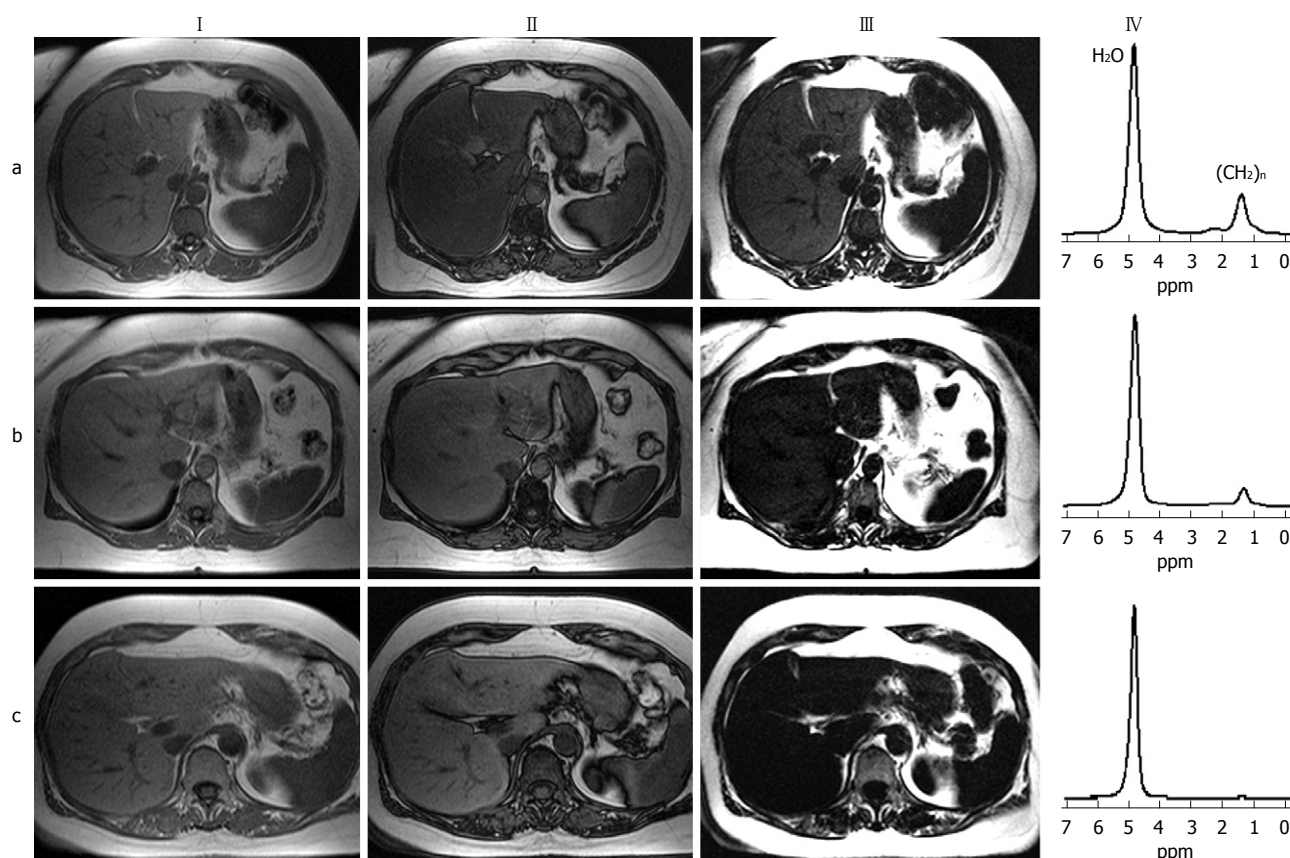


Figure 3 Three subjects with different intrahepatic lipid contents are compared. The subject in row (a) shows intrahepatic lipid content of about 20%; the subject in row (b) shows intrahepatic lipid content of about 10%; and the subject in row (c) shows intrahepatic lipid content in the normal range (about 1%). The figure shows IP (column I) and OP (column II) images of a T1-weighted gradient echo sequence. Column III shows the results of a fat-selective spectral-spatial imaging sequence, and column IV shows the results from single-volume ^1H -MRS using a STEAM sequence.

circumstances, as well as for hard- and software capabilities. Correctly applied, each technique (MRS/MRI) provides accurate data on intrahepatic fat fraction, correlating well with findings in liver biopsies, which is often considered as the current gold standard. The methods described above provide non-invasive quantification of the intrahepatic fat fraction, and give a reliable basis for longitudinal clinical and research studies. Thus, the influence of various medical treatments and diseases on intrahepatic lipid storage can be easily investigated in a non-invasive way.

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Diffusion weighted imaging in the liver

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Abstract

Diffusion weighted magnetic resonance imaging (DWI) is an imaging technique which provides tissue contrast by the measurement of diffusion properties of water molecules within tissues. Diffusion is expressed in an apparent diffusion coefficient (ADC), which reflects the diffusion properties unique to each type of tissue. DWI has been originally used in neuroradiology. More recently, DWI has increasingly been used in addition to conventional unenhanced and enhanced magnetic resonance imaging (MRI) in other parts of the body. The reason for this delay was a number of technical problems inherent to the technique, making DWI very sensitive to artifacts, which had to be overcome. With assessment of ADC values, DWI proved to be helpful in characterization of focal liver lesions. However, DWI should always be used in conjunction to conventional MRI since there is considerable overlap between ADC values of benign and malignant lesions. DWI is useful in the detection of hepatocellular carcinoma in the cirrhotic liver and detection of liver metastases in oncological patients. In addition, DWI is a promising tool in the prediction of tumor responsiveness to chemotherapy and the follow-up of oncological patients after treatment, as DWI may be capable of detecting recurrent disease earlier than conventional imaging.

INTRODUCTION

Magnetic resonance imaging (MRI) is an imaging technique which is used to visualize the internal structure and function of the body. MRI provides excellent tissue contrast, which is much greater than that of any other imaging modality^[1,2]. Tissue contrast is realized by a wide range of pulse sequences. For example, tissue contrast on T1- and T2-weighted images is based on the rate at which signals from protons in water molecules in a static magnetic field decay following excitation by a sequence of radiofrequency (RF) pulses. Diffusion weighted imaging (DWI) is another mechanism for developing image contrast and relies on changes in the diffusion properties of water molecules in tissues. DWI is a widely accepted technique in neuroradiology for detecting early ischemia in cerebrovascular accidents and characterization of brain tumors and intracranial infections^[1-3]. The use of DWI in other parts of the body is relatively new, but very promising for the detection and differentiation of benign and malignant lesions, imaging for dissemination (i.e. staging) in oncological patients before treatment and

for follow-up after treatment of liver tumors. Besides this, DWI is thought to be capable of predicting the response to therapy of malignant tumors (especially chemotherapy)^[4].

BASIC PRINCIPLES OF DWI

Diffusion

Diffusion is a physical property, which describes the microscopic random movement of (water) molecules driven by their internal thermal energy. This movement is known as Brownian motion. In biological tissues, water diffusion is movement of water molecules in intracellular, extracellular and intravascular spaces. Diffusion is affected by the biophysical properties of tissue cell organization (cell membranes, fibers and macromolecules), density, microstructure and microcirculation. Intracellular water diffusion is more hindered than that in the extracellular spaces which are lacking natural barriers. Pathological processes which change the volume ratio or physical nature of intra- and extracellular spaces affect the diffusion of water molecules. Restricted or impeded diffusion is seen in tissues with high cellularity, e.g. tumors, abscesses, fibrosis and cytotoxic edema. Relative free or unimpeded diffusion is encountered in tissues with low cellularity or tissues with disrupted cell membranes, for example in cysts and necrotic tissues^[3-5] (Figure 1A and B).

DWI

DWI relies on measuring diffusion of water molecules in the tissue by MRI. It uses a pulse sequence (T2-weighted spin echo sequence) and 2 strong motion probing gradients on either side of the 180° refocusing pulse, known as the Stejskal-Tanner sequence. The first gradient, prior to the 180° RF pulse is the dephasing (diffusion sensitizing) gradient. The second gradient, after the RF pulse, is the rephasing gradient. In tissues with restricted diffusion, the effect of the dephasing gradient is cancelled out by the rephasing gradient. This causes little impact on the overall T2 decay, reflected as a maintained T2 signal in the tissue. When diffusion is not impeded, water molecules can move a considerable distance between the dephasing and rephasing gradients. The mobile water molecules will not be fully rephased and a reduction in overall T2 signal intensity follows.

DWI is sensitive to very small scale motion of water molecules at a microscopic level. The sensitivity of a DWI sequence is characterized by its b-value, expressed in s/mm². The b-value summarizes the influence of the gradients in DWI. The higher the b-value, the more sensitive the sequence is to diffusion effects. DWI is performed with at least two b-values. Diffusion is quantitatively reflected in a diffusion coefficient. The diffusion coefficient is related to the molecular mobility of water molecules and reflects tissue properties such as the size of the extracellular space, viscosity and cellularity. Diffusion coefficients in DWI are reflected in the *apparent diffusion coefficient* (ADC, expressed in mm²/s), apparent because it is a mean value of diffusion contributed by

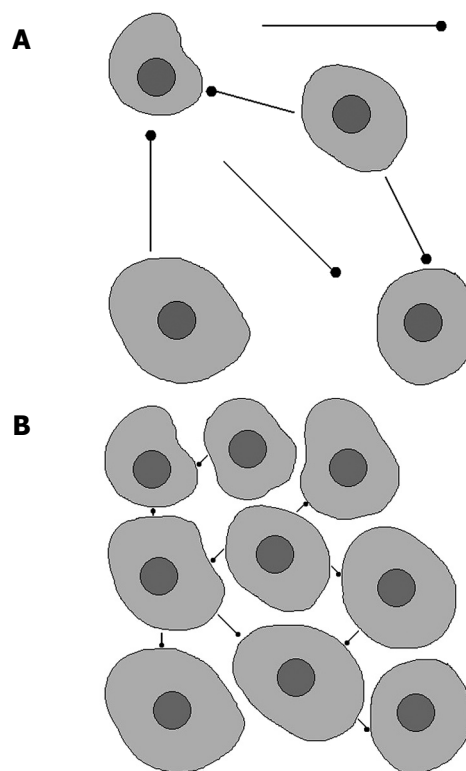


Figure 1 Brownian movements in hypocellular (A) and hypercellular (B) environment. A: Tissue with low cellularity permits movement of the water molecules; B: Tissue with high cellularity restricts the movement of water molecules.

movement of intracellular, extracellular and vascular water molecules within an image voxel (volumetric pixel) at different b-values. Analysis of ADC is an automated process, available as an application on most scanners or workstations. Calculation of ADC is made for each voxel of an image and can be displayed as a parametric (ADC) map. ADC measurements are then recorded for a given region by drawing regions of interest (ROIs) on the ADC map. Low ADC values mean restricted diffusion, thus in tissues which are highly cellular. High ADC values are seen in areas with relative free diffusion, thus in tissues with low cellularity^[1,3-5].

Problems encountered in DWI

DWI can be performed with different techniques, including spin-echo (SE), fast spin echo (FSE), gradient echo (GE) and echo-planar imaging (EPI). EPI is the gold standard DWI technique. When DWI is performed in the body, scanning can be carried out with free breathing, breath hold or respiratory triggered. There are some important limitations of DWI^[2,6].

Firstly, the signal-to-noise ratio (SNR, describes the relative contributions of the true signal and background noise to a detected signal) and spatial resolution are low due to hardware limitations and high bandwidth (a measure of frequency range, the range between the highest and lowest frequency allowed in the signal), inherent to the technique and EPI sequence. SNR will be decreased in incomplete spin echo formation, as is the case with

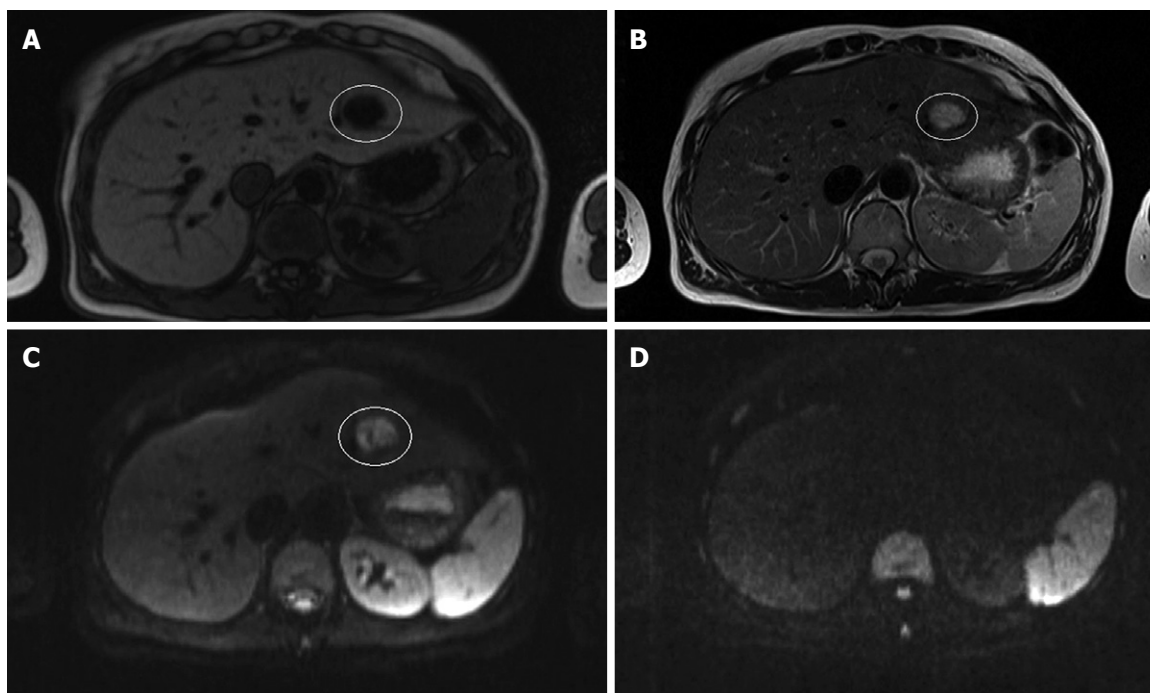


Figure 2 Magnetic resonance imaging (MRI) and diffusion weighted imaging (DWI) of a cyst. A: T1-weighted MRI; B: T2-weighted MRI; C: Diffusion weighted image (b-value 50 s/mm²); D: Diffusion weighted image (b-value 1000 s/mm²) in a 33-year old woman with multiple liver lesions. The cyst is hypo-intense on the T1-weighted image, hyper-intense on the T2-weighted image and the diffusion weighted image at a b-value 50 s/mm². Note that the cyst totally disappears on the diffusion weighted image at a b-value 1000 s/mm².

the most common DWI technique, the EPI sequence. SNR can be increased, but then spatial resolution is sacrificed^[2,3,6-8].

Secondly, DWI is susceptible to a number of artifacts. Ghosting images and blurring may arise from motion, caused by respiratory, cardiac and voluntary movements. Tissue contrast is maintained during free-breathing scanning, but breath hold and respiratory triggered techniques reduce image blurring. Breath hold scans have very short acquisition times of 20-30 s (i.e. the time the patient holds his or her breath) and are theoretically less likely to be degraded by motion-related artifacts. A disadvantage of breath-hold scanning is that the patient needs to hold his or her breath for a considerable time, which may be difficult when the physical condition does not allow him or her to do so. Respiratory triggering will make the acquisition time of the images longer (5 min), especially when the patient is breathing irregularly or slowly, but this is a minimal time penalty. Respiratory triggering provides substantially improved signal, spatial resolution and the ADC values are comparable to breath-hold DWI. In respiratory triggering, multiple b-values can be used to reduce errors in ADC calculation^[2,3,6,7,9-11].

Motion artifacts caused by the heart beating alter ADC in the left lobe of the liver, making measurements unreliable. Cardiac motion can be overcome by using electrocardiographic-triggering, but cardiac gating is not always reliable and increases acquisition times significantly^[2,3,6,7,12].

Susceptibility artifacts are due to magnetic field inhomogeneity (to be overcome by shimming techniques) or metal artifacts and are seen as bright spots, spatial

distortion or signal drop out. Susceptibility artifacts occur especially in fast imaging techniques like EPI^[2,6,7].

Artifacts caused by air-tissue interfaces or fat-water interfaces (chemical shift) appear as black or bright bands at the edge of an anatomical structure. Other artifacts are eddy currents, resulting from the rapid on and off switching of the gradients, leading to geometrical distortion and image shearing artifacts^[2,6,7].

Pathological diffusion

When evaluating diffusion on DWI images, the radiologist focuses on the measurement of extracellular diffusion. As mentioned earlier, the higher the cellularity in a tissue, the less far is extracellular water able to diffuse during the MR observation period without being blocked by cell membranes. Highly cellular tissues provide a short path of diffusion, resulting in low ADC values, as is seen in solid liver lesions and abscesses. Low cellularity means that there are fewer structural barriers, making the diffusion path longer. This results in high ADC values as is seen in cysts and necrotic lesions. In summary, ADC maps, derived from DWI provide a non-invasive measure of cellularity. This makes DWI a potential tool in diagnosis, treatment planning and monitoring, especially in oncology^[3-5]. Examples of different types of lesions and their diffusion weighted images are seen in Figures 2-5.

DWI IN THE LIVER

There are an increasing number of studies dealing with quantitative measurements of ADC in liver lesions, but

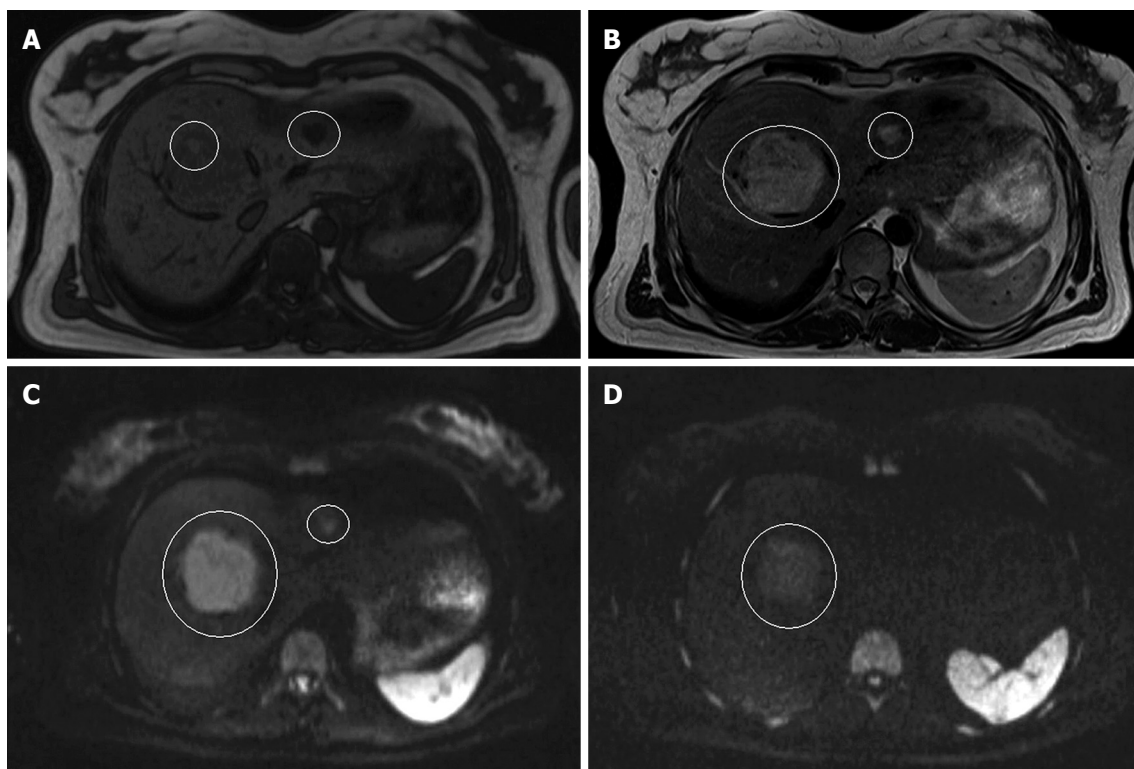


Figure 3 MRI and DWI of an adenoma and a hemangioma. A: T1-weighted MRI; B: T2-weighted MRI; C: Diffusion weighted image (b-value 50 s/mm²); D: Diffusion weighted image (b-value 1000 s/mm²) in a 41-year-old woman with multiple liver lesions. The large lesion in segment 8 is an adenoma and the small one in segment 2-3 is a hemangioma. On the T1-weighted image, the adenoma is slightly hypo-intense to the normal liver parenchyma, and hyper-intense on the T2-weighted image. On the diffusion weighted images, it remains hyper-intense at both b-values. The hemangioma is also hypo-intense on the T1-weighted image and hyper-intense on the T2-weighted image. However, in contrast to the adenoma, it totally disappears at a b-value of 1000 mm/s².

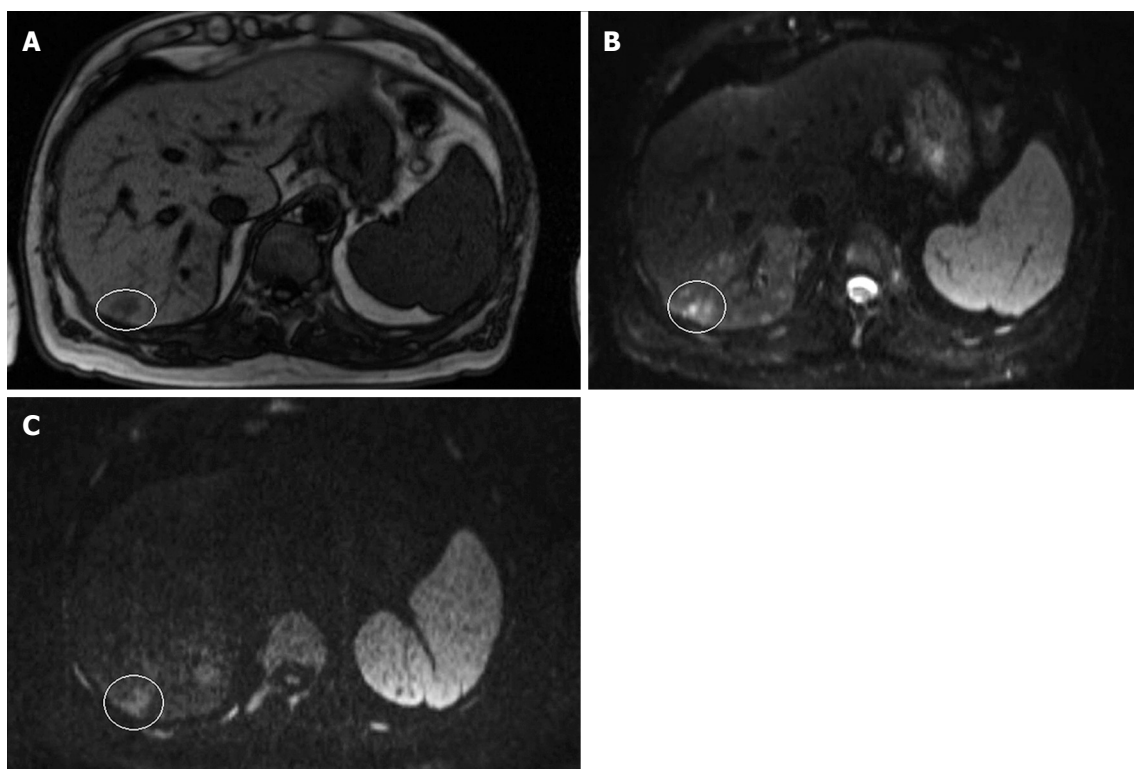


Figure 4 MRI and DWI of hepatocellular carcinoma. A: T1-weighted; B: DWI (b-value 50 s/mm²); C: Diffusion weighted image (b-value 1000 s/mm²) in a 67-year-old male with hemophilia, hepatitis C-based liver cirrhosis and HCC in segment 7. The HCC is hypo-intense on the T1-weighted image and hyper-intense on the diffusion weighted images at both b-values. Note that the lesion remains hyper-intense on the image with a b-value 1000 s/mm².

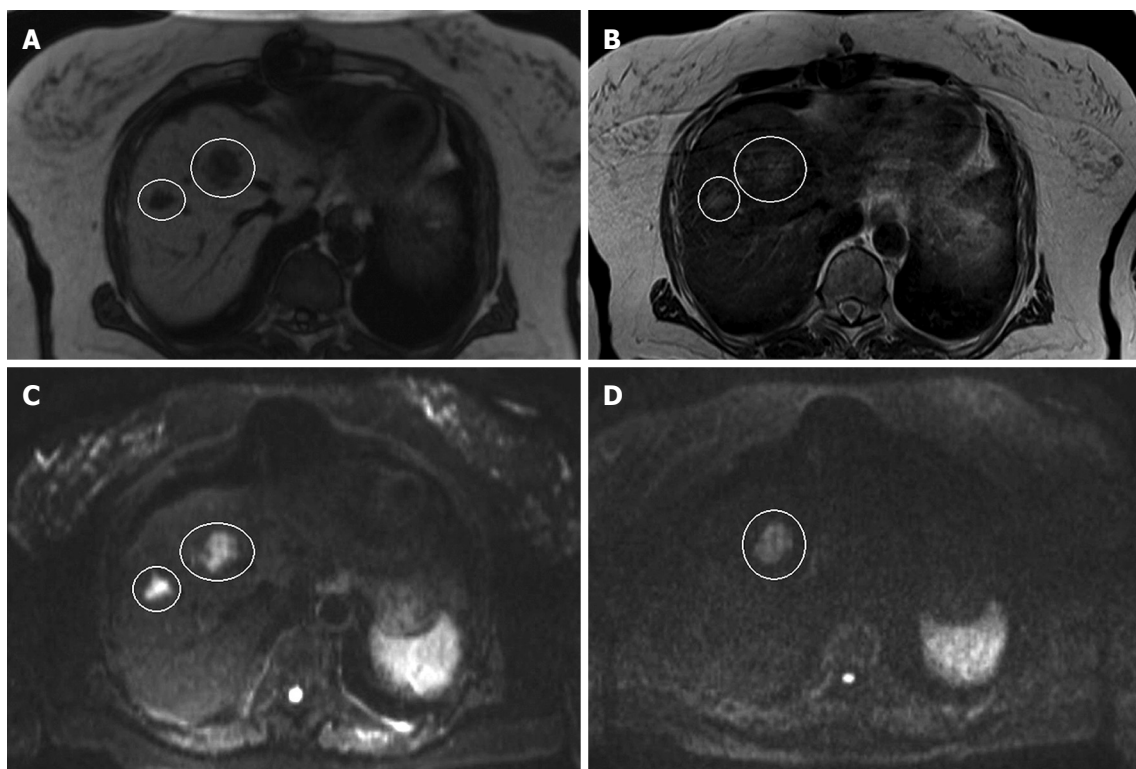


Figure 5 MRI and DWI of hepatic metastases. A: T1-weighted; B: T2-weighted; C: Diffusion weighted image (b-value 50 s/mm²); D: Diffusion weighted image (b-value 1000 s/mm²) in a 74-year-old woman with a history of rectal cancer, recently diagnosed with lung and liver metastases in segment 4 and 8, respectively. The lesions are hypo-intense to the liver parenchyma on the T1-weighted image and hyper-intense on the T2-weighted image. On the diffusion weighted image at a b-value 50 s/mm², both lesions appear hyper-intense, but at a b-value 1000 s/mm², only the lesion in segment 4 remains hyper-intense. The lesion in segment 8 has completely disappeared.

there are as many discrepancies in the reported ADC values (Table 1). This is often associated with the choice of b-values and other technical parameters. Low b-values lead to overestimation of the ADC due to the contribution of perfusion to the diffusion measurement. Large b-values underestimate ADC due to increasing contributions from low ADC components and SNR.

DWI as a tool for characterization of liver lesions

Several studies have suggested that the measurement of ADC values is useful in the characterization of focal liver lesions^[13-26]. Reduced ADC values have been reported for most malignant tumors. This finding is thought to be the result of cellular membranes impeding the mobility of water molecules. However, solid benign lesions, which are also highly cellular, exhibit decreased ADC values as well. Abscesses do so too because their viscous content with bacteria, inflammatory cells, mucoid proteins and cell debris result in restricted diffusion, thus low ADC values. On the other hand, necrotic and cystic malignancies show high ADC values resulting from larger diffusion distances as a consequence of lost membrane integrity. Benign lesions as simple cysts and hemangiomas show high ADC values because of their liquid content and large extracellular spaces. However, ADC values cannot discriminate between solid benign and malignant lesions, since there is considerable overlap. According to Feuerlein *et al*^[15], the pretest probability of malignancy is very important in the determination to which degree a large

ADC value is predictive for a malignancy, i.e. the history, demography and clinical picture of the individual patient. Even ADC values of lesions of the same kind show overlap and there is no cut-off value for ADC values in normal parenchyma, benign and malignant lesions. In the literature, ADC values vary between $0.94\text{--}2.85 \times 10^{-3} \text{ mm}^2/\text{s}$ for metastases and $0.69\text{--}2.28 \times 10^{-3} \text{ mm}^2/\text{s}$ for normal liver parenchyma. This is mainly because every study group uses their own scanning parameters. Differences in b-values are the main cause of non-equivocal results. Breath-hold, respiratory triggered and navigator echo techniques can also give different ADC values. There is need for an uniformly applicable scanning protocol to eliminate discrepancies in ADC values caused by different scanning parameters^[13].

DWI alone is not suitable for the characterization of liver lesions, because solid benign lesions also can show restricted diffusion, and cystic or necrotic malignant lesions have unimpeded diffusion. DWI can help direct the attention of the radiologist to findings that may otherwise be overlooked. Unenhanced and dynamic MRI contrast series alone are very capable in the discrimination of different types of liver lesions, but a combination of DWI and MRI increases the accuracy of the characterization of benign and malignant lesions^[15-17].

Detection of hepatocellular carcinoma

Multiphase multidetector contrast enhanced computed tomography (CT) has reached a high standard for the

Table 1 Reported ADC values in different types of lesions in the liver

Study	Type of lesion	n	ADC value mm ² /s, BH (SD)	ADC value mm ² /s, RT (SD)
Taouli <i>et al</i> ^[10] b-values 0, 50, 500 s/mm ²	Benign	18	2.21 (0.60)	2.39 (0.44)
	Malignant	11	1.04 (0.27)	1.16 (0.33)
Goshima <i>et al</i> ^[13] b-values 100, 200, 400, 800 s/mm ²	Hemangioma	12	1.23-2.23 (0.2-1.2) ²	
	Cyst	15	3.70-4.72 (0.9-1.2) ²	
	Metastases	7	0.99-1.70 (0.5-1.1) ²	
	HCC	21	1.08-1.79 (0.3-10.9) ²	
Kandpal <i>et al</i> ^[14] b-value 0, 500 s/mm ²	Hemangioma	11	2.22 (0.45)	2.36 (0.48)
	Cyst	11	2.66 (0.44)	2.90 (0.51)
	FNH	3	2.03 (0.24)	2.15 (0.18)
	Abscess	6	1.21 (0.36)	1.13 (0.43)
	Metastases	38	1.06 (0.36)	1.13 (0.41)
	HCC	12	1.22 (0.34)	1.27 (0.42)
Gourtsoyianni <i>et al</i> ^[18] b-values 0, 50, 500, 1000 s/mm ²	Hemangioma	7		1.90
	Cyst	15		2.55
	Metastases	13		0.99 (0.22)
	HCC	2		1.38
Oner <i>et al</i> ^[19] b-values 0, 500 s/mm ²	Hemangioma	5	1.72 (0.30)	
	Cyst	3	2.34 (0.36)	
	Metastases	6	1.03 (0.24)	
Demir <i>et al</i> ^[23] 0, 1000 s/mm ²	Benign	24	1.09-3.36 (0.32/0.28) ³	
	Malignant	17	0.54-1.24 (0.07/0.14) ³	
Bruegel <i>et al</i> ^[24] b-values 50, 300, 600 s/mm ²	Hemangioma	56		1.92 (0.34)
	Cyst	51		3.02 (0.31)
	FNH	4		1.40 (0.15)
	Metastases	82		1.22 (0.31)
	HCC	11		1.05 (0.09)
Holzapfel <i>et al</i> ^[25] b-values 50, 300, 600 s/mm ²	Hemangioma	18		1.69 (0.34)
	Cyst	71		2.61 (0.57)
	FNH/adenoma	6/9		1.43 (0.22)
	Metastases	76		1.08 (0.32)
	HCC	17		1.12 (0.28)

¹These studies found significantly higher ADC values in benign liver lesions than in malignant liver lesions; ²This study evaluated the ADC values at different b-values. The lowest and highest ADC values are reported here. The lowest ADC value corresponds with the highest b-value, the highest ADC value with the lowest b-value; ³This study evaluated the ADC values of different types of benign and malignant lesions. The lowest and highest ADC values are reported here. ADC: Apparent diffusion coefficient; BH: Breath-hold; RT: Respiratory triggered; SD: Standard deviation; FNH: Focal nodular hyperplasia; HCC: Hepatocellular carcinoma.

evaluation of the cirrhotic liver and for the detection of hepatocellular carcinoma (HCC). On CT images, diagnosis of HCC is made based on neovascularization with increased arterial enhancement and rapid portal venous wash-out. In the last few years, (liver-specific) contrast enhanced multiphase dynamic MRI has increasingly been used for the detection of HCC. MRI proved to be superior to CT in the detection of HCC and for the characterization of nodules in patients with liver cirrhosis because of the high tissue contrast provided by MRI and the available liver-specific contrast agents. Contrast enhanced MRI is now regarded as the best non-invasive imaging modality. However, even with liver-specific contrast enhanced MRI, there is a diagnostic problem for small HCC lesions (< 10 mm) as well as in

the differentiation from other non-malignant nodules. Large HCC lesions are well-recognized on conventional MRI by their rapid enhancement in the arterial phase and their contrast agent wash-out in the portal-venous phase. Small HCC is less typical on conventional MRI, and differentiation of atypical nodules in the cirrhotic liver is challenging^[27-31].

Xu *et al*^[27,28] found that ADC values were not useful in cirrhotic livers, because cirrhotic parenchyma and solid benign lesions have low ADC values. They cannot be differentiated from lesions with malignant diffusion restriction because of the considerable overlap among their ADC values. Necrosis and vascularization within HCC also alter diffusion, often seen as a false increase in the ADC values.

Zech *et al*^[29] reported a higher sensitivity for DWI compared to conventional MRI in the detection of HCC in the cirrhotic liver (98% for DWI *vs* 83%-85% for MRI). Vandecaveye *et al*^[30] concluded that DWI provided higher sensitivity and positive predictive value for the detection of HCC < 20 mm compared to conventional contrast enhanced MRI (sensitivity and specificity 91.2% and 82.9% *vs* 67.6% and 61.6%, positive predictive value 81.6% and 59.0%, respectively). DWI did not show significantly better results than conventional MRI in detecting HCC > 20 mm. These findings can be explained by the better contrast-to-noise ratio and background suppression of normal liver parenchyma and vascular or bile structures in DWI, which make small lesions more visible, especially when they are in close vicinity to vessels or bile ducts. DWI provides a high negative predictive value on the presence or absence of HCC and reduces the rate of unnecessary invasive diagnostic procedures and follow-up.

Detection of liver metastases

Several studies have demonstrated the usefulness of DWI in the detection of liver metastases. They compared DWI to unenhanced and dynamic liver specific contrast enhanced MRI (Table 2).

Coenegrachts *et al*^[20] showed that lesion conspicuity of hemangiomas and metastases is significantly higher with respiratory triggered DWI at low b-values compared to conventional unenhanced MRI imaging. This is due to an excellent lesion to liver contrast and suppression of background signals from vessels.

Koh *et al*^[32] compared the diagnostic accuracy of DWI and mangafodipir trisodium (MnDPDP)-enhanced MRI alone and in combination in the detection of colorectal liver metastases. They found that a combination of MnDPDP MRI and DWI resulted in the highest diagnostic accuracy (0.94-0.96 *vs* 0.88-0.92 for MnDPDP alone and 0.83-0.90 for DWI alone) with an increased sensitivity, but no loss of specificity. DWI alone is not useful because the sequence is very susceptible to motion artifacts, which obscure lesions and make images difficult to interpret. This is especially the case in the left lobe of the liver. They also stated that experience is needed to interpret DWI correctly, mainly because of the large numbers of artifacts on the images^[33].

Parikh *et al*^[34] reported a significantly higher overall lesion detection rate for breath-hold or respiratory triggered DWI than for conventional T2-weighted MRI (88% *vs* 70%). Bruegel *et al*^[35] compared respiratory DWI-EPI with T2-TSE. They found a sensitivity and specificity for T2-TSE MRI of 45%-62% for unenhanced MRI and 88%-91% for DWI-EPI for lesions > 10 mm. When considering only small metastases < 10 mm, the differences between DWI and conventional MRI with and without contrast are even more pronounced: a sensitivity of 85% for DWI-EPI and 26%-44% for T2-TSE. Lesion detection on T2-TSE is hindered by low lesion to liver contrast and by the interfering bright signal from intrahepatic vessels. Lesion conspicuity with DWI is excellent and limitation

Table 2 Performance of DWI and conventional MRI in the detection of liver metastases *n* (%)

Study	Sensitivity	Specificity	Accuracy	PPV	NPV
Vandecaveye <i>et al</i> ^[30]					
> 20 mm					
B600 SI ratio	100	81.8	94.9	93.3	100
T2-CE MRI	96.4	81.8	92.3	93.1	90.0
< 20 mm					
B600SI ratio	91.2	82.9	86.7	81.6	91.9
T2-CE MRI	67.6	61.0	64.0	59.0	69.4
Koh <i>et al</i> ^[32]					
MnDPDP MRI	81.3	93.0	88-92		
DWI 0, 150, 500 BH	78.3	95.0	83-90		
MnDPDP MRI and DWI	92.2	97.0	94-96		
DWI RT	88-91				
T2 MRI	45-62				
Nasu <i>et al</i> ^[36]					
DWI RT (0, 500)	82	94			
SPIO MRI	66	90			

¹In this study, 2 observers reviewed the images. The given values in sensitivity, specificity and diagnostic accuracy refer to the separate results of both observers. PPV: Positive predictive value; NPV: Negative predictive value.

of the DWI sequence is predominantly referred to lesion characterization rather than to lesion detectability.

Nasu *et al*^[36] assessed the diagnostic accuracy of respiratory triggered DWI in combination with unenhanced MRI *vs* superparamagnetic iron oxide (SPIO)-enhanced imaging. On the basis of a receiver operator characteristic analysis (ROC), averaged over 3 observers, they found a sensitivity and specificity for SPIO-enhanced images of 66% and 90% and for DWI 82% and 94%, respectively.

Predicting response to therapy of primary and secondary liver malignancies by DWI

Tumor responses to chemotherapy and radiation therapy are conventionally assessed by measurement of percentage reduction in tumor size after chemotherapy. However, tumor size measurement on CT or MRI is insensitive to early treatment changes. Theoretically, DWI is sensitive to microenvironmental changes in tumors that occur after treatment. Studies on the predictive value of DWI in primary cancer demonstrated a strong negative correlation between mean pre-treatment ADC values and percentage size reduction of tumors after chemotherapy and chemoradiation. High pretreatment ADC values in tumors were associated with a poor response to chemotherapy^[37,38].

Koh *et al*^[37] showed that high pre-treatment ADC values in colorectal liver metastases were predictive of a poor response to oxaliplatin and 5-fluorouracil-based chemotherapy. They determined with ROC that a mean pretreatment ADC150-500 (ADC map with b-values 150 s/mm² and 500 s/mm²) of 1.69×10^{-3} mm²/s had 60% sensitivity and 100% specificity for identification of non-responding metastatic lesions. They found also a significant linear regression relation between mean ADC150-500 and percentage in tumor size reduction

after treatment. Responding tumors showed a significant increase in ADC values at the end of the treatment. Non-responding tumors and liver parenchyma did not show significant changes in ADC values. Cui *et al*^[38] analyzed 87 liver metastases of colorectal and gastric origin in 23 patients. They also found significant lower ADC values in responding tumors than in non-responding ones. ADC increased in responding metastases, but not in non-responding ones. They found a weak correlation between tumor size reduction and pretreatment ADC values. The theoretical background for these findings is that higher ADC values are observed in necrotic tissue, and in tissue with loss of cell membrane integrity. When these changes are present before chemotherapy, it may indicate a more aggressive phenotype. Necrotic regions within a tumor are usually poorly perfused, resulting in less delivery of chemotherapeutic agents to these areas. Necrotic regions are also exposed to a more hypoxic and acidic environment, which diminishes the effect of chemotherapy. Necrosis in hepatic metastases is present in almost half of the cases. A possible explanation for non-responding tumors with lower ADC values may be the fact that necrosis is not always associated with high ADC values, especially in the case of coagulation necrosis without cell lysis or liquefaction. The increase in ADC values at the end of the treatment suggests a change from a more cellular pretreatment to a less cellular or necrotic phenotype.

Only one study assessed DWI in HCC treatment with sorafenib. Schraml *et al*^[39] showed that ADC values with sorafenib, an angiogenesis inhibitor for treatment of HCC, actually showed a decrease instead of an increase. This may be a result of ischemia, induced by inhibition of angiogenesis. The extracellular volume is decreasing, leading to lower ADC values during treatment. They also often observed hemorrhage within the tumors (55%), which may contribute to a decrease in ADC. However, progression of HCC more than 3 mo after therapy was related to a decrease in ADC values. Only conventional MRI could differentiate between hemorrhage and tumor progression. Pre-treatment HCC had high ADC values, because of rich vascularization of the tumors. ADC changes early after chemotherapy seem to reflect the underlying mechanisms in tumor necrosis, most probably hemorrhagic, induced by the novel targeted agent sorafenib early after therapy and may indicate tumor reactivation in the later follow-up period.

DWI after locoregional treatment for liver tumors

Locoregional therapy for liver tumors is used in patients who are not eligible for surgery. The most commonly used local ablative therapies are radiofrequency ablation (RFA) and transcatheter arterial chemoembolization (TACE). The success of these procedures is determined by the rate of ablation site recurrences (ASR), i.e. tumor recurrence as result of incomplete ablation. Close and careful follow-up is needed in patients who underwent treatment by RFA and TACE to detect ASR at an early stage. Unfortunately, diagnostic management remains an issue in these patients,

because of difficulties in differentiating ASR from non-tumoral tissue changes after thermal therapy. Since DWI can provide information about molecular tissue characteristics, it may have an additional value in the evaluation and follow-up of local ablative therapies in patients with liver tumors.

There is one study which evaluated the time-related diffusion alterations after hepatic RFA with regard to potential diagnostic information for the detection of ASR. Schraml *et al*^[40] reviewed 54 oncological patients treated by RFA for liver metastases from different origins. The ablation zone did not show significant alterations at different time points. Measurement of the ADC value of the entire ablation zone was not suitable for the detection of ASR, because locally changing ADC values were masked by the heterogeneous appearance of the entire ablation zone. Peripheral zones should be analyzed separately. An important technical limitation of ADC measurements after RFA is that the limited spatial resolution does not allow exact ROI positioning in the narrow peripheral rim. Viable tumors after RFA appeared as hyper-intense, and necrotic regions were recognized as hypointense areas on DWI. ASR showed significantly lower ADC values than the ablation zone and normal liver parenchyma ($1.02 \times 10^{-3} \text{ mm}^2/\text{s}$ vs $1.31 \times 10^{-3} \text{ mm}^2/\text{s}$). Suspected areas on DWI were more easily identified and analyzed in conjunction to conventional MR. Signal alterations in the periphery, especially ones with lower ADC values should raise suspicion on ASR. Edema, inflammation, fibrosis and necrosis are associated with higher ADC values. DWI together with conventional imaging is a promising tool in the evaluation of the post-RFA liver and may contribute to the detection of ASR.

The use of DWI after TACE in HCC has recently been investigated. Goshima *et al*^[41] reported significant increases in ADC values after TACE, but they varied widely and did not contribute to the accurate diagnosis of tumor necrosis by any cut-off points. Yu *et al*^[42] found that DWI added to conventional MRI could increase the sensitivity for determining ASR especially in the case of atypical lesions. However, they also noticed an increase in the number of false positive findings by adding DWI which affected the overall accuracy of MRI. This was caused by perilesional inflammation and arterial reperfusion of the perilesional atrophic area after TACE. ADC measurement was not helpful for distinguishing viable tumors from perilesional nontumorous changes, since there was great overlap between the ADC values of both entities.

CONCLUSION

DWI in the liver is a relative new and increasingly used imaging technique in addition to conventional unenhanced and contrast enhanced MRI. DWI proved to be helpful in the characterization of focal liver lesions, but should always be used in conjunction with traditional MRI since there is great overlap between ADC values of benign and malignant lesions. DWI is useful in the detection of

small HCC in the cirrhotic liver, with higher sensitivity, specificity and positive predictive value compared to conventional contrast enhanced imaging due to better lesion to liver contrast and background suppression of signals arising from vessels and bile ducts. This is also the case for the detection of metastases in the liver. However, it should be noted that DWI images are difficult to interpret since DWI is very sensitive to artifacts. It seems reasonable to use DWI in conjunction to conventional imaging. DWI is not yet commonly used in the follow-up after treatment of liver malignancies. Pre-treatment ADC values in tumors treated with chemotherapy seem to be useful in the prediction and evaluation of the treatment response of primary and secondary liver malignancies. DWI in the follow-up after RFA and TACE shows promising results in the detection of ablation site recurrences, especially in combination with conventional contrast enhanced imaging.

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Magnetic resonance spectroscopy to study hepatic metabolism in diffuse liver diseases, diabetes and cancer

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phosphodiester using proton decoupling, especially if combined with price reductions for stable isotope tracers, will lead to intensified research into metabolic syndrome, cardiovascular disease, hepato-biliary diseases, as well as non-metastatic liver metabolism in patients with a distant malignant tumor.

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Abstract

This review provides an overview of the current state of the art of magnetic resonance spectroscopy (MRS) in *in vivo* investigations of diffuse liver disease. So far, MRS of the human liver *in vivo* has mainly been used as a research tool rather than a clinical tool. The liver is particularly suitable for static and dynamic metabolic studies due to its high metabolic activity. Furthermore, its relatively superficial position allows excellent MRS localization, while its large volume allows detection of signals with relatively low intensity. This review describes the application of MRS to study the metabolic consequences of different conditions including diffuse and chronic liver diseases, congenital diseases, diabetes, and the presence of a distant malignancy on hepatic metabolism. In addition, future prospects of MRS are discussed. It is anticipated that future technical developments such as clinical MRS magnets with higher field strength (3 T) and improved delineation of multi-component signals such as phosphomonoester and

INTRODUCTION

The liver is a particularly suitable and interesting organ for metabolic studies as it plays a central role in intermediary metabolism, has a high metabolic activity with rapid response to metabolic insults, and is profoundly altered in acute and chronic diseases. Magnetic resonance spectroscopy (MRS) has been established as a non-invasive technique to study cellular biochemistry and metabolism, both at high magnetic strengths *in vitro* and in the whole body *in vivo* at field strengths of up to 3 T.

MRS has a number of important advantages over conventional approaches when studying metabolism in diffuse liver disease in humans. The non-invasive character of the technique allows valid assessment of the metabolic profile of many hepatic metabolites *in vivo* which cannot

be accurately measured using invasive biopsy techniques, due to the instability of these compounds and the invasiveness of biopsies. In addition, the non-destructive character of MRS, combined with the large hepatic blood flow, allows repeated measurements over time in the same subject, facilitating long-term longitudinal (observational/intervention) studies as well as short-term dynamic metabolic intervention studies with real-time monitoring of biochemical or metabolic alterations after a meal or an oral or intravenous metabolic challenge by nutrients (sugars, amino acids, lipids), hormones *etc.* Moreover, the technique can be complemented by dynamic stable isotope tracer studies, allowing the simultaneous assessment of hepatic metabolite concentrations by MRS and turnover measurements in plasma. Of note, although challenge techniques are also used in clinical practice for diagnostic purposes, for instance, to diagnose inherited metabolic errors of metabolism, MRS of the human liver *in vivo* has so far mainly been used as a research tool rather than a clinical tool.

However, one limitation of MRS studies is that only compounds present at mmol/L concentrations can be detected, due to the low inherent sensitivity of the MR signal *in vivo*.

The liver is a particularly suitable and interesting organ for metabolic studies for a number of reasons: first, it plays a central role in intermediary metabolism, has a high metabolic activity with a rapid response to metabolic insults, and is profoundly altered in acute and chronic diseases. Second, its superficial location in the right upper abdomen, covered only by a thin layer of skin, adipose tissue and muscle, allows excellent MRS localization using a double-tuned surface coil, by different localization techniques such as chemical shift imaging (spectroscopic imaging, CSI/SI)^[1-3] and image-selected *in vivo* spectroscopy (ISIS)^[4,5]. Third, the relatively large volume of the liver allows the detection of signals with relatively low intensity in volumes of up to 1 L; of note, for the study of diffuse liver disease, the inherent low spatial resolution of MRS does not play a restrictive role.

Different nuclei such as ³¹P, ¹H, ¹³C and ¹⁹F have been applied in studies of diffuse liver disease. So far, ¹H MRS has mainly been applied to detect hepatic lipid levels, ³¹P MRS to investigate intracellular energy metabolism, phospholipid metabolism and gluconeogenesis, and ¹³C MRS to study liver lipid and glycogen metabolism. ¹H and ³¹P are naturally abundant isotopes, whereas ¹³C represents only 1% of total carbon (the majority being ¹²C). Therefore, in many ¹³C studies, investigators have infused ¹³C-labeled compounds to increase the MR signal in dynamic metabolic studies.

The present review provides an overview of the current state of the art of MRS in *in vivo* investigations of diffuse liver disease. First, we discuss publications regarding the application of MRS to study diffuse and chronic liver diseases including cirrhosis, fibrosis and alcoholic liver disease, followed by the application of MRS in congenital diseases and pediatrics. We then discuss the

application of MRS to study the metabolic involvement of the liver in diseases, such as diabetes or the presence of a malignant tumor elsewhere in the body. Finally, future prospects including potential clinical applications of MRS are discussed.

From the present review, we excluded T2 and MRS studies on liver iron and fat content as well as *in vitro* applications of MRS in hepato-biliary disease^[6,7], as well as MRS studies of focal liver diseases such as secondary liver tumors, lymphomas and adenomas, and transplantation. We also excluded ¹⁹F MRS studies of metabolites of fluorinated chemotherapeutic drugs such as 5-FU and capecitabine^[8].

MRS IN DIFFUSE AND CHRONIC LIVER DISEASE

Over the last two decades, the usefulness of ³¹P MRS for the diagnosis of liver disease has been investigated as a non-invasive alternative to liver biopsy, which is still the gold standard and carries significant morbidity^[9]. The *in vivo* spectra of the human liver reflect metabolic and biochemical alterations in disease. Several publications on liver functionality have addressed spectral changes related to the underlying liver disease. However, in order to be helpful as a diagnostic tool, MR spectra should provide both sensitive and specific information for different types of liver disease, such as hepatitis, steatosis, fibrosis or cirrhosis.

The ³¹P MRS spectrum provides information on phosphorylated compounds of hepatic metabolism: alpha-, beta- and gamma peaks of nucleotide triphosphates (NTP), inorganic phosphate (Pi), phosphomonoesters (PME) and phosphodiester (PDE). The NTP, PME and PDE peaks are multicomponent and individual resonances of their components cannot be distinguished by the majority of techniques currently used. PME reflects components from glucose metabolism (gluconeogenesis and glycolysis) and cell membrane precursors such as phosphoethanolamine (PE) and phosphocholine (PC)^[10]. The majority of the NTP resonance contains ATP components; below, we will use ATP and NTP as synonyms, following the nomenclature of the cited publications. The PDE peak contains information on cell membrane breakdown products, such as glycerophosphorylethanolamine (GPE) and glycerophosphorylcholine (GPC), and endoplasmic reticulum^[11].

The diagnostic value of ³¹P MRS for various types of diffuse liver disease has been investigated in several studies. Overall, diffuse liver disease was associated with increasing levels of PME and decreasing levels of PDE. These changes have been attributed to hepatocyte damage, increased phospholipid turnover in hepatocyte membranes, and/or altered glucose metabolism. In general, the magnitude of MRS changes increased significantly with increased disease severity and increased functional impairment.

In one of the earliest reports^[12], liver metabolite concentrations were studied in 24 patients with various types of diffuse liver disease as compared to healthy control subjects. The authors reported high PME and low PDE levels in patients with acute viral hepatitis, high PME levels in patients with alcoholic hepatitis, and decreased Pi and Pi/ATP ratios in primary biliary cirrhosis and in some patients with hepatitis^[12]. However, they noted that these changes were not present in all patients.

Cox *et al.*^[11] studied 49 patients with liver disease of varying etiology, including 25 patients with diffuse liver disease such as cirrhosis and non-hepatic malignancies. A non-specific elevation in PME/PDE was observed in the ³¹P MR spectra of 10 (40%) out of these 25 patients with mixed diffuse liver disease. Even though the spectral pattern did not distinguish between diseases of varying etiologies, there was a linear correlation between increasing PME/PDE and a reduction in plasma albumin concentrations ($P = 0.03$).

Subsequent MRS studies showed elevated PME/NTP levels compared to healthy controls in patient populations with primary biliary cirrhosis^[13] and compensated/decompensated cirrhosis of various etiology^[14]. Jalan *et al.*^[13] studied 23 patients with primary biliary cirrhosis of varying functional severity and healthy subjects using ³¹P MRS. PME/NTP, Pi/NTP, PME/PDE, and PME/Pi ratios were higher and PDE/NTP ratios significantly lower in patients compared with healthy volunteers. Significant correlations were seen between PME/Pi ratios and prognostic indicators such as the Christensen index, the Mayo R value, and the Pugh score.

Taylor-Robinson *et al.*^[14] compared 14 patients with compensated cirrhosis (Pugh's score ≤ 7) and 17 with decompensated cirrhosis (Pugh's score ≥ 8) of various etiology with healthy subjects. Worsening liver function was associated with increased PME/NTP and decreased PDE/NTP ratios. In freeze-clamped tissue, elevated PE and PC, and reduced GPE and GPC mirrored these *in vivo* changes, but no distinction was noted between compensated and decompensated cirrhosis. In contrast, electron microscopy showed that functional decompensation was associated with reduced endoplasmic reticulum (ER) in parenchymal liver disease, but elevated ER in biliary cirrhosis. In a more recent study in only 14 cirrhotic subjects^[15], reduced ATP and elevated PME/PDE levels were detected in patients with decompensated cirrhosis only.

The capability of ³¹P MRS to detect pathological processes was further investigated by comparing *in vivo* MRS results with histological samples. In 38 patients with various types of diffuse liver disease, van Wassenae-van Hall *et al.*^[5] showed that the degree of elevation of PME/Ptotal in individual patients was significantly correlated with necrosis, intralobular degeneration, and portal inflammation scorings in liver biopsies^[5], but not with fibrosis. However, ³¹P MRS was not able to classify patients into diagnostic categories, such as fibrosis *vs* cirrhosis, and no diagnostic value of MRS was found with respect to steatosis and cholangitis.

Dezortova *et al.*^[16] studied 80 patients with liver cirrhosis of different etiology and functional status, described by Child-Pugh score, and a control group of healthy subjects. Patients with both alcoholic and viral etiology had lower absolute PDE and ATP levels than healthy subjects. Patients with alcoholic etiology, but not viral etiology, also had lower Pi levels than controls. Patients with cholestatic disease had elevated PDE levels. Thus, these authors were able to distinguish alcoholic, viral and cholestatic etiologies of liver cirrhosis based on MR spectra.

Noren *et al.*^[17] studied patients with non-alcoholic fatty liver disease (NAFLD) and none to moderate inflammation ($n = 13$), patients with severe fibrosis or cirrhosis ($n = 16$), and healthy controls. All patients underwent liver biopsy and extensive biochemical evaluation. Absolute concentrations and the anabolic charge (AC), defined as $(PME)/[(PME)+(PDE)]$, were calculated. AC was increased and PDE reduced in the cirrhotic group relative to healthy subjects, whereas NAFLD patients showed values similar to controls. Using a PDE concentration of 10.5 mmol/L as a cut-off value to discriminate between mild, (F0-2) and advanced (F3-4) fibrosis, the sensitivity and specificity of PDE were 81% and 69%, respectively. AC, using a cut-off value of 0.27, showed a sensitivity of 93% and a specificity of 54%. The authors concluded that PDE is a potential marker of liver fibrosis, whereas AC is a potentially clinically useful parameter discriminating mild from advanced fibrosis.

Interestingly, few authors have performed dynamic ³¹P MRS studies with a metabolic challenge, in contrast with diabetes and cancer (see below). In healthy subjects, it is well known that fructose infusion induces a rapid rise in PME and marked reductions in ATP and Pi^[3,18,19]. An early Japanese study^[20] evaluated changes in the metabolic state of the liver after an intravenous fructose load (250 mg/kg) in six patients with liver cirrhosis and eight healthy volunteers. The cirrhotic livers did not show the usual increase in PME after the fructose load, suggesting that fructose metabolism in the cirrhotic livers had impediments before the fructose-1-phosphate stage. Furthermore, the spectra of the cirrhotic livers showed a significant drop in Pi, PDE and ATP peak after the fructose challenge. Dufour and colleagues^[21] studied nine patients with nonalcoholic cirrhosis. Fructose (250 mg/kg) was injected intravenously, and further spectra were collected sequentially every 6 min for 1 h. PME formation and utilization of Pi were markedly attenuated in cirrhotic patients; these measures correlated with the impairment of liver function as measured by galactose-elimination capacity^[21].

In summary, the general observation in cirrhosis and fibrosis is an increase in PME, often combined with reductions in PDE and ATP. Of note, the observed changes were correlated with classical markers such as Pugh score and plasma albumin. However, ³¹P MRS was not able to classify patients into diagnostic categories, such as fibrosis *vs* cirrhosis, and no diagnostic value of MRS was found with respect to steatosis and cholangitis.

An intravenous fructose load induced a rapid rise in PME and reductions in ATP and Pi.

VIRAL HEPATITIS AND HIV

A study of 26 patients with an acute viral hepatitis A infection showed increased PME/PDE ratios^[22]. After 6 wk of recovery, these abnormalities in liver metabolites were restored to normal levels, as reflected by decreasing PME and increasing PDE^[22].

Lim and colleagues^[9] used ³¹P MRS to assess disease severity in 48 patients with hepatitis C virus (HCV)-related liver disease. Worse liver function was correlated with an *in vivo* elevation in PME and decrease in PDE. PME/PDE ratios showed an increase from control (0.15), *via* mild disease (0.18) and moderate disease (0.25), to the cirrhosis group (0.38). An 80% sensitivity and specificity was achieved when using a PME/PDE ratio less than or equal to 0.2 to denote mild hepatitis and a corresponding ratio greater than or equal to 0.3 to denote cirrhosis^[9]. In a subsequent study^[23], the same authors applied ³¹P MRS to prospectively study 47 patients with biopsy-proven hepatitis C undergoing viral eradication treatment with interferon and ribavirin at 6-mo intervals over a total period of 6-18 mo. In 25 out of 32 patients with virological response to HCV treatment, this was accompanied by decreasing PME/PDE ratios over time, whereas in 15 patients without virological response, PME/PDE ratios increased^[23], suggesting that PME and PDE can be used to monitor treatment response to HCV.

Elevated levels of PME were also observed in a study of 75 chronic hepatitis B and C infected patients using ¹H-MRS^[24]. In addition, compared to healthy control subjects, glutamine/glutamate and glycogen/glucose resonances were increased, whereas lipids were decreased^[24]. As in studies using ³¹P MRS, increases in metabolite levels were correlated with disease severity^[24]. In a study in patients with hepatitis C virus infection (HCV, *n* = 14) and 20 HIV/HCV co-infected individuals^[25], a significant increase in glutamine/glutamate and PME, both measured relative to hepatic lipid levels, was observed in both groups when compared with healthy individuals. These changes in metabolite ratios were attributed to an increase in the particular metabolite contents and a decrease in lipid levels. HIV/HCV-infected patients treated with anti-retroviral therapy showed elevated PME and glutamine/glutamate levels and decreased total lipid levels compared to patients not undergoing anti-retroviral treatment^[25]. The authors concluded that ¹H-MRS could be used to detect even slight alterations in hepatic metabolite ratios in this type of patient.

Orlacchio *et al*^[26] also applied ¹H-MRS but used the water signal as a reference instead of the lipid signal. These authors studied 23 patients with biopsy-proven precirrhotic HCV-related liver disease, graded by the Ishak fibrosis (F) scoring system. Similar to the previous studies, increasing disease severity correlated with a significant increase in choline-containing compounds and glutamine/glutamate. In contrast, lipid levels in this study were found

to be increased in patients relative to healthy subjects^[26].

In summary, ³¹P MRS studies have demonstrated that PME/PDE ratios in viral hepatitis are increased, and normalization of PME/PDE over time correlates with treatment effectiveness. ¹H MRS studies showed increased glutamine/glutamate and glycogen/glucose resonances. The observation of either increased or decreased lipid resonances in different studies requires further investigation.

ALCOHOL ABUSE

Although the above data show that different ³¹P MRS studies have demonstrated elevated PME/NTP levels in patient populations with alcoholic liver disease compared to healthy subjects, there are few reports on the effects of alcohol abuse and alcohol abstinence *per se*. Menon *et al*^[27] studied 26 chronic alcohol abusers by ³¹P MRS 6-12 h after their last alcoholic drink and following abstinence from alcohol. Results showed that in patients with minimal liver injury, recent drinking was associated with a significant elevation in PDE/ATP and a non-significant rise in PME/ATP, and abstinence with normalization of both metabolite ratios. In contrast, in patients with alcoholic cirrhosis, recent drinking was associated with a significant elevation in mean PME/ATP and a non-significant increase in PDE/ATP, whereas abstinence was associated with no significant change in PME/ATP but with a reduction in PDE/ATP. The authors suggested that the changes in PDE/ATP most likely reflected the induction of hepatocyte ER.

In summary, recent alcohol consumption is associated with elevated PME and PDE. In patients with minimal liver injury, both metabolites are normalized during abstinence; in contrast, in patients with cirrhosis, only PDE is normalized during alcohol abstinence.

OTHER LIVER DISEASES

Several studies have been performed on acute poisoning, inherited diseases and pediatrics using ³¹P MRS. A study in 18 patients after acetaminophen overdose showed that liver metabolites including PME, PDE and ATP, were all dramatically decreased with increasing liver damage expressed as the international normalized ratio (INR) of prothrombin^[28]. Repeated MRS measurements in the same patients would be particularly valuable to see whether improvement in liver damage could also be confirmed by ³¹P MRS.

The presence of hemolysis, elevated liver enzymes and low platelets in pregnant women (HELLP syndrome) can be associated with disturbed hepatic metabolism. To investigate whether or not women with HELLP syndrome have detectable abnormalities of hepatic energetics, Magee *et al*^[29] studied seven patients with HELLP syndrome. One pregnancy was later terminated but the other women gave birth to healthy infants. One patient with the most clinically severe HELLP syndrome by laboratory criteria exhibited MR spectra which showed a relative increase in phosphomonoester and an absolute decrease in hepatic

ATP (to 62% of control)^[29]. Most patients with HELLP syndrome had normal liver metabolism as assessed by MRS, although these results show that clinically severe HELLP syndrome can be associated with disturbed hepatic metabolism consistent with that seen in hepatic ischemia and/or granulocytic infiltration of the liver.

In ten patients with severe hypothyroidism, prospective ³¹P MRS measurements before and after thyroid hormone treatment were performed. In contrast with striated muscle, which showed a marked normalization of phosphocreatine/Pi ratios within several weeks of thyroid treatment, no changes in hepatic metabolism during treatment were detected by ³¹P MRS^[30].

In a single patient with amyloid light chain (AL) amyloidosis, hepatic ¹H MR spectra were characterized by small line widths, a striking increase in trimethylammonium compounds, and the presence of a further resonance at 3.8 ppm^[31]. None of the healthy control subjects showed trimethylammonium levels of comparable intensity^[31].

Changes in liver metabolites in inherited liver diseases have been reported using ³¹P MRS. In a report by Dixon *et al.*^[32] on an infant with galactosemia, these authors showed increased levels of galactose-1-phosphate in the liver which decreased during diet therapy, paralleling the falling level of galactose-1-phosphate in red blood cells.

Elevated plasma uric acid concentrations (hyperuricemia), which are a characteristic feature in gout patients, may be caused by altered liver fructose metabolism. As hereditary fructose intolerance is known to be associated with hyperuricemia, this concept has been used in ³¹P MRS studies^[33-35]. The effect of a fructose challenge on liver metabolism was studied by ³¹P MRS in five patients with hereditary fructose intolerance (HFI) and eight heterozygotes for HFI^[33]. In patients with HFI, ingestion of small amounts of fructose was followed by an increase in sugar phosphates (PME) and a decrease in Pi in hepatic ³¹P MR spectra, combined with a rise in plasma uric acid. ³¹P MRS could be used to diagnose fructose intolerance in heterozygotes. Oral fructose (50 g) also led to sugar phosphate accumulation and Pi depletion in the liver, which was associated with a larger increase in plasma uric acid than in control subjects. The effect of fructose on liver Pi and plasma uric acid was most pronounced in heterozygotes with gout ($n = 3$). In a subsequent study in 18 additional subjects from different families with familial gout^[34], the authors demonstrated a positive association between response in ³¹P MR spectra (i.e. increase in PME, decrease in Pi) and response in serum uric acid after oral glucose, suggesting ³¹P MRS as a method for initial screening of this defect.

Limited MRS data are available in the pediatric population. Nevertheless, ³¹P MR spectra of infants have been compared with adult spectra in an attempt to investigate changes in hepatic metabolism with age^[36]. Spectra from three infants showed that PME/ATP levels were markedly higher than in adults, whereas PDE/ATP levels were decreased when compared with adults. Spectral hepatic concentrations of a single adolescent studied were

intermediary between the neonates and the adults. The authors hypothesized that this indicated an increased rate of membrane synthesis in the infant livers, and concluded that these differences should be taken into account when comparing studies using varying ages^[36].

In summary, a considerable amount of data on the use of MRS for the study of liver metabolism in disease is currently available, although most studies were performed in relatively small groups. Static measurements mainly reveal alterations in relative PME and/or PDE concentrations as compared to healthy controls. In contrast, dynamic applications of the MRS technique to obtain information during a challenge have remained limited to date. Challenge tests will be discussed in more detail in the following sections.

LIVER METABOLISM IN PATIENTS WITH DIABETES

Impairment of hypoglycemic counterregulation, which occurs even in intensively treated type diabetes, has traditionally been attributed to deficits in counterregulatory hormone secretion in type I diabetes, and to impaired hormone sensitivity of target organs in type II diabetes. In the normal fed state, the liver takes up glucose for energy production (*via* glycolysis and TCA cycle) and stores excess energy as glycogen, whereas in the fasted state, the liver releases glucose produced from glycogen and *via* gluconeogenesis; in fact, the liver is almost exclusively responsible for endogenous glucose production (EGP). After a glucose load, liver glycogen can be synthesized both directly from glucose (*via* glucose-6P, glucose-1P and UDP-glucose) and indirectly from 3 carbon units (*via* phosphoenolpyruvate-glucose-6P *etc.*). ¹³C MRS, either at natural abundance or combined with ¹³C tracer infusion, provides a tool to study glycogen content in the liver and thus, in dynamic studies, net glycogen synthesis and breakdown over time. Moreover, combined with turnover measurements using deuterated glucose tracer infusions and measurement of isotopic enrichment of UDP-glucose using acetaminophen (measured as acetaminophen glucuronide in plasma/urine) allows an estimation of relative contributions of the direct and indirect pathways of glycogen synthesis. Noninvasive sampling of hepatic glutamine pools by oral administration of phenylacetate allows simultaneous estimation of the contribution of pyruvate oxidation to the TCA cycle flux^[37].

Over the past decades, ¹³C MRS studies have significantly contributed to the notion that the liver plays a critical role in the derangements of plasma glucose regulation in both type I and type II diabetes. Most MRS studies to date have been performed in diabetes type I. To determine alterations in the direct and indirect pathways of glycogen synthesis in diabetes, Cline and coworkers^[37] studied subjects with poorly controlled diabetes type I using a 5 h hyperglycemic-hyperinsulinemic clamp (plasma glucose: 9 mmol/L, insulin: 400 pmol/L) and [1-¹³C]-glucose. Hepatic pools of UDP-glucose and glutamine

were noninvasively sampled by oral administration of acetaminophen and phenylacetate, respectively. Although total hepatic glycogen synthesis was similar in both groups, the flux through the indirect (gluconeogenic) pathway was found to be proportionately about twice as active in the diabetic subjects compared to the control subjects. Moreover, the relative contribution of pyruvate oxidation to the TCA cycle flux in diabetic subjects was decreased by circa 30%, indicating reduced glycolysis. The abnormalities were not immediately reversed by normalizing intraportal concentrations of glucose, insulin and glucagon, and might contribute to postprandial hyperglycemia.

Hwang *et al.*^[38] studied poorly controlled diabetes type I patients and weight-matched control subjects during a day in which three isocaloric mixed meals were ingested. Although fasting hepatic glycogen levels were identical in the two groups, the diabetic subjects synthesized less glycogen over the day than healthy subjects. Again, the flux through the gluconeogenic pathway relative to the direct pathway of glycogen synthesis was markedly reduced in diabetic subjects.

Bischof *et al.*^[39] studied poorly controlled diabetes type I patients (HbA1C: 8.8% and matched non-diabetic subjects (HbA1C: 5.5%) in an experiment over 24 h with 3 isocaloric mixed meals. Over 24 h, the mean plasma glucose concentration was 2.4-fold higher in diabetic subjects. Net liver glycogen synthesis and breakdown were calculated from linear regression of the glycogen concentration time curves from 19:30-22:30, and 22:30-8:00, respectively. Glycogen synthesis was reduced by 74% and glycogen breakdown by 47%, and both were partly but not completely normalized by intensified insulin treatment.

In a subsequent study in long-term well-controlled diabetes type I patients, Bischof *et al.*^[40], showed that tight glycemic control in diabetes type I patients normalized overall glycogen synthesis and breakdown as well as glucose production. However, the relative contribution of the indirect pathway of glycogen synthesis remained elevated in diabetic subjects, a finding which indicated augmented gluconeogenesis in type I diabetic patients, which would be consistent with experimental models showing increased PEP-carboxykinase and/or reduced glucokinase activity in the liver of diabetic animals.

Also, in 6 to 12-year-old children with diabetes type I^[41], the capacity to replenish hepatic glycogen after an overnight fast was at least as good as in age-matched healthy children.

Kishore *et al.*^[42] compared the hepatic response to hypoglycemia in well-controlled type I diabetes patients and healthy subjects. In the overnight fasted state, diabetes type I patients had decreased hepatic glycogen levels compared to controls. In insulin-induced hypoglycemia, the normal response of glycogenolysis observed in healthy subjects was virtually absent in diabetic subjects.

Type II diabetes patients were studied by Magnusson *et al.*^[43], who compared seven diabetic (mean duration 13 years) and five healthy subjects matched for age and body mass index (BMI) during 23 h of fasting after an initial liquid meal. Oral medication with sulfonylurea agents (n

= 5) was discontinued 3 d before the study and insulin treatment ($n = 2$) was discontinued the evening before the study. Blood glucose levels in the diabetic subjects were consistently higher throughout the experiment. Four hours after the meal, hepatic glycogen levels in diabetic subjects were < 50% of those in control subjects. During the subsequent fasting period, glycogen breakdown was reduced in diabetic subjects; instead, their rate of gluconeogenesis was markedly increased, resulting in a circa 20% overall increase in glucose output compared to healthy subjects.

Krssak and colleagues^[44] studied hepatic glycogen synthesis, glycogen breakdown and gluconeogenesis in patients with type II diabetes (mean duration 6 years) and age- and weight-matched healthy subjects before and after a mixed meal. Hepatic glycogen concentrations were lower in diabetic patients before and after dinner, and post-meal glycogen synthesis was reduced by circa 44%. Overnight, rates of glycogenolysis were also circa 50% lower in diabetic patients than controls. Endogenous glucose production was elevated in diabetic patients before dinner and remained so for 3 h after dinner; also, the nadir of glucose production was delayed in diabetic patients (240 min *vs* 60-90 min); thereafter, glucose production in patients and controls was similar. At 6-9 h after dinner, gluconeogenesis amounted to 67% of EGP in diabetic patients *vs* 43% in controls.

During a subsequent hyperglycemic-hyperinsulinemic clamp, glycogen synthesis was circa 46% lower in diabetic patients, with a similar contribution from the direct and indirect pathways. Glycogen breakdown was similar, resulting in circa 54% lower net glycogen synthesis. EGP was circa 30% elevated in diabetic patients both before and during the clamp. Hepatocellular lipid content was three times higher in type II diabetic patients and negatively correlated with rates of net glycogen synthesis and whole-body glucose uptake during the clamp test (which was circa 37% reduced in diabetic patients)^[44]. Petersen *et al.*^[45] demonstrated that an average weight loss of 8 kg in type II diabetic patients over a period of 7 wk led to normalization of plasma glucose concentrations as well as hepatic glycogenolysis, glucose production and gluconeogenesis, indicating marked restoration of hepatic insulin sensitivity. These changes were associated with a reduction in hepatic lipid content from 12% to 3%, whereas muscle lipid content and muscle insulin resistance remained unchanged^[45].

In summary, the presence of marked alterations in hepatic glucose and glycogen metabolism in type I and type II diabetes has been substantiated by ¹³C MRS in combination with isotope tracer studies. When compared with non-diabetic humans, both patients with type I and type II diabetes exhibit elevated endogenous glucose production by increased gluconeogenesis, combined with reduced glycogen synthesis and breakdown. In type I diabetes, this defect can be partly restored by combined long- and short-term optimized treatment with insulin. In contrast, in type II diabetes, increased gluconeogenesis appears to be the main cause of elevated glucose

production and fasting hyperglycemia, and it is normalized by weight reduction.

LIVER METABOLISM IN PATIENTS WITH A DISTANT MALIGNANT TUMOUR

The cancer-bearing state is generally associated with profound alterations in host metabolism. Many patients lose weight due to decreasing fat and muscle mass, a condition known as cancer cachexia, which not only leads to functional decline but is also an important predictor of poor prognosis. Although cancer cachexia is partly caused by decreased appetite, altered metabolism in host organs such as liver and muscle are now thought to play a major role in the pathogenesis of the condition. So far, most studies have concentrated on experimental animal models of cancer cachexia. To further explore the role of altered liver metabolism in the etiology of cancer cachexia in humans, Dagnelie *et al.*^[46] and Leij-Halfwerk *et al.*^[47-51] performed a series of studies in patients with advanced lung and breast cancer. Importantly, patients were only included if they were free of liver metastases, as confirmed by CT and/or ultrasound. In all studies, weight-stable and weight-losing cancer patients were compared with healthy subjects. Dietary energy intake was similar in all subjects, and all spectra were acquired in the overnight-fasted state. Since ATP peak areas differed between patients and controls, total phosphorous content of the liver (which was demonstrated to be stable) was used as a reference.

In one study, ATP peak areas were significantly reduced in both weight-stable (WS) and weight-losing (WL) cancer patients^[46]; in the second study, the reduction was only statistically significant in WL but not WS cancer patients^[47]. During a 90-min infusion of 2.8 mmol/kg per minute of the gluconeogenic amino acid alanine, liver ATP levels decreased in healthy subjects and patients to a similar extent for 60 min; however, from 60-90 min, ATP levels in WS cancer patients and healthy subjects recovered to baseline values despite continued infusion, but further decreased in WL patients, indicating impaired ATP recovery^[47]. Intravenous infusion of ATP at 75 µg/kg per minute over 24 h induced complete normalization of liver ATP levels both in WL and WS patients to levels similar to healthy subjects^[51].

As shown in Figure 1, baseline PME values in the liver were markedly increased in WL cancer patients relative to WS patients and healthy controls, due to increased glucose cycling and gluconeogenesis as indicated by both the observed downfield alteration of the PME chemical shift^[46] and the significant correlation of PME levels with glucose turnover and gluconeogenesis from alanine^[49]. Baseline alanine turnover as measured by tracer techniques was elevated in WL cancer patients, but not in WS patients^[48]. L-alanine infusion induced a steady 33% rise in PME in healthy subjects (Figure 1). In WS cancer patients, a markedly faster and higher (i.e. 69%) response of PME to alanine infusion was observed, whereas WL patients showed virtually no further rise in PME (7%) relative to their already elevated baseline PME levels (Figure 1)^[48].

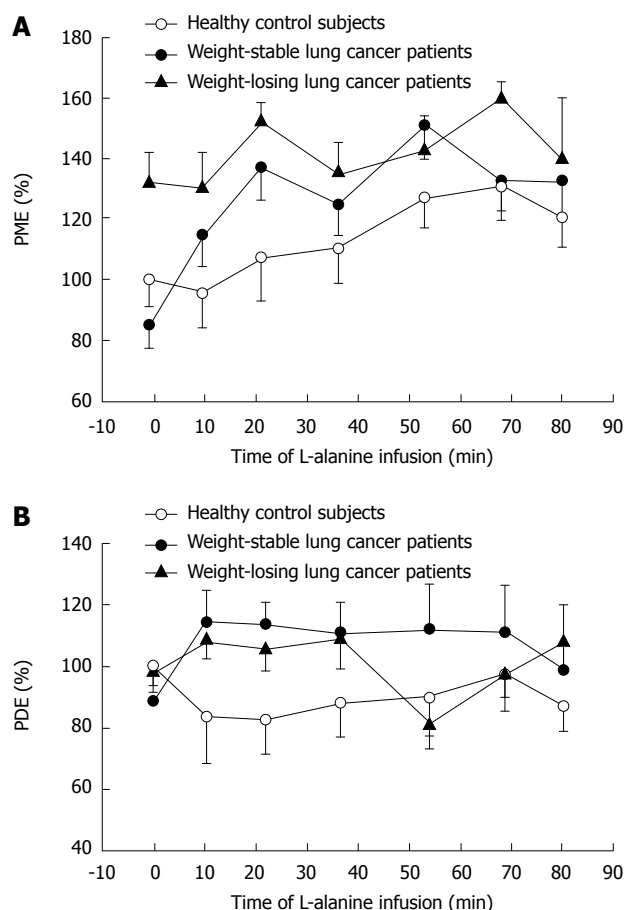


Figure 1 PME (A) and PDE (B) concentrations in the liver of healthy control subjects ($n = 9$) as well as weight-stable ($n = 10$) and weight-losing ($n = 7$) lung cancer patients during a primed-constant infusion of L-alanine (initial priming, 1.4-2.8 mmol/kg; infusion, 2.8 mmol/kg per hour). Curves represent means; bars, SE. Values are expressed as percentage of mean baseline value of healthy subjects (100%). Times during L-alanine infusion are mid-time points of ^3P MRS data collection referenced to the start of the L-alanine infusion (0 = baseline). From^[50], with permission.

In cancer patients, but not in healthy subjects, the rise in PME levels during alanine infusion was strongly inversely correlated with baseline PME ($r = -0.82$). Liver intracellular pH increased in the order: healthy < cancer-WS < cancer-WL^[46], suggesting that the host liver may have a similar intra-extracellular pH gradient as reported for cancerous tissue^[52-55].

In summary, using ^3P MRS, marked alterations in hepatic glucose metabolism were demonstrated in the non-metastatic liver of cancer patients, as shown by elevated hepatic concentrations of gluconeogenic intermediates in the overnight-fasted state, as well as during infusion of a gluconeogenic challenge. Of note, the rapid response of the PME peak to alanine infusion demonstrated rapid induction of the gluconeogenic pathway in WS cancer patients, corroborating the power of MRS as a tool to detect enzymatic alterations within the liver.

DISCUSSION

So far, MRS has primarily been used as a research tool.

MRS *in vivo* allows rapid assessment of the metabolic profile and function, including alterations in liver metabolism and physiology under different conditions. Dynamic studies have so far mainly been performed in diabetes and cancer. The limitation of these studies is the relatively high cost of the equipment and, even more, the cost of ^{13}C enriched compounds which are infused to increase hepatic ^{13}C content and to directly monitor the signal increase of ^{13}C in different metabolites above the 1% natural abundance of ^{13}C . As high-field machines become more available in the next decades, it is expected that the application of ^{13}C MRS studies in the liver may continue. However, the possibilities of ^{31}P MRS have by no means been exhausted, as is shown by the dynamic studies demonstrating altered host liver metabolism in patients with a malignant tumor elsewhere in the body. As the studies in cancer and diabetes demonstrate, metabolic challenges such as carbohydrates (like as glucose and fructose), amino acids (such as alanine) and lipids are likely to yield substantial new knowledge in the near future.

Comparing individual papers investigating disease states is difficult as there is no overall standard for representation of data or methods. Interpretation of part of the studies in diffuse liver disease is hampered by the notion that healthy controls were not always matched for age, weight/body mass index (BMI), gender, *etc.*; often, healthy subjects were younger than patients. As it has been shown that these factors may impact the quality of the spectra^[56] and concentrations of metabolites^[56], attempts should be made to minimize their bias. Many studies showed substantial between-subject variability within patient and control groups, e.g. with regard to PME.

Another important issue is the difference in magnetic field strengths and techniques used in different studies with regard to spatial resolution and differences in repetition times (TR), which lead to T1 weighting. T1 weighting in quantification of metabolites in liver MRS studies has so far been relatively neglected^[2]. Each data acquisition step in MRS comprises a brief excitation of nuclei of interest by irradiation with non-ionizing radiofrequency energy, followed by measurement of the signal derived from relaxing nuclei in the tissue of interest. TR is defined as the time between two subsequent radiofrequency pulses. The full information is only derived when the nuclei of interest are allowed sufficient time to fully relax. T1 weighting occurs when a new radiofrequency pulse is administered before full relaxation of the nuclei of interest. Although depending on the pulse angle, a rule of thumb is that the applied TR must be circa 5 times T1 or longer in order to obtain fully relaxed spectra. Sijens *et al.*^[2], using chemical shift imaging with 1 D phase encoding for localized measurement of ^{31}P metabolites to measure a large liver voxel, showed that, with a pulse angle of 70° at TR 1 s, concentrations of liver metabolites are only 54%-84% of those at full relaxation (TR 50 s). Importantly, T1 relaxation times may change in disease: Dagnelie *et al.*^[46] showed ATP concentrations in WL-cancer patients to be reduced relative to healthy subjects at TR 5 s and TR 20 s, but not at TR 1 s. In a later study, Leij-Halfwerk *et al.*^[51]

showed that ATP infusion induced an increase in hepatic ATP levels at TR 15 s; however, this was not observed at TR 1 s (Leij-Halfwerk *et al.*, 2002, unpublished data). Thus, accurate assessment of changes in metabolite concentrations in different conditions is only possible if TR is chosen to be sufficiently long to allow a large degree of relaxation. As the measured MR signal becomes larger as TR increases, signal to noise ratios will improve, allowing a smaller number of acquisitions per phase encoding step, thereby relatively reducing measurement time. Thus, using 4 phase encoding steps, Sijens *et al.*^[2] showed the following number of acquisitions and measurement time at TR 1, 5 and 20 s, respectively: 60 (4:08 min), 20 (7:20 min), and 8 acquisitions (13:20 min).

What are the prospects for the clinical application of MRS in diffuse liver disease? For use as a diagnostic tool, not only high sensitivity and specificity at an individual patient levels are essential, but also practicality of application including patient burden and costing. These requests are most likely to be fulfilled in inherited diseases, where marked metabolic alterations are typical, however, for the majority of diseases, other, less invasive tools will remain methods of first choice. However, MRS may be an excellent tool for assessing disease severity and for monitoring disease progress or recuperation, as shown e.g. in diabetes^[45] and patients with primary biliary cirrhosis^[13].

Finally, altered findings in MRS may predict disease progression, weight loss or survival, and thus assist in the estimation of patients' prognosis. For instance, preliminary analyses (Leij-Halfwerk & Dagnelie, unpublished observations) demonstrated that elevated baseline PME levels in WS cancer patients predicted subsequent weight loss and shorter survival.

CONCLUSION

MRS of diffuse liver disease has given important new insight in a number of diseases, including inherited diseases, hepatitis, steatosis and cirrhosis. Importantly, MRS has also allowed new insights in metabolic derangements in non-liver diseases such as diabetes and non-liver cancer. Based on the progress achieved so far by MRS studies in diabetes, it is anticipated that future technical developments such as clinical MRS magnets with higher field strength (3 T) and improved delineation of multi-component signals such as PME and PDE using proton decoupling, especially if combined with price reductions for stable isotope tracers, will lead to intensified research into metabolic syndrome, cardiovascular disease, hepatobiliary diseases, as well as non-metastatic liver metabolism in patients with a distant malignant tumor.

Of note, there are also potential drawbacks: thus, tightening regulations related to preparing and administering compounds by intravenous infusion or orally threaten to make metabolic research extremely costly (or perhaps even impossible) in the near future: a development which could have dramatic negative consequences for scientific and clinical progress in the field.

Emphasis should be placed on the development of standards of the techniques used in order to be able to compare data and to obtain valid estimates of absolute metabolite concentrations. In order to allow MRS to compete with standards of care, efforts should be made to validate results in larger patient cohorts and to minimize bias in distinguishing diseased states from healthy states.

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Liver iron content determination by magnetic resonance imaging

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Abstract

Accurate evaluation of iron overload is necessary to establish the diagnosis of hemochromatosis and guide chelation treatment in transfusion-dependent anemia. The liver is the primary site for iron storage in patients with hemochromatosis or transfusion-dependent anemia, therefore, liver iron concentration (LIC) accurately reflects total body iron stores. In the past 20 years, magnetic resonance imaging (MRI) has emerged as a promising method for measuring LIC in a variety of diseases. We review the potential role of MRI in LIC determination in the most important disorders that are characterized by iron overload, that is, thalassemia major, other hemoglobinopathies, acquired anemia, and hemochromatosis. Most studies have been performed in thalassemia major and MRI is currently a widely accepted method for guiding chelation treatment in these patients. However, the lack of correlation between liver and cardiac iron stores suggests that both organs should be evaluated with MRI, since cardiac disease is the leading cause of death in this population. It is also unclear which MRI method is the most accurate since there are no large studies that have directly compared the different available techniques. The role of MRI in

the era of genetic diagnosis of hemochromatosis is also debated, whereas data on the accuracy of the method in other hematological and liver diseases are rather limited. However, MRI is a fast, non-invasive and relatively accurate diagnostic tool for assessing LIC, and its use is expected to increase as the role of iron in the pathogenesis of liver disease becomes clearer.

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Key words: Thalassemia major; Iron overload; Magnetic resonance imaging; Liver; Hemochromatosis; Desferrioxamine; Deferiprone; Deferasirox; Thalassemia intermedia; Myelodysplastic syndromes

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INTRODUCTION

Iron homeostasis in humans depends exclusively on the modulation of iron absorption since iron excretion is passive (by shedding of intestinal and skin cells, and additionally, in women by menstruation), and cannot be actively upregulated^[1]. Therefore, patients with excessive iron absorption (hemochromatosis) or with transfusion-dependent congenital or acquired anemia are at increased risk for developing iron overload^[1]. Excessive iron is toxic because it generates free radicals and induces oxidative stress^[2]. Iron overload can result in the development of diabetes and other endocrinopathies, liver cirrhosis and hepatocellular carcinoma (HCC), cardiomyopathy and

premature death in patients with thalassemia major^[3-6] and hemochromatosis^[7-10]. In turn, the management of iron overload with chelation therapy in thalassemia major^[11-14] and with phlebotomy in hemochromatosis reduces the risk of diabetes, liver and cardiac disease and death^[7-9].

The accurate evaluation of iron overload is necessary to establish the diagnosis of hemochromatosis and guide chelation treatment in transfusion-dependent anemia^[3,15-17]. Serum ferritin levels are not an accurate measure of total body iron stores, particularly in patients with high iron burden, because concurrent conditions, particularly inflammation and liver disease, increase these levels independently of iron burden^[16,18,19]. Serum iron, transferrin, transferrin saturation and transferrin receptor levels are also imprecise markers of body iron stores^[18]. The liver is the primary site for iron storage in patients with hemochromatosis or transfusion-dependent anemia, therefore, liver iron concentration (LIC) accurately reflects total body iron stores^[1,19,20]. Liver biopsy with measurement of iron concentration by atomic absorption spectroscopy is considered the gold standard for LIC assessment^[16]. However, hepatic iron distribution appears to be uneven, particularly when cirrhosis is present, but also in the absence of cirrhosis^[21-25]. In addition, liver biopsy is an invasive procedure and complications requiring hospitalization are observed in approximately 0.5% of patients with thalassemia major who undergo liver biopsy, even in experienced centers^[26]. The risks of liver biopsy also preclude repeated biopsies, which are necessary in patients with thalassemia major in order to adjust chelation treatment and avoid iron overload and chelation-associated toxicity^[3,16,17,19].

It is apparent that there is a pressing need for non-invasive methods that can provide accurate LIC measurements. Superconducting quantum interference devices (SQUIDS) has been used for this purpose, but appear to underestimate LIC and are available in only a few centers^[16]. In the past 20 years, magnetic resonance imaging (MRI) has emerged as a promising method for measuring LIC in a variety of diseases. We review the potential role of MRI in LIC determination in the most important disorders characterized by iron overload, namely, thalassemia major, other hemoglobinopathies, acquired anemia, and hemochromatosis.

LITERATURE SEARCH

A literature search (using PubMed) was performed using the following key words: “thalassemia major”, “iron overload”, “MRI”, “liver”, “hemochromatosis”, “desferrioxamine”, “deferiprone”, “deferasirox”, “thalassemia intermedia”, “sickle cell disease”, “myelodysplastic syndromes”, “bone marrow transplantation”, “hepatitis C”, “alcoholic liver disease” and “non-alcoholic liver disease” up to 13 January 2010. The authors also manually reviewed the references of retrieved articles for any pertinent material.

MRI METHODS FOR ASSESSING LIC

The measurement of iron overload with MRI is based

on the shortening effect of the interaction of iron-containing molecules (particularly ferritin and hemosiderin) with hydrogen nuclei (mainly in water molecules) on T2 relaxation time^[19,27,28]. Hepatic iron deposition with MRI can be quantified by measuring the ratio of the signal intensity of the liver and of a reference tissue (mainly paraspinous muscle, which does not develop siderosis)^[27,28]. These signal intensity ratios (SIRs) can be derived from either spin-echo (SE) T2-weighted or from gradient recalled-echo (GRE) T2*-weighted sequences^[27,28]. Liver siderosis can also be determined by the direct measurement of relaxation time (relaxometry), either T2 [or 1/T2 (R2)] from SE sequences or T2* [or 1/T2* (R2*)] from GRE sequences; it is also possible to measure both R2 and R2* relaxation times (hybrid method)^[27,28]. SIR-measuring methods are faster than relaxometry but less sensitive, particularly in patients with severe iron overload^[27,28]. In addition, SIR-measuring methods appear to have smaller interscanner reproducibility than do relaxometry methods^[29,30]. Among relaxometry methods, R2 acquisition time is longer than R2* acquisition time^[30].

MRI IN THALASSEMIA MAJOR

LIC determination

Some early studies have assessed the accuracy of LIC quantification by liver/muscle SIR derived from either GRE or SE sequences^[21,31,32]. The correlation between LIC measured in liver biopsy and SIR was stronger when GRE sequences were used^[21,31,32]. However, both methods were inaccurate in patients with severe iron overload or liver fibrosis, who represent a sizable percentage of the thalassemia population^[21,31,33]. In contrast, the presence of viral hepatitis did not affect the correlation between MRI and liver-biopsy-based LIC measurements^[31]. More recent studies also have reported moderate correlations between SIR and LIC ($r = 0.65-0.89$)^[34-36].

Regarding relaxometry methods, two large studies ($n = 80$ and $n = 106$) have reported moderate correlations between liver T2 and T2* measured in 1.5 T scanners with LIC ($r = -0.82$ and $r = -0.81$, respectively)^[37,38]. The correlation coefficient between liver T2* and LIC was stronger in patients without hepatic fibrosis (-0.93 vs -0.68 in patients with liver fibrosis)^[38]. Two smaller studies ($n = 46$ and $n = 52$, respectively) reported relatively stronger correlations between LIC and liver R2 determined in a 1.5 T and 0.5 T imager, respectively ($r = 0.874$ and $r = 0.94$, respectively)^[39,40]. Interestingly, the presence of liver fibrosis reduced the accuracy of the method only in the 1.5 T scanner^[39,40]. Liver inflammation and chronic hepatitis C virus (HCV) infection also had no effect on the correlation between R2 and LIC in the 0.5 T unit^[39,40]. A recent study also has suggested that measuring liver R2* in higher field strength imagers (i.e. 3 T vs 1.5 T) yields less accurate measurements, particularly in patients with more severe iron overload^[41]. However, the latter studies evaluated patients with different characteristics and their results are not directly comparable^[39-41].

Several studies have shown that relaxometry methods are more accurate than SIR-measuring methods for LIC determination^[30,36,42,43]. In an early comparative study, liver R2 correlated more strongly with biopsy-determined LIC than liver/paraspinal muscle SIR measured in SE sequences ($r = 0.97$ and 0.71 , respectively)^[42]. Moreover, the presence of liver fibrosis or inflammation did not affect the correlation between liver R2 and LIC^[42]. Liver/subcutaneous fat SIR did not correlate significantly with LIC^[42]. In another early small study ($n = 10$), liver R2 relaxation time measured with a 0.5 T MRI unit was better correlated with LIC than was R2* relaxation time^[43]. In a large study in patients with thalassemia major ($n = 57$), sickle cell disease (SCD) ($n = 34$), thalassemia intermedia ($n = 6$) and other causes of iron overload (aplastic anemia, hemochromatosis and heme-metabolism defects; $n = 5$), liver R2 and R2* measured with a 1.5 T scanner showed a strong correlation with LIC ($r = 0.98$ and 0.97 , respectively; Figure 1)^[30]. R2 showed less variability between imaging slices and better reproducibility between examinations compared with R2*^[30]. Combined measurement of R2 and R2* did not improve diagnostic accuracy^[30]. We also recently showed in 94 patients with thalassemia major a strong correlation between liver R2, R2* and GRE-derived liver/muscle SIR in a 1.5 T unit^[36]. Liver R2 was more accurate than the other methods in patients with more severe iron overload^[36]. According to current guidelines for the management of patients with thalassemia major, MRI is a feasible alternative to liver biopsy for determining LIC^[16]. The use of R2 sequences and local individual calibration is recommended^[16].

LIC and iron overload in other organs in thalassemia major

Myocardial iron-overload-induced heart failure is the cause of death in approximately 60% of patients with thalassemia major^[4]. Measurement of cardiac T2* is the currently recommended method for assessing cardiac iron overload^[16]. However, several large cross-sectional studies (total, $n = 429$) have not identified a significant correlation between cardiac and liver T2*^[38,44-47]. Only one large study ($n = 180$) has reported a weak, albeit significant, correlation between liver T2* and cardiac T2* ($r = 0.18$, $P < 0.05$)^[48]. In smaller studies ($n = 46$ and 38 , respectively), liver R2 and R2* did not correlate significantly with cardiac R2 and R2*^[39,49,50]. We also found no correlation between myocardial R2* and liver R2, and R2* and GRE-derived liver/muscle SIR in 94 patients^[36]. Only a few studies have reported a significant correlation between liver T2 and heart T2^[37], or between liver R2* and cardiac R2*^[51]; however, the correlation was weak ($r = 0.34$ and 0.23 , respectively)^[37,51]. Cardiac iron overload has also been reported in patients with minimal iron deposition in the liver^[50-52]. Finally, in a recent large study in 652 patients with thalassemia major, liver T2* was very weakly correlated with cardiac T2* ($R^2 = 0.003$, $P = 0.04$)^[53]. More importantly, liver T2* was not associated with the development of heart failure or arrhythmia, whereas cardiac T2* predicted heart failure and arrhythmia^[53].

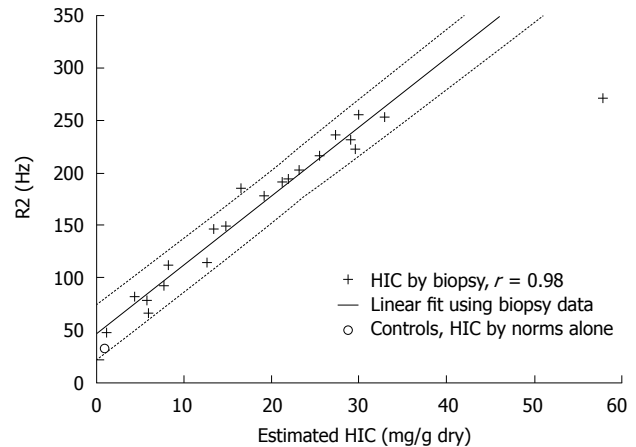


Figure 1 Plot of transverse relaxivity R2 (1/T2) vs biopsy-measured LIC in 20 patients (22 biopsies). R value was 0.98, and dotted lines indicate 95% prediction intervals for the regression. Average R2 value for 13 healthy controls is shown by "o", plotted using an LIC value estimated from normative data (no biopsy) (reproduced with permission from^[30]).

The lack of correlation between liver and cardiac iron load might be explained by different mechanisms of iron uptake in the two organs^[28]. In addition, small studies have suggested that iron deposition and chelation-induced clearance of iron from the heart lag behind liver iron changes^[50,54]. Moreover, patients receiving treatment with different chelators might experience iron clearance from the liver and heart at different rates^[46,55-58]. It appears that desferrioxamine is more or similarly effective than deferiprone in removing iron from the liver, but less effective than the latter in removing iron from the heart^[46,55-58]. Deferasirox, another oral chelator, appears to be similarly effective to desferrioxamine in reducing liver iron (assessed with liver biopsy or SQUID) when appropriately dosed^[59,60]. In uncontrolled studies, deferasirox also has reduced cardiac iron deposition (assessed with cardiac T2*)^[61,62]. These findings suggest that myocardial iron overload should also be monitored closely in patients with thalassemia major^[16]. According to recent guidelines for the management of these patients, myocardial iron deposition should be monitored with T2* MRI every year in patients with a poor chelation history, or in those with LIC, who show a non-optimal response to chelation therapy^[16].

Endocrine disorders, particularly hypogonadism and diabetes, are frequently observed in patients with thalassemia major^[63,64]. However, liver iron load does not appear to predict the extent of iron deposition in the pancreas or the pituitary gland, as assessed by SIR^[65-67], T2^[68], R2^[49] or T2*^[69]. However, in the largest study that has assessed the relationship between hepatic and pancreas/pituitary gland iron stores ($n = 180$), liver T2* correlated weakly, albeit significantly, with pancreas and pituitary gland T2* ($r = 0.35$ and 0.17 , respectively)^[48]. Liver T2* also correlated significantly with pituitary gland T2 and pituitary gland/muscle SIR ($r = 0.21$ and 0.63 , respectively)^[48]. However, liver T2* was not associated with the presence of endocrine disorders (diabetes, hypothyroidism, hypo-

gonadism and hypoparathyroidism)^[48]. No differences in liver iron overload between diabetic and non-diabetic patients were reported in other studies using T2*^[69,70] or SIR obtained from SE or GRE sequences^[34]. There was also no correlation between liver T2* and insulin resistance or pancreatic β -cell reserve^[69]. Only one small study ($n = 31$) has reported more severe liver iron overload (assessed with liver/paraspinous muscle SIR in GRE sequences) in diabetic patients^[66]. Regarding hypogonadotropic hypogonadism, one study of 36 patients has reported no difference in liver/subcutaneous fat SIR between patients with hypogonadotropic hypogonadism and those without pituitary gland dysfunction^[67], whereas another larger study ($n = 50$) has identified low liver T2* as a predictor of the presence of hypogonadism (but not of diabetes)^[70]. The apparent lack of association between the iron burden in liver, pancreas and pituitary gland might be partly due to different mechanisms of iron accumulation in these organs^[49,65-69]. In addition, pancreatic T2 and R2 are also reduced by the development of fatty degeneration, which might also confound the relationship between liver and pancreatic relaxation times in MRI^[49,66,68].

Liver MRI in patients with thalassemia major who have undergone bone marrow transplantation

Allogeneic bone marrow transplantation (BMT) from an HLA-identical donor is a potentially curative treatment for thalassemia major, particularly when pre-transplantation chelation treatment is adequate and there is no portal fibrosis or hepatomegaly^[71-74]. In these patients, liver iron overload progressively decreases without phlebotomy but may persist for 4-6 years after BMT in older patients^[75,76]. In patients who have undergone BMT for thalassemia major, higher LIC independently predicts progression of liver fibrosis^[77]. In these patients, phlebotomy reduces liver iron overload and improves liver and myocardial function^[78-80]. Liver MRI has also been used in these patients to assess iron overload^[81]. Measurement of liver T2 and T2* with a 1.5 T imager revealed iron overload in four out of eight patients, even though a mean time of 11.3 years had elapsed since BMT, and seven patients had received iron chelation treatment with phlebotomy, desferrioxamine or their combination for a mean 39 mo^[81]. In contrast, heart T2 and T2* were normal in all patients^[81].

LIC DETERMINATION WITH MRI IN OTHER HEMOGLOBINOPATHIES

Thalassemia intermedia is a diverse group of hemoglobinopathies that are characterized by less severe transfusion dependency and iron overload than in thalassemia major^[82,83]. Liver iron overload assessed with measurement of the T2* relaxation time is less pronounced in patients with thalassemia intermedia than in those with thalassemia major^[84]. Even though patients with thalassemia intermedia have liver iron overload compared with healthy controls, cardiac T2* does not differ between the former and the latter^[84]. In a study of 26 patients with thalassemia

intermedia, liver T2 strongly correlated with LIC ($r = -0.82$, $P = 0.0003$) but not with heart T2 ($r = -0.17$, $P = 0.41$)^[37]. Iron overload can develop in patients with thalassemia intermedia who have not been regularly transfused^[82,83]. In patients with thalassemia intermedia who received < 10 red blood cell (RBC) units, liver R2 revealed iron overload in two-thirds of the patients, whereas cardiac T2* was normal in all patients^[85]. In a very recent study in 49 patients with thalassemia intermedia, liver T2* revealed the presence of hepatic iron overload in 77.5% of patients, even though 44.9% had never been transfused and only 8.2% were being regularly transfused^[86]. In contrast, no patient had cardiac iron overload and liver T2* did not correlate with cardiac T2*^[86].

Beta thalassemia/hemoglobin E, a frequent hemoglobinopathy in Asia, manifests as thalassemia intermedia in half of the patients and as thalassemia major in the other half^[87,88]. Iron overload can develop even in patients who do not require regular transfusions and is due to increased intestinal absorption of iron^[87,88]. In a pivotal study in patients with beta thalassemia/hemoglobin E ($n = 41$), thalassemia major ($n = 9$) or hereditary hemochromatosis ($n = 23$), liver R2 strongly correlated with LIC measured in biopsy ($r = 0.98$, $P < 0.0001$)^[89]. However, R2 variability increased with increasing LIC^[89].

Hemoglobin H (Hb H) disease is another frequent hemoglobinopathy in Asia, which results from the deletion of three of the four α -globin genes (deletional Hb H disease) or from deletion of two α -globin genes and a non-deletional mutation of a third α -globin gene (non-deletional Hb H disease). In all cases, there is an excess of β -globin chains that form β_4 tetramers (Hb H)^[88,90,91]. The clinical phenotype of Hb H disease is variable and often resembles thalassemia intermedia^[88,90,91]. Regular transfusions are infrequently required but iron overload can develop even in never-transfused patients^[88,91]. Increased intestinal iron absorption due to hemolysis and ineffective erythropoiesis appears to explain the development of iron overload in these patients^[91]. Non-deletional Hb H disease has more severe presentation and more frequently leads to iron overload than does deletional Hb H disease^[91,92]. In an early study in 36 non-transfusion-dependent patients with Hb H disease, liver/paraspinous muscle SIR obtained from GRE sequences was more sensitive in detecting iron overload than SIR determined from SE sequences^[93]. Liver iron overload was observed in 33/36 patients, whereas iron overload in the heart and pancreas were present in only one and six patients, respectively^[93]. In a large study in 114 Chinese patients with Hb H disease, liver/paraspinous muscle SIR measured from GRE sequences revealed liver iron overload in 85% of the patients^[92]. Iron overload was present even though only one patient was transfusion-dependent and only eight had received more than five transfusions (median number of transfusions: 5.5; range: 5-20)^[92]. Patients with deletional Hb H disease had more severe iron overload than those with non-deletional Hb H disease^[92]. In a smaller recent study in 37 patients with Hb H

disease or thalassemia intermedia and serum ferritin levels > 1000 pmol/L, liver T2* was abnormal in most patients (84%)^[94]. Log-liver T2* correlated with serum ferritin levels but not with heart, pancreas or pituitary siderosis, as assessed by MRI, or with abnormalities in pancreatic function or the growth hormone axis^[94].

Chronic RBC transfusion therapy is increasingly being used in patients with SCD, particularly for the primary or secondary prevention of stroke^[95]. However, iron overload frequently develops in patients with SCD who are receiving chronic RBC transfusion therapy^[96]. The development of iron overload in these patients increases the risk for hospitalization^[97] and death^[98], whereas chelation treatment reduces mortality^[97]. In a recent study in patients with SCD, thalassemia major or bone marrow failure, liver R2* was strongly correlated with LIC assessed with liver biopsy ($r = 0.96-0.98$, $P < 0.001$), regardless of the presence of fibrosis^[99]. In another study in 35 patients with SCD, liver T2 was moderately correlated with LIC ($r = -0.80$, $P = 0.00001$) but not with heart T2 ($r = 0.10$, $P = 0.56$)^[37].

LIC DETERMINATION WITH MRI IN OTHER HEMATOLOGICAL DISORDERS

Iron overload frequently develops in patients with myelodysplastic syndromes (MDSs), which can result in abnormal liver function, diabetes and heart failure and increase the risk of death^[100-104]. In these patients, treatment with desferrioxamine improves liver, pancreas and pituitary gland function^[100,102,105]. Several small studies ($n = 10$ or 11) have assessed liver iron deposition by measuring liver T2* and have revealed liver iron overload in almost all patients^[106-109]. Log liver T2* correlates with RBC units transfused^[106,107]. In contrast, myocardial siderosis was present in only 10%-15% of patients and cardiac T2* did not correlate with liver T2*^[106,108,109]. Only one study has assessed pancreas and pituitary gland siderosis with MRI in patients with MDS, and has reported no association between liver T2* and pancreas or pituitary gland siderosis, but a correlation between log liver T2* and indices of insulin resistance and reduced beta cell reserve^[106]. According to recent guidelines, iron overload should be monitored in patients with MDS, using serum ferritin and transferrin levels, whereas liver MRI is considered useful but not essential^[92].

Iron overload is frequently observed in patients who have undergone allogeneic BMT for hematological diseases other than thalassemia major, and results primarily from RBC transfusions^[110-112]. Iron overload in these patients appears to be associated with increased risk for infections, veno-occlusive disease, hepatic dysfunction^[110,113,114] and death^[111,114-118]. Phlebotomy improves liver function in this population^[118]. It is recommended that patients who have undergone BMT and have iron overload should be treated with phlebotomy and/or chelation therapy^[112]. Liver MRI is a useful tool for quantifying body iron stores in this population^[110]. In an early study in 13 children who had

undergone autologous BMT, mostly for non-hematological disorders (neuroblastoma in 8 patients), liver iron overload assessed with liver/paraspinous muscle SIR measured in SE sequences was present in 10 patients (77%)^[119]. Liver iron overload is correlated with RBC units transfused^[119]. Three small studies ($n = 32$, 19 and 20) have revealed liver iron overload in the majority of patients (97%, 95% and 85%, respectively) who had undergone allogeneic BMT for leukemia, MDS, multiple myeloma, lymphoma, aplastic anemia or myelofibrosis, who had serum ferritin levels greater than the upper limit of the normal range, > 1000 ng/mL or > 1600 pmol/L, respectively^[120-122]. Liver iron deposition was assessed by liver/paraspinous muscle SIR measured from GRE sequences^[122], R2^[121] or T2*^[120]. Liver siderosis correlated with RBC units transfused^[122]. Patients with more advanced liver siderosis more frequently exhibited elevated transaminases, which normalized in most of them after phlebotomy^[122]. However, liver iron deposition did not correlate with heart, pancreas or pituitary siderosis as assessed by MRI, or with abnormalities in pancreatic function or the growth hormone axis^[120]. In addition, the duration of post-BMT follow-up, type of graft or conditioning, and the presence of chronic graft-versus-host-disease had no effect on the presence of liver iron overload^[121].

Two small case-series ($n = 11$ and 3 , respectively) have evaluated iron overload with MRI in patients with acute leukemia or solid tumors who received multiple RBC transfusions due to chemotherapy-induced anemia^[123,124]. Liver/muscle SIR identified liver iron overload in all patients, whereas cardiac T2* was marginally reduced and pancreas/skeletal muscle SIR was normal in all patients^[123,124].

LIC DETERMINATION WITH MRI IN NON-HEMATOLOGICAL DISEASES

Hemochromatosis is an autosomal recessive disease with variable penetrance. In most patients, it results from a mutation of the *HFE* gene, which leads to iron overload by increasing intestinal iron absorption and by enhancing iron release from macrophages after phagocytosis of erythrocytes^[7,8,15]. In an early study in 20 patients with hemochromatosis and 18 with hematological diseases (mainly MDS), liver/muscle SIR obtained from SE sequences correlated strongly with LIC ($r^2 = 0.98$)^[125]. However, in subsequent studies in patients with suspected hemochromatosis, SIR obtained from GRE sequences has provided a more accurate determination of LIC than from SE sequences^[126,127]. In a more recent and larger study in 174 patients with suspected hemochromatosis or with chronic HCV infection, Gandon *et al.*^[128] have shown that liver/muscle SIR obtained from GRE sequences identifies liver iron overload with high sensitivity and specificity. The severity of hepatic siderosis and the presence of cirrhosis does not affect the accuracy of MRI measurements. The diagnostic accuracy of the technique proposed by Gandon *et al.*^[128] has been replicated in other studies in patients

with suspected hemochromatosis, chronic HCV infection, or persistent elevation of transaminase levels^[129,130]. However, it should be mentioned that this method does not appear to be accurate in assessing LIC in patients with thalassemia major or MDS, who have higher LIC than patients with hemochromatosis^[135]. Two smaller studies ($n = 11$ and 23 , respectively) have reported a strong correlation between liver R2 and biopsy-determined LIC in hemochromatosis^[89,131]. Some experts consider liver MRI to be the method of choice for documenting iron overload in patients with suspected hemochromatosis^[15]. Nevertheless, others argue that MRI has limited sensitivity to detect mild iron overload in patients with suspected hemochromatosis^[7].

Besides hemochromatosis, mild to moderate iron overload is also present in some patients with other liver diseases, including chronic hepatitis B or C, alcoholic liver disease and non-alcoholic fatty liver disease^[1,132-136]. In chronic hepatitis B or C and alcoholic liver disease, low levels of hepcidin, a protein that regulates iron absorption, may play a role in the development of iron overload^[1,132-134,136]. Increased iron uptake from hepatocytes due to upregulation of transferrin receptors might also contribute^[136]. In non-alcoholic fatty liver disease, insulin-induced redistribution of intracellular transferrin receptors to the hepatocyte membrane, and downregulation of the iron exporter protein due to the pro-inflammatory state present in these patients, may contribute to the development of liver iron overload^[132]. MRI is a sensitive method for evaluating liver iron deposition in patients with chronic HCV infection^[128-130]. However, liver biopsy is required in most of these patients to assess the presence of inflammation, fibrosis and cirrhosis.

Increased LIC is frequently observed in patients with non-biliary cirrhosis (not due to hemochromatosis) and can occasionally be severe; in contrast, liver siderosis is rare in biliary cirrhosis^[1,23,134]. Liver/paraspinous muscle SIR obtained from SE or GRE sequences has revealed liver iron overload in 40% of cirrhosis patients^[137]. However, in studies in patients with cirrhosis of various etiologies in whom liver biopsy had been performed, there was only a moderate correlation between liver/muscle SIR in GRE sequences and histological grading of liver siderosis ($r = 0.515$, $P < 0.001$)^[138]. Moreover, others did not find a difference in liver iron load (assessed with SIR obtained from GRE sequences) between patients with viral hepatitis and cirrhosis^[139]. In addition, liver siderosis in either SE or GRE sequences did not increase with the progression of viral hepatitis-induced cirrhosis^[140]. Despite these discrepant findings, MRI might be particularly useful when cirrhosis is present because of the risk of bleeding in these patients during liver biopsy and because of the uneven iron distribution in the cirrhotic liver^[21-25].

Finally, MRI might be useful for the evaluation of iron stores in porphyria cutanea tarda, a familial or sporadic disease that is characterized by decreased activity of hepatic uroporphyrinogen decarboxylase (UROD), which is frequently associated with iron overload^[141]. In these patients, iron inhibits UROD activity, and phlebotomy is

the treatment of choice^[136,141]. A recent study in 20 patients with porphyria cutanea tarda measured liver/paraspinous muscle SIR from GRE sequences and identified liver iron overload in 11 (55%)^[142].

CONCLUSION

MRI is a potentially useful non-invasive method for evaluating liver iron stores in a wide spectrum of hematological and liver diseases. Most studies have been performed in thalassemia major and MRI is currently a widely accepted method for guiding chelation treatment in these patients. However, the lack of correlation between liver and cardiac iron stores suggests that both organs should be evaluated with MRI, since cardiac disease is the leading cause of death in this population. It is also unclear which MRI method is the most accurate because there are no large studies that have directly compared the different techniques. Another issue is the reproducibility of the various methods in different centers. Liver/muscle SIR obtained from GRE sequences^[128-130] and liver and heart T2* have been shown to be reproducible in different scanners^[143-145] but more data are needed. The role of MRI in the era of genetic diagnosis of hemochromatosis is also debated, whereas data on the accuracy of the method in other hematological and liver diseases are rather limited. However, MRI is a fast non-invasive and relatively accurate diagnostic tool for assessing liver iron content, and its use is expected to increase as the role of iron in the pathogenesis of liver disease becomes clearer.

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Perfusion magnetic resonance imaging of the liver

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INTRODUCTION

Perfusion magnetic resonance imaging (MRI) refers to imaging of tissue blood flow (i.e. tissue microcirculation), which is beyond the resolution of the MR scanner to directly visualize. In the liver, perfusion MRI can be applied to measure microcirculation in the liver parenchyma or in tumors. Whilst there are techniques available for direct measurement of blood flow in macroscopic vessels, such as the portal vein and hepatic artery, such as Doppler ultrasound (US), contrast-enhanced US, xenon computed tomography (CT) or phased contrast MR angiography; these are beyond the scope of the current review. Instead, our discussions are primarily focused on dynamic contrast-enhanced (DCE) MRI techniques with tracer kinetic modeling, which allows for the quantitative characterization of parenchymal and tumor microcirculatory alterations in the liver. Selected DCE liver perfusion studies using other imaging modalities are used to illustrate the value of perfusion imaging.

In the liver, conventional characterization of focal liver lesions is reliant on observing the rate and pattern of contrast enhancement assessed visually on DCE scans. The rate and pattern of contrast enhancement reflects the time evolution of the contrast agent within the liver tissue, which occurs as a result of the microcirculatory

Abstract

Perfusion magnetic resonance imaging (MRI) studies quantify the microcirculatory status of liver parenchyma and liver lesions, and can be used for the detection of liver metastases, assessing the effectiveness of anti-angiogenic therapy, evaluating tumor viability after anti-cancer therapy or ablation, and diagnosis of liver cirrhosis and its severity. In this review, we discuss the basic concepts of perfusion MRI using tracer kinetic modeling, the common kinetic models applied for analyses, the MR scanning techniques, methods of data processing, and evidence that supports its use from published clinical and research studies. Technical standardization and further studies will help to establish and validate perfusion MRI as a clinical imaging modality.

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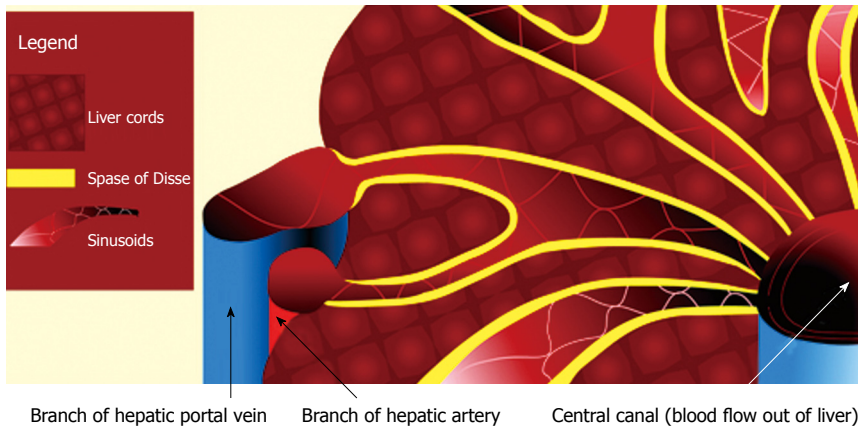


Figure 1 Schematic diagram illustrating the vascular architecture of the liver. Note the dual blood supply into the liver derived from the portal vein and hepatic artery. The vascular inflow is channeled into the hepatic sinusoids, which normally communicate freely with the Space of Disse (yellow). The Space of Disse is an interstitial space that lies between the sinusoids and the liver cords. From the hepatic sinusoids, blood is drained out of the liver via branches of the hepatic vein.

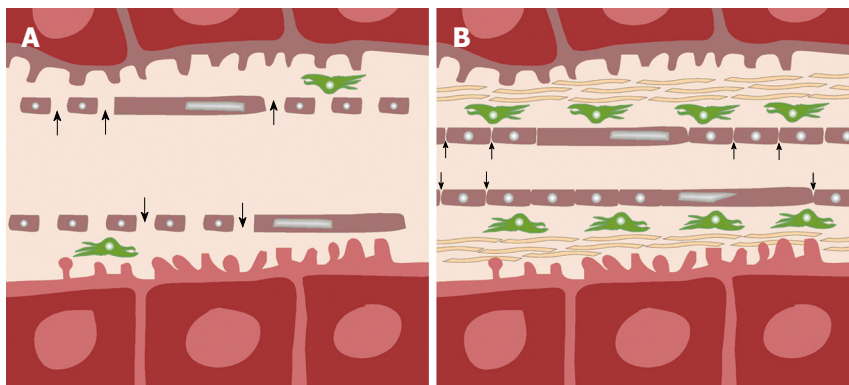


Figure 2 Schematic diagram showing pathological differences between normal (A) and cirrhotic (B) liver. In normal liver (A), normal fenestrae along the hepatic sinusoids allow free passage of blood (arrows) into the Space of Disse, in which, stellate cells (green) are found. In liver cirrhosis (B), there is an increase in the number of stellate cells, associated with deposition of collagenous fibers in the Space of Disse, and loss of fenestrae as the sinusoids become more capillary-like. As a result, transfer of low-molecular-weight compounds (e.g. contrast medium) from the sinusoids into the Space of Disse becomes more impeded (small arrows).

pathophysiological changes. Perfusion MRI could extend the currently used qualitative assessment applied for the differential diagnosis of focal liver lesions, by applying quantitative metrics to describe their vascular behavior.

In the treatment of liver tumors, current therapy for hepatocellular carcinoma (HCC) includes novel anti-angiogenic agents such as sorafenib. As these drugs may have significant clinical effects without causing tumor shrinkage, the microcirculatory characteristics, such as blood flow and tumor capillary permeability, have the potential to be response biomarkers, which allow these drug effects to be confidently assessed. Furthermore, it may also be possible to explore whether the quantitative microcirculatory parameters correlate with drug exposure and whether they can predict response. Quantitative vascular measurements could also be applied to assess the efficacy of local tumor ablation such as by trans-arterial chemo-embolization (TACE), radiofrequency ablation (RFA) or yttrium-90 microsphere embolization.

The early detection of liver metastases remains challenging, but changes in the relative hepatic arterial *vs* portal venous blood supply allow earlier detection of microscopic liver metastases^[1]. Likewise, changes in the relative contribution of hepatic arterial and portal blood flow are also observed in patients with liver cirrhosis^[2-8].

Central to the assessment of liver perfusion are model-based or model-free methods that analyze the contrast concentration-time curve in focal liver lesions or liver parenchyma, derived from the DCE-MRI images. Although similar concentration-time curves may be obtained using

CT and nuclear medicine studies, radiation burden is a practical concern, which becomes even more significant on repeated measurements. The ability of MRI to acquire such information without radiation burden and in a potentially more favorable scan plane (e.g. oblique coronal), to demonstrate the vascular input into the liver, are important advantages.

NORMAL AND ABNORMAL LIVER CIRCULATION

The liver is a highly vascular organ that consists of a series of porous vascular channels (sinusoids with fenestrae) that are predominantly supplied by the portal vein (75%) and supplemented by the hepatic artery (25%)^[9]. The two arterial inputs mix in the sinusoids at different time intervals to supply the liver cords. There is a small space (Space of Disse) that separates the sinusoids from the tightly ordered hepatic cords, which comprise two rows of closely apposed hepatocytes. The Space of Disse may be considered as an interstitial space within the liver. However, due to the large size of the fenestrae of the sinusoids, there is usually free exchange of low-molecular-weight compounds (e.g. gadolinium contrast medium) between the vascular space (sinusoids) and the interstitial space (Space of Disse) (Figures 1 and 2)^[10].

In cirrhosis, due to sinusoidal capillarization, there is loss of normal fenestrae, due to deposition of basement membrane and new formation of capillary tight junctions along the sinusoids. There is also deposition of fibers by

activated Ito cells (hepatic stellate or antigen presenting cells), which results in enlargement of the Space of Disse. Consequently, transfer of low-molecular-weight gadolinium contrast medium from the vascular sinusoids into the interstitial space becomes increasingly impeded (Figure 2)^[11].

In liver metastases and HCC, tumor blood supply is initially derived from proliferation of the sinusoidal cells that become capillarized with loss of fenestrae and formation of basement membrane. This also results in a significant barrier to the free passage of low-molecular-weight contrast medium between the sinusoidal space and interstitial space of the tumor. As the tumor continues to grow, there is recruitment of new vessels directly supplied by the hepatic artery (neoarteriogenesis). This is a prominent feature of HCC but can also be seen in the peritumoral area of liver metastases^[12-15]. Such arterIALIZATION of the vascular supply is typical of malignant liver tumors.

MRI TECHNIQUE FOR MEASURING HEPATIC PERFUSION

For perfusion MRI of the liver, injection of a low-molecular-weight gadolinium-chelate contrast is necessary, and this is administered through a wide bore (20G or larger) intravenous cannula sited within a large antecubital vein. Contrast medium is injected using a programmable pump injector, which ensures uniform and rapid contrast delivery as a tight bolus. The amount of contrast medium administered is based on body weight. For example, using Gd-DTPA (Magnevist®, Bayer-Schering, Germany), 0.1-0.2 mmol of contrast medium/kg body weight is typically administered. Once the contrast medium is injected, imaging of the liver commences using an MRI sequence that is capable of rapid and repeated measurements, to enable the passage of contrast medium through the liver to be tracked accurately. Liver perfusion imaging is typically performed using T1-weighted MRI sequences. On T1-weighted imaging, liver or tumor perfusion is observed as increasing enhancement as contrast medium passes through the liver or tumor.

With regard to technical details, a T1-weighted 3D spoiled gradient echo technique with variable flip angles is useful. Parallel imaging could be applied to reduce scan time and improve temporal resolution. Compared with 2D imaging sequences, the 3D technique eliminates inaccuracies due to the radiofrequency excitation pulse profile, and also has the advantage of better signal-to-noise ratio. However, the peripheral image sections may still have to be excluded from analysis because they may have unfavorable slice profiles or be degraded by wrap artifacts that result from phase under-sampling. T1 mapping or calibration, a necessary step for quantitative analysis, can be performed using the variable flip angle method described by Wang *et al.*^[16]. As the gadolinium contrast concentration is inversely proportional to change in the reciprocal of T1, a gadolinium contrast concentration-time curve of the liver or tumor can be generated, which is then used to derive quantitative vascular perfusion

Table 1 Illustrative example of a perfusion MRI sequence performed on a 1.5 T MR platform

MRI platform	Avanto (Siemens, Erlangen, Germany)
Type of pulse sequence	3D FLASH
TR	2.72 ms
TE	1 ms
Partition thickness	8 mm
Slices per slab	10
Matrix	256 × 159
Phase encode direction	Anterior to posterior
Number of averages	1
Sensitivity encoding factor	2
Flip angle before contrast	2° and 14°
Flip angle after contrast	14°
Bandwidth	490 Hz
RF spoiling	Yes
Temporal resolution	1.98 s per slab of 10 slices.
Precontrast scans	10 measurements of each flip angle averaged for calculation of native T1
Gadolinium injection	0.2 mmol/kg at 3 mL/s followed by 20 mL flush
Patient respiration	Quiet breathing
Post contrast scans	A total of 180 consecutive measurements. Inject contrast only when the 20th measurement has been completed
Scan sections to use for processing	Center 6 image sections only

MRI: Magnetic resonance imaging; FLASH: Fast low-angle shot; TR: repetition time; TE: Echo time.

indices. Table 1 provides an example of a liver perfusion MRI protocol implemented on a commercial scanner. It is important to note that such an imaging sequence may vary from one imaging platform to another, and it is important to engage the help of an experienced clinical scientist to ensure that the performance of the sequence is optimized. Although some workers have advocated simpler methods of estimating gadolinium concentration by measuring differences in signal intensities before and after contrast arrival in the liver, we would like to caution that the success of this simplified approach might be dependent on the imaging sequence, as well as the range of signal intensity and tracer concentration encountered.

In order to track liver perfusion reliably, MRI of the liver should employ a high temporal resolution technique (i.e. repeated imaging of the same area in the liver about every 4 s), in a scan plane that shows the lesion or area of interest. Ideally, the aorta and the portal vein should be included in the same image sections. Hence, an oblique imaging plane (e.g. oblique coronal) afforded by the MRI technique would be particularly helpful to ensure all these structures are included. The signal intensity changes within these structures, together with the T1 calibration maps, are used to derive the gadolinium contrast concentration-time curves in the liver and tumors. Once the gadolinium contrast concentration-time curve in an area of interest is known, knowledge of the contemporaneous contrast enhancement within the aorta and portal vein allows analytic methods to be applied to extract quantitative or semi-quantitative parameters that describe the vascular

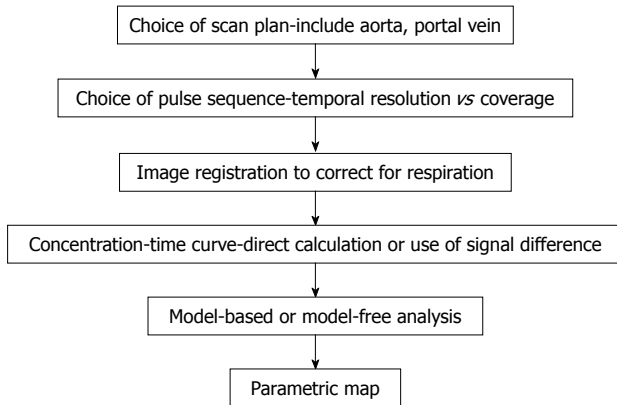


Figure 3 Chart shows workflow involved from data acquisition to obtaining vascular information by perfusion MRI of the liver.

properties. The MR images may also be qualitatively assessed by visual survey for areas of increased enhancement relative to the normal liver parenchyma. However, such comparisons can be subjective. The various approaches to extracting quantitative and/or semi-quantitative liver perfusion data are discussed in the next section.

One of the challenges to acquiring high quality liver perfusion MRI is respiratory motion, which can substantially degrade image quality. It is currently a subject of debate whether to acquire images during breath-hold or quiet respiration. Imaging in quiet respiration is widely performed, which allows rapid uninterrupted image acquisition for high temporal resolution data. However, the images often need to be aligned using image registration techniques, which can be complex, prior to quantitative analysis. Furthermore, respiration can result in through-plane motion and non-linear tissue deformation that cannot be easily overcome. For these reasons, imaging in sequential breath-hold has been advocated as a method to minimize the effects of respiratory motion. Images are acquired during suspended respiration (usually expiration), followed by a short period of normal breathing, after which respiration is again suspended for image acquisition. Imaging during expiration can be monitored by navigator control. Breath-hold imaging minimizes the need for complex image registration, but the main potential disadvantage is decreased temporal sampling because images cannot be acquired continuously. One method which can be used to improve temporal sampling of breath-hold studies is to acquire two datasets (instead of one) during each breath-hold^[17]. Single breath-hold studies have also been reported. In single breath-hold studies, patients are required to breath-hold for 40–60 s during which the first passage of gadolinium contrast is observed^[18].

EXTRACTING LIVER PERFUSION INFORMATION FROM MRI DATA

Once the MRI data have been acquired, they have to be analyzed in a meaningful way to extract information

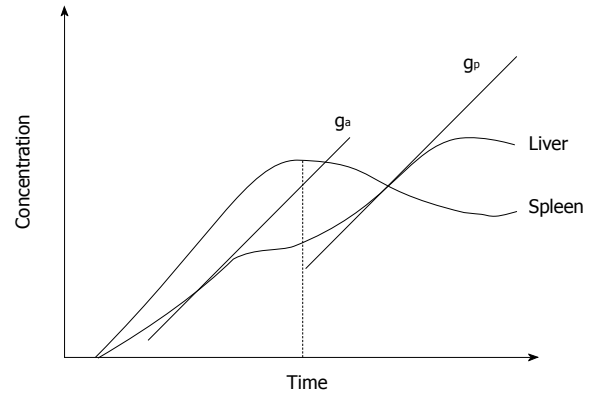


Figure 4 Schematic plot of gadolinium concentration-time curve in liver tissue obtained from a perfusion MRI study. The diagram illustrates how the maximum gradient for arterial perfusion (G_a) and portal perfusion (G_p) are derived (based on Miles *et al.*^[2]). Note that the peak splenic enhancement is used to define the transition between arterial and portal phase of liver parenchyma enhancement. The maximum slope after the peak splenic enhancement is used to define portal perfusion.

that describes tissue vascularity. Dynamic MRI data can be processed using a model-free or a model-based approach, with the former being simpler to implement.

Using model-free approaches, semi-quantitative descriptions of liver perfusion can be derived by observing the rate of liver tissue signal change in the arterial and portovenous phases of contrast enhancement. One semi-quantitative description of liver vascularity is the hepatic perfusion index (HPI), which describes the relative contribution of arterial vs portovenous flow to the total liver perfusion. The HPI has been investigated using different imaging techniques, and appears to provide biologically meaningful information despite its relative simplicity.

However, the quantitative model-based approach is appealing because it may provide more sophisticated descriptions of tissue vascular properties, by underpinning the data analysis on mathematical assumptions that reflect alterations in the underlying pathophysiology. Both model-free and model-based approaches are being widely investigated; each has provided unique information that has shown to improve liver disease assessment.

A simplified schema which shows the workflow for performing DCE-MRI is shown in Figure 3.

Model-free approaches for vascular quantification

These methods are based on simply observing the MR signal changes that result from the passage of contrast agent through the liver parenchyma or liver tumor, but may not directly relate these to the contemporaneous change in the aorta or portal vein. Disease characterization is based on the fact that the perfused liver or tumor shows enhancement with the arrival of contrast agent and therefore perfusion can be estimated by the rate of tissue enhancement. Hence, most model-free approaches use parameters derived from the initial slope of the tissue signal intensity-time or contrast concentration-time curve (Figure 4).

HPI

One semi-quantitative index that can be calculated using the model-free approach is the HPI. The HPI was first proposed by Miles *et al*^[2]. HPI refers to the proportion of hepatic perfusion that is derived from the hepatic artery, and it can be calculated using the following formula: arterial perfusion/arterial perfusion + portal venous perfusion). Miles *et al*^[2] have used the time to peak in the splenic enhancement curve to distinguish between the arterial and portal venous phases of the liver. Liver arterial perfusion is then estimated by the maximum slope in the liver enhancement curve before the splenic peak, divided by the peak aortic enhancement (Figure 4). Correspondingly, liver portal venous perfusion is estimated by dividing the maximum slope in the liver enhancement curve after the splenic peak by the peak aortic enhancement (Figure 4).

Blomley *et al*^[3] have further refined calculation of portal venous perfusion by removing the contribution of the hepatic artery from the liver contrast concentration-time curve by subtracting from it a scaled splenic contrast concentration-time curve. This is because the spleen is predominantly supplied by the aorta and not by the portal vein. Using the arterially subtracted liver contrast concentration-time curve, the portal venous perfusion is estimated by dividing the maximum slope of the arterially subtracted liver curve by the peak portal enhancement. This more direct method of estimating portal vascular contribution has also been advocated by Tsushima *et al*^[19]. However, liver arterial perfusion can be estimated in a similar way as proposed by Miles *et al*^[2].

The key advantage of using model-free approaches is that they are relatively easy to derive and do not require complex computation.

Understanding tracer kinetic modeling for perfusion MRI

Applying tracer kinetic modeling enables quantitative vascular information to be extracted from temporally sampled MRI data, when the passage of contrast medium through the liver parenchyma or tumor is observed over time. Generally, an assumption is made that the imaged voxel contains a supplying impermeable artery that leads to a permeable capillary that leaks tracer (gadolinium contrast) into the interstitial space (Figure 5). The tracer is cleared from the voxel *via* an impermeable vein. However, the vessels and the interstitial space are beyond the resolution of the MRI scanner to directly image. What is measured by the MRI scanner is the average concentration of the tracer at any one time (reflected by the measured signal intensity) within the image voxel, which changes as contrast medium courses through.

Tracer kinetic modeling uses mathematical curve fitting to describe the tissue contrast concentration-time curves. If the contrast concentration-time curve of the vascular supply is known (arterial input function), subsequent mathematical operations by convolution or deconvolution allow quantitative vascular parameters to be derived that best fit the tumor or tissue contrast

concentration-time curves. Such an approach could be applied to tumor and non-tumor tissues, although the mathematical and pathophysiological assumptions may be different for each. Using one particular kinetic model (e.g. distributed parameter model), quantitative parameters such as hepatic arterial flow, portal venous flow, fraction of total flow contributed by hepatic artery, capillary permeability-surface area product (PS), percentage of intravascular space (v_1), percentage of interstitial space (v_2), and mean transit time (MTT) are derived. However, depending on the mathematical model applied and physiological assumptions made, variants of such quantitative parameters are obtained. Hence, when applying tracer kinetic modeling to clinical studies, it is important to state the choice of kinetic model employed at the outset. Currently, there is no consensus as to which kinetic model is best suited to evaluate the liver, and development of an international consensus in this area would be welcomed.

It has been observed that a hypervascular tumor (usually a tumor with a larger vascular space relative to the interstitial space) shows a pattern of rapid arterial enhancement followed by washout, whereas a hypovascular tumor (usually a tumor with a larger interstitial space relative to the vascular space) shows progressive enhancement. These observations could also be explained by considering tracer kinetic modeling.

A hypervascular tumor is predominantly supplied directly by hepatic arterial neovessels. As the intravascular space is relatively larger than the interstitial space, the average concentration imaged by the voxel predominantly reflects changes in the intravascular space. In such lesions, there is rapid and strong enhancement in the arterial phase. However, in the equilibrium phase, the contrast redistributes to the interstitium and the rest of the body. This reduces the concentration of tracer in the tumor vascular space and is visualized as contrast wash-out within the voxel.

A hypovascular tumor may be supplied by the hepatic artery and the portal vein. As the interstitial space is relatively larger than the intravascular space, the averaged contrast medium concentration within the image voxel predominantly reflects changes in the interstitial space. Hence, there is faint enhancement in the arterial phase as contrast agent diffuses into the relatively large interstitial space. With continued blood flow in the portal venous phase, more contrast medium diffuses outwards and the concentration in the interstitium increases. In the equilibrium phase, although the tracer concentration in the vascular space has reduced due to redistribution to the rest of the body, it is still higher than the concentration in the tumor interstitium. Hence, there may even be a net efflux of contrast medium into the interstitium in the equilibrium phase, and such a tumor may demonstrate a pattern of progressive enhancement.

Clearly, the above explanations could be simplistic, as other measurement factors determine the degree of tumor enhancement. Nevertheless, these descriptions

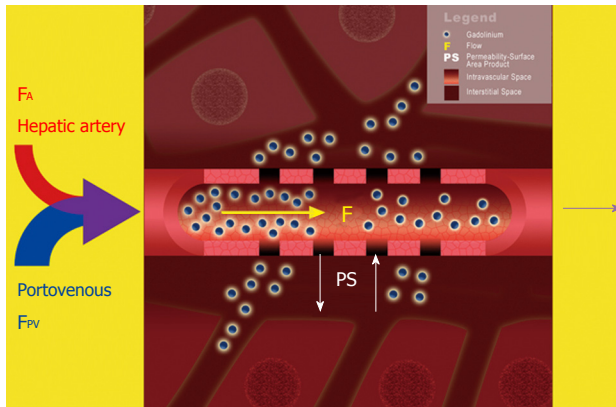


Figure 5 Schematic diagram illustrating a dual-input dual-compartment tracer kinetic model. Dual blood supply carrying gadolinium contrast molecules (blue spheres) from the hepatic artery (F_A) and portal vein (F_{PV}) enters the hepatic sinusoids (intravascular space). From here, the contrast molecules can leak outwards into the Space of Disse (interstitial space). Using a dual-input, dual compartment tracer kinetic model allows the estimation of intravascular properties (e.g. blood flow, F), as well as characteristics of the interstitial space (e.g. PS).

can help us to appreciate how the pathophysiological differences in different disease processes could account for their imaging behavior.

Model-based vascular quantification

Several kinetic models are currently in use for the assessment of liver perfusion. A detailed mathematical analysis of these is beyond the scope of this review. Kinetic models applied to the liver vary according to the physiological assumptions made, and can broadly differ in the following ways.

Single-input vs dual-input: Single-input models assume that the vascular input is derived from the hepatic artery only, whereas dual-input models assume that the vascular input is derived from both the hepatic artery and the portal vein. Dual-input models are more physiological, although in tumors, where the blood supply is highly arterialized, assumption of a single vascular input may suffice to approximate the vascular behavior.

Single-compartment vs dual-compartment: Single compartment models assume that the contrast is confined to only one compartment (i.e. vascular space), whereas dual compartment models assume that there is dynamic distribution of contrast between two compartments (i.e. the vascular space and the interstitial space). Single-compartment models are computationally simpler and could be applied as an approximation for the normal liver, because the Space of Disse communicates freely with the sinusoids. Dual-compartment models are computationally more demanding but may give a better reflection of the microcirculation of the diseased liver, resulting from tumor or cirrhosis^[4,20] (Figure 6).

Conventional compartment (CC) model vs distributed parameter (DP) model: In several kinetic models,

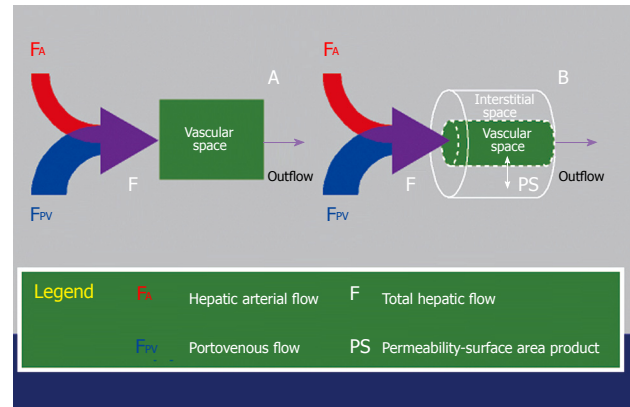


Figure 6 Schematic diagram illustrating the key difference between a single-compartment model (A) and a dual-compartment tracer kinetic model (B). Using a single-compartment model, only the vascular compartment is considered and kinetic properties related to this (e.g. blood flow, F) can be estimated. The behavior of the normal liver can be approximated by a single-compartment model. Using a dual-compartment model, kinetic properties that describe the interstitial space (e.g. PS) can be quantified in addition. In disease states (e.g. liver cirrhosis and tumors), the vascular behavior of these tissues are better described using a dual-compartment model.

the assumption is made that there is instantaneous mixing or equilibrium of the contrast medium along the entire course of a vessel. We term these as CC models. One example of a CC model is Toft's model^[21]. Another approach takes into account a concentration gradient within the vascular space. This approach is called the DP model. CC models are simpler to implement and computationally easier to solve but make more assumptions about the microcirculation. By comparison, DP models are more complex, which attempts to make fewer assumptions about the microcirculation, but are computationally more intensive and require higher temporal resolution data to derive meaningful results.

Derived microcirculatory parameters: Generally, dual compartment models are necessary to extract parameters that describe the interstitial space, e.g. v_2 , PS and extraction fraction (EF) using the DP model; or extracellular extravascular volume using the Toft's model. Such parameters cannot be derived using a single-compartment model. Single compartment models allow for estimates of blood flow, volume of distribution and mean transit time.

Clearly, the choice of the kinetic model depends on many factors including local expertise, available software to perform the perfusion analysis, understanding of the disease pathophysiology, MRI measurement technique applied, and the quality (spatial and temporal resolution) of the MRI data. At the time of writing, only a few commercial softwares are available to undertake such analyses, and many research groups are therefore reliant on self-scripted software for data evaluation. Not surprisingly, this has led to disparate efforts in developing analysis tools, which are often institution-specific and non-standardized. The lack of standardization in the methodologies applied

Table 2 Examples of the types of tracer kinetic models that have been applied for perfusion MRI of the liver

Study	Diseases	Comment
Single-input, single compartment, CC model		
Scharf <i>et al.</i> ^[48]	Preclinical study in pigs	Experimental model of normal liver in pigs. Only arterial input from hepatic artery taken into account. Such a model may lack physiological realism, especially when there is substantial vascular input contribution from the portal vein
Single-input, dual-compartment, DP model		
Sahani <i>et al.</i> ^[30]	HCC	Single input assumed because majority of vascular input to HCC is derived from hepatic artery. Dual-compartment model used to probe interstitial space and PS, which can be substantial in tumors. DP model implemented as standard on General Electric (GE) perfusion software 2.0 used for analysis
Dual-input, single-compartment, CC model		
Materne <i>et al.</i> ^[45-47]	Normal and cirrhotic livers	Assumption of single compartment based on understanding that the fenestra in the sinusoids of liver are extremely porous and allows free exchange of low-molecular-weight contrast tracers between the vascular and the sinusoidal interstitial space. To simplify calculations, assumption was made that there was instantaneous mixing of contrast medium from the dual input ^[6,7,27,45-47,49] within the single compartment. In this way, quantitative parameters such as arterial perfusion, portal venous perfusion, MTT and volume of distribution (Ve) could be derived. Cuenod <i>et al.</i> ^[27,49] applied a deconvolution technique to fit these parameters, and variants of such a model were also used by Funabasam <i>et al.</i> ^[50] and Miyazaki <i>et al.</i> ^[51]
Cuenod <i>et al.</i> ^[27,49]	Metastatic disease	
Dual-input, dual-compartment, DP model		
Koh <i>et al.</i> ^[4,20]	Metastases, HCC and cirrhosis	The DP model applies a concentration gradient within the vascular space. Parameters derived include, arterial flow, portal venous flow, fractional arterial flow, permeability, fractional intravascular space, fractional interstitial space, MTT, contrast arrival time. A dual-input dual-compartment approximation of the DP model is used commercially (CT Perfusion 3.0; General Electric, Milwaukee, USA) and was also adopted by Chen <i>et al.</i> ^[42,52,53] in perfusion studies of the liver

CC: Conventional compartment; HCC: Hepatocellular carcinoma; DP: Distributed parameter; MTT: Mean transit time; CT: Computed tomography.

for data analysis is an acknowledged issue by researchers in the field, and there is fortunately increasing international efforts towards the harmonization and standardization of imaging acquisition and data analysis. Such developments are paramount for the wider clinical adoption of the technique and institution-vendor partnerships are being developed to address such challenges.

Some of the kinetic modeling approaches that have been applied in the published literature are summarized in Table 2.

CLINICAL APPLICATIONS OF PERFUSION MRI IN THE LIVER

Liver metastases

Model-free approach: Several studies have demonstrated the potential of perfusion imaging to detect changes in the liver of patients at risk of liver metastases. Totman *et al.*^[22] have shown that there is a difference in the portal perfusion index (PPI) between patients with and without cancer. Leggett *et al.*^[11] have observed that the HPI is elevated in patients with overt metastases compared with those without metastases. In another study, Tsushima *et al.*^[23] have found that the normal looking liver in a patient with liver metastases shows a reduction in the PPI, which suggests that there is potential for using liver perfusion studies to detect microscopic metastases. These cross-sectional imaging observations have been corroborated by nuclear scintigraphy studies^[24,25]. Despite the reported relationship between high HPI and low PPI with increased likelihood of liver micrometastases in patients with cancer, few studies have had longitudinal follow-up to validate the subsequent development of macroscopic liver metastases. For example, in the study by Leggett

et al.^[11], follow-up data were only available in eight patients, of which three who subsequently developed macroscopic liver metastases showed decreased portal perfusion. Although there is great potential to use liver perfusion studies to detect or predict microscopic liver metastases, more work involving longitudinal studies is required to establish its clinical role (Figure 7).

Meijerink *et al.*^[26] have found functional liver perfusion maps to be helpful supplements to the routine radiological diagnosis of liver metastases. Compared with routine four-phase CT, total-liver-volume CT perfusion studies with calculation of the HPI increased the sensitivity of metastases detection to 89.2% from 78.4% and specificity to 82.6% from 78.3%. Four out of a total of 37 metastases were detected with the help of perfusion maps. Perfusion CT maps increase the conspicuity of metastatic disease because of the increased perfusion at the tumor rims, which makes liver metastases appear larger, thus facilitating the detection of smaller lesions.

Model-based approach: There have been few studies using a model-based approach for evaluating liver metastases. Cuenod *et al.*^[27] have applied a dual-input single-compartment model in rats and have observed that the presence of micrometastases in an apparently macroscopically normal liver resulted in a 34% decrease in portal blood flow and a 25% increase in the MTT for blood to pass through the liver. The changes were similar for macrometastases but more marked.

Koh *et al.*^[20] have employed a dual-input dual-compartment DP model in three patients with liver metastases for a technical validation study. Using the particular model, they found that the normal liver had near zero interstitial space volume and PS (which may be explained by the

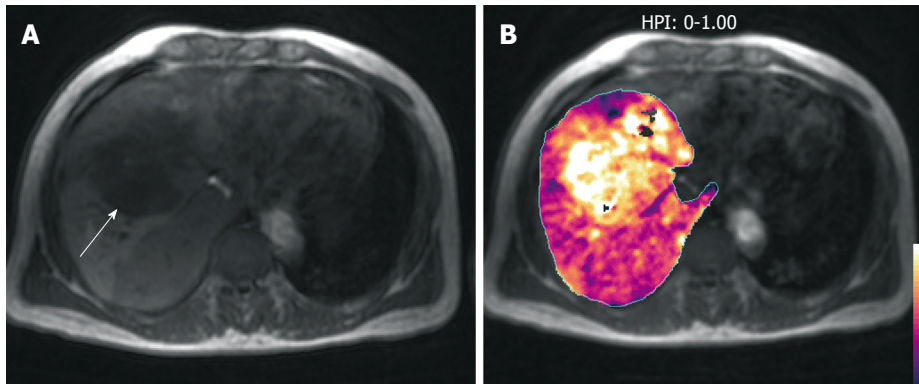


Figure 7 A middle-aged man with colorectal liver metastases to the liver. A: T1-weighted axial MR image demonstrates a hypointense liver metastasis in the right liver lobe (arrow); B: HPI map (calculated by the method described by Miles *et al*^[23]) overlaid on the T1-weighted image shows increased HPI within the metastasis, typical of malignant disease.

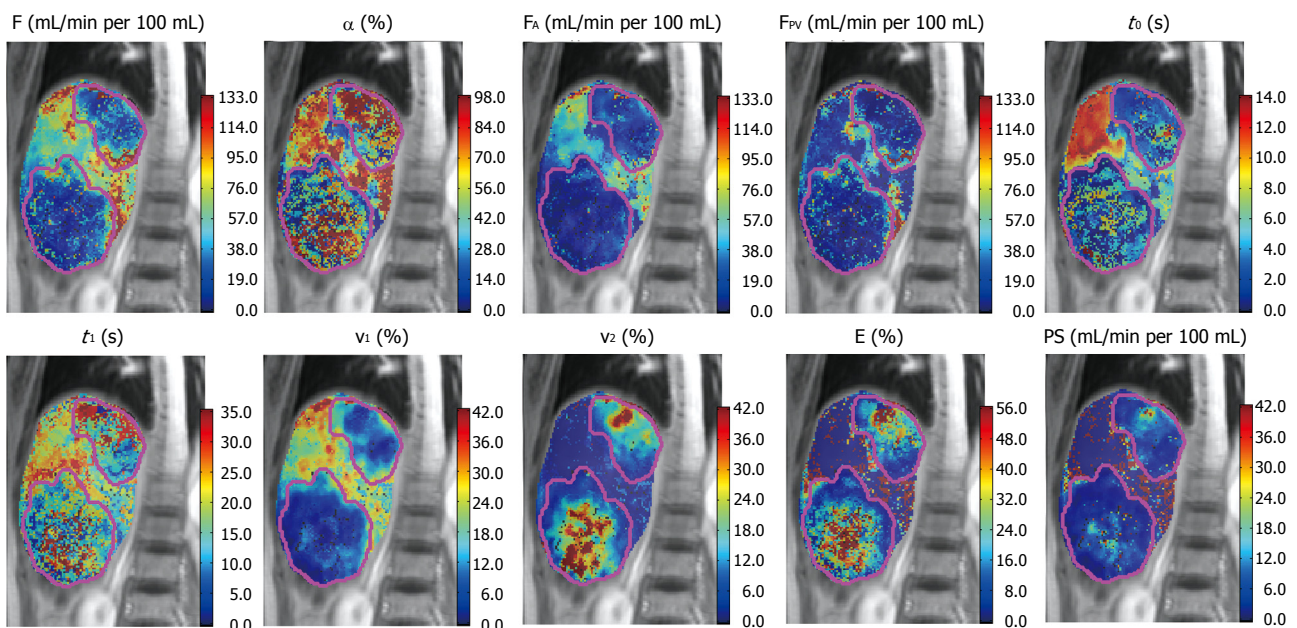


Figure 8 Parametric maps of a patient with colorectal metastases derived from a dual-input, dual-compartment, DP tracer kinetic model. F (blood flow), α (arterial fraction, or % hepatic arterial flow from total hepatic blood flow), F_A (hepatic arterial blood flow), F_{pv} (hepatic portal venous blood flow), t_0 (contrast arrival time), t_1 (MTT), v_1 (fractional intravascular volume), v_2 (fractional interstitial volume), E (extraction fraction), PS (permeability-surface area product). Note that in the two liver metastases demonstrated (outlined in pink), the lesions were characterized by lower total blood flow, but higher arterial fraction and fractional interstitial volume.

large fenestrae allowing free contrast medium exchange between the Space of Disse and the liver sinusoids), and that liver metastases have a non-zero and finite interstitial space volume and PS. Using this quantitative technique, there is an increase in the arterial fraction (arterial flow/sum of arterial and portal flow) to the metastases, although the portal flow remains significant (Figure 8). These observations of the difference in kinetic modeling behavior between the normal liver and liver metastases are supported by histopathological observations reported by Liu and Matsui^[12]. Thus, the dual-input dual-compartment DP model appears to have the potential to characterize microcirculatory pathophysiology but larger studies are required to confirm the initial findings.

HCC

Model-free approach: Abdullah *et al*^[28] have studied the differences between colorectal metastases and HCC and have found no significant difference in HPI between the two malignant entities. However, there is an increase in

arterial flow, portal flow, and total blood flow in HCC compared to colorectal metastases. The distribution volume is also observed to be higher in HCC.

Model-based approach: Fournier *et al*^[29] have applied the dual-input single-compartment model in a rat HCC model and have found that HCC is characterized by higher arterial flow and lower portal flow.

Sahani *et al*^[30] have employed a single-input dual-compartment DP model in patients with HCC and have found that blood flow, blood volume and PS are higher in well-differentiated HCC compared with moderately or poorly differentiated HCC, which suggests that such vascular quantification could yield information on tumor grade.

Koh *et al*^[4] have used the dual-input dual-compartment DP model in four patients with HCC and have measured the fractional interstitial space and associated extravasation parameters (PS and extraction ratio). HCC is characterized by increased arterial flow, increased total blood flow,

as well as early contrast agent arrival time. The early arrival of contrast agent is postulated to be related to arteriogenesis and direct tumor supply by branches of the hepatic artery. Tumor vascularity (fractional intravascular volume) was higher for two out of four patients. Portal venous flow decreased but remained significant. The derived microcirculatory parameters were supported by histopathological findings of arteriogenesis in HCC^[15,31]. Together with other imaging modalities such as diffusion-weighted MRI, MR perfusion imaging has the potential to contribute significantly to the multi-parameteric functional assessment of the liver to improve the diagnosis and characterization of HCC^[32].

Assessment of treatment response

Anti-angiogenic agents have emerged as a class of anti-tumor agents that target tumor vasculature. The rationale of anti-angiogenic therapy is based on the observation that tumors require new blood vessels for growth and survival. Based on the diffusion distances of oxygen and nutrients, tumor cells cannot survive if they are further than 2 mm from a blood vessel. Thus, effective anti-angiogenic therapy acts by depriving a growing tumor of its nutrients, and can thus curb the growth of the primary tumor as well as its metastasis.

However, current methods of assessing efficacy of chemotherapy, such as the Response Evaluation Criteria in Solid Tumors (RECIST), are based on observing a decrease in tumor size^[33]. Effective anti-angiogenic therapy often manifests as lack of tumor growth rather than decrease in tumor size because the therapy is not cytotoxic. Hence, there is a need for a reliable response biomarker to assess the efficacy of such therapy.

The challenge for a reliable response biomarker holds true for HCC that is treated by local tumor ablation. Ablated tumor differs from viable tumor in its blood supply. Previous international consensus conferences have recommended that the RECIST criteria be modified for HCC to assess only the viable tumor^[34-36]. Viable tumor is defined as tumor that shows enhancement in the arterial phase. Thus, MR perfusion imaging also has the potential to provide valuable functional information that can be used to distinguish viable tumor from necrosis in HCC^[37].

Model-free approach: Wang *et al.*^[38] have analyzed the arterial uptake slope in MR liver perfusion studies of patients with HCC treated with thalidomide. They have found that there was a greater decrease in the peak enhancement, maximum enhancement, and slope of enhancement in patients without disease progression compared to those who progressed on treatment. Miyazaki *et al.*^[39] have applied HPI to assess the efficacy of anti-angiogenic therapy in patients with liver metastases and have found a median 15% decrease in HPI at 28 d after anti-angiogenic treatment in patients who responded to treatment. In another study, Meijerink *et al.*^[40] have evaluated liver HPI and have found that HPI decreased in liver tumor treated with combination anti-angiogenic therapy with AZD2171 and gefitinib. However, most of these

studies involved relatively small numbers of patients, and more studies are therefore required to establish whether MR liver perfusion indices can serve as a reliable response biomarker for anti-angiogenic drug therapy.

Meijerink *et al.*^[41] also have evaluated HPI for assessing local recurrence in liver metastases treated with RFA. The authors have found that an increase in HPI parallels disease recurrence detection by ¹⁸F-fluorodeoxyglucose positron emission tomography.

Model-based approach: There are few published studies that have applied a model-based approach for the evaluation of treatment response in hepatic malignancy. Chen *et al.*^[42] have studied the effects of transarterial chemo-embolization using a dual-input dual-compartment DP model and have found that the hepatic arterial fraction, hepatic arterial perfusion, and hepatic blood volume are significantly reduced by effective embolization. These indices could also help to indicate the presence of viable tumor. The potential of using model-based approaches for assessing the effects of anti-angiogenic or antivascular treatment is currently being investigated (Figure 9).

Cirrhosis

Model-free approach: Increase in hepatic arterial perfusion and decrease in portal venous perfusion have been reported in perfusion scintigraphy studies in patients with liver cirrhosis^[43]. Miles *et al.*^[2] and Blomley *et al.*^[3] also have found an increase in HPI and decreased PPI respectively in cirrhotic liver.

Model-based approach: Guan *et al.*^[44] have applied a dual-input dual-compartment DP model in rats treated with diethylnitrosamine. As diethylnitrosamine induces a continuum of hepatitis, hepatic fibrosis and eventually cirrhosis, a gradual increase in hepatic arterial flow and MTT are observed across these groups, accompanied by a corresponding gradual decrease in blood volume and blood flow.

Koh *et al.*^[4] have utilized a dual-input dual-compartment DP model and have found that cirrhotic livers return a measurable fractional interstitial space, whereas normal liver shows a near-zero fractional interstitial space. In another study, Hashimoto *et al.*^[5] have applied a dual-input dual-compartment DP model and have found that the hepatic arterial fraction increases with the extent of fibrosis and cirrhosis.

The use of a single-compartment kinetic model has also been found to be useful for the assessment of liver cirrhosis.

Annet *et al.*^[6] have investigated a dual-input single-compartment CC model and have found that the microcirculatory parameters derived from such a technique correlate with the severity of cirrhosis and portal pressure. The measured portal pressure, a reflection of the degree of portal hypertension, is correlated with the calculated portal fraction, portal perfusion and MTT. Furthermore, the severity of cirrhosis as assessed by Child-Pugh class has been found to correlate with portal fraction, portal

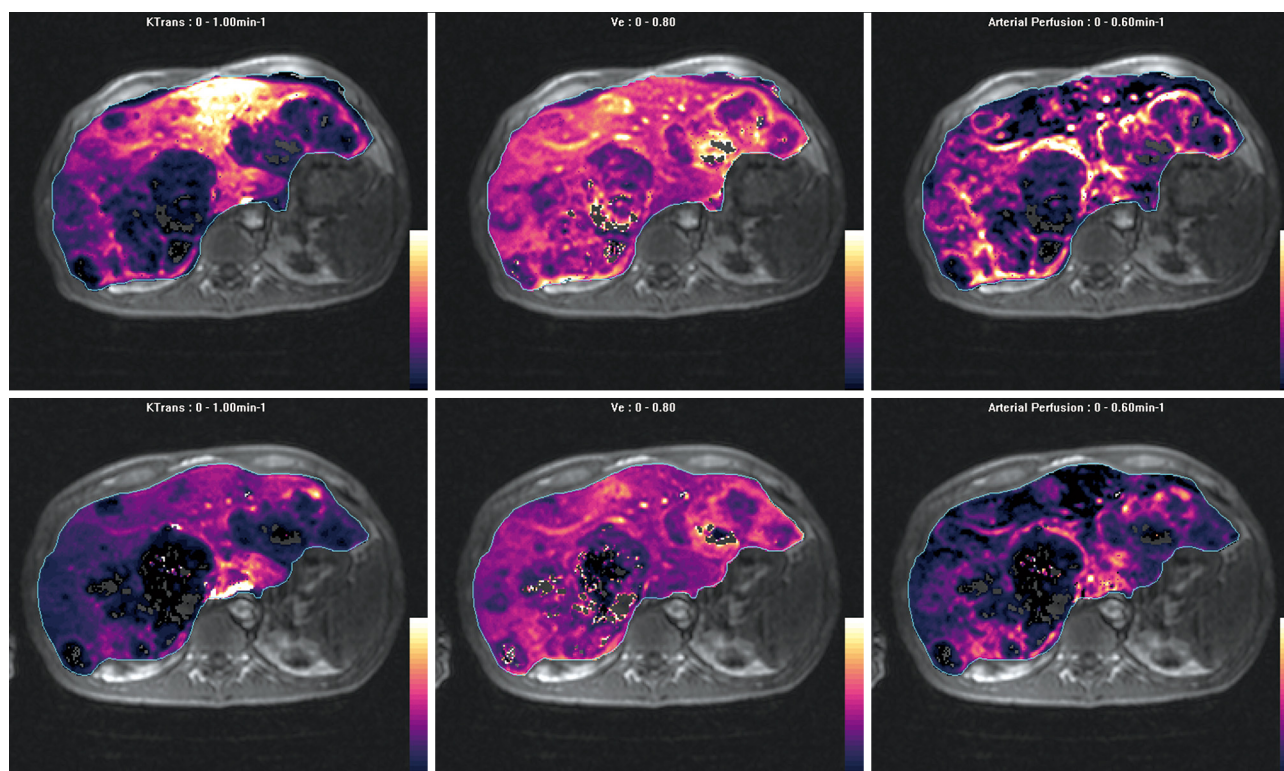


Figure 9 Parametric maps of efflux constant (Ktrans), extravascular extracellular space (Ve) and arterial perfusion fraction before (top row) and after (bottom row) anti-angiogenic therapy obtained using a dual-input, single-compartment, conventional compartment model (after Materne *et al*^[46]). Note multiple metastases in the liver, which are largely hypovascular but show increased arterial perfusion at the edge of the lesions. The metastases also show moderate Ve. After treatment with anti-angiogenic drug, arterial perfusion is reduced at the tumor rims, also with a decrease in tumor Ve. The Ktrans in the left lobe of the liver is also decreased, which may reflect drug effects on microscopic disease.

perfusion, arterial perfusion and MTT. Cirrhotic livers have shown an increase in arterial perfusion, decrease in portal perfusion, decrease in total blood flow, and an increase in MTT.

These findings can be corroborated with other published studies. In an earlier study by Van Beers *et al*^[7], a cutoff threshold of 22.6 s for the MTT in the liver enabled the diagnosis of liver cirrhosis to be made with a sensitivity and specificity of 81%. In another study, Hagiwara *et al*^[8] have used a dual-input single-compartment CC model and have found that there was an increase in the arterial blood flow, arterial fraction, distribution volume, and MTT in patients with advanced hepatic fibrosis. Receiver operating curve analysis has shown that the MTT, distribution volume and arterial flow are good predictive parameters with an area under the receiver operating characteristic curves that ranges from 0.791 to 0.824, and reported sensitivity of 76.9%-84.6% and specificity of 71.4%-78.5%. Thus, MR liver perfusion appears to be a promising method for the non-invasive diagnosis of liver cirrhosis and for assessing the severity of the condition.

CONCLUSION

MR liver perfusion imaging provides functional information about the microcirculation of liver parenchyma and focal liver lesions and appears to be a promising technique for evaluating liver metastases and HCC;

for assessing the efficacy of anti-angiogenic or local tumor ablation therapy; and for diagnosing cirrhosis and assessing its severity. However, standardization of imaging acquisition and analysis techniques need to be actively addressed for the technique to be widely adopted.

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Techniques for colorectal anastomosis

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Abstract

Colorectal anastomotic leak remains one of the most feared post-operative complications, particularly after anterior resection of the rectum with, the shift from abdomino-peritoneal resections to total mesorectal excision and primary anastomosis. The literature fails to demonstrate superiority of stapled over hand-sewn techniques in colorectal anastomosis, regardless of the level of anastomosis, although a high stricture rate was noted in the former technique. Thus, improvements in safety aspects of anastomosis and alternatives to hand-sewn and stapled techniques are being sought. Here, we review alternative anastomotic techniques used to fashion bowel anastomosis. Compression anastomosis using compression anastomotic clips, endoluminal compression anastomotic rings, AKA-2, biofragmental anastomotic rings, or Magnamosis all involve the concept of creating a sutureless end-to-end anastomosis by compressing two bowel ends together, leading to a simultaneous necrosis and healing process that joins the two lumens. Staple line reinforcement is a new approach that reduce the drawbacks of staplers used in colorectal practice, i.e. leakage, bleeding, misfiring, and inadequate tissue approximation. Various non-absorbable, semi or fully absorbable materials are now available. Two other techniques can provide alternative anastomotic support to the suture line: a colorectal

drain and a polyester stent, which can be utilized in ultra-low rectal excision and can negate the formation of a defunctioning stoma. Doxycycline coated sutures have been used to overcome the post-operative weakness in anastomosis secondary to rapid matrix degradation mediated by matrix metalloproteinase. Another novel technique, the electric welding system, showed promising results in construction of a safe, neat, smooth sutureless bowel anastomosis. Various anastomotic techniques have been shown to be comparable to the standard techniques of suturing and stapling. However, most of these alternatives need to be accepted and optimized for future use.

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Key words: Alternative anastomosis; Compression anastomotic clip; Compression anastomotic ring; Biofragmental anastomotic ring; AKA-2; Magnamosis (magnetic anastomosis); Matrix metallo-proteinase; Sutureless

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INTRODUCTION

Since the first reports of laparoscopic colectomy in the 1990's, this technique has matured into a well-developed mode of therapy. It has introduced the colorectal surgical world to the advantages, and the unique perspectives and concerns of minimal access surgery. Colorectal anastomotic leakage remains one of the most feared post-operative complications, particularly after anterior resection of the rectum, with the shift from abdomino-peritoneal resections to total mesorectal excision and primary

anastomosis. It is also associated with a higher local recurrence rate and lower long-term survival. Moreover, long-term functional outcome might be adversely affected by anastomotic leakage^[1-4]. The importance of surgical technique is underscored by the wide variations of anastomotic leakage rates among surgeons. The frequency of anastomotic leakage ranges from 1% to 24%^[5-7]. The rate of leakage is generally considered to be higher for elective rectal anastomosis (12% to 19%) than for colonic anastomosis (11%)^[8-10].

PHYSIOLOGY OF GASTROINTESTINAL HEALING

Better appreciation of the principles of intestinal healing will lead to a better adoption of techniques to overcome the risk factors inherent to the laparoscopic approach and hence anastomotic dehiscence. The environment for wound healing is substantially different in an anastomosis, due to the presence of shear stress (secondary to intraluminal bulk transit and peristalsis), as well as the presence of aerobic and anaerobic bacteria.

The process of intestinal anastomotic healing can be arbitrarily divided into an acute inflammatory (lag) phase, a proliferative phase, and, finally, a remodeling or maturation phase. Collagen is the single most important molecule for determining intestinal wall strength, which makes its metabolism of particular interest for understanding anastomotic healing. After surgery, degradation of mature collagen begins in the first 24 h and predominates for the first four days. This is caused by the upregulation of matrix metalloproteinases (MMPs), which are an important class of enzymes involved in collagen metabolism. *In vivo* use of MMP inhibitors has been found to increase the strength of intestinal anastomoses by up to 48% at postoperative day three, which suggests that these enzymes are important in determining the risk of leakage. By postoperative day seven, collagen synthesis takes over, especially proximal to the anastomosis. After five to six weeks, there is no significant increase in the amount of collagen in a healing wound or anastomosis, though turnover and, thus synthesis, are extensive. The strength of the scar continues to increase progressively with time. The orientation and the cross-linking between collagen fibers maintain the tensile strength of the tissues^[11,12].

Bursting pressure is used as a quantitative measure to grade the strength of an anastomosis *in vivo*. This pressure has been found to increase rapidly in the early postoperative period, reaching 60% of the strength of the surrounding bowel by three to four days and 100% by 1 wk^[11]. In 1887, Halsted^[13] discovered that the submucosa provides the GI tract with the majority of its tensile strength. The bulk of collagen is contained within this layer, along with blood vessels, lymphatics, and nerve fibers. Type I collagen predominates (68%), followed by type III collagen (20%), and type V collagen (12%). The serosa is a thin layer of connective tissue that covers the muscularis propria. When creating an anastomosis, direct

apposition of this layer minimizes the risk of leakage^[14,15]. During the first postoperative days, anastomotic strength is limited, and hence the risk of wound failure is greatest, as collagen breakdown increases. Early anastomotic strength is therefore dependent on the suture- or staple-holding capacity of existing collagen, until a large amount of new collagen can be synthesized by both fibroblasts and smooth muscle cells. Postoperatively, anastomosis will be weak for one or two days until this occurs^[16-19].

FASHIONING ANASTOMOSES

Over the past two decades, numerous different materials have been used to join one bowel end to another, including catgut, stainless steel, and newer monofilamentous and absorbable sutures. In the past 30 years, stapling devices have been embraced enthusiastically by the surgical community^[11]. However, the choice of either technique in fashioning anastomoses is a matter of controversy among various schools^[18].

Apart from inert substances, most foreign materials will evoke an inflammatory reaction in the human body and surgical sutures are no exception. It is now known that silk has the ability to evoke an inflammatory reaction that can persist for weeks after implantation. Substances such as polypropylene (Prolene), catgut, and polyglycolic acid (Dexon) evoke a milder response. There is little difference between absorbable and nonabsorbable sutures with respect to the strength of the anastomosis^[11].

STAPLING

Surgical stapling devices were first introduced by Hüttl, Humer (Budapest) in 1908; but their use has grown since the introduction of new and reliable disposable instruments in the past 30 years^[11]. However despite comparable results in terms of mortality, anastomotic dehiscence, and wound infection, the rate of stricture at the anastomotic site is considerably higher with staples than with sutures: 8% *vs* 2%, respectively, for colorectal anastomosis^[20].

Lim *et al*^[21] confirmed the presence of foreign body reaction in stapled human GI anastomoses. The source of the foreign materials eliciting this reaction was the stapler cartridges.

The literature fails to demonstrate superiority of stapled over hand-sewn techniques in colorectal anastomosis, regardless of the level of anastomosis, although a high stricture rate was noted with the former technique.

The use of staplers for intraperitoneal anastomosis has been questioned^[20]; Matos systematically reviewed (Cochrane Database) nine studies involving 1233 patients (622 stapled and 611 hand-sewn) and found that overall leaks were 13% *vs* 13.4%, clinical 6.3% *vs* 7.1%, radiological 7.8% *vs* 7.2%. There was insufficient evidence to demonstrate superiority of either technique^[20]. The decision over which technique to use must be judged on the basis of previous experience, clinical circumstances, and available resources. Another systematic review showed that both techniques (stapler *vs* Hand-sewn) are effective,

and the choice may be based on personal preference^[22]. Other prospective and randomized trials have shown different results. No significance intergroup difference was found in regard to time for anastomosis construction or occurrence of complications in colorectal anastomosis^[23]. In addition, the routine use of stapling instruments for intraperitoneal colorectal anastomosis could not be recommended because of a higher incidence of mishaps and strictures, even though the operation took less time to perform and anastomotic leakage occurred less often^[24].

Therefore, there is an ongoing search for an ideal method of anastomosis that would not only lower the incidence of dangerous complications, but also avoid the need for a defunctioning colostomy or ileostomy. Based on the aforementioned data, there is still controversy between surgeons.

It is therefore necessary to review all relevant studies and trials to resolve this issue. Multi-center, well-designed, randomized controlled trials are required to build a link between new technology and practice. As technology advances, the use of newer techniques should allow improvements in the quality of patient care.

COMPRESSION ANASTOMOSIS

Connecting sections of the intestine after the surgical removal of a diseased portion has been the subject of research and invention since the 19th century. The goal has been to find a method to eliminate the leakage associated with anastomosis. The principle of compression anastomosis consists of two opposing rings trapping the cut ends of the transected bowel with subsequent ischemia and eventual sloughing of the trapped bowel, thus releasing the rings into the fecal stream^[25]. Despite its technical safety, it was not accepted^[26].

The idea of compression anastomosis was first reported in 1826 by Denan, who conceived a sutureless bowel anastomosis that encompassed the inverting technique proposed by Lembert. The idea was to compress two bowel walls together and cause a simultaneous necrosis and healing process leading to the joining of the two lumens. In 1892, Murphy introduced a mechanical device known as “Murphy’s button” that was used for years^[27].

It comprises a pair of metal rings that hold circular segments of intestine together under continuous pressure; the rings are expelled several days after surgery. However, its clinical success was limited and the results were mediocre. Moreover, it was a metallic foreign body that remained in the lumen of the bowel for several days until it was spontaneously discharged from the body with the necrotic tissues^[28].

In the 1980s a device comprising two magnetic rings was used for intestinal anastomosis, but this concept was not further pursued^[29].

In 1984, Kanshin *et al*^[30] developed the AKA-2 device (Seidel Medipool, Munich, Germany) for colorectal surgery. In 1985, Hardy *et al*^[31] introduced the Valtrac biofragmentable anastomotic ring (BAR) (Davis and

Geck/Cyanamid, Danbury, CT). Numerous publications, including prospective randomized controlled trials (RCTs), reported that the BAR was safe and efficacious in both emergency and elective surgery^[31-39]. Both devices adopted the concept of compression anastomosis, and incorporated some of the basic features of Murphy’s button. However, in contrast to the BAR, the AKA-2 ring is not absorbable and it is usually disconnected from the anastomosis after four to six days. In addition, it was made exclusively for transanal application^[40].

Years later a novel device for performing compression anastomosis using the shape memory alloy (SMA) of nickel-titanium was introduced. The device is available both as a clip (Compression Anastomosis Clip or CAC, NiTi Medical Technologies, Netanya, Israel), and as a ring (Compression Anastomosis Ring or CAR, NiTi Medical Technologies). After approximately one week, the entire device, together with the necrosed tissue, detaches and is naturally expelled from the body^[41-44]. A summary of the four main types of compression devices is presented in Table 1.

CLASSIFICATION OF DIFFERENT COMPRESSION DEVICES

Valtrac™ BAR

Valtrac™ BAR is composed of two segments containing absorbable polyglycolic acid (87.5%) and barium sulfate (12.5%). It comes in a size range (25, 28, 31, and 34 mm). The two components interdigitate on a central frame; a 6-mm gap is seen between the scalloped edges of the BAR in the open position, and a 1.5-mm, 2-mm, or 2.5-mm gap is made in the fully closed position to accommodate different thicknesses of bowel wall. This also limits the amount of tissue necrosis^[45].

Each ring is securely placed into the cut bowel ends with the aid of a purse-string suture, and the device snapped shut. Between two and three weeks after the operation, the BAR rings fragment and are passed into the stool. This results in the production of a sutureless, inverted, serosa-to-serosa intestinal anastomosis^[46]. The BAR has been used for construction of various types of anastomosis, including procedures involving the upper and lower GI tract. Prior to the development of the transanal applicator, early studies often excluded patients with low rectal anastomosis^[47].

In a randomized control trial comparing BAR with stapling devices in extra-peritoneal mid-rectal anastomosis, surgeons did not consider the use of BAR to be more difficult than a stapled anastomosis. The time required to create a BAR anastomosis was slightly shorter than the time needed for a stapled anastomosis, although this was not statistically significant. The overall operating time, intraoperative blood loss, and postoperative complication rates were similar with both anastomotic techniques^[48]. Correspondingly, there were no statistical differences in the complication rates between the BAR and a sutured anastomosis in elective and emergency procedures^[48].

Table 1 Characteristics of the four main compression devices

	BAR	AKA-2	CAC	EndoCAR ²⁷
Absorbable	Yes	No	No	No
Application	Laparoscopy, laparoscopy, transanal	Transanal	Laparotomy, laparoscopy, hand-assisted lap	Laparotomy, laparoscopy, hand-assisted lap
Internal Lumen	11-20	25, 28, 31	8	One ring size (27 mm) replaces a number of competitive sizes (25-34 mm)
Average time to expulsion (d)	14-21	4-6	7	7-10
Type of surgery				
Elective/emergency	Yes/yes	Yes/yes	Yes/no	Yes/no
Foreign body reaction	No	Possible to metal pins	No	No
Tissue healing	Extensive fibrosis/may cause stricture	Extensive fibrosis/may cause stricture	Primary intention/no strictures reported	Primary intention/no strictures reported (recovery of multi-layer lumen structure)
Anastomotic index	Lumen capacity depends upon standardized ring size			Full lumen capacity within 8-12 wk
Efficacy	Safe and secure and can be applied to achieve multiple anastomosis (in case requiring rapidity and security)			
Learning curve ¹	Technically difficult than the other three devices		Technically simple after education	
Cost ²	About \$600	NA	About \$3	NA (however higher than conventional staples)
Tissue thickness accommodation	Selecting ring size to be compatible with diameter and thickness of bowel wall	Same as BAR	Only one size, shape memory alloy that accommodates varies tissue thickness. Unique thermo-mechanical properties and super elasticity	
Type of anastomosis	End-to-end, end-to-side, side-to-side		Side-to-side	End-to-end
Site of anastomosis	Suitable for intestinal, colonic and rectal anastomosis	Distal colon and rectal only	Intestinal, colonic and rectal anastomosis	

¹Galizia *et al*^[47] described a learning curve of nine patients for BAR anastomosis. A meta-analysis of over 500 cases in North America, Europe and Israel, 75% of surgeons rated the CAR²⁷ device to be very easy or easy to use^[83]; ²Cost-effectiveness depend upon a number of factors namely learning curve and post operative morbidity. However, as multiple staplers are used for construction of most colonic/rectal anastomosis, there might be a cost advantage for compression devices. BAR: Biofragmentable anastomotic ring; CAC: Compression anastomotic clip.

An initial study with large animal models (300 dogs and 31 pigs) presented a randomized analysis of 28 pigs, comparing sutured, stapled and BAR anastomosis. They found the “Burst” pressure at day 0 was highest with the BAR and equal at days seven and 16 in all three types of anastomosis. The authors stated that the BAR anastomoses were performed more easily and quickly than the other two anastomoses. Microscopic examination also revealed the least amount of tissue necrosis for the BAR anastomosis^[46].

In 1987, Hardy *et al*^[49] published results of the first 27 patients who had colorectal anastomoses using the BAR device. They reported no difficulties and all patients tolerated a regular diet before fragmentation of the rings.

In the 1990s, the device gained popularity and a number of prospective studies confirmed that the results with the device were satisfactory, although there were reports of intraoperative problems, such as failure of the purse-string suture, incorrect estimation of the diameter of the colon lumen, subsequent mucosal tears, and failure of the device to lock. Many of these might simply have been because of the operator's learning curve^[33,50-53].

Based on the previously mentioned studies, possible limitations of the device include: (1) Failure of purse string sutures; (2) Incorrect estimation of colon lumen diameter; (3) Subsequent mucosal tears; (4) Failure of the device to lock (the bowel might be contused by closing maneuver from outside the gut); (5) Excessive snapping

pressure could shatter the friable device; (6) fragmentation delay; (7) possibility of postoperative tenesmus and frequent stool passage before excretion of fragments; (8) bulky and uncomfortable device to deploy; and (9) potential risk of relative obstruction due to smaller inner diameter of the ring.

AKA-2

In 1984, Kanshin *et al*^[30] developed the AKA-2 device (Seidel, Medipool) to address the transanal approach for compression anastomosis. The AKA-2 is composed of two rings: a base ring, which includes metal pins and metal springs, attached on a plastic ring (the “distal ring”), and a proximal plastic ring (the “proximal ring”). The rings are applied with a transanal applicator. The AKA-2 works on a similar principle to that of endoanal stapling devices, though the bowel edges are pressed together with intraluminal rings and held in place by metal pins. Circular blades cut the central cuff of bowel, and the metal pins ensure constant compression on the inverted bowel edges. The two plastic rings and the compressed resection margins separate from the anastomosis after four to six days and are expelled with the feces^[46]. The technique had an advantage in that it created a good lumen size for stool passage.

There is only one report in English of the use of this device^[40]; the majority of the literature being in Russian or German. A prospective audit presented the results of

442 patients undergoing colorectal surgery for benign and cancerous disease. There was a 5.4% overall complication rate, with 11 patients (2.5%) developing clinical features of an anastomotic leak, which is relatively low compared to other series using various anastomotic techniques^[48,49,54,55]. Fourteen of the 442 patients died (3.2%), of which three cases were related to anastomotic leak (0.7%). Among 442 patients who underwent AKA-2 anastomosis only two patients developed a stricture^[40].

The authors maintain that the advantage of this device is that it produces a good size lumen for the passage of feces. The plastic ring sizes are 25, 28, and 31 mm, respectively. In addition, necrosis of inverted resection margins is the only biological factor leading to the rejection of the plastic rings, which is an advantage in cases with delayed healing. However, early device exclusion raised the possibility of higher leak rate, as it is concurrent with the maximal breaking strength of anastomosis^[46].

Compression anastomotic clip

Nitinol^[60] (Nickel Titanium Naval Ordnance Laboratory), an alloy of nickel and titanium, is a temperature-dependent, shape-memory alloy (SMA) that has been used in the formation of compression anastomoses^[56]. The metal is shaped under high temperatures, and when it is ice cooled (to less than 0°C), it loses its rigidity and becomes flexible. At or above room temperature, it resumes its preset configuration. It has been used mostly for vascular prostheses, orthodontic braces, and for internal fixation of bone fractures for its inherent advantage of controlled compression with a constant force^[46].

The Nitinol CAC device (Niti Medical Technologies) has been approved by the food and drug administration (FDA) for use in GI surgery^[41]. The device consists of a double-ring that, in the open and flexible state (at 0°C), has a diameter of 30 mm and an opening angle of 30 degrees. At body temperature, the rings return to their closed configuration and hold bowel tissue under a constant compressive force, regardless of the thickness of intervening tissue. This leads to ischemia of the entrapped bowel wall and the formation of a compression anastomosis. The internal diameter of the rings is 8 mm, and is pierced by a 5-mm blade built into the applicator to restore bowel continuity in the early period. The device is elastic, pliable, and easy to manipulate^[46].

Initial reports on both animal and human studies using the CAC device to create a side-to-side anastomosis in upper and lower GI tracts revealed no signs of anastomotic stricture or leakage, with formation of a uniform, completely re-epithelialized anastomotic line^[41]. There were no reported postoperative complications, and colonoscopic examination at six months demonstrated a satisfactory anastomosis^[43].

The safety of this device has been documented in numerous animal studies^[41,57], and the safety of the alloy has been demonstrated by its extensive uses in other medical procedures. The CAC was considered to be

safe, simple, and effective in colon surgery in a study that evaluated the thermo-mechanical properties of the device^[58].

In line with this conclusion, a randomized control trial studied the clinical effects of using the CAC device in small intestinal anastomosis proximal to the ileocecal valve. CAC anastomosis was performed in 33 out of 66 patients, with the other 33 patients being used as a control group for whom a stapled anastomosis was constructed. The main indication was gastric cancer in both groups. Anastomosis was fashioned to reconstruct a Roux-en-Y loop, entero-entrostomy, Billroth II gastro-jejunostomy, and gastro-jejunostomy. The authors found no post-operative complications whatsoever in terms of leakage, obstruction, bleeding, or stenosis after six months of follow-up^[59].

Clinical trials for SMA of nickel-titanium in intestinal anastomosis are scarce, and all of the clinical reports are of CAC from a single center that included only elective cases performed both by laparotomy and laparoscopically^[42-44]. None of the patients who underwent surgery with CAC had a protective stoma. None of the patients reported so far in published clinical studies experienced a clinical leak and initial experience with a laparoscopic technique had similar results, thus precluding the learning curve among surgeons.

The consensus among the published studies was that microscopic examination of the CAC anastomosis showed minimal inflammation and no foreign body reaction, with very little scar tissue at the anastomotic line.

The specific advantages of the CAC include a one sized clip with a wide external diameter, preprogrammed round shape negating the need to forcefully close the rings and therefore diminish the risk of shattering the device. It exerts constant compression of the bowel ends, regardless of the intervening tissue thickness; coils exert a constant stress plateau at about 400 Mpa. The result is a smooth homogenous anastomosis formed by the gradual controlled necrosis of the tissue, limited by the coil perimeter while the external edges become sealed^[46]. A drawback of this device is the need for suture closure of the insertion incisions made in the bowel wall.

Endoluminal compression anastomotic ring, EndoCAR²⁷

The endoluminal compression anastomotic ring, EndoCAR²⁷ (spectrum of the shape memory alloy of nickel-titanium), utilizes two separate synthetic rings that are mounted on an instrument very similar to a circular stapler. An anvil containing one ring is fixed to the proximal bowel end, and the instrument with the other ring is inserted trans-anally for a rectal anastomosis. When engaged, the rings are locked together by Nitinol springs that exert the desired constant controlled pressure force (7.7 Newtons or 1.65 Pounds), and a circular knife resects the access tissue. As in the side-to-side device, a simultaneous necrosis-healing process takes place, and at the completion of this process (seven to ten days), the

Table 2 Compression anastomosis: clinical experience and complications

Study	Device	Emergency/elective	Anastomotic leakage	Obstruction	Stricture
Bubrick <i>et al</i> ^[34]	BAR	0/395	12 (3.2%)	18 (5%)	-
Cahill <i>et al</i> ^[35]	BAR	0/101	2 (2%)	4 (4%)	-
Corman <i>et al</i> ^[36]	BAR	0/222	6 (2.7%)	9 (4%)	2 (0.9%)
Gullichsen <i>et al</i> ^[37]	BAR	-	-	13 (16%)	-
Seow-Choen <i>et al</i> ^[39]	BAR	-	0	0	2 (10%)
Di Castro <i>et al</i> ^[49]	BAR	90/424	17	0	4 (1%)
Thiede <i>et al</i> ^[33]	BAR	0/1360	34 (2.5%)	-	-
Pahlman <i>et al</i> ^[38]	BAR	24/26	2 (4%)	3 (6%)	-
Ghitulescu <i>et al</i> ^[53]	BAR	23/136	7 (4.2%)	13 (7.9%)	3 (1.8%)
Kim <i>et al</i> ^[45]	BAR	101/515	5 (0.8%)	13 (2.1%)	1 (0.5%)
Wullstein <i>et al</i> ^[40]	AKA-2	70/372	11 (2.5%)	-	2 (0.5%)
Nudelman <i>et al</i> ^[43]	CAC	0/5	0	0	0
Nudelman <i>et al</i> ^[42]	CAC	0/30	0	0	0
Nudelamm <i>et al</i> ^[44]	CAC	0/10	0	0	0
Liu <i>et al</i> ^[59]	CAC	0/33	0	0	0

device is detached and expelled naturally. Furthermore, the longitudinally orientated metal prongs further fixate both bowel ends and prevent tissue slippage from axial movements. An advantage of this contemporary device is that there is no anastomotic-scarred lip inside lumen and a safe applicator removal without fishtailing^[60].

Two separate studies looked at bursting strength in a porcine model. Kopelman *et al*^[57] measured a mean bursting strength of 247.7 mmHg (range 100-300 mmHg) in nine animals at time zero (immediately after the excision of the fashioned anastomosis). Furthermore Stewart *et al*^[61] revealed a significantly higher bursting pressure after compression anastomosis in comparison with a conventional double stapling technique (103, 75.3 mmHg *vs* 3, 23 mmHg, respectively). Four of the nine compression anastomoses failed at the anastomotic line whereas nine of nine stapled anastomoses failed at the staple line (Fishers' exact test, $P < 0.01$). Bursting pressures measured at two weeks after the anastomosis revealed equal pressures (266, 32.2 mmHg and 230, 87.5 mmHg, respectively). Compression therefore seems to be capable of overcoming anastomotic weakness during the 'classical' lag-phase and to result in equal strength after detachment of the ring^[62].

Kopelman *et al*^[57] looked at the anastomotic index (ratio of the mean bowel diameter 5 cm proximal and distal to the anastomosis and on antero-lateral and posterior view), which was 0.81 (0.60-0.92) at two months.

An early clinical trial was performed in Israel using the EndoCAR²⁷ device to construct a left-side anastomosis. Four patients were enrolled. No device related complications were noted in these patients and no anastomotic leak reported (unpublished data). Based upon that experience, a pilot study was started in May 2007 in Uppsala (Sweden) and in Leuven (Belgium) to obtain clinical data in a consecutive group of 40 patients^[62]. The recruited patients had either malignant or benign (diverticular) disease requiring resection with a high colorectal anastomosis (between 10 and 15 cm from the anal verge). Preliminary results from that pilot study showed that of the first ten patients, nine underwent high

anterior resection, and left colectomy was performed on one patient. No leak age occurred in this first group of patients. No other data is available yet.

These promising results demonstrate that this device could be a revolutionary invention in colorectal practice; however, there are still doubts regarding its efficacy in low/ultralow rectal anastomoses. The location of the ring above the pelvic floor could induce persistent anal sensation (urge) and it is still unknown whether a spontaneous evacuation will occur in diverted patients.

Magnamosis

Controlled magnetic approaches have shown promise in biliary and vascular anastomoses (although the latter involves permanent implantation). A specially designed self-orienting device has been put into a trial to test the hypothesis of creating a magnetically mediated intestinal anastomosis using a temporary device that is expelled some time after creating the desired compression-necrosis effect (Department of Surgery, University of California, San Francisco)^[63].

Two topologies were evaluated; namely the uniform and the gradient compression device. The study was conducted on 16 pigs with the creation of a side-to-side anastomosis. Half of these were created with the uniform device and the rest with the gradient. They also created hand-sewn and stapled side-to-side anastomosis for comparison. Devices were designed with surface fields of approximately 3000 Gauss (G). Preliminary experimentation had revealed that combinations of 3000/6000 G and 6000/6000 G uniformly caused necrosis and perforation within 48 h independent of device geometry. The results were promising, with the creation of successful patent anastomosis using the magnetic devices and no leaks reported^[63].

The mechanical integrity of the magnetic anastomoses was not statistically significantly different from stapled or hand-sewn; however, there was a trend toward greater strength with the gradient type device and earlier patency. No evidence of stenosis was reported^[63].

Table 2 presents the past clinical experience with

Table 3 List of reinforcement materials^[64]

	Material	Stapler type	Company
Non-absorbable	ePTFE	Linear	W.L. Gore, Elkton, MD, USA
Semi-absorbable	Bovine pericardium (peristrips dry)	Circular linear	Synovis Life Technologies, Inc.
	Porcine small bowel (surgisis)	Linear	Cook Biotech Inc.
Absorbable	Polyglycolic acid:trimethylene carbonate (seamguard bioabsorbable)	Linear circular	W.L. Gore & Associates, Inc.
	Cellulose (Xcell)	Linear	Xylos Corp.
	Knitted calcium alginate (foreseal)	Linear	Laboratoires Brothier, Nanterre, France

compression anastomosis devices and their related complications.

BUTTRESSING OF INTESTINAL ANASTOMOSIS

Many staple devices are commercially available, however all the different types and models have inherent drawbacks that contribute to post-operative complications. Complications such as enteric leakage, bleeding, inadequate tissue approximation, and misfiring (technical failures) have been reported. However, complications related to colorectal anastomosis are the most devastating in terms of morbidity and mortality^[64].

A new approach to reduce this is to use staple line reinforcement materials. Gastrointestinal reinforcement is well known, but its application in colorectal surgery is relatively new^[65]. The application of buttressing materials is thought to moderate the tension of the stapler line because it acts as a neutralization plate. It reinforces the stapler line by sealing the gaps between staples and narrowing the spaces, thus reducing tearing of tissues, bleeding, and leakage^[64]. Reinforcement materials can be applied exogenously to the staple line or incorporated into it. The material is composed of two regions, one that secures it to the stapler prior to activation, and is later discarded, and the other forms the seal. It has an adhesive surface and is readily packed in a sterile manner^[65].

Reinforcement material can be non-absorbable, semi- or fully-absorbable. Studies have shown diminished incidence of leakage and stapler line failure in gastrointestinal and pulmonary surgery. Although all types of materials seem equally adequate in reducing staple line complications, the material itself can cause problems^[65]. Therefore, the choice of material must be considered from a safety point of view, although there seems to be advantages of absorbable material over the other two types.

The effects of the materials in colorectal anastomosis have been tested in a small clinical pilot study by Franklin *et al.*^[66,67] using bio-absorbable seamguard (BSG) with a linear stapler. Published data revealed no bleeding, or apparent bleeding, at the staple line.

Several reports support the theoretical benefits of reinforcement materials in increasing the burst pressure^[68-71]. It

was also hypothesized that buttressing of stapler line can have a positive effect on tumor recurrence^[66]. Although published studies showed a decrease in complications with these materials, no previous studies have shown significant results for reducing bleeding or leak rates at the stapler line. Thus, further research and investigations are required. Table 3 refers to the list of materials used as staple line adjuncts.

Non-absorbable materials

ePTFE: ePTFE is a very easy and quickly employed material composed of non-absorbable expanded polytetrafluoroethylene. It is constructed like a sleeve that can be slid over both arms of the stapling device negating the need for additional fixing to the stapler before firing. After firing, the material is released from the arms by pulling a ripcord. The potential benefits of this material include a low host response and biocompatibility. There are no reports of strip erosion or migration with this material, which evokes a minimal tissue inflammatory reaction. It provides thick tissue coverage for an extended period of time with no extra handling time required for its preparation and use. Its application suits open and laparoscopic procedures^[72,73].

Semiabsorbable material

Bovine pericardium: This material is composed of bovine pericardium (peristrips dry). It is temporarily attached to the stapler with gel (which is applied to inner surface of both stapler arms), after which the stapler is positioned and locked over the strips. It can be applied on linear, as well as circular, staplers. The material is then incorporated by the host tissue after firing the stapler. Apart from increasing the burst pressure, this material demands relatively more handling time than other materials. However, it has the potential of to reduce the time required to stop staple line bleeding. Possible limitations include a high risk of animal source contamination, resulting in an inflammatory reaction to the xenomaterial (non-biocompatible). This makes it prone to erosion and migration^[64]. Recently, its combination with Veritas technology results in remodeling of the material into indistinguishable host tissue.

Porcine small intestinal submucosa: This is a completely resorbable, acellular xenograft composed of

porcine small animal submucosa. It is suitable for anastomotic and non-anastomotic staplers. A potential advantage is that it provides a bioscaffold for tissue growth, inducing submucosal regeneration and also achieving an increase in burst pressure^[74,75]. However, its efficacy in human staple-line reinforcement is undocumented.

Absorbable material

Polyglycolic acid:trimethylene carbonate: This is a synthetic fiber web that is composed of polyglycolic acid: trimethylene carbonate Maxon polymer. It is formed like a sleeve to be fitted over the stapler arms and released by pulling the suture that holds the sleeve in place. It can also be affixed as discs onto circular-type staplers. The material is strongly biocompatible, simple and easy to apply on the stapler and is non antigenic. It maintains its strength for four to six weeks and is fully resorbed after six months (hydrolytic and enzymatic reactions lead to the breakdown of the material)^[76,77]. Overall, it minimizes staple-line bleeding, leakage, and operative time^[78-80].

Cellulose: Cellulose (XylosT M Surgical Reinforcing Material. Xcell SDMC surgical film) was originally developed as a wound dressing. This dry sterile material is composed of a microbially-derived cellulose matrix having multilayered, three-dimensional structures. The cellulose is produced by *Acetobacter xylinum* bacteria and is processed into a resorbable form. Research is in progress to evaluate and construct it as a possible staple line reinforcing material in GI surgery^[81].

Knitted calcium alginate: This material is composed of polysaccharidic polyglycuronates biopolymers (highly purified fractions from calcium alginates), originating from seaweeds. The device consists of preformed coated knitted bio-absorbable sheets held into the form of sleeves (one cartridge device, one anvil device) sized to fit snugly onto the forks of the surgical stapler. When applied to wet surfaces, the material becomes highly conformable and acquires bio-adhesive and sealant properties. It contains no additives or preservatives, and therefore no presoaking or rinsing is required as a preparatory step. The device is easy to handle and simple to apply, eliciting minimal foreign body response^[82]. However, clinical trials are scarce.

OTHER FORMS OF ANASTOMOTIC SUPPORT

C-seal (polyganics)

The colorectal drain (C-seal) is applied with a circular stapler. It is a single use tubular device, closed at one end and composed of biodegradable synthetic material. It is a thin walled tube with an approximate diameter of 3 cm and an approximate length of 20 cm. This drain works as a shield covering the newly formed anastomosis, preventing contact between the bowel contents and the

anastomosis. Degradation process starts gradually, and the material is expelled from the bowel after approximately 10-15 d. Its theoretical benefits lie in the ability to protect a low rectal anastomosis, preventing leakage. It can also be used as a staple line adjunct. It is microbiologically safe and is completely expelled after two weeks, negating the use of a protective defunctioning stoma in low rectal excision^[83].

Polyester stent

Most recently, covered intraluminal stents have been successfully introduced to manage anastomotic leaks after esophagectomy and gastric bypass operations.

A randomized control trial in a large animal model addressed this issue in stapled colorectal anastomosis. The study found that placement of a covered polyester stent across a colorectal anastomosis prevents leak-related complications and supports healing of an anastomotic leak^[84].

It consists of a polyflex self-expandable covered plastic stent (25 mm proximal flare and 12 cm long) and a delivery system. The outer layer is composed of braided polyester and the inner layer is silicone (no gaps in this layer). It is applied through a standard flexible colonoscope using a guide wire and a delivery system, and is deployed under fluoroscopic control after reconstruction of the end-to-end anastomosis. The components of the material allow it to adapt elastically to the lumen wall, exerting a well-balanced radial force. The silicone membrane provides a reliable leak occlusion, preventing ingrowth of granulation tissue; hence allowing stent repositioning or removal. Similarly to C-Seal, it negates the need for a diverting stoma in low rectal excision. However, the main disadvantage for the future of these types of stents is migration^[84].

NOVEL TECHNIQUES

Doxycycline coated sutures

Experimental studies revealed that the strength of an intestinal anastomosis diminishes postoperatively reaching a nadir on the third postoperative day. This is mediated by the increased activity of MMP, causing local matrix degradation in the tissue surrounding the sutures. This activity is higher still in concurrent bacterial peritonitis, with subsequently greater deterioration of anastomotic strength. Several experimental studies showed that MMP inhibitors administered systemically alleviate postoperative weakening of intestinal anastomoses. Other studies have shown the beneficial effects of treatment with systemic MMP inhibitors, e.g. doxycycline, most notably on the critical third postoperative day^[85-87].

Potent MMP inhibitors administered systemically can cause joint stiffness, swelling^[88], and other toxic reactions^[89]. Additionally, there are concerns about detrimental effects of broad-spectrum hydroxamate MMP inhibitors on secondary healing of cutaneous wounds^[90], although these types of MMP inhibitors can increase tensile strength of primary skin wounds^[91]. The less

potent MMP inhibitor doxycycline does not appear to delay wound closure^[92]. Due to adverse systemic effects, local delivery of an MMP inhibitor in humans would be advantageous over systemic administration^[93].

This hypothesis was studied by Pasternak *et al*^[93] in 2008. They implemented a novel method for coating sutures with a cross-linked fibrinogen film and then bound the MMP inhibitor (doxycycline) into this film. The sutures were then used in a standard rat model for evaluating mechanical properties of colonic anastomosis three days after surgery. The breaking strength of the anastomosis was higher with the doxycycline-coated sutures than with the controls. This might inspire further studies involving pharmacological manipulation of intestinal healing by local drug delivery^[93].

Electric “Welding” of soft tissues

Experience of the application of electric surgery for cutting tissues and hemostasis is about one hundred years long. It has been established that under certain conditions, it is possible to join incisions in different organs and soft tissues by a method based on heating the joint zone by a high-frequency current. Electric welding to join incisions of live tissues and organs during surgery was applied for the first time by the team of researchers of the E.O. Paton Electric Welding Institute of NASU in cooperation with the scientists and specialists of the experimental department of the Institute of Surgery and Transplantology (IS&T) of AMSU with participation of International Association (Welding) and financial support of CSMG Company, USA^[94].

They developed a novel welding system that includes a power unit comprising a power source (High frequency coagulator) with an adaptive automatic control system and special software, bipolar welding tools (forceps, clamps and laparoscopes) connected to a power source, and special assembly devices. The control system is based on feedbacks. The tissue layers being joined are brought into contact over their surface layers by means of the welding tool. The surgeon clamps the tissue to be welded by the electrodes of the tool and switches on the welding current source. Upon completion of the process (i.e. thermal denaturation of albumin molecules), control program power is turned off. Clamped tissue is then released and process repeated until complete wound closure^[94].

The device has been tested in multiple experimental trials and on more than 2000 patients in the clinics and hospitals of Kiev, Ukraine. The author maintains that the advantage of the device is in the formation of an attractive, neat, smooth thin welded anastomosis. In addition, it is a fumeless and odorless technology, causing no burns to surrounding tissues. The report demonstrates a reduction of blood loss and no organ deformation or stenosis. It also shortens the average operative time (20–40 min)^[94].

CONCLUSION

Although alternatives to the conventional methods have

been sought, many have been abandoned by the surgical community.

Compression anastomosis, although existing for decades, has not gained worldwide popularity. This concept seems to be difficult for surgeons to accept, as it includes relying on a device to create an anastomosis and letting it be spontaneously discharged from the body. Re-institution of this concept using new technology, such as Nitinol, could be a potential replacement for the current available techniques. Controlled magnetic anastomosis is no exception to this. It is a promising novel technique for creation of a side-to-side anastomosis but requires to be optimized for future use.

The theoretical benefits of colorectal seals and the polyester stents as adjuncts to creating an end-to-end anastomosis could alleviate the need for a defunctioning stoma for lower rectal tumor resections. This is another concept that needs to be accepted and subjected to further research to optimize its use. In line with this, staple line reinforcement is an effective technique for reducing perioperative complications in stapled resection and anastomoses, with absorbable materials having a considerable advantage over semi or non-absorbable material(s). However, there has been little experience with absorbable staple line reinforcement materials.

A contemporary and sophisticated technique, such as electric tissue welding shows a promising future in modern surgical techniques. It is a revolutionary technique that still needs acceptance and research, utilizing greater patient samples in colorectal surgical practice.

Finally, overcoming the postoperative anastomotic weakness due to over activity of matrix metalloproteinases, and hence the risk of dehiscence, using doxycycline coated sutures should also be explored.

In summary, these various techniques fulfill the requirements of creating a safe anastomosis (overcoming the lag-phase, increasing the bursting pressure, and decreasing the rate of leakage, bleeding and stricture). They revealed great differences in avoiding dramatic complications that can occur with the conventional methods; an outcome that every colorectal surgeon would advocate. Surgeons need to widen their scope of practice, and further trials and research are required to overcome “dogmas” in traditional colorectal practice. Keeping abreast of technological advances is considered vital in every surgeons training and daily practice; failure to do so could lead to reduced quality of patient care.

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Therapeutic effect of *Streptococcus thermophilus* CRL 1190-fermented milk on chronic gastritis

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Abstract

AIM: To investigate the potential therapeutic effect of exopolysaccharide (EPS)-producing *Streptococcus thermophilus* (*S. thermophilus*) CRL 1190 fermented milk on chronic gastritis in Balb/c mice.

METHODS: Balb/c mice were fed with the fermented milk for 7 d after inducing gastritis with acetyl-salicylic acid (ASA, 400 mg/kg body weight per day for 10 d). Omeprazole was included in this study as a positive therapeutic control. The gastric inflammatory activity was evaluated from gastric histology and inflammation score, number of interleukin-10 (IL-10), interferon- γ (INF γ) and tumor necrosis factor- α (TNF- α) cytokine-producing cells in the gastric mucosa, and thickness of the mucus layer.

RESULTS: Animals receiving treatment with the EPS-

producing *S. thermophilus* CRL 1190 fermented milk showed a conserved gastric mucosa structure similar to that of healthy animals. Inflammation scores of the fermented milk-treated mice were lower than those of mice in the gastritis group (0.2 ± 0.03 vs 2.0 ± 0.6 , $P < 0.05$). A marked decrease in INF γ^+ (15 ± 1.0 vs 28 ± 1.2 , $P < 0.05$) and TNF- α^+ (16 ± 3.0 vs 33 ± 3.0 , $P < 0.05$) cells and an increase in IL-10 $^+$ (28 ± 1.5 vs 14 ± 1.3 , $P < 0.05$) cells compared to the gastritis group, was observed. Also, an increase in the thickness of the mucus gel layer (2.2 ± 0.6 vs 1.0 ± 0.3 ; 5.1 ± 0.8 vs 1.5 ± 0.4 in the corpus and antrum mucosa, respectively, $P < 0.05$) compared with the gastritis group was noted. A milk suspension of the purified EPS from *S. thermophilus* CRL1190 was also effective as therapy for gastritis.

CONCLUSION: This study suggests that fermented milk with *S. thermophilus* CRL 1190 and/or its EPS could be used in novel functional foods as an alternative natural therapy for chronic gastritis induced by ASA.

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Key words: Lactic acid bacteria; Probiotics; Gastritis; *Streptococcus thermophilus*; Exopolysaccharides

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INTRODUCTION

Gastritis is a common disorder where discontinuity of the gastric mucosa is observed. It is caused by several factors,

such as alcohol, stress, infection with *Helicobacter pylori* (*H. pylori*)^[1-3], resulting in an imbalance between offensive acid-pepsin secretion and defensive mucosal factors like mucin secretion and cell shedding^[4]. Non-steroidal anti-inflammatory drugs (NSAIDs) such as acetyl-salicylic acid (ASA) are used worldwide as anti-inflammatory and analgesic agents in the treatment of chronic diseases such as rheumatoid arthritis and osteoarthritis^[5] as well as for the prevention of cardiovascular diseases. However, gastrointestinal injury is a serious adverse effect of NSAIDs producing a broad range of toxic effects mainly in the stomach^[6], the toxicity of ASA being attributed to direct damage of mucosal cells^[7]. Furthermore, ASA affects various mucosal defense lines such as bicarbonate secretion, mucus synthesis, decrease of mucosal blood flow^[8,9] with amplification of the inflammatory process by expression of pro-inflammatory cytokines^[10].

Among the most conventional drugs employed for the treatment of gastritis are proton-pump inhibitors such as omeprazole (OM)^[11]; however, most of these drugs also produce undesirable side effects and drug interactions^[12].

Probiotics are “live microorganisms which when consumed in adequate numbers confer a health benefit on the host”^[13]. Probiotic foods containing lactic acid bacteria (LAB) have been used in the treatment of various gastrointestinal disorders, such as gastric ulcers and inflammation related to *H. pylori* infection, gastrointestinal infections or antibiotic-associated diarrhea^[14-16], providing beneficial effects to the host by modulating immune functions, e.g. systemic cytokine production^[17]. The mucosal immune system is functionally divided into sites where foreign antigens are taken up and meet immune cells to initiate the immune response through a network of signals among different cell populations. This cell network is highly integrated by cytokine production, and finely regulated by the selective expression of cytokine receptors. The T-helper (Th) cell subsets and cytokine patterns determine the nature of the immune response^[18].

Some LAB strains secrete exocellular carbohydrate polymers named exopolysaccharides (EPS). A large diversity of EPS from LAB strains exists regarding their chemical characteristics, yield, technological and functional properties^[19-21]. EPS play an important role in the dairy industry mainly in yogurt production and certain kinds of cheeses such as reduced-fat cheddar and mozzarella^[22], improving the textural, melting and sensory characteristics of the products. The health-promoting effects ascribed to probiotic strains or foods arise not only from the bacteria themselves but also from the metabolites produced during fermentation.

EPS from LAB have been claimed to participate in various regulatory processes such as immunomodulatory, cholesterol-lowering and anti-ulcer activities^[23,24]. In previous work^[25], we demonstrated that Balb/c mice fed a fermented milk with the EPS-producing *S. thermophilus* CRL 1190 was efficient in gastritis prevention through the modulation of the immune response and maintenance of the mucus layer. The present study addressed the potential

therapeutic application of fermented milk prepared using the EPS-producing *S. thermophilus* CRL 1190 strain for the treatment of ASA-associated chronic gastritis.

MATERIALS AND METHODS

Strain, culture conditions and preparation of the fermented milk

S. thermophilus CRL 1190 {EPS⁺ and producing also capsular EPS, CPS⁺; [Centro de Referencia para Lactobacilos (CERELA) culture collection, Tucumán, Argentina]} was used in this study. This strain was previously selected for the physicochemical properties of its polysaccharide^[20], for displaying no secondary effects such as bacterial translocation (liver and spleen), and for its effectiveness in preventing gastritis induced by ASA^[25]. The strain was cultured (10 mL/L inoculum) in LAPTg broth (peptone, 15 g/L; tryptone, 10 g/L; yeast extract, 10 g/L; glucose, 10 g/L; and tween 80, 1 mL/L) and sub-cultured at least twice in reconstituted skim milk (RSM, 100 g/L) just prior to experimental use. The strain was maintained at -20°C in RSM containing 100 mL/L glycerol, 10 g/L glucose, and 5 g/L yeast extract.

Fermented milk was prepared in sterile RSM (sterilized at 115°C for 20 min and cooled down to 37°C) using a 10 mL/L inoculum of an active culture of the EPS⁺ strain *S. thermophilus* CRL 1190 (named FM 1190), incubated at 37°C for 16 h and maintained at 4°C prior to experimental use. Non-fermented milk was used as a control.

Animals

Six week-old Balb/c male mice (25-30 g) were obtained from a closed colony kept at the animal facilities of CERELA and maintained in a room with a 12-h light/dark cycle at 20 ± 2°C. Animals were individually housed in cages (20 cm × 30 cm × 15 cm) with litter tray (20 cm × 30 cm × 6 cm) and allowed to have free access to conventional balanced diet and water *ad libitum*. All mice received no food for 24 h before the assays but had free access to water. Animal protocols were approved by the Ethical Committee for animal care of CERELA.

Experimental protocol

Chronic gastritis was induced following the protocol previously standardized in our laboratory^[25]. Oral administration of ASA (BAYER[®]) supplied in the drinking water given at an approximate daily dose of 400 mg/kg per day for 10 d induced chronic gastritis in Balb/c mice (gastritis group, G). The administered dose was twice the analgesic dose for mice and was applied to induce gastritis in a short experimental time period. Healthy mice (H) received drinking water without ASA during the same experimental period (negative control group).

To evaluate the therapeutic effect of FM 1190 on the chronic gastritis model, animals were randomly divided into 6 groups (*n* = 5 each): (1) H group: received drinking water without ASA for 10 d; (2) G group: received ASA for 10 d as described above; (3) FM 1190 group: received

FM with the EPS-producing strain CRL 1190 for 7 d after gastritis induction. FM 1190 was administered *ad libitum* at an approximate dose of 10^8 cfu/mL; daily fermented milk consumption was monitored and intake was set at 5 mL/d; (4) Omeprazole (OM) group: received OM (used as positive control in ASA-induced gastric lesions) at a daily dose of 30 mg/kg per day^[26] for 7 d after gastritis induction; (5) Milk group (M): received non-fermented milk for 7 d after gastritis induction; and (6) Water group (W): received water for 7 d after gastritis induction (used as negative control).

To determine whether the EPS produced by *S. thermophilus* CRL 1190 (EPS 1190) had an anti-gastritis effect, the polymer was isolated from 16-h milk cultures grown at 37°C by using a deproteinization/precipitation technique with 200 g/L (final concentration) trichloroacetic acid, and ethanol (ratio 1:3)^[20], was further purified as described previously^[19], and freeze-dried and stored at 4°C until use. The EPS 1190 was resuspended in RSM (M-EPS 1190) or in water (W-EPS 1190) and administered to mice intragastrically at a dose of 4 mg/kg per day for 7 d after gastritis induction. The administered EPS amount was calculated based on the EPS quantity received by the animals when they were fed with FM 1190.

After the experimental period (day 11 for groups 1 and 2, and day 18 for the remaining groups), mice were sacrificed by cervical dislocation and weighed. Stomachs were aseptically removed, weighed and rinsed several times with saline solution and used for the assays described below.

Histopathological evaluation of gastric samples

Stomachs were fixed in 10% paraformaldehyde in 0.1 mol/L phosphate-buffered saline (PBS) pH 7.0 and embedded in paraffin following the Sainte-Marie technique^[27]. Three serial paraffin sections (4 µm) of each sample were cut from each specimen and stained with hematoxylin-eosin followed by light microscopy examination (Leica DM LS2, Wetzlar, Germany). The pathologic characteristics and degree of inflammation of the gastric mucosa were assessed according to the updated Sydney system^[28] by microscopic observation without knowledge of the experimental groups and expressed as follows: normal appearance of scattered mononuclear cells in the lamina propria (same degree as healthy control mice): none = score 0; mild infiltration of mononuclear cells in the lamina propria and the submucosa, and no erosion in the epithelium: mild = score 1; moderate infiltration of mononuclear cells in the lamina propria and the submucosa, and no erosion in the epithelium: moderate = score 2; and severe infiltration of mononuclear cells in the lamina propria and the submucosa, and erosion in some parts of the epithelium: severe = score 3.

Determination of the number of IL-10, INF-γ, and TNF-α-producing cells in gastric mucosa by indirect immunofluorescence assay

Histological slices of the antral and corpus regions of the stomach, processed as described earlier, were depar-

affinized and rehydrated in a graded series of ethanol. After incubation at room temperature for 30 min in 10 g/L blocking solution of bovine serum albumin (Sigma Chemical Co.), histological slices were incubated at 37°C for 60 min with rabbit anti-mouse IL-10 or INF-γ (Peprotech Inc., NJ, USA) or TNF-α (eBioscience, San Diego, CA, USA) polyclonal antibodies. Then, sections were washed twice with saline solution and treated with a 1/10 dilution of a goat anti-rabbit antibody conjugated with fluorescein isothiocyanate (FITC) (Jackson Immuno Research Inc., PA, USA) at 37°C for 45 min; washed again with saline solution and examined with a fluorescent light microscope (Leica DM LS2). Results were expressed as the number of IL-10, INF-γ and TNF-α-producing cells (fluorescent cells) per 10 fields (magnification × 1000)^[29]. Data were obtained by counting 30 fields from 3 histological slices for each animal group.

Mucus layer determined by periodic acid-Schiff staining

The mucus layer was identified by periodic acid-Schiff (PAS) staining^[30]. Briefly, after deparaffinization and rehydration, tissue sections were oxidized in 10 mL/L periodic acid for 5 min. Then they were rinsed in distilled water and stained with Schiff's reagent for 10 min. After a second washing with distilled water, tissue sections were counterstained with hematoxylin and rinsed in running tap water. Finally, they were dehydrated, cleared and mounted. Sections were viewed under a microscope (Leica M LS2) and the thickness of the mucus-secreting layer in the corpus and antrum mucosa was assessed with an image analyzer (× 1000) and the ratio of the mucus gel layer thickness to that of the lamina propria mucosa was calculated as a percentage.

Statistical analysis

Experimental data were expressed as mean ± SD and statistically evaluated by analysis of variance (ANOVA) with the SPSS software. Multiple group data were analyzed using one-way ANOVA and the Tukey multiple comparison test. Differences were considered statistically significant at $P < 0.05$.

RESULTS

Body weight and stomach weight

No significant changes in body weight or stomach weight were found during the entire experimental period in animals of any group other than those of the FM 1190 and M-EPS 1190 groups, which showed an increase (between 30%-40% with respect to H and G, respectively) in the stomach weight at the end of the experimental period (data not shown). No correlation between stomach weight and induced gastritis was found.

Histopathological evaluation of gastric samples

Mice subjected to oral administration of ASA at doses of 400 mg/kg per day for 10 d (gastritis group, G) showed moderate infiltration with scattered lymphocytes and

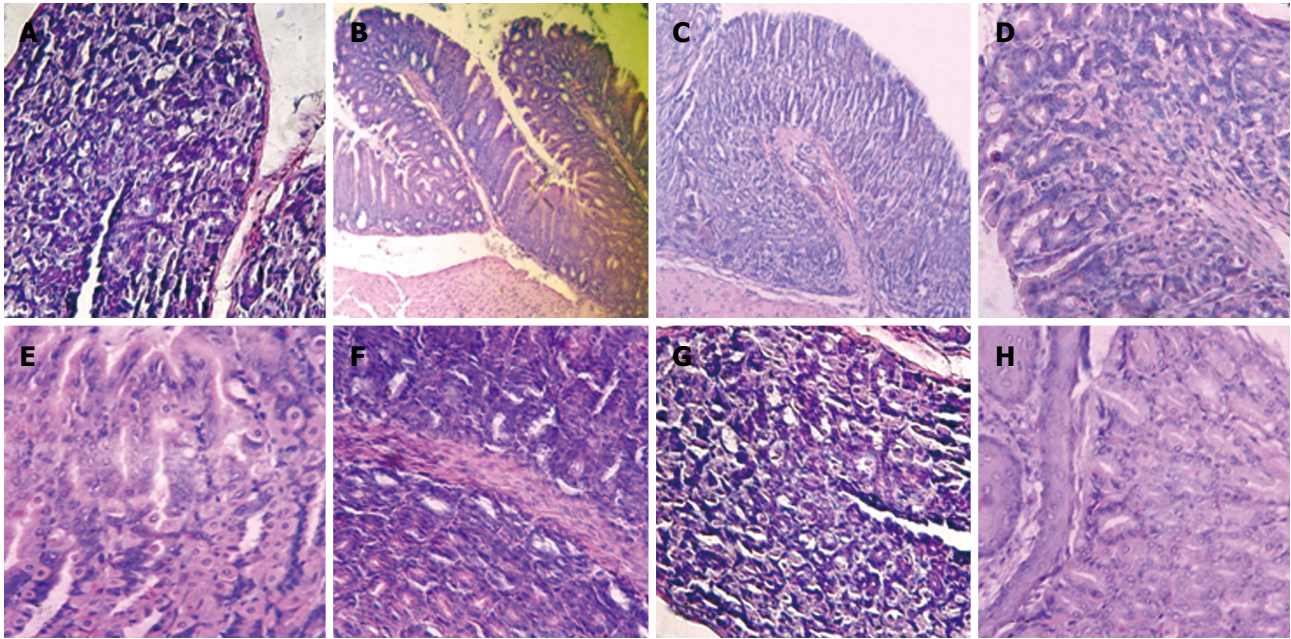


Figure 1 Histological micrographs of mice stomachs. A: H (Healthy) group; B: G (Gastritis) group; C: FM 1190 group (fermented milk with *Streptococcus thermophilus* CRL 1190); D: OM (omeprazole) group; E: M (Milk) group; F: W (Water) group; G: M-EPS 1190 group (EPS produced by *S. thermophilus* CRL 1190 resuspended in milk) showed conserved histological structures; and H: W-EPS 1190 group (EPS produced by *Strep. thermophilus* CRL 1190 resuspended in water) displayed chronic inflammatory infiltrations in the mucosa surface (Hematoxylin & eosin, light microscope, $\times 100$).

macrophages in the surface of the mucosa and folds of mucosa, in the direction of the submucosa, without formation of lymphoid follicles, and predominance in the gastric corpus region (Figure 1B). The induced lesions were classified as superficial chronic gastritis with inflammation score = 2 (Figure 2). No significant increase in polymorphonuclear infiltration in the normal gastric mucosa was observed. Healthy animals (group H) showed an absence of gastritis (Figure 1A). Interestingly, the stomachs from animals treated with FM 1190 and M-EPS 1190 displayed no leukocyte infiltration in the gastric mucosa immediately after treatment (day 18) (Figure 1C and G). Similar stomach structures of mice therapeutically treated with OM (Figure 1D) were observed, showing lower inflammatory scores than the gastritis group (0.2 ± 0.02 vs 2.2 ± 0.4 , $P < 0.05$, Figure 2). In contrast, the animals treated with milk, water and W-EPS 1190 showed high inflammation scores (1.8 ± 0.5 , 2.0 ± 0.5 , 1.7 ± 0.4 , respectively, $P < 0.05$), compared to those of the gastritis group.

Determination of the number of regulatory and pro-inflammatory cytokine-producing cells in the gastric mucosa

The stomachs from group G mice showed a significant decrease in the regulatory cytokine-producing cells ($IL-10^+$: 14 ± 1.3 vs 22 ± 1.7 , $P < 0.05$) and an increase in the pro-inflammatory cytokine-producing cells ($INF\gamma^+$: 28 ± 1.2 vs 14 ± 1.0 and $TNF-\alpha^+$: 33 ± 3.0 vs 15 ± 2.0 , $P < 0.05$) with respect to healthy animals (Figure 3A-C).

The therapeutic administration of FM 1190 to mice regulated the gastric inflammatory process, significantly

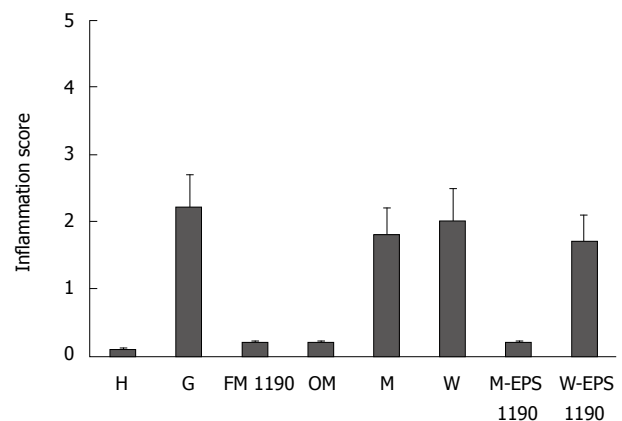


Figure 2 Inflammation score of stomachs in different groups (mean \pm SD).

decreasing the number of pro-inflammatory cytokine-producing cells ($INF\gamma^+$: 15 ± 1.0 vs 28 ± 1.2 , $P < 0.05$ and $TNF-\alpha^+$: 16 ± 3.0 vs 33 ± 3.0 , $P < 0.05$) and increasing the regulatory cytokine-producing cells ($IL-10^+$: 28 ± 1.5 vs 14 ± 1.3 , $P < 0.05$), as compared to the G group. Furthermore, the number of cytokine-producing cells were similar to those of OM and H groups showing $IL-10^+$ values slightly higher than these 2 groups (28 ± 1.5 vs 20 ± 2.9 ; 28 ± 1.5 vs 22 ± 1.7 , respectively, $P < 0.05$). In contrast, a significant decrease in the number of $IL-10^+$ cells and an increase in the number of both pro-inflammatory cytokine-producing cells ($INF\gamma^+$ - and $TNF-\alpha^+$) similar to those of the G group (Figure 3) were found in the M and W groups, used as controls.

M-EPS 1190 but not W-EPS 1190 was able to modulate the induced gastritis in a similar way to FM

1190, showing a decrease in the number of pro-inflammatory cytokine-producing cells ($\text{INF}\gamma^+$: 15 ± 2.0 vs 28 ± 1.2 , $P < 0.05$ and $\text{TNF-}\alpha^+$: 20 ± 2.6 vs 33 ± 3.0 , $P < 0.05$) and an increase in the regulatory cytokine-producing cells (IL-10^+ : 21 ± 2.1 vs 14 ± 1.3 , $P < 0.05$), as compared to the G group (Figure 3).

A correlation between the histopathological structure and the number of regulatory and pro-inflammatory cytokine producing-cells was observed for all groups.

Mucus layer determined by PAS staining

The thickness ratio in the corpus and antrum mucosa, and representative photomicrographs of PAS-stained corpus mucosa from control (H), gastritis (G) and different therapeutic groups (FM 1190, OM, M and W) are shown in Figure 4A and B. The animals treated with ASA (G group) displayed a disruption in the protective mucus layer and exhibited a significant decrease of 80%-85% in the thickness of the mucus gel layer in both the corpus and antrum gastric regions as compared to healthy animals (corpus: 1.07 ± 0.3 vs 5.4 ± 1.0 ; antrum: 1.47 ± 0.4 vs 10.0 ± 1.3 , $P < 0.05$).

In general, the different treatments for gastritis revealed a marked depletion (approximately 80%) in the mucus layer with concomitant reduction in the volume of PAS-positive intramucosal mucus in either the corpus or antrum mucosa of animals of most groups compared to the H group. A different behavior was observed in animals of group FM 1190, which showed a decrease of 49%-59% in the mucus layer of the antrum and corpus mucosa, compared with healthy animals (2.2 ± 0.6 vs 5.4 ± 1.0 ; 5.1 ± 0.8 vs 10.0 ± 1.3 , respectively, $P < 0.05$). However, an increase in the thickness of the mucus gel layer as compared to animals displaying gastritis (G group) was observed (2.2 ± 0.6 vs 1.0 ± 0.3 ; 5.1 ± 0.8 vs 1.5 ± 0.4 , in corpus and antrum mucosa respectively, $P < 0.05$). The volume of intramucosal mucus was maintained compared to the G group, suggesting that FM 1190 was able to protect the stomach mucosal barrier.

Mice treated with M-EPS 1190 showed an increase in the thickness of the mucus gel layer compared to animals with gastritis (2.1 ± 0.5 vs 1.0 ± 0.3 ; 5.0 ± 0.75 vs 1.5 ± 0.4 , in corpus and antrum mucosa, respectively, $P < 0.05$) and was similar to the FM 1190 group.

DISCUSSION

It has been demonstrated previously that ASA administration to mice caused chronic inflammation of the gastric mucosa^[25]. Mononuclear cell infiltration, an increase in the number of pro-inflammatory cytokine-producing cells, a decrease in the regulatory cytokine-producing cells, and depletion of both the mucus gel layer and volume of intramucosal mucus were observed. The use of probiotics has been proposed to ameliorate different gastrointestinal tract disorders including inflammatory bowel diseases, diarrhea, pancreatitis, irritable bowel syndrome and colorectal cancer^[31,32]; however, little attention has been paid to gastric disease. Uchida and Kurakazu^[33] reported

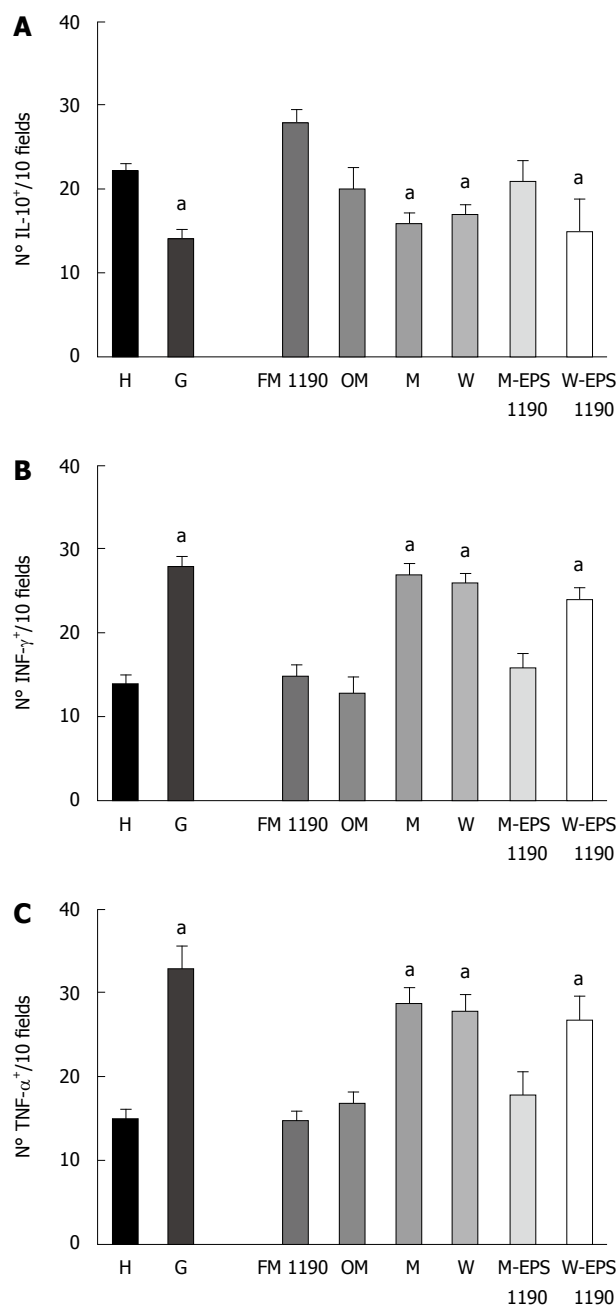


Figure 3 Number of (A) IL-10-, (B) INF- γ - and (C) TNF- α -producing cells on histological slices of stomachs of mice of different groups. Results are presented as means of 3 determinations and are expressed as the number of IL-10-, INF- γ - and TNF- α -producing cells per 10 fields (magnification $\times 100$). ^a $P < 0.05$ compared to the controls.

that yogurt LG21 significantly inhibited the formation of acute gastric lesions caused by HCl in rats; the beneficial effect being dose-dependent. Recently, Liu *et al.*^[34] reported that continuously feeding LAB-fermented soy-skim milk to rats for 28 d inhibited acute gastric lesions induced by ethanol and pylorus ligation in a dose-dependent manner, and improved prostaglandin E2 and superoxide dismutase activities. In addition, some authors have demonstrated the anti-ulcer properties of LAB-fermented milks on *H. pylori*-induced gastric lesions^[14,35]. Based on the multiple functional effects of probiotics on the gastrointestinal

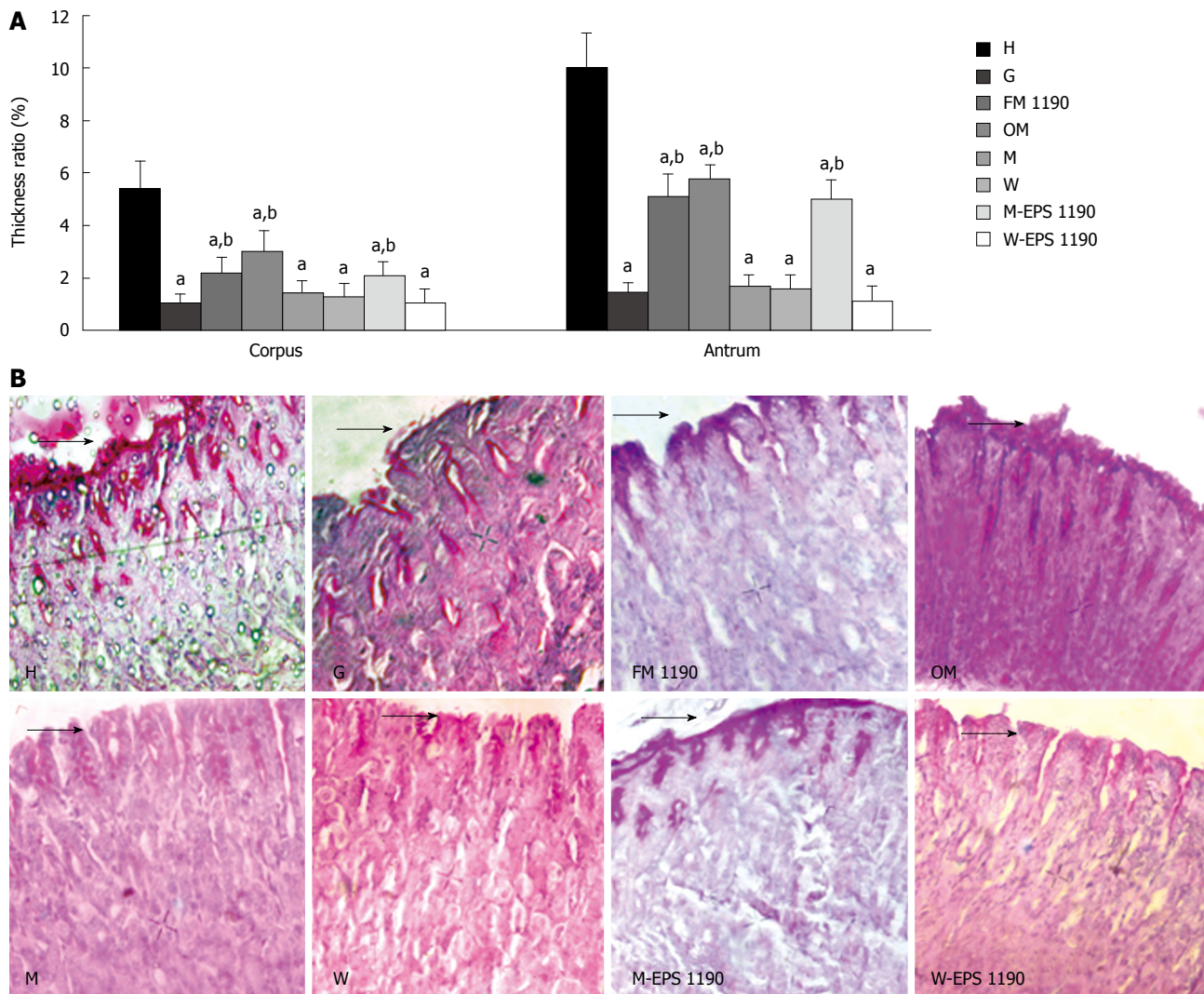


Figure 4 Periodic acid-Schiff (PAS)-positive mucus on the mice gastric mucosa. A: Thickness ratio of the mucus gel layer to the lamina propria mucosa in corpus and antrum mucosa. B: Representative photomicrographs of corpus mucosa from the various groups. Sections were stained using PAS reagent. Values represent mean \pm SE of the mean for 5 mice. ^a $P < 0.05$ compared to the healthy group (H), ^b $P < 0.05$ compared to the gastritis group (G). Original magnification: $\times 400$. Arrows indicate thickness of mucus layer.

tract, we have previously studied the capability of 2 EPS-producing LAB strains to protect the gastric mucosa from challenges produced by ASA; only the strain *S. thermophilus* CRL 1190 successfully prevented gastric damage by modulation of inflammation with significant preservation of the mucus gel layer^[25]. *S. thermophilus* CRL 1190 produces a slime heteropolysaccharide of high molecular mass (MM) composed of D-glucose and D-galactose (molar ratio 1.0:1.5) as well as CPS in milk cultures^[20]. In this work, we evaluated the therapeutic effect of milk fermented with *S. thermophilus* CRL 1190 on ASA-induced chronic gastritis. The therapeutic administration of FM 1190 showed a marked ($P < 0.05$) immunomodulatory effect when comparing the pro-inflammatory cytokine-producing cells (TNF- α and INF- γ) of healthy animals, as a result of inhibition of IL-10 on Th1^[36]. Similar immunological modulation of the preventive effect of *S. thermophilus* CRL 1190 on chronic gastritis was observed in our previous work. Bibiloni *et al.*^[37] demonstrated that the Th1 cell response could be modulated by probiotic bacteria

in pathological processes such as inflammatory bowel disease or colon cancer^[38]. Th1 cytokines such as INF- γ and TNF- α , released by lymphocytes and macrophages that infiltrate the gastric mucosa are associated with immune activation and tissue injury. TNF- α has been shown to be a crucial mediator of NSAID-induced gastric mucosal damage^[39]. In contrast, IL-10 (Th2 type cytokine) suppresses the differentiation and effector functions of Th1 cells and the production of pro-inflammatory cytokines by dendritic cells and macrophages, thus maintaining immune homeostasis^[40]. The proton pump inhibitor drugs used clinically, such as omeprazole, exert an anti-inflammatory action beyond strong acid suppression^[41]. It has been reported that omeprazole regulates the cytokine profile in *H. pylori*-infected patients with duodenal ulcer disease by suppressing cytokine synthesis of the Th1 cells^[42]. In our study, this drug was used as a positive control in the treatment of ASA-induced gastritis, and it displayed similar therapeutic effectiveness to FM 1190, though the fermented milk displayed slightly higher

numbers of regulatory cytokine-producing cells (IL-10⁺) than did omeprazole. On the other hand, this drug has diverse adverse effects such as diarrhea, abdominal pain, cutaneous reactions, decreased bone density and microscopic colitis^[43,44] as well as drug interactions^[45]. Thus, the use of FM 1190 as a therapeutic agent constitutes a safe and nutritional alternative for gastritis treatment.

Myeloperoxidase activity as a neutrophil infiltration marker in gastric tissue was assayed but it could not be used as an inflammatory parameter in the chronic gastritis model as the values obtained were similar to that of the healthy groups (data not shown).

Milk alone, water, omeprazole or the prepared fermented milk did not cause any damage *per se* on the gastric mucosa of healthy animals.

To determine if the therapeutic effect obtained after administration of FM 1190 resulted from the presence of its EPS, the produced biopolymer was isolated, purified and resuspended in milk or water, and assayed for its potential therapeutic effect. As previously observed^[25], only the EPS dissolved in milk (M-EPS 1190) displayed a similar behavior to the fermented milk with respect to the cytokine profile and the histological structures, suggesting that EPS-milk protein interactions play a major role in the immune response modulation and consequently, in the therapeutic effect observed. Whey proteins have been reported to possess biological functions including immunomodulatory activities^[46] in addition to their nutritional value. Rosaneli *et al.*^[47] reported the protective effect of bovine milk whey protein concentrate on the ulcerative lesions caused by administration of indomethacin. Moreover, the gastro-protective effect of α -lactalbumin, one of the major whey proteins, against gastric injury induced by ethanol was demonstrated by enhancing the gastric defense mechanisms such as mucin synthesis and secretion in mucus-producing cells^[48-50].

The mucus gel layer is an important defense barrier, covering gastric epithelial cells and holding bicarbonate ions to neutralize hydrogen ions that diffuse back into the gastric mucosa. However, this layer is frequently disrupted by acid, pepsin, alcohol, and other injurious agents in the gastric lumen resulting in damage to gastric epithelial cells^[51]. The mucus gel layer thickness in the mouse gastric mucosa as well as the volume of intramucosal PAS-positive mucus were evaluated, as a continuous supply of mucus from the intramucosa is important to preserve the surface gel layer. Therapeutic administration of FM 1190 and M-EPS 1190 increased the thickness of the mucus gel layer in both corpus and antrum mucosa without reducing intramucosal mucus. Thus, the activation of mucin synthesis by FM 1190 and M-EPS 1190 led to the increase in the mucus gel layer and a stable mucus supply from the intramucosa. The stimulation of mucus metabolism contributes to the gastroprotective action. *L. rhamnosus* GG, a probiotic EPS-producing strain widely used in dairy products, is able to increase the mucus layer thickness in the gastric glandular mucosa^[29]. Also, it has been observed that the

fungus polysaccharide from *Ganoderma lucidum* reinstated the gastric mucus levels^[52].

Nagaoka *et al.*^[53] reported anti-ulcer effects of EPS produced by bifidobacteria, *Lactobacilli* and *Streptococci* strains, which were attributed to the high rhamnose content (> 60%) of the polysaccharides. Conversely, the fermented milk with the EPS-producing strain *S. thermophilus* CRL 804, which produced an EPS with rhamnose and galactose in its monomer composition^[20], did not show any anti-gastritis effect in contrast to the EPS 1190 that contained galactose and glucose and displayed a gastroprotective effect. Sengül *et al.*^[54] showed that a high MM EPS produced by the probiotic strain *L. delbrueckii* subsp. *bulgaricus* B3 significantly ameliorated experimental colitis in rats. Gao *et al.*^[52,55] found that treatment of acetic acid-induced ulcers in rats with high MM polysaccharide from *Ganoderma lucidum* suppressed or restored the decreased gastric mucus levels, increased gastric prostaglandin concentrations and partly suppressed the TNF- α gene. In addition, a high MM pectin polysaccharide from Chinese herbs has shown to be a potent anti-ulcer compound in experimental HCl-ethanol induced ulcers^[56]. Moreover, a high MM-homopolysaccharide from marine microalga *Gyrodinium impudicum* strain KG03 presented immunostimulatory effects, enhancing the tumoricidal activities of macrophages and natural killer cells *in vivo*^[57]. In this case, the beneficial effect was attributed to the sulfate groups present in the polymer, which also contained galactose and uronic acids. In our work, the anti-gastritis effect observed for FM 1190 may be ascribed to the large polymer size of EPS 1190, independently of its monomer composition. In addition, it was recently demonstrated^[58] that EPS 1190 was partially degraded when the polymer was submitted to the harsh conditions of an *in vitro* gastric system, indicating that this polymer may still exert its beneficial properties *in vivo*.

The present findings indicate that the milk fermented with *S. thermophilus* CRL 1190 and/or its EPS was effective in the therapeutic treatment of chronic gastritis by modulating the immune response of the mice and by increasing the thickness of the gastric mucus gel layer. Thus, the application of this fermented milk and/or its EPS constitutes a potential natural alternative for the prevention and treatment of ASA-associated gastric damage.

COMMENTS

Background

Gastritis is a common disorder where there is discontinuity in the gastric mucosa. It is caused by several factors including the intensive consumption of anti-inflammatory drugs such as acetyl-salicylic acid (ASA), commonly used in the treatment of chronic diseases and prevention of cardiovascular pathologies. The conventional drugs employed as therapies against gastritis often produce undesirable side effects. The administration of specific probiotics provides a new therapy against gastric disease.

Research frontiers

Gastritis affects 80% of the worldwide population according to data of the Worldwide Health Organization. The use of probiotics has been proposed to ameliorate different gastrointestinal tract disorders such as inflammatory bowel disease, diarrhea, irritable bowel syndrome, etc.; however, little attention has been

paid to gastric disease. Thus, the authors decided to investigate the therapeutic effect of milk fermented with exopolysaccharide (EPS)-producing *S. thermophilus* CRL 1190 and its polymer on chronic gastritis induced by aspirin in mice.

Innovations and breakthroughs

This research demonstrates for the first time the therapeutic effect of the fermented milk with the polymer-producing strain *S. thermophilus* CRL 1190 and/or its EPS on chronic gastritis induced by ASA in mice. Both the fermented milk and the EPS were able to modulate the immune response in mice and increased the thickness of the gastric mucus gel layer. Furthermore, the therapeutic effectiveness observed was similar to omeprazole®, a commercial drug commonly employed in the treatment of gastritis.

Applications

The fermented milk with the EPS-producing strain *S. thermophilus* CRL 1190 and/or its EPS constitutes a potential natural alternative for the prevention and treatment of ASA-associated gastric damage.

Terminology

EPS are carbohydrate polymers naturally produced by certain bacteria, algae, yeasts and fungi. These polymers are extensively used in several industries mainly due to their thickening, texturizing, and gelifying properties.

Peer review

The research is a well carried out study.

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Effects of thymidine phosphorylase on tumor aggressiveness and 5-fluorouracil sensitivity in cholangiocarcinoma

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Abstract

AIM: To evaluate the role of thymidine phosphorylase (TP) in cholangiocarcinoma using small interfering RNA (siRNA).

METHODS: A human cholangiocarcinoma-derived cell line KKK-M139, which has a naturally high level of endogenous TP, had TP expression transiently knocked down using siRNA. Cell growth, migration, *in vitro* angiogenesis, apoptosis, and cytotoxicity were assayed in TP knockdown and wild-type cell lines.

RESULTS: TP mRNA and protein expression were decreased by $87.1\% \pm 0.49\%$ and $72.5\% \pm 3.2\%$, respectively, compared with control cells. Inhibition of TP significantly decreased migration of KKK-M139, and suppressed migration and tube formation of human umbilical vein endothelial cells. siRNA also reduced the ability of TP to resist hypoxia-induced apoptosis, while suppression of TP reduced the sensitivity of KKK-M139 to 5-fluorouracil.

CONCLUSION: Inhibition of TP may be beneficial in decreasing angiogenesis-dependent growth and migration of cholangiocarcinoma but may diminish the response to 5-fluorouracil chemotherapy.

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Key words: Liver fluke; Cholangiocarcinoma; Thymidine phosphorylase; 5-fluorouracil; siRNA; Tumor aggressiveness; Cell migration

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INTRODUCTION

Cholangiocarcinoma is a common hepatobiliary malignancy among the Northeastern Thai population and it is an important public health problem because the incidence and fatality rates are high^[1,2]. Liver fluke (*Opisthorchis viverrini*) infection is a risk factor for cholangiocarcinoma, which accounts for about 89% of all liver cancer cases in Khon Kaen province, where the liver fluke is highly endemic and the incidence of cholangiocarcinoma is the highest in the world (97.4 per 100 000 males and 39 per 100 000 females)^[1]. A number of different genes have been implicated in carcinogenesis of cholangiocarcinoma, e.g. *p53*^[3], *MDM2*^[3], *bMLH1* and *bMSH2*^[4,5], and *TFE1*^[6]. We previously demonstrated the high prevalence of thymidine phosphorylase (TP) gene amplification (53.8%; 35 of 65 cases) in cholangiocarcinoma tumor tissues, suggesting that TP may play an important role in carcinogenesis of liver fluke-related cholangiocarcinoma^[7].

TP (EC 2.4.2.4) is located in chromosomal region 22q13.33 and encodes a protein that catalyzes the reversible phosphorolysis of thymidine, deoxyuridine, and their analogs to their respective bases and 2-deoxyribose-1-phosphate, which is then dephosphorylated to 2-deoxy-D-ribose^[8]. TP has been proposed to function in DNA synthesis, cell growth, chemotaxis stimulation in endothelial cells *in vitro*, and to enhance angiogenesis *in vivo*^[9,10]. TP is abnormally expressed in certain cancers of the gastrointestinal tract e.g. pancreatic cancers^[11], colon carcinomas^[12], gallbladder adenocarcinomas^[13], and intrahepatic cholangiocarcinoma^[14]. Interestingly, TP expression was found to be elevated from 10- to 260-fold in nearly all biopsies examined from carcinomas of the stomach, colon, ovary, and bladder when compared to non-neoplastic regions of these organs^[15,16]. Nevertheless, how TP expression is up-regulated in human tumors remains unclear.

During the past few years the utility of RNA interference (RNAi) and post-transcriptional gene silencing has significantly advanced the study of the effects of loss of individual gene functions. Therefore, we investigated the functions and roles of TP in carcinogenesis of liver fluke-related cholangiocarcinoma using small interfering RNA (siRNA) to suppress TP expression. Understanding the functions of TP involved in cholangiocarcinoma will help us elucidate the molecular mechanisms of chol-

angiocarcinogenesis and thus could potentially provide important strategies for prevention, early diagnosis, and early treatment to reduce the cancer incidence and increase cholangiocarcinoma patient survival.

MATERIALS AND METHODS

Cell lines and culture condition

The KKKU-M139 cell line was established from the primary tumor of a 53-year-old Thai woman with squamous cell type cholangiocarcinoma. It was selected for this work after prior screening of TP expression in 5 cholangiocarcinoma-derived cell lines (data not shown). KKKU-M139 was maintained in RPMI-1640 medium containing 100 IU/mL penicillin, 100 µg/mL streptomycin, and 100 mL/L heat-inactivated fetal bovine serum (FBS) as a complete medium. The human umbilical vein endothelial cells (HUVECs) isolation procedure followed the protocol of Jaffe *et al.*^[17]. HUVECs were maintained in M199 medium containing 100 U/mL penicillin, 100 µg/mL streptomycin, 50 mL/L pooled human serum, and 300 mL/L heat-inactivated FBS. Culturing was carried out at 37°C in a humidified 50 mL/L CO₂ incubator. Confluent HUVEC monolayers (passages 2-4) were used in the assays.

Transient knockdown of TP using siRNA

siRNA sequences against human TP were designed and synthesized by Stealth RNAi (Invitrogen, Carlsbad, CA, USA). The siRNA sequences were 5'-AUAGACUCCA GCUUAUCCAAGGUGC-3' (sense) and 5'-GCACCU UGGUAUAGCUGGAGUCUAU-3' (antisense). A non-related control siRNA that lacked identity with known gene targets was used as a control for non sequence-specific effects. TP siRNA or control siRNA (20 pmol) was transiently transfected into 1 × 10⁵ KKKU-M139 using OligofectAMINE (Invitrogen) according to the manufacturer's instructions. Briefly, siRNA:OligofectAMINE complexes were mixed gently in Opti-MEM I reduced-serum medium and incubated for 20 min at room temperature. Then 200 µL of the complex mixture was added to cells growing in a 35-mm Petri dish containing 800 µL Opti-MEM I and mixed gently. After 24 h incubation at 37°C in a 50 mL/L CO₂ incubator, the complexes were replaced by complete medium. Cells were incubated further for 24 h and gene function assays performed within 72 h post-transfection. The intensity of TP expression, either mRNA or protein, was analyzed using ImageJ software version 1.32j (NIH, Bethesda, MD, USA). To determine the knockdown efficiency, the density of TP expression was normalized with its corresponding β-actin for mRNA or α-tubulin for protein. The normalized intensity was compared between TP siRNA-transfected cells (TP-deficient cells) and control siRNA-transfected cells (control cells).

Reverse transcription and semi-quantitative polymerase chain reaction

Total RNA from the KKKU-M139 cell line was reverse transcribed using Omniscript reverse transcriptase (QIA-

gen, Hilden, Germany). The forward and reverse primer sequences of TP were 5'-GGCATGGATCTGGAG-GAGAC-3' and 5'-CTCTGACCCACGATACAGCA-3', respectively. The forward and reverse primer sequences for β -actin as control were 5'-ACTGGGACGACATG-GAGAAA-3' and 5'-ATAGCACAGCCTGGATAGCA-3', respectively. Polymerase chain reaction (PCR) amplification was carried out in a final volume of 20 μ L containing first-strand cDNA, 10 pmol of each primer, 5 U Taq polymerase in 1 \times Taq buffer (67 mmol/L Tris-HCl, 16.6 mmol/L ammonium sulfate, and 10 mL/L Tween 20), 2.5 mmol/L $MgCl_2$, and 0.2 mmol/L dNTPs. The amplification was initiated by incubation at 94°C for 5 min followed by 40 cycles of denaturation at 94°C for 30 s, annealing at 59°C (for TP) or 60°C (for β -actin) for 30 s, and extension at 72°C for 30 s, with a final 10-min extension at 72°C. PCR products were separated on a 20 g/L agarose gel containing 100 ng/mL ethidium bromide. Gels were visualized and photographed on a UV Transilluminator.

Western blot analysis

Total protein was separated on 10% SDS-PAGE then transferred onto a nitrocellulose membrane and blocked with TBST buffer (20 mmol/L Tris-HCl, 150 mmol/L NaCl, 0.5 mL/L Tween 20) plus 50 g/L skim milk. The membrane was probed with anti-TP (Taiho Pharmaceutical Co., Ltd. Japan) or anti- α -tubulin (Zymed Laboratories Inc., San Francisco, CA, USA) as a control at a dilution of 1:1000 in TBST followed by a dilution of 1:2000 of a horseradish peroxidase-conjugated secondary antibody. Immunopositive bands were detected with freshly prepared ECL chemiluminescent solution (Amersham Biosciences, Buckinghamshire, England) and visualized by exposure to X-ray film.

Cell growth assay

To evaluate the cellular growth rates, 1×10^4 wild-type, TP-deficient, or control cells were seeded in 6-well cell culture plates and grown in complete medium at 37°C in a 50 mL/L CO_2 incubator. The cells were counted in triplicate every day for up to 6 d following a trypan blue dye exclusion assay. The population doubling time was calculated from the exponential growth phase.

Migration assays

The migration of HUVECs stimulated by TP-expressing KKU-M139 cells was evaluated by a 2-chamber assay using a 96-well modified Chemotaxicell chamber (Kurabo, Japan) with an 8- μ m pore size membrane. In brief, 100 μ L total protein of wild-type, TP-deficient or control cells (PBS alone as a negative control) was added to the lower chamber. HUVECs (1×10^5 resuspended in 60 μ L PBS) were placed in the upper chamber then incubated at 37°C in a 50 mL/L CO_2 incubator for 4 h. The number of cells that moved through the pores toward the attractant—that is, TP protein produced by KKU-M139 cells—and settled on the bottom chamber was counted using trypan blue staining. The assays were performed in triplicate. The

migration assay for KKU-M139 cells was carried out using the same protocol, but the lower chamber contained 100 μ L of a complete medium as a chemoattractant. RPMI-1640 medium containing 10 mL/L FBS served as a negative control. Wild-type, TP-deficient or control cells (1×10^5 in suspension in 60 μ L fresh RPMI-1640) were placed in the upper chamber.

In vitro angiogenesis assay

The HUVEC tube-like structure was determined using the Endothelial Tube Formation Assay (Cell Biolabs Inc., San Diego, CA, USA) according to the manufacturer's protocol. In brief, HUVECs were harvested and resuspended in M199 culture medium containing 100 mL/L FBS and angiogenesis mediators, i.e. serum of endometriosis patients as a positive control, total protein from KKU-M139 cells before and after treatment with TP-siRNA. Cell suspension (150 μ L, 1.5×10^4 cells) was layered on the 50 μ L of solidified extracellular matrix (ECM) gel in each 96-well plate, and the plate was incubated at 37°C for 18 h to allow the HUVECs to reorganize into a 3-dimensional tubular structure. The tube-like structures were stained with Calcein AM fluorescent dye, and examined and captured under a fluorescence microscope (Olympus CKX41, Tokyo, Japan). The tube length was quantified using ImageJ (NIH, Bethesda, MD, USA) and represented as total tube length (μ m) for 3 photographic fields per experimental condition. Each treatment was performed in duplicate, and the set of experiments was repeated twice independently.

TUNEL apoptosis assay

The DeadEnd™ Colorimetric TUNEL System (Promega, Madison, WI, USA) was used to detect apoptosis in cultured cell lines. In brief, cells grown in a 35-mm petri-dish were exposed to hypoxic conditions for 48 h. Cells were allowed to undergo apoptosis at 37°C in a humidified 50 mL/L CO_2 incubator for 12 h. Treated cells were fixed with 40 g/L buffered formaldehyde, permeabilized by 2 mL/L Triton X-100, and equilibrated with Equilibration buffer (Promega). The cells were incubated with recombinant terminal deoxynucleotidyl transferase (rTdT) reaction mix, containing biotinylated nucleotides, inside a humidified chamber at 37°C for 1 h to allow end-labeling of the fragmented DNA of apoptotic cells to occur. The reactions were terminated by addition of 2 \times SSC buffer, and endogenous peroxidases were blocked with 3 mL/L H_2O_2 . To detect the incorporated biotinylated nucleotides, streptavidin-horseradish peroxidase solution (Promega) in PBS was used. A total of 100 μ L diaminobenzidine chromogen was added to each plate and developed until a light brown background occurred. Cells stained brown was counted per total number of cells in the 100 \times power field of inverted microscope. The assays were performed in duplicate, and the experiment was repeated at least 3 times.

MTS cytotoxicity assay

To determine the concentration of 5-fluorouracil that

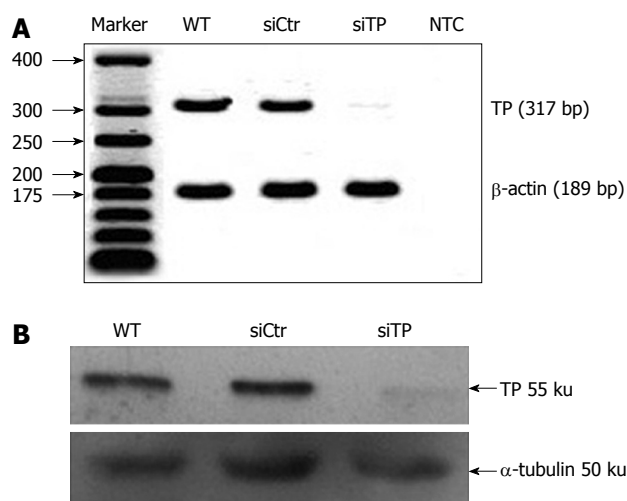


Figure 1 Small interfering RNA (siRNA) significantly reduced thymidine phosphorylase (TP) mRNA analyzed by semi-quantitative polymerase chain reaction (A) and protein (B) analyzed by Western blot of TP-deficient (siTP) cells compared with wild-type (WT) and control (siCtr) cells. For negative control (NTC), no cDNA was present in the reaction.

inhibited cell proliferation by 50% (IC_{50}), the MTS assay (Promega) was used according to the manufacturer's instructions. Briefly, at one day post-transfection, 100 μ L of cell suspension (1×10^5 cells/mL) was added to each well of a 96-well flat-bottom culture plate then incubated at 37°C in a humidified 50 mL/L CO_2 incubator. After 24 h incubation, 100 μ L medium containing various concentrations of 5-fluorouracil or fresh medium for untreated controls was added to each well for 48 h. The culture medium containing dead cells was then removed, and 100 μ L of medium containing 20 μ L MTS solution (317 μ g/mL) was added to each well for 2 h to allow living cells to catalyze the MTS to a colored formazan product which was measured at 490 nm using a microplate reader (Tecan Austria GmbH, Salzburg, Austria). The assays were performed in duplicate, and the experiment was repeated twice.

Statistical analysis

Values are presented as mean \pm SD. The statistical significance of the data was analyzed by one-way ANOVA using SPSS statistical software version 10.0 for Windows (SPSS Inc, Chicago, IL, USA). $P < 0.05$ was considered statistically significant.

RESULTS

TP is not a pivotal factor for tumor cell proliferation in cholangiocarcinoma

After TP-siRNA transfection, expression of TP mRNA (Figure 1A) and protein (Figure 1B) was suppressed by $87.1\% \pm 0.49\%$ and $72.5\% \pm 3.2\%$, respectively, compared with control cells. To evaluate TP function on the proliferation of KKU-M139, the cell growth assay was performed. The population doubling time calculated from exponential growth phase (day 1-day 4) of wild-

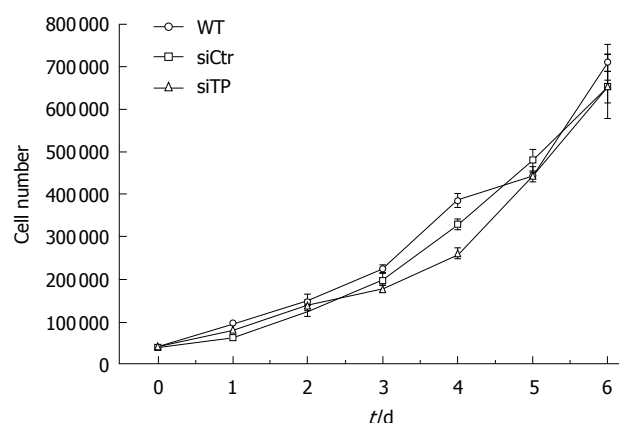


Figure 2 Kinetic growth curves of KKU-M139 WT, control (siCtr), and TP-deficient (siTP) cells. The doubling time of TP-deficient cells was not significantly different from that of WT or control cells.

type, TP-deficient, and control cells was 25.7 ± 4.7 , 29.7 ± 2.4 , and 29.6 ± 6 h ($n = 4$), respectively (Figure 2). Doubling time of TP-deficient cells was not significantly different from that of wild-type or control cells.

TP-siRNA decreases TP-induced migration of HUVECs and KKU-M139

Angiogenesis, which is crucial for the development and progression of various cancers, requires migration and proliferation of endothelial cells in order to form the vasculature. Therefore, we tested the ability of TP to activate migration of endothelial cells using a 2-chamber assay. Migration of HUVECs toward total protein of TP-deficient cells was similar to that of PBS (Figure 3A). Use of total protein of TP-expressing KKU-M139 cells as a chemoattractant induced the migration of HUVECs by more than 2-fold relative to PBS ($P < 0.05$). Migration of cancer cells is important for invasion and metastasis. We investigated the migration of wild-type, TP-deficient, and control cells directed to 100 mL/L FBS as a chemoattractant. Migration towards 100 mL/L FBS of TP-deficient cells was significantly reduced by 2.4-fold compared with wild-type ($P < 0.001$) and 1.8-fold compared with control cells ($P < 0.01$) (Figure 3B).

TP-siRNA attenuates TP-induced tube formation of HUVECs

To assess the effect of TP on the formation of blood vessels, we analyzed the ability of TP produced from KKU-M139 to induce HUVECs to form tube structures using an ECM gel angiogenesis assay. Tube-like structures were formed by HUVECs activated by serum of endometriosis patients as a positive control (Figure 4A) but not by the PBS as a negative control (Figure 4B). HUVECs exhibited significant tube formation when total protein from wild-type KKU-M139 cells (Figure 4E and F), which endogenously express TP, was added ($P < 0.001$ vs PBS). Pre-treatment of KKU-M139 cells with TP-siRNA (Figure 4D) to suppress TP expression reduced tube formation of HUVECs compared with control cells (Figure 4C) (P

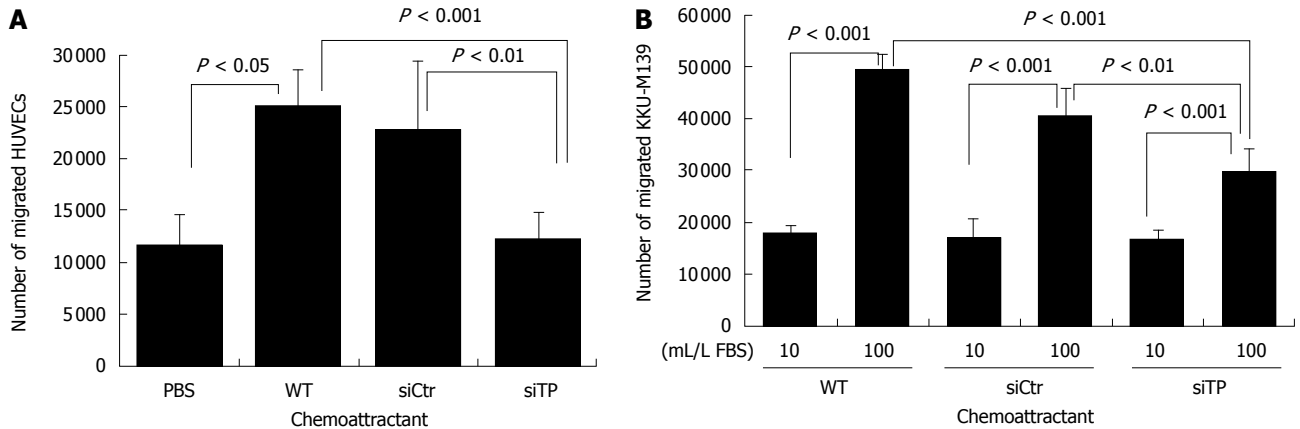


Figure 3 Knockdown of TP reduces migration of HUVECs and KKKU-M139. **A:** Migration of HUVECs toward protein produced from TP-deficient cells (siTP) was similar to that of PBS, and 2-fold less than that of wild-type (WT); **B:** TP-deficient KKKU-M139 cells are less migratory in response to FBS compared with wild-type, and control.

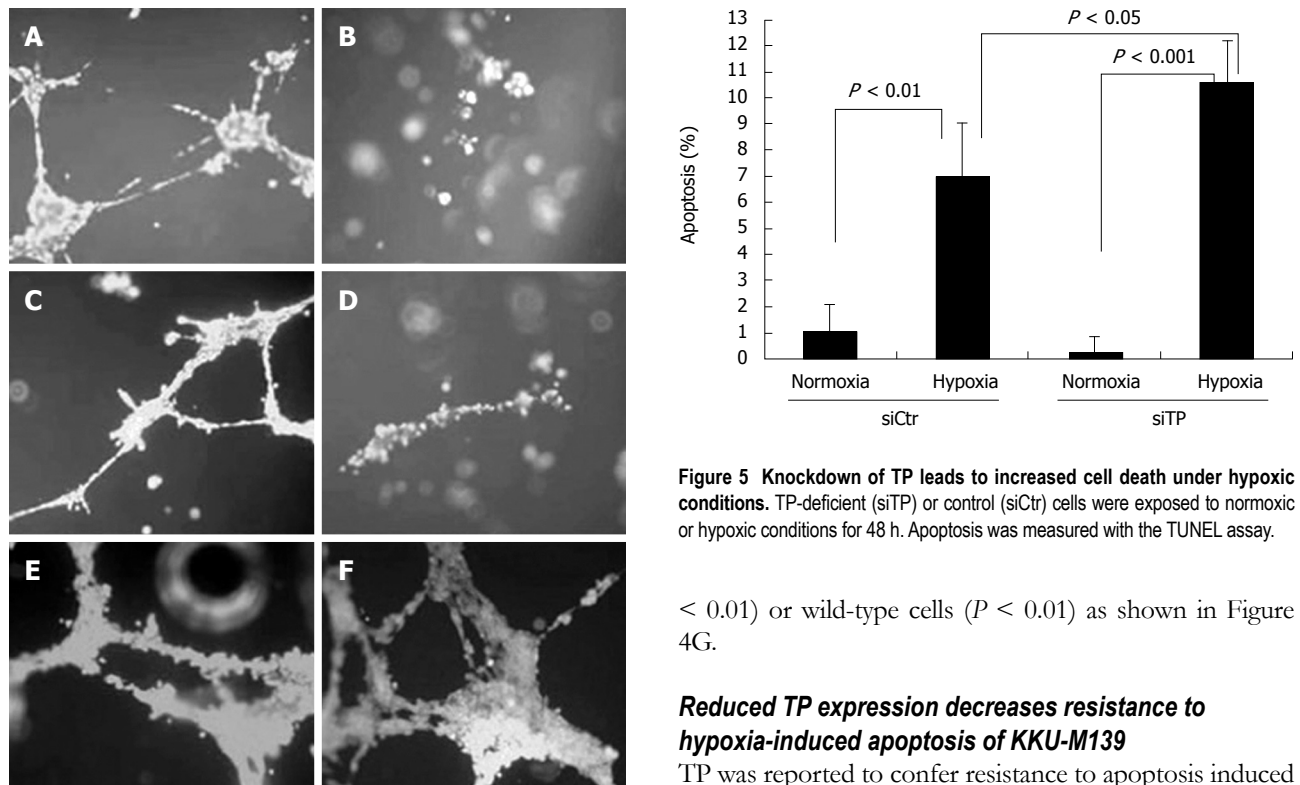


Figure 4 Knockdown of TP decreases the formation of tube-like structures in an ECM gel angiogenesis assay. Serum of endometriosis patients was used as a positive control (A), and PBS as a negative control (B). Total protein of control (siCtr) (C), TP-deficient (siTP) (D), or WT cells (E and F) were assayed. The tube length was quantified (G).

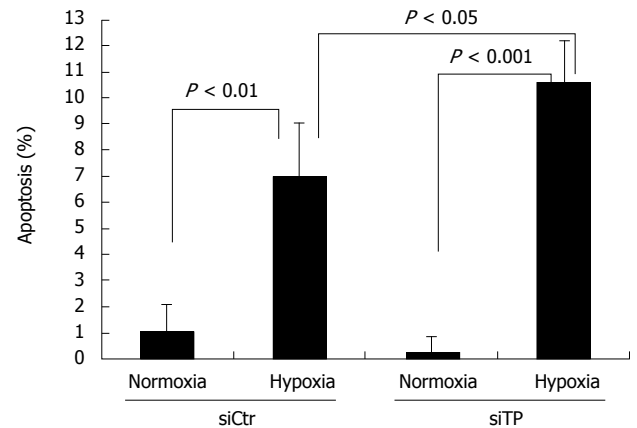


Figure 5 Knockdown of TP leads to increased cell death under hypoxic conditions. TP-deficient (siTP) or control (siCtr) cells were exposed to normoxic or hypoxic conditions for 48 h. Apoptosis was measured with the TUNEL assay.

< 0.01) or wild-type cells ($P < 0.01$) as shown in Figure 4G.

Reduced TP expression decreases resistance to hypoxia-induced apoptosis of KKKU-M139

TP was reported to confer resistance to apoptosis induced by hypoxia. Under hypoxic conditions, the TP-deficient cells exhibited significantly more cell death than did the control cells ($P < 0.05$). Thus, TP seems to confer resistance to hypoxia-induced apoptosis in control cells. For both control and TP-deficient cells, the percent apoptosis under normoxic conditions was less than that under hypoxic conditions ($P < 0.01$ and $P < 0.001$, respectively). Anti-apoptotic activity of KKKU-M139 under 48 h hypoxic conditions was suppressed by TP knockdown (Figure 5).

TP-siRNA decreases the sensitivity of KKKU-M139 cells to 5-fluorouracil

5-fluorouracil is a commonly used chemotherapeutic drug for cholangiocarcinoma, and TP is needed for 5-fluorouracil activation. To determine to what extent TP contributes to the cytotoxic effect of 5-fluorouracil, cells were

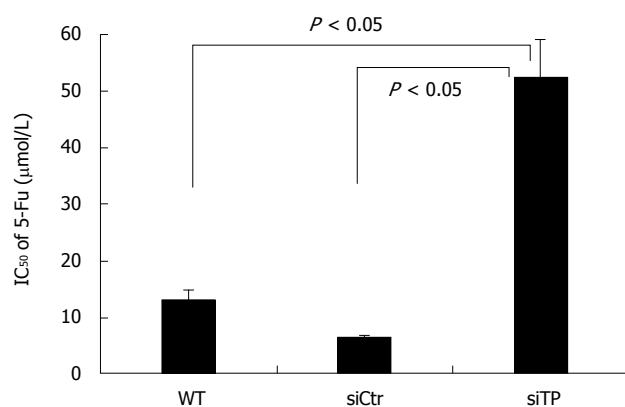


Figure 6 Effects of TP-siRNA on 5-fluorouracil (5-Fu) cytotoxicity of KKKU-M139. KKKU-M139 WT, control (siCtr), and TP-deficient (siTP) cells were incubated with various concentrations of 5-fluorouracil for 48 h. Sensitivity of the cells to 5-fluorouracil was then determined with a MTS cytotoxicity assay.

exposed to a range of concentrations of 5-fluorouracil in the presence or absence of siRNA against TP. The IC₅₀ of control and wild-type cells were 6.4 ± 0.36 and 13 ± 1.70 μmol/L 5-fluorouracil, respectively. As expected, TP-deficient cells required 52.3 ± 6.8 μmol/L 5-fluorouracil, an 8.2-fold increase over that in control cells ($P < 0.05$), to inhibit cell proliferation by 50% (Figure 6).

DISCUSSION

We previously reported that 35 of 65 (53.8%) liver fluke-related cholangiocarcinoma cases exhibited TP amplification, suggesting that TP may contribute to the pathogenesis of cholangiocarcinoma^[7]. Here, we report that TP confers resistance to apoptosis, induces migration of a cholangiocarcinoma-derived cell line, and mediates migration and tube formation of HUVECs. However, TP is essential for activation of 5-fluorouracil, a commonly used anti-cancer drug for treatment of cholangiocarcinoma. In fact, TP amplification and overexpression have been reported for various solid tumors that exhibited invasion, metastasis, angiogenesis, and poor prognosis, including cholangiocarcinoma^[13-16,18,19]. In this study, transfection with TP-siRNA suppressed TP mRNA expression by approximately 87% and protein expression by 72% (Figure 1), indicating effective gene silencing. The population doubling time of TP-deficient cells was not significantly different from that of wild-type or control cells (Figure 2), suggesting that TP is not a pivotal factor for tumor cell proliferation in cholangiocarcinoma.

Increased TP expression has been shown to increase invasiveness of KB epidermoid carcinoma cells compared with control cells^[20]. Migration is an important characteristic of metastatic cancer cells. TP-expressing KKKU-M139 also exhibited greater migration than TP knockdown cells (Figure 3B). Nakajima *et al.*^[21] determined the molecular basis for induction of invasive activity by TP. They suggested that TP and 2-deoxy-D-ribose induces the expression, secretion, and activity of matrix metalloproteinase-9 (MMP-9) that subsequently confers

invasiveness on cancer cells, but the exact mechanism by which TP and one of its degradation products, 2-deoxy-D-ribose, enhance MMP-9-mediated invasive activity is not known. The expression level of TP was reported to significantly correlate with that of the invasion-related genes MMP-1, MMP-7, and MMP-9^[22]. Taken together, these data indicate that TP enhances the invasion of tumor cells through the induction of invasion-related genes in cholangiocarcinoma.

The study by Hotchkiss *et al.*^[23] demonstrated that TP-expressing cells mediated HUVEC migration via the intracellular metabolism of thymidine by TP and the subsequent extracellular release of 2-deoxy-D-ribose, which formed a chemotactic gradient. Later, they proved that both TP and 2-deoxy-D-ribose stimulated HUVEC migration by increasing cell surface expression of the integrins $\alpha_5\beta_1$ and $\alpha_v\beta_3$ to form focal adhesions. Integrins bind to the ECM and directly activate focal adhesion kinase signaling pathways, mediating cell attachment and migration^[24]. KKKU-M139, which endogenously expresses TP, also strongly induced HUVEC migration, an early step in angiogenesis. Our study showed that TP-expressing KKKU-M139 could stimulate the migration and formation of tube-like structures of HUVECs to a much greater degree than TP-silenced KKKU-M139 (Figure 3A and 4). In addition, 2-deoxy-D-ribose can mediate an oxidative stress mechanism and upregulate other angiogenic factors^[25]. Attracted endothelial cells proliferate upon stimulation by growth factors released from cancer cells and subsequently form new blood vessels. Suppression of TP-mediated migration and angiogenesis of HUVECs by TP-siRNA thus can be explained by the fact that TP and its product, 2-deoxy-D-ribose, could not exert their effect on cell migration and tube formation. Chemically synthesized TP inhibitor (TPI) has been demonstrated to suppress tumor growth by increasing the proportion of apoptotic cells and probably by inhibiting angiogenesis; TPI completely suppressed angiogenesis by TP-cDNA transfected KB cells^[26]. 2-deoxy-L-ribose, the stereoisomer of 2-deoxy-D-ribose, is also a TPI, as shown by the ability of 2-deoxy-L-ribose to inhibit 2-deoxy-D-ribose-mediated angiogenesis and metastasis of tumor cells expressing TP^[20,27]. Our study also demonstrated that TP-siRNA was able to act as TP suppressor or inhibitor.

Resistance to apoptosis is a key process in tumor formation. 2-deoxy-D-ribose was found to be involved in a hypoxia-induced apoptotic pathway. 2-deoxy-D-ribose inhibited hypoxia-induced phosphorylation of p38 mitogen-activated protein kinase but not c-jun NH₂-terminal kinase/stress-activated protein kinase in human leukemia HL-60 cells^[28]. In addition, 2-deoxy-D-ribose and thymine partially prevented hypoxia-induced apoptosis^[29]. 2-deoxy-D-ribose may be an important energy source under hypoxic conditions^[30]. The ability of TP-siRNA to increase the proportion of apoptotic KKKU-M139 cells (Figure 5) was most likely attributable to an inhibition of TP pathways, however, further studies are needed to de-

termine the exact role of TP in these tumor phenotypes.

The enzymatic activity of TP is also indispensable for the activation of prodrug 5'-deoxy-5-fluorouridine (5'-DFUR) to active 5-fluorouracil and fluorodeoxyuridine in tumors. The sensitivity of TP cDNA-transfected MCF-7 breast cancer cells to 5'-DFUR was increased approximately 20-fold compared to the parent cells or cells with control vector alone, and sensitivity to 5-fluorouracil was also somewhat increased^[31]. The sensitivity of a TP-transfected SMMC-7721 hepatocellular carcinoma cell line to 5'-DFUR was also significantly enhanced; however, endothelial cell migration was also promoted at the same time^[32]. We demonstrated that TP-siRNA was able to suppress TP expression *in vitro* and that this treatment impaired the therapeutic efficacy of 5-fluorouracil in KKU-M139 (Figure 6), indicating that TP enzyme activity is needed for 5-fluorouracil activation.

In conclusion, we found that TP plays a dual role in development and therapy of cholangiocarcinoma. TP confers apoptotic resistance and migration of a cholangiocarcinoma-derived cell line, and also induces migration and tube formation of HUVECs. On the other hand, TP is essential for the therapeutic efficacy of 5-fluorouracil chemotherapy. It may be useful to examine the expression level of TP in tumors of early stage cholangiocarcinoma patients to select those likely to respond well to 5-fluorouracil. However, most cholangiocarcinoma patients are diagnosed at a late stage, and there are several chemotherapeutic drugs of choice in addition to 5-fluorouracil for cholangiocarcinoma. Inhibition of TP activity may be helpful in decreasing tumor aggressiveness i.e. migration, angiogenesis and anti-apoptosis, and improving the poor prognosis of cancer patients who show high TP expression.

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COMMENTS

Background

Liver fluke-related cholangiocarcinoma is the most common hepatobiliary malignancy found in Northeast Thailand. Amplification of thymidine phosphorylase (TP) copy number was found in most cholangiocarcinoma patients suggesting that it has a significant role in tumor progression.

Research frontiers

TP catalyzes the reversible phosphorolysis of thymidine, deoxyuridine, and their analogs to their respective bases and 2-deoxyribose-1-phosphate which is then dephosphorylated to 2-deoxy-D-ribose. TP is abnormally expressed in certain cancers of the gastrointestinal tract including cholangiocarcinoma. However, how TP expression is upregulated in human tumors remains unclear. In this study, the authors investigated the roles of TP in liver fluke-related cholangiocarcinoma using RNA interference to suppress TP expression.

Innovations and breakthroughs

Suppression of TP not only reduces angiogenesis, resistance to apoptosis, and

tumor migration but also diminishes chemosensitivity to 5-fluorouracil in cholangiocarcinoma.

Applications

Because of its significance in tumor aggressiveness and 5-fluorouracil sensitivity, TP expression may be used as a prognostic and predictive marker as well as targeted therapy in cholangiocarcinoma.

Peer review

The TP gene is aberrantly expressed in different human malignancies including cholangiocarcinoma. In the current manuscript submitted by Thanasai *et al.*, the authors made several interesting observations. This article reports the effects of TP expression on tumor migration and 5-fluorouracil sensitivity in cholangiocarcinoma cells, as well as tumor cell-induced angiogenesis of endothelial cells *in vitro*.

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Hepatic osteodystrophy and liver cirrhosis

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Abstract

AIM: To investigate the correlation between hepatic osteodystrophy and osteoporosis in patients with liver cirrhosis.

METHODS: Bone mineral density of the patients ($n = 55$) and that of the control group ($n = 30$) were measured by dual-energy X-ray absorptiometry. All the women in the study were premenopausal. Deoxypyridinoline, pyridinoline and urinary Ca^{2+} were measured as bone destruction markers, while alkaline phosphatase (ALP), osteocalcin and insulin-like growth factor-1 (IGF-1) were measured as bone formation markers. Furthermore, interleukin-1 (IL-1), IL-6, tumor necrosis factor α (TNF- α), vitamin D3, direct bilirubin, albumin, cortisol and parathyroid hormone (PTH) levels were measured. The independent Student t test and χ^2 test were employed in comparing both groups, and the Pearson correlation test was used to determine associations.

RESULTS: Comparing cirrhosis and control groups, lumbar total T-score ($-1.6 \pm 1.2 \text{ g/cm}^2$ vs $-0.25 \pm 1.3 \text{ g/cm}^2$, $P < 0.001$), lumbar total Z-score ($-1.2 \pm 1.23 \text{ g/cm}^2$ vs $-0.6 \pm 1.3 \text{ g/cm}^2$, $P < 0.001$), total femur T-score ($-0.05 \pm 1 \text{ g/cm}^2$ vs $-0.6 \pm 0.9 \text{ g/cm}^2$, $P = 0.003$) and total femur Z-score ($-0.08 \pm 1.5 \text{ g/cm}^2$ vs $0.7 \pm 0.9 \text{ g/cm}^2$, $P =$

0.003) showed significantly lower values in the cirrhosis group. Blood ALP level ($109.2 \pm 57 \text{ U/L}$ vs $62.6 \pm 32.5 \text{ U/L}$, $P < 0.001$), IL-6 level ($27.9 \pm 51.6 \text{ pg/mL}$ vs $3.3 \pm 3.1 \text{ pg/mL}$, $P = 0.01$), TNF- α level ($42.6 \pm 33.2 \text{ pg/mL}$ vs $25.3 \pm 12.3 \text{ pg/mL}$, $P = 0.007$) and direct bilirubin level ($0.9 \pm 0.7 \text{ mg/dL}$ vs $0.3 \pm 0.2 \text{ mg/dL}$, $P < 0.001$) were significantly higher in the cirrhosis group. IGF-1 level ($47.7 \pm 26.2 \text{ ng/mL}$ vs $143.4 \pm 53.2 \text{ ng/mL}$, $P < 0.001$), osteocalcin level ($1.05 \pm 2.5 \text{ ng/mL}$ vs $7.0 \pm 13 \text{ ng/mL}$, $P = 0.002$) and 24 h urinary Ca^{2+} ($169.6 \pm 227.2 \text{ mg/dL}$ vs $287 \pm 168.6 \text{ mg/dL}$, $P = 0.003$) were significantly lower in the cirrhosis group. Urinary deoxypyridinoline/creatinine ($9.4 \pm 9.9 \text{ pmol}/\mu\text{mol}$ vs $8.1 \pm 5.3 \text{ pmol}/\mu\text{mol}$, $P = 0.51$), urinary pyridinoline/creatinine ($51.3 \pm 66.6 \text{ pmol}/\mu\text{mol}$ vs $29 \pm 25.8 \text{ pmol}/\mu\text{mol}$, $P = 0.08$), blood IL-1 level ($3.4 \pm 8.8 \text{ pg/mL}$ vs $1.6 \pm 3.5 \text{ pg/mL}$, $P = 0.29$), vitamin D3 level ($18.6 \pm 13.3 \mu\text{g/L}$ vs $18.4 \pm 8.9 \mu\text{g/L}$, $P = 0.95$), cortisol level ($11.1 \pm 4.8 \mu\text{g/dL}$ vs $12.6 \pm 4.3 \mu\text{g/dL}$, $P = 0.15$) and PTH level ($42.7 \pm 38 \mu\text{g/dL}$ vs $34.8 \pm 10.9 \mu\text{g/dL}$, $P = 0.27$) were not significantly different.

CONCLUSION: Hepatic osteodystrophy is an important complication encountered in patients with liver cirrhosis and all patients should be monitored for hepatic osteodystrophy.

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Key words: Liver cirrhosis; Osteoporosis; Hepatic osteodystrophy

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INTRODUCTION

Liver cirrhosis develops when the liver parenchyma takes a nodular form as a result of fibrosis arising from delayed wound-healing in chronic liver damage. Osteoporosis, characterized by a reduced bone mass and an increase in bone fragility due to distortion of bone tissue microstructure, is a multifactorial disease and the most common bone disease. Dual energy X-ray absorptiometry is a method of measuring bone mineral density which has been become the standard in many centers and is a highly accurate X-ray method.

It is known that liver diseases may lead to bone disease^[1]. Although chronic liver disease is associated with a broad spectrum of bone diseases, the most common type of hepatic osteodystrophy is osteoporosis. If an increase in bone resorption exceeds bone formation, or decreased bone formation is present together with normal bone destruction, then in advanced cases, bone mass will decrease and the risk of fracture will increase^[2]. Increased cytokine levels in chronic liver disease and liver cirrhosis contribute to the development of hepatic osteodystrophy. Levels of interleukin-1 (IL-1), IL-6 and tumor necrosis factor α (TNF- α) are higher in patients with alcoholic hepatitis and liver cirrhosis than gender- and age-matched controls^[3]. It is thought that a reduction in growth factors, such as insulin, or an excess of growth inhibitors such, as bilirubin, in patients with cirrhosis causes osteoblastic function disorder^[4]. Low bone formation and a high resorption rate in chronic liver disease patients results in osteoporosis. It has been shown that serum osteocalcin decreases depending on reduction of osteoblast function. A decrease in serum osteocalcin levels and an increase in deoxypyridinoline (DPD) level can be explained by lower bone turnover^[4-7].

Osteopenia is more frequent than osteomalacia in the primary biliary cirrhosis (PBC)-associated metabolic bone diseases in patients in North America^[8]. Osteoclastic activity may be increased in premenopausal PBC women. In these people there is a disorder in the formation of new bone. Calcium and vitamin D metabolism is often normal in anicteric PBC patients^[8]. Osteoporosis is a common complication of cholestatic liver disease. In primary sclerosing cholangitis (PSC), the cause of osteoporosis is multifactorial. The pathophysiological mechanism of osteoporosis has not been identified clearly yet^[9,10].

The aim of this study was to establish the osteoporosis risk in liver cirrhosis, and to investigate the role of IL-1, IL-6, TNF- α , osteocalcin, insulin-like growth factor-1 (IGF-1), alkaline phosphatase (ALP), parathyroid hormone (PTH), cortisol and 25(OH)D3 in bone metabolism, and to investigate urinary DPD, pyridinoline and Ca²⁺ levels, which are biochemical markers of bone cycling.

MATERIALS AND METHODS

Our study comprised a cohort of 85 patients, 55 men and 30 premenopausal women (older than 18 years) with liver cirrhosis, and a control group (15 men and 15 women).

The etiology was 37 hepatitis B, 10 cryptogenic, 2 PBC, 2 cardiac, 2 hepatitis C and 2 Wilson diseases in the patients with liver cirrhosis. The diagnosis of liver cirrhosis was done by biochemical and serological tests, abdominal ultrasonography, upper gastrointestinal endoscopy. Liver biopsy could not performed because of ascites. Twenty-four patients had Child-Pugh stage C (score 11.2), 17 patients had Child-Pugh stage B (score 7.9), 14 patients had Child-Pugh stage A (score 5.6) cirrhosis. Previous diseases, fracture anamnesis, smoking, using of alcohol or coffee were investigated in all cases included in the study. There was no history of diabetes mellitus, hypertension, goitre, early menopause, surgical menopause, hyperparathyroidism or Cushing's syndrome in any of the study subjects, and none took any drug associated with increased risk of osteoporosis (anticoagulant, oral contraceptive, steroid, thiazide, diuretics, *etc.*).

Height, weight and body mass indexes (BMI) of all cases were calculated. Blood samples were analyzed in the laboratory department. Urea, creatinine, aspartate aminotransferase (AST), alanine aminotransferase (ALT), ALP, gamma glutamyl transpeptidase, Ca²⁺, P⁺, bilirubin and albumin levels were measured on an Abbott Aeroset device. Plasma cortisol, thyroid stimulating hormone, free T4, parathyroid hormone (PTH) and alpha-fetoprotein (AFP) levels were determined on a Modulator E 170 autoanalyzer by electrochemiluminescence method. IGF-1 was measured on an Immulite 2000 autoanalyser, cytokines (IL-1, IL-6 and TNF- α) on an Immulite 1000 autoanalyser and vitamin D3 on an Agilent 1100 autoanalyser by HPLC.

Hydroxychloric acid was added to 24 h urine for determination of pyridinoline and DPD, markers of bone destruction, by HPLC. Calcium was also determined in 24 h urine. Clearance of creatinine was calculated as (24 h urine volume \times urine creatinine)/(plasma creatinine \times 1440). Bone mineral density measurements were conducted in the triangle of L1-L4 vertebrae and femoral neck, trochanteric major, intertrochanteric region. The results were calculated as g/cm². T and Z scores for all subjects were analyzed.

Statistical analysis

Statistical assessment was carried out by SPSS 13.0. The independent Student *t* test and χ^2 test were used for comparing the groups. The Pearson correlation test was used to determine associations.

RESULTS

Seventeen female and 38 male patients with liver cirrhosis and 15 female and 15 male controls were included in the study. There were no significant differences in BMI and gender between the patients and the controls. The mean age of the patients was significantly higher than in the control group (mean, 44.8 years *vs* 34.8 years, Table 1). All the women included in the study were premenopausal, and none took alcohol or coffee. There was no fracture anamnesis. T- and Z-scores were significantly lower in

the cirrhosis group than in controls when bone mineral densities were compared (Table 1). IL-6 and TNF- α were significantly higher in the liver cirrhosis group, but there was no significant difference in IL-1 (Table 1). ALP levels were significantly higher in the cirrhosis group, but IGF-1 and osteocalcin were significantly lower compared to the control group (Table 1). There were no significant differences in DPD/creatinine and pyridinoline/creatinine levels, nor in blood cortisol, PTH and vitamin D levels (Table 1). There were no significant differences in sedimentation rate, AFP and C-reactive protein levels. Prothrombin time and direct bilirubin were significantly higher and albumin level significantly lower in the cirrhosis group (Table 1).

DISCUSSION

Hepatic osteodystrophy is an important health problem encountered in patients with liver cirrhosis^[1,2]. The reported prevalence of osteoporosis among patients with liver cirrhosis ranges from 20% to 50% depending on patient selection and diagnostic criteria, and the prevalence of fracture ranges from 5% to 20%^[9]. In the present study, osteoporosis was found in 37% of patients in accordance with the literature. The fact that all the female patients were premenopausal allowed reliable examination of the effect of liver cirrhosis on osteoporosis. Diamond *et al*^[5] found in their 2 separate studies that the prevalence of osteoporosis was 30%-48% in patients with chronic liver disease of different etiologies.

Hepatic osteodystrophy is defined as bone disease associated with chronic liver disease^[5,6,11,12]. The mechanism of hepatic osteodystrophy encountered in liver cirrhosis patients has not been clearly determined. Osteoporosis has been reported in patients with cholestatic liver, chronic viral hepatitis, alcoholic liver, hemochromatosis and benign and malignant tumors of the liver^[13]. In our study, the prevalence of osteoporosis was determined as 37% from the T-scores in lumbar vertebrae. Lumbar total BMD T-score and Z-score in patients with cirrhosis were significantly lower than those of the control group ($P = 0.003$).

IL-1, IL-6 and TNF- α cytokines are the cytokines most associated with postmenopausal osteoporosis^[14]. These cytokines can affect bone metabolism through either increasing osteoclast formation or increasing osteoclast activity. In addition, the cytokines can block osteoblast function directly and increase formation of other cytokines^[9]. IL-1, IL-6 and TNF- α levels were higher in patients with alcoholic hepatitis or liver cirrhosis compared to gender- and age-matched controls^[13]. The incidence and severity of osteoporosis is increased in patients with chronic hepatitis or intestinal diseases. Inadequate nutrition, use of steroids, and cytokines have roles in this outcome. Pfeilschifter *et al*^[15] concluded that these cytokines were strong resorptive agents. In the present study, IL-1, IL-6 and TNF- α levels were higher than those of the control group. It is thought that increased IL-6 and TNF- α levels were effective in promoting hepatic osteodystrophy in our patients with liver cirrhosis.

Table 1 Clinical features and laboratory test results of the liver cirrhosis and control groups

	Cirrhosis (n = 55)	Control (n = 30)	P-value
Lumbar total T-score (g/cm ²)	-1.6 \pm 1.2	-0.25 \pm 1.3	< 0.001
Lumbar total Z-score (g/cm ²)	-1.2 \pm 1.2	-0.6 \pm 1.3	< 0.001
Total femur T-score (g/cm ²)	-0.05 \pm 1	0.6 \pm 0.9	0.003
Total femur Z-score (g/cm ²)	-0.08 \pm 1.5	0.7 \pm 0.9	0.003
Age (yr)	44.8 \pm 12.9	34.8 \pm 8.2	< 0.001
BMI (kg/m ²)	25.5 \pm 4.3	25.5 \pm 3.7	0.970
IL-1 pg/mL	3.4 \pm 8.8	1.6 \pm 3.5	0.290
IL-6 pg/mL	27.9 \pm 51.6	3.3 \pm 3.1	0.010
TNF- α pg/mL	42.6 \pm 33.2	25.3 \pm 12.3	0.007
AFP (ng/mL)	2.6 \pm 16.9	2.6 \pm 1.1	0.100
Direct bilirubin (mg/dL)	0.3 \pm 0.2	0.9 \pm 0.7	< 0.001
CRP (mg/L)	5.8 \pm 5.9	5.4 \pm 11.3	0.800
Osteocalcin (ng/mL)	1.05 \pm 2.5	7.0 \pm 13	0.002
IGF-1 (ng/mL)	47.7 \pm 26.2	143.4 \pm 53.2	< 0.001
ALP (IU/L)	109.2 \pm 57	62.6 \pm 32.5	< 0.001
24 h urinary Ca ²⁺ (mg/dL)	169.6 \pm 227.2	287 \pm 168.6	0.003
Deoxypyridinoline/creatinine (pmol/ μ mol)	9.4 \pm 9.9	8.1 \pm 5.3	0.510
pyridinoline/creatinine (pmol/ μ mol)	51.3 \pm 66.6	29 \pm 25.8	0.080
Cortisol level μ g/dL	11.1 \pm 4.8	12.6 \pm 4.3	0.150
PTH level μ g/dL	42.7 \pm 38	34.8 \pm 10.9	0.270
Vit D3 level μ g/L	18.6 \pm 13.3	18.4 \pm 8.9	0.950
Albumin (g/dL)	2.5 \pm 0.5	4 \pm 0.4	0.001
Prothrombin time (s)	17.1 \pm 5.2	11.7 \pm 1.7	< 0.001
Sedimentation rate (h)	18.8 \pm 10.6	11.6 \pm 9.6	0.030

BMI: Body mass index; IL: Interleukin; TNF: Tumor necrosis factor; AFP: α -fetoprotein; CRP: C-reactive protein; ALP: Alkaline phosphatase; IGF: Insulin-like growth factor.

Menon *et al*^[16] have shown that there is a positive correlation between serum bilirubin level and bone destruction ratio. In the present study, increased serum bilirubin levels in patients with osteoporosis concur with studies in the literature. Gillberg *et al*^[17] have established that idiopathic osteoporosis exists together with a reduced IGF-1 level. It was thought that reducing growth factors, such as insulin, or increasing bilirubin in cirrhosis patients induces an osteoblastic function disorder^[4]. In our study, we found significantly lower IGF-1 levels in the liver cirrhosis group ($P < 0.001$), and direct bilirubin levels were significantly higher ($P < 0.001$).

In recent studies it has been reported that there is no abnormality in Ca²⁺, phosphorus and vitamin D metabolism in patients with PBS, which was unexpected^[18]. However in some studies vitamin D was lower. In our study, in accordance with the literature, vitamin D levels were not higher than the healthy group suggesting that vitamin D metabolism was normal in the liver cirrhosis group.

In another study conducted by Karan *et al*^[19], 24 patients with liver cirrhosis developing after hepatitis were compared with 22 healthy controls, and osteocalcin levels were non-significantly lower in the patient group. In the study of Capra *et al*^[20], comprising 20 people with cirrhosis and 22 healthy controls, osteocalcin levels in the cirrhosis group were lower than in the control group,

whereas plasma calcitonin levels in the cirrhosis group were higher than in the control group^[20].

In most studies, ALP levels in chronic liver patients were elevated, including the study of Karan *et al.*^[19]. It is necessary to measure enzyme function of osteoblasts (bone ALP) for diagnosis of osteoporosis. If liver-gallbladder disorders can be excluded, serum total ALP levels only can be used as an index of bone formation. In contrast, bone specific isoenzyme of ALP (BAP) is inside the osteoblast membrane and if osteoblast activation exists it is excreted into the circulation. Thus, measurement of BAP is more accurate and is affected less by non-bone pathologies.

Low bone formation and a high resorption ratio in chronic liver patients results in osteoporosis. It has been shown that serum osteocalcin decreases with reduction in osteoblast function. A reduction in serum osteocalcin and an increase in urine DPD can be explained by lower bone turnover^[4-7]. Monegal *et al.*^[21] have established that serum osteocalcin in patients with PBC is low. They emphasized that this was associated with a reduction in bone formation. Guañabens *et al.*^[22] have found that the volume of bone decreases because of a reduction in bone formation in PBC.

Osteoclastic activity may be increased in premenopausal women with PBC. In these patients there is a disorder in formation of new bone. Calcium and vitamin D metabolism in anicteric patients with PBC is often normal^[9]. Osteoporosis is a common complication of cholestatic liver disease. The causes of osteoporosis in PSC are multifactorial. The pathophysiological mechanism of osteoporosis has not been identified clearly yet^[10].

In the present study, we investigated osteocalcin and ALP as measures of bone formation. Osteocalcin levels were lower than those of the control group ($P = 0.002$), whereas ALP levels were higher ($P < 0.001$). Lower osteocalcin levels exhibit lower osteoblastic activity. A higher ALP level, which is a bone formation marker, can be associated with cholestasis, while lower serum osteocalcin levels may be associated with a reduction in bone formation. Urine 24 h calcium levels in the liver cirrhosis patients were significantly reduced, and could be associated with inadequate nutrition in these patients, or might indicate that osteoclastic activity did not increase, or even decrease, to reduce bone formation.

PTH stimulates both bone resorption and bone formation, but it is known that if it remains at a high level, osteoclasts are stimulated, whereas osteoblasts are inhibited. Some authors have been reported that hepatocellular dysfunction increases serum PTH levels, while some authors reported no change or even a reduction^[23]. In addition to the studies reporting that serum PTH levels in liver cirrhosis patients are higher than that of controls, there exists some studies indicating that the PTH level is lower^[23]. In the present study, although there was an increase in PTH levels in the liver cirrhosis group, it was not statistically significant.

Urinary DPD/creatinine and pyridinoline/creatinine level were non-significantly increased in the cirrhosis

patients. This finding suggests there may be more bone resorption than normal. Both insulin and IGF-1 have an influence on osteoblast function and contribute to bone formation. IGF-1 levels in the cirrhosis group were significantly lower in the study. This implied that IGF-1 reduction is an important factor in progression to hepatic osteodystrophy. There is an increase in the urinary hydroxyproline/creatinine ratio especially in advanced stages of liver cirrhosis^[20]. The urinary deoxypyridine/creatinine ratio and serum bone ALP levels increase in osteoporosis developing from hepatitis C virus-associated liver cirrhosis^[4]. Increased activity of the osteoprotegerin system can be associated with increased TNF- α and IL-6 levels^[24,25].

In conclusion, metabolic bone diseases are important complications of chronic liver diseases. The bone mineral density of patients with liver cirrhosis is reduced. A decreased osteocalcin level in patients with liver cirrhosis means reduced bone formation, which, in turn, may contribute to development of osteoporosis. Increased TNF- α and IL-6 levels in patients with cirrhosis may contribute to the development of hepatic osteodystrophy. Decreased IGF-1 levels in patients with liver cirrhosis may contribute to the development of osteoporosis. Early scanning for osteoporosis in patients with liver cirrhosis will reduce the risk of morbidity and mortality. As advanced hepatic osteodystrophy is difficult to treat and adversely affects both the quality of life and the long-term prognosis of patients with chronic liver disease, special care is required in order to prevent the development of clinical bone disease in individuals with advanced hepatic disease.

COMMENTS

Background

Liver cirrhosis develops when the liver parenchyma takes a nodular form as a result of fibrosis arising from delayed wound-healing in chronic liver damage. Osteoporosis, characterized by a reduced bone mass and an increase in bone fragility due to distortion of bone tissue microstructure, is a multifactorial disease and the most common bone disease. In North America, osteopenia is more frequent than osteomalacia in patients with metabolic bone diseases associated with primary biliary cirrhosis. In primary sclerosing cholangitis, the causes of osteoporosis are multifactorial. The pathophysiological mechanism of osteoporosis has not been identified clearly yet.

Research frontiers

Metabolic bone diseases are important complications of chronic liver diseases. Increased cytokine levels in chronic liver disease and in cirrhosis contributes to the development of hepatic osteodystrophy. This study compared bone mineral density and cytokines in patients with liver cirrhosis and controls.

Innovations and breakthroughs

Cytokines have a role in hepatic osteodystrophy. Screening and treatment of symptoms are very useful for patients with liver cirrhosis.

Applications

Early diagnosis of osteoporosis by scanning in patients with liver cirrhosis will reduce the risk of morbidity and mortality. Therefore life quality will be better in patients with liver cirrhosis.

Peer review

The manuscript investigated the correlation between hepatic osteodystrophy and osteoporosis by comparing 55 patients with liver cirrhosis and 30 healthy controls and there are several comments and suggestions. This is an interesting study as the pathogenesis of hepatic osteodystrophy has not been studied extensively.

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Clinicopathological significance and prognostic value of LRP16 expression in colorectal carcinoma

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Abstract

AIM: To explore the expression of leukemia related protein 16 (LRP16) in colorectal carcinoma, and analyze its correlation with clinicopathologic features and prognosis.

METHODS: Immunohistochemistry for LRP16 was performed in 201 cases of colorectal carcinoma and 60 cases of distal normal mucosa. Medical records were reviewed and clinicopathological analysis was performed.

RESULTS: LRP16 expression was detected in 117 of 201 cases of the colorectal carcinoma and in 21 cases of 60 distal normal mucosa. The expression of LRP16 in carcinoma was significantly higher than that in normal mucosa ($\chi^2 = 9.999$, $P = 0.002$). LRP16 protein expression was found in 43.3% (52/120) of carcinoma at stage I and II, and 80.2% (65/81) of carcinoma at stage III and IV ($\chi^2 = 27.088$, $P = 0.001$). Correlation

between LRP16 expression and clinicopathological factors was significant in differentiation ($P = 0.010$), tumor size ($P = 0.001$), infiltrative depth ($P = 0.000$) and distant metastasis ($P = 0.027$). The difference of median survival time between cancer patients with LRP16 expression (38.0 mo) and those without was statistically significant (105.0 mo, Log rank = 41.455, $P = 0.001$). The multivariate survival analysis revealed that LRP16 expression was correlated significantly (Cox's regression: $P = 0.001$, relative risk = 2.082) with shortened survival in the patients with colorectal cancer.

CONCLUSION: The expression of LRP16 is related to the degree of differentiation, invasiveness, metastasis and prognosis of colorectal carcinoma.

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Key words: Colorectal neoplasms; Immunohistochemistry; Leukemia related protein 16; Prognosis; Clinicopathology

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INTRODUCTION

Leukemia related protein 16 (LRP16), which was originally recognized and isolated from human lymphocytes in 1999, was identified as an estrogen-responsive gene. It

localizes on chromosome 11q12.1 and encodes nuclear factor^[1-5]. It expresses in testicle, ovaries, mucosa of colon, prostate, small intestine, spleen, thymus^[2,3] and gastric carcinoma^[6]. LRP16 is also an estrogen receptor α (ER α) coactivator. Its expression level is strongly dependent on the estrogen activities. It is involved in estrogen signaling pathway and can strengthen the ER α responsive gene activation.

Colorectal carcinoma is a major cause of cancer-related morbidity and mortality. In the Western countries, it is the second leading cause of death from cancer^[7]. The incidence of colorectal cancer has increased in China recently, therefore, some new molecular markers are necessary to raise the efficiency of tumor diagnosis and to predict prognosis of the patients or even for therapeutic application. It has been reported that ER is identified in colorectal cancer^[8-10]. However, no immunohistochemical and clinicopathological studies of LRP16 have been performed in colorectal carcinoma. Here, we investigated the expression of LRP16 in colorectal cancer specimens and normal mucosa by an immunohistochemistry (IHC)-based technique and analyzed the relationship between LRP16 expression and the clinicopathological features of Chinese patients with colorectal cancer.

MATERIALS AND METHODS

Patients and specimens

A series of 201 consecutive colorectal carcinoma patients who were treated surgically in Chinese People's Liberation Army General Hospital (Beijing, China) between August 1999 and September 2003 and 60 cases with distal normal mucosa were recruited in this study. There were 127 men and 74 women with ages ranging from 20 to 81 years (median, 62 years; mean, 57.8 years). The carcinomas were located in the cecum ($n = 9$), ascending colon ($n = 29$), transverse colon ($n = 14$), descending colon ($n = 9$), sigmoid colon ($n = 48$) and rectum ($n = 92$). Of these patients, 24 were grade I, 110 grade II and 67 grade III, according to histological grading; and 48 were stage I, 72 were stage II, 70 were stage III and 11 were stage IV, according to clinical TNM stage revised by International Union Against Cancer in 2003. All the patients were followed up for survival. During the follow-up period from the date of surgery until April 30, 2009, 126 patients died and 75 were alive (median survival time, 51 mo, range, 0.10-115 mo).

Histological examination

The resected specimens were fixed in 10% formalin. The gross appearance and the size of tumor were recorded. Patients' medical records and histopathology of each specimen were reviewed. The tissues (cancer and normal) were cut into 5-mm slices, embedded in paraffin for histological and immunohistochemical examinations. Each paraffin block was cut at 4 μ mol/L in thickness, and sections were mounted onto adhesive-coated slides. Slides were deparaffinized in xylene twice for 10 min and

rehydrated through descending concentration of ethanol. Antigen retrieval was performed in 0.01 mol/L citrate buffer (pH 6.0) by microwave oven for 2 min and 30 s at 100°C. Endogenous peroxidase activity was blocked with 0.3% hydrogen peroxidase for 15 min. After washing with phosphate-buffered saline (PBS), the sections with primary polyclonal rabbit antibody to human LRP16 (recognized and isolated in 1999 by Department of Molecular Biology of our hospital) that was diluted 1:1000 in blocking solution were incubated at 4°C overnight, in a humidified chamber. After washing three times with PBS, the sections incubated for 30 min with biotinylated secondary antibody (polyperoxidase-anti-mouse/rabbit IgG, Zymed) then washed again with PBS. 3,3'-Diaminobenzidine was used as the chromogen. Sections were then counterstained with hematoxylin for 1 min, raised in water, dehydrated in ascending concentrations of ethanol followed by clearance with xylene, and cover slipped permanently for light microscopy. Normal ovary tissue was used as a positive control, whereas the primary antibody was replaced by PBS as a negative control.

Evaluation of score

Immunohistochemically stained slides were reviewed by two investigators independently who were blinded to all clinical data. Slides were analyzed under light microscopy using manual methods. Staining was graded for intensity of staining (0, negative; 1, weak; 2, moderate; 3, strong) and percentage of cells stained (0, < 5%; 1, 6%-25%; 2, 26%-50%; 3, > 50%). The final score was determined by the combined staining score (extent + intensity). Score 0 was defined as negative expression (-), scores 1-3 as weak staining pattern (+), and 4-6 as strong expression (++)

Statistical analysis

Statistical analysis was performed using SPSS 13.0 software (SPSS Inc., Chicago, Illinois). The χ^2 test was used to examine the various clinicopathological characteristics and LRP16 expression. Cumulative survival curves were drawn by the Kaplan-Meier method. The difference between the curves was analyzed by the Log-rank test. Multivariate survival analysis was based on Cox proportional hazard model. $P < 0.05$ was considered statistically significant.

RESULTS

Pattern of LRP16 expression in colorectal carcinoma and normal mucosa

LRP16 staining was performed in 201 colorectal cancer patients and 60 cases of normal tissues by IHC. The result showed that in cancer tissues, LRP16 staining was negative (-) in 41.8% (84/201) cases, weak positive (+) in 21.4% (43/201) cases and strong positive (++) in 36.8% (74/201) cases; whereas in normal mucosas, LRP16 was negative in 65% (39/60) cases, weak positive in 13.3% (8/60) and strong positive in 21.7% (13/60) cases. As a whole, LRP16 expression was detected in 58.2%

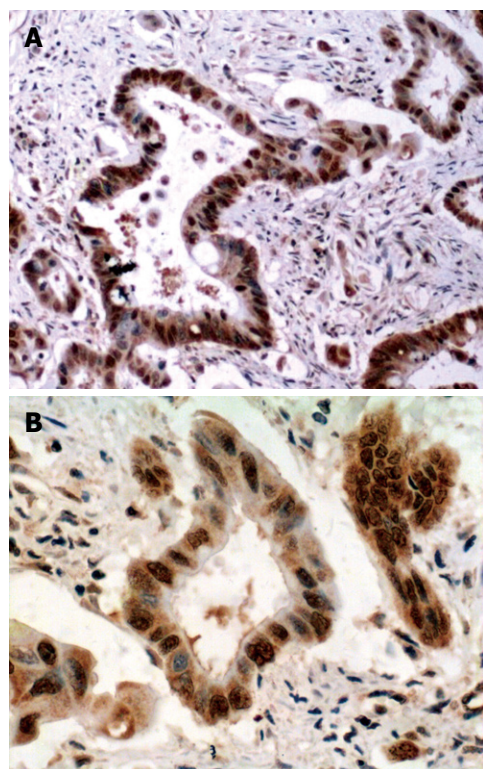


Figure 1 Positive leukemia related protein 16 (LRP16) expression in colorectal adenocarcinoma. The final score was 6, including 3 scores (strong) related to intensity of staining and 3 scores (> 50%) related to extent of staining. Thus it was defined as strong staining pattern (++). A: Immunohistochemistry (IHC), $\times 100$; B: IHC, $\times 400$.

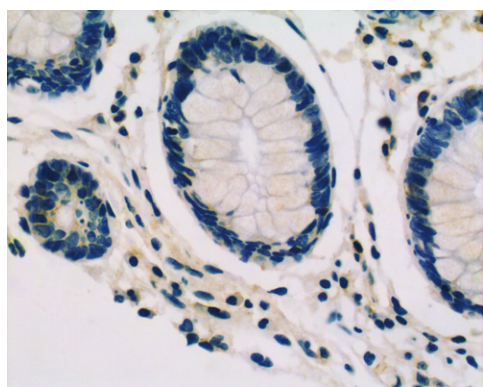


Figure 2 Negative LRP16 expression in normal colorectal mucosa (IHC, $\times 400$). The final score was 0, including 0 score (negative) related to intensity of staining, and 0 scores (< 5%) related to extent of staining. Thus it was defined as negative expression (-).

(117/201) colorectal carcinoma, and in 35% (21/60) distal normal mucosa (Figures 1 and 2). The expression of LRP16 protein was found in cell nucleus or both cell nucleus and cytoplasm. The difference of LRP16 expression between colorectal cancer and normal mucosa was statistically significant ($\chi^2 = 9.998$, $P = 0.002$).

Correlation of LRP16 expression and clinicopathological features in colorectal cancer

Upon clinicopathological analysis, more LRP16 expres-

Table 1 Correlation between LRP16 expression and clinicopathological factors in colorectal cancer n (%)

Variables	LRP16		Statistical value
	Positive group ($n = 197$)	Negative group ($n = 139$)	
Gender			
Male	78 (61.4)	49 (38.6)	$\chi^2 = 1.460^a$
Female	39 (52.7)	35 (47.3)	$P = 0.227$
Age (yr)			
< 45	20 (64.5)	11 (35.5)	$\chi^2 = 0.640^a$
$45 \leq n < 60$	36 (58.0)	26 (42.0)	$P = 0.726$
≥ 60	61 (56.5)	47 (43.5)	
Tumor size (cm)			
$d < 5$	68 (50.0)	68 (50.0)	$\chi^2 = 13.604^a$
$5 \leq d < 10$	42 (72.4)	16 (27.6)	$P = 0.001$
$d \geq 10$	7 (100.0)	0 (0.0)	
Tumor location			
Cecum	5 (55.6)	4 (44.4)	$\chi^2 = 6.027^a$
Ascending colon	22 (75.9)	7 (24.1)	$P = 0.304$
Transverse colon	8 (57.1)	6 (42.9)	
Descending colon	6 (66.7)	3 (33.3)	
Sigmoid colon	29 (60.4)	19 (39.6)	
Rectum	47 (51.1)	45 (48.9)	
Histologic differentiation			
Well differentiated	12 (50.0)	12 (50.0)	$\chi^2 = 9.210^a$
Moderately differentiated	56 (50.9)	54 (49.1)	$P = 0.010$
Poorly differentiated	49 (73.1)	18 (226.9)	
Depth of invasion, T stage			
T1	2 (28.6)	5 (71.4)	$\chi^2 = 25.470^a$
T2	25 (36.8)	43 (63.2)	$P = 0.001$
T3	79 (69.9)	34 (30.1)	
T4	11 (84.6)	2 (15.4)	
Lymph node metastasis			
LN = 0	58 (45.3)	70 (54.7)	$\chi^2 = 24.735^a$
LN = 1-3	43 (78.2)	12 (21.8)	$P = 0.001$
LN > 3	16 (88.9)	2 (11.1)	
Distant metastasis			
Negative	107 (56.3)	83 (43.7)	$\chi^2 = 5.115^a$
Positive	10 (90.9)	1 (9.1)	$P = 0.027$
TNM stage			
I - II	52 (43.3)	68 (57.7)	$\chi^2 = 27.088^a$
III-IV	65 (80.2)	16 (19.8)	$P = 0.001$

^a $P < 0.05$, statistically significant. LRP16: Leukemia related protein 16.

sion was present in bigger tumors ($P = 0.001$). When comparing the LRP16 status with clinicopathological variables, we found significant positive correlations between LRP16 expression and degree of differentiation ($P = 0.010$), depth of invasion ($P = 0.001$), lymph node metastasis ($P = 0.001$) and distant metastasis ($P = 0.027$). The level of LRP16 in cases of low TNM stage (I + II) was lower than that of high stage (III + IV) ($\chi^2 = 27.088$, $P = 0.001$) (Table 1).

Relationship between LRP16 expression and overall survival of colorectal cancer patients

All 201 patients were followed up for survival to assess LRP16 expression as a prognostic factor. Kaplan-Meier survival curves and Log-rank test demonstrated that LRP16 positive cases showed a significantly shortened median survival time (38.0 mo) in comparison with other patients (105.0 mo) (Log rank = 41.455, $P = 0.001$) (Figure 3).

Table 2 Cox regression analysis of prognostic factors in colorectal carcinoma

Prognostic variables	B	SE	Wald value	P value	RR
Gender	0.261	0.197	1.756	0.185	1.298
Age (yr)	0.191	0.124	2.366	0.124	1.211
Tumor location	0.071	0.060	1.338	0.239	1.074
Histologic differentiation	0.747	0.162	21.128	0.001	2.110
Tumor size	0.123	0.188	0.433	0.510	1.131
Depth of invasion	0.508	0.199	6.523	0.011	1.661
Lymph node metastasis	0.060	0.206	0.085	0.771	1.062
Distant metastasis	1.450	0.526	7.589	0.006	4.264
TNM stage	0.717	0.236	9.236	0.002	2.049
LRP16 expression	0.741	0.225	10.868	0.001	2.099

B: Partial regression coefficient; RR: Relative risk. $P < 0.05$, statistically significant.

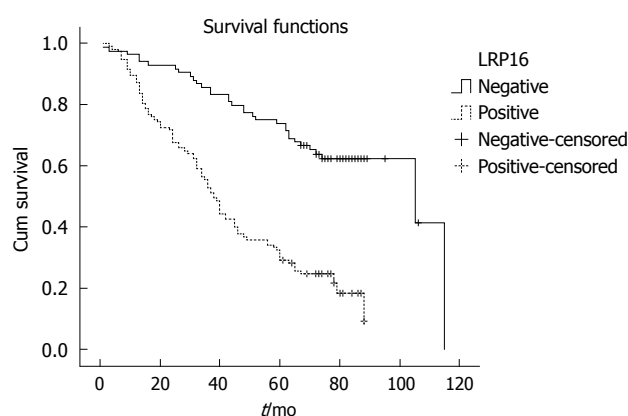


Figure 3 Cumulative survival according to LRP16 expression in colorectal cancer patients. The Kaplan-Meier plot of colorectal carcinoma patients ($n = 201$) demonstrates a significantly lower survival with LRP16 positive expression, $P = 0.001$.

The overall 5-year survival rate of the LRP16 negative patients (71.4%) was higher than that of the LRP16 positive group (29.1%). LRP16 expression appeared as a significant independent prognostic factor ($P = 0.001$) with a relative risk of 2.082 (confidence interval, 1.3-3.2) in the multivariate survival analysis of the patients. Other independent prognostic factors in multivariate survival analysis included tumor shape, degree of differentiation, depth of invasion and distant metastasis (Table 2).

DISCUSSION

LRP16 was originally recognized and isolated from human lymphocytes in 1999^[1]. It was identified as an estrogen responsive gene^[1-5]. The expression of LRP16 was found in different tissues in varying degrees, including ovary, testicle, prostate, small intestine, spleen, thymus and stomach^[2-3,6]. Furthermore, LRP16 is overexpressed in tumors, compared with their matched normal tissues^[2]. Some studies have indicated that LRP16 may play an important role in the carcinogenesis and progression of hormone-dependent breast cancer^[5,11]. Overexpression

of LRP16 significantly stimulated MCF-7 cell proliferation by promoting G1/S transition^[4]. The suppression of the endogenous LRP16 in ER α -positive MCF-7 cells not only inhibits cell growth but also significantly attenuates the cellular estrogen-responsive proliferation ability and sensitizes tumor cells to radiation^[11-13]. However, some authors thought that expression of LRP16 in ER α -negative cells had no effect on proliferation^[11]. The expression of LRP16 gene was dependent on the estrogen activities^[14,15], LRP16 was also involved in estrogen signaling and could strengthen the ER α -responsive gene activation, therefore, it is also considered as an ER α coactivator^[11]. Previous studies have demonstrated that ER α /PR status, tumor size and auxiliary lymph node metastasis were closely correlated with LRP16 overexpression^[16]. ER includes two subtypes, ER α and ER β . They have been identified in colorectal cancer tissue and normal mucosa^[8-10]. It was also reported that the ER expression level of carcinoma was higher than that of non-cancerous colon tissues^[10]. Activation of ER signaling pathway plays an important role in multi-tissue development^[17-20]. Therefore, we propose that LRP16, a coactivator of ER α , may display an important function in the carcinogenesis and progression of colorectal cancer as in breast cancer.

Although the detailed molecular mechanism involved in this process is unclear, our study have potentially clinical benefits. LRP16 expression that could be detected by IHC may be a useful molecular marker to predict the prognosis in colorectal carcinoma patients. Moreover, LRP16 and ER α could inter-regulate each other, as LRP16 is an ER α coactivator. This study raises the possibility that anti-estrogen therapy could be used in the patients with high LRP16 expression. This information may help us individualize patient care (e.g. progression and prognosis of patients after operation). In this study, LRP16 expression was commonly up-regulated in colorectal carcinoma and was associated with shortened survival time of the patients in univariate and multivariate analyses. However, further investigations and clinicopathological correlation are necessary.

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COMMENTS

Background

Colorectal cancer is a major cause of cancer-related morbidity and mortality. In the Western world it is the second leading cause of death from cancer. The evidence of colorectal cancer has increased recently in China. Although this disease is curable by surgical interventions together with chemotherapy and radiation in its early stages, the patients are often asymptomatic before the metastasis occurred. Therefore, effective screening and preventive strategies for colorectal cancer are necessary to enhance our capability to predict the clinical outcome of the disease.

Research frontiers

Leukemia related protein 16 (LRP16) gene plays an important role in the

carcinogenesis and progression of hormone-dependent breast cancer. LRP16 expression was also reported to be associated with invasion, metastasis and prognosis of gastric carcinoma. But no comprehensive description of LRP16 protein expression in colorectal cancer has been reported. In this study, the authors investigated the expression patterns of LRP16 protein in human colorectal cancers and compared the clinical and pathological variables including survival time of the patients.

Innovations and breakthroughs

To date, only limited data on LRP16 protein expression in solid tumors are available. This study is believed to be the first trial for verifying the relationship between LRP16 expression and clinicopathological factors of colorectal carcinoma.

Applications

The authors presented some evidences to show a significant association between high LRP16 expression in colorectal cancer and early disease progression or the disease related death, and they believe that the LRP16 expression status detected by immunohistochemistry (IHC) may be a molecular marker to predict the prognosis of colorectal carcinoma patients.

Peer review

In this paper the authors have analyzed by IHC the expression of LRP16 protein in 201 cases of colorectal carcinoma and 60 cases of distal normal mucosae and they have correlated the expression of this marker with the degree of differentiation, invasiveness, metastasis and prognosis of colorectal carcinoma. The study is well performed and the results are quite interesting, and are potentially helpful for clinical application, the quality of the research and presentation, including photomicrographs and figures, is high, the discussion is clear and well written.

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Long-term efficacy of perioperative chemoradiotherapy on esophageal squamous cell carcinoma

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Abstract

AIM: To investigate the role of perioperative chemoradiotherapy (CRT) in the treatment of locally advanced thoracic esophageal squamous cell carcinoma (ESCC).

METHODS: Using preoperative computed tomography (CT)-based staging criteria, 238 patients with ESCC (stage II-III) were enrolled in this prospective study between January 1997 and June 2004. With informed consent, patients were randomized into 3 groups: preoperative CRT (80 cases), postoperative CRT (78 cases) and surgery alone (S) (80 cases). The 1-, 3-, 5- and 10-year survival were followed up. Progression-free survival (PFS) was chosen as the primary endpoint by treatment arm measured from study entry until documented progression of disease or death from any cause. The secondary endpoint was overall survival (OS) determined as the time (in months) between the date of therapy and the date of death. Other objectives were surgical and adjuvant therapy complications.

RESULTS: With median follow-up of 45 mo for all the enrolled patients, significant differences in the 1-, 3-, 5-, 10-year OS (91.3%, 63.5%, 43.5%, 24.5% vs 91%, 62.8%, 42.3%, 24.4% vs 87.5%, 51.3%, 33.8%, 12.5%, $P = 0.0176$) and PFS (89.3%, 61.3%, 37.5%, 18.1% vs 89.1%, 61.1%, 37.2%, 17.8% vs 84.5%, 49.3%, 25.9%, 6.2%, $P = 0.0151$) were detected among the 3 arms. There were no significant differences in OS and PFS between the preoperative CRT and postoperative CRT arm ($P > 0.05$). For the patients who had radical resection, significant differences in median PFS (48 mo vs 61 mo vs 39.5 mo, $P = 0.0331$) and median OS (56.5 mo vs 72 mo vs 41.5 mo, $P = 0.0153$) were detected among the 3 arms, but there were no significant differences in OS and PFS between the preoperative CRT and postoperative CRT arm ($P > 0.05$). The local recurrence rates in the preoperative CRT, postoperative CRT group and S group were 11.3%, 14.1% and 35%, respectively ($P < 0.05$). No significant differences were detected among the 3 groups when comparing complications but tended to be in favor of the postoperative CRT and S groups ($P > 0.05$). Toxicities of CRT in the preoperative or postoperative CRT arms were mostly moderate, and could be quickly alleviated by adequate therapy.

CONCLUSION: Rational application of preoperative or postoperative CRT can provide a benefit in PFS and OS in patients with locally advanced ESCC.

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Key words: Esophageal cancer; Surgery; Esophagectomy; Chemotherapy; Radiation therapy

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INTRODUCTION

Esophageal squamous cell carcinoma (ESCC) is one of the common malignancies and the seventh leading cause of cancer-related deaths in the world^[1]. The 5-year survival rate is only 15%-20% in patients with locally advanced esophageal cancer^[2]. Treatment failure mainly results from recurrence or metastasis. A standardized comprehensive treatment is still under development. Radiotherapy can control local-regional esophageal cancer and chemotherapy has both local and systemic antineoplastic activity. With increasing enthusiasm for multidisciplinary treatment modalities to improve outcome, a commonly employed treatment approach for esophageal cancer is chemoradiotherapy (CRT) in addition to surgical resection^[3]. Preoperative CRT had been applied to patients with esophageal carcinoma in an effort to reduce the relapse rate and improve survival. Many studies have demonstrated the effectiveness of neoadjuvant CRT^[4-9]. In contrast to data available on preoperative CRT, although a number of trials compared adjuvant chemotherapy or radiotherapy with surgery alone, there are almost no randomized trials comparing adjuvant CRT with surgery alone. The only exception is a trial that compared surgery alone with surgery and adjuvant CRT for patients with resectable cancers of the stomach and gastroesophageal junction^[10]. Although the results suggest that resection followed by concurrent CRT should be considered for patients with adenocarcinoma of the stomach and the gastroesophageal junction, the question remains whether postoperative CRT can improve overall survival in patients with ESCC.

To our knowledge, as yet no study is available to determine the best sequencing of CRT and surgery. To resolve this deficiency, the present prospective study was conducted in our center from January 1997 to June 2004.

MATERIALS AND METHODS

Patients

Two hundred and seventy one patients with ESCC were diagnosed by endoscopic biopsy and histopathology between January 1997 and June 2004 in our center. According to the preoperative computed tomography (CT) staging criteria, 238 patients with stage II-III thoracic ESCC were enrolled in this prospective study. The CT staging criteria were as follows: Stage I, the tumor was limited to the esophageal lumen or the thickness of the esophageal wall varied between 3 and 5 mm; Stage II, the thickness exceeded 5 mm but there was no invasion of the mediastinum or distant metastasis; Stage III, the tumor invaded the adjacent mediastinal structure; and

Table 1 Characteristics of the preoperative patients (*n* = 238)

	Pre-CRT (<i>n</i> = 80)	Post-CRT (<i>n</i> = 78)	S (<i>n</i> = 80)	Statistics	
				χ^2	<i>P</i>
Sex (M:F)	52:28	48:30	50:30	3.1326	0.209
Age (yr)				1.2375	0.975
40-	12	13	11		
50-	24	25	24		
60-	28	29	30		
70-	16	11	15		
Location				0.1920	0.996
Upper	11	10	12		
Middle	45	45	44		
Lower	24	23	24		
CT staging				0.1165	0.943
II	35	33	36		
III	45	45	44		

Pre-CRT: Preoperative chemoradiotherapy; Post-CRT: Postoperative CRT; S: Surgery alone; CT: Computed tomography.

Stage IV, there was distant metastasis. All patients gave informed consent prior to their inclusion in the study. The form had been reviewed by the appropriate ethics committee and had been developed and was administered in accordance with the ethical standards laid down in an appropriate version of the 1964 Declaration of Helsinki. The enrolled patients were randomized into 3 groups: preoperative CRT, postoperative CRT, and surgery alone (S). The randomization method was based on random numerals produced by computer. Characteristics of the preoperative patients are shown in Table 1.

Treatment

The surgical procedure used in this study was either a radical resection, which involved an esophagectomy through a left or right thoracotomy with 2-field lymphadenectomy, or palliative resection or esophageal bypass. All the patients who underwent palliative resection or esophageal bypass had taken traditional Chinese medicine themselves.

For patients in the preoperative arm, surgical resection was to be performed 4-6 wk after induction of CRT. The patients treated with postoperative CRT underwent surgery and received CRT 4-6 wk later. For patients treated with preoperative CRT, radiation was delivered in a total dose of 40 Gy (20 fractions at 2 Gy per fraction) in anteroposterior fields including esophageal tumors and enlarged lymph nodes, with a 4-5 cm proximal and distal margin and a 1-2 cm radial margin. For 30 out of 78 patients treated with postoperative CRT, radiation was delivered in daily fractions of 2 Gy to a total dose of 40 Gy over 4 wk by using the same double field technique as the preoperative CRT group, and the anteroposterior fields of the following 48 patients were extended from the sixth cervical vertebrae to the first lumbar vertebrae, including the origin of esophagus and lymph drainage that encompassed the supraclavicular regions and left gastric lymph nodes. Then a 10 Gy boost was delivered through parallel opposed lateral or oblique portals for limitation of spinal

cord radiation dose. Radiotherapy was carried out by linear accelerators with 6 MV photons; treatment ports were designed to include enlarged regional nodes based on CT evaluation and endoscopic ultrasound.

For chemotherapy, 2 cycles were administered on days 1-3 and days 22-24 of radiotherapy. A paclitaxel (PTX) + cisplatin (DDP) regimen was used, including PTX (135 mg/m² per day) administered as a short-term infusion on day 1 of each cycle, while DDP (20 mg/m² per day) was delivered as a continuous infusion over 24 h on days 1-3 of each cycle. The patients received the antiemetics granisetron and metoclopramide before and after the cisplatin infusion. The dose of chemotherapy in the second cycle was adjusted according to hematological toxicities.

Efficacy assessment

Any perioperative complications were observed and recorded. During treatment, the patients were monitored weekly with a physical examination and blood chemistry evaluation. Clinical evaluation was carried out by endoscopy, endoscopic ultrasonography, and CT scans. All results of the examination were collected and evaluated by the oncology experts. The 1-, 3-, 5- and 10-year survival were followed up.

Follow-up

Clinical follow-up after completion of treatment was based on periodic visits (every 3 mo during the first 2 years, every 6 mo after 2 years). The follow-up time for survivors ranged from 5 to 124 mo (median 45 mo).

Statistical analysis

STATA 10.0 for Windows (StataCorp, College Station, Texas 77845, USA) was used for statistical analysis. The differences in age group, sex, tumor location, and tumor staging, complications, and cause of death among groups were compared by the χ^2 test or Fisher's Exact test. The survival among groups were described by Kaplan-Meier curves and analyzed by the log-rank test. *P* values less than 0.05 were considered statistically significant. Progression free survival (PFS) was chosen as the primary endpoint by treatment arm measured from study entry until documented progression of disease or death from any cause. The secondary endpoint was overall survival (OS) determined as the time (in months) between the date of therapy and the date of death. Other factors determined were surgical and adjuvant therapy complications.

RESULTS

By June 30 2009, 228 out of all cases were followed up by means of telephone or outpatient service, and 10 cases were lost. There were 3.8% treatment-related deaths in preoperative CRT but no treatment-related deaths in the postoperative CRT and S group, and 51.3% of patients died from the local tumor recurrence and distant metastasis.

Median PFS was 46.5 mo (95% CI: 37.4-66.3) in the preoperative CRT group, 45 mo (95% CI: 35.8-67) in the postoperative CRT group, and 32.5 mo (95% CI:

25.4-42.3) in the S group. The 1-year PFS in preoperative CRT, postoperative CRT and S groups was 89.3%, 89.1% and 84.5%, respectively, with no significant differences ($\chi^2 = 0.64$, $P = 0.4123$). However, the 3-, 5- and 10-year PFS of the preoperative CRT (61.3%, 37.5%, 18.1%, respectively) and postoperative CRT groups (61.1%, 37.2%, 17.8%, respectively) were significantly different ($\chi^2 = 4.16$, $P = 0.0319$; $\chi^2 = 4.14$, $P = 0.0321$; $\chi^2 = 5.38$, $P = 0.0203$; respectively) from those of the S group (49.3%, 25.9%, 6.2%, respectively). There was no significant difference in PFS between the neoadjuvant and adjuvant therapy groups ($\chi^2 = 0.14$, $P = 0.7060$) (Figure 1A). Median OS was 53 mo (95% CI: 43-64.3) in the preoperative CRT group, 48 mo (95% CI: 38.5-72.5) in the postoperative CRT group, and 36 mo (95% CI: 29.7-47.3) in the S group. The 1-year OS in the preoperative CRT, postoperative CRT and S groups was 91.3%, 91% and 87.5%, respectively, which were not significantly different ($\chi^2 = 0.72$, $P = 0.3970$). However, the 3-, 5- and 10-year survival rates of the preoperative CRT (63.5%, 43.5%, 24.5%, respectively) and postoperative CRT groups (62.8%, 42.3%, 24.4%, respectively) were significantly different ($\chi^2 = 3.98$, $P = 0.0453$; $\chi^2 = 4.76$, $P = 0.0402$; $\chi^2 = 4.27$, $P = 0.0389$; respectively) from those of the S group (51.3%, 33.8%, 12.5%, respectively). There was no significant difference in OS between the neoadjuvant and adjuvant therapy groups ($\chi^2 = 0.46$, $P = 0.4978$) (Figure 1B).

For the patients who experienced radical resection, median PFS of the preoperative CRT group, postoperative CRT group and S group was 48 mo (95% CI: 40-67.0), 61 mo (95% CI: 44.0-75.4), and 39.5 mo (95% CI: 30-60), respectively. A significant difference in PFS was detected among the 3 arms ($\chi^2 = 6.82$, $P = 0.0331$), however, there was no significant difference in PFS between neoadjuvant and adjuvant therapy groups ($\chi^2 = 0.22$, $P = 0.6416$) (Figure 1C). There was a significant difference in OS among the 3 arms ($\chi^2 = 8.36$, $P = 0.0153$). The median OS of the preoperative CRT group, postoperative CRT group and S group was 56.5 mo (95% CI: 44-72), 72 mo (95% CI: 49.6-88.4), and 41.5 mo (95% CI: 31.6-57.7), respectively. However, there was no significant difference in OS between neoadjuvant and adjuvant therapy groups ($\chi^2 = 0.16$, $P = 0.6873$) (Figure 1D).

The local recurrence rates of the preoperative CRT group, postoperative CRT group and S group were 11.3%, 14.1% and 35% respectively, and showed a statistically significant difference ($P = 0.011$). There was no significant difference among the 3 groups when comparing perioperative complications but there was a tendency in favor of the postoperative CRT and S groups ($P = 0.179$).

For the 158 patients receiving CRT, incidence of grade 3 or greater leucopenia, thrombocytopenia, anemia and vomiting were 11.4% (18/158), 6.3% (10/158), 1.3% (2/158) and 6.3% (10/158), respectively. Postoperative pathologic staging and perioperative complications, and the causes of death are presented in Table 2.

DISCUSSION

Patients with resectable ESCC should receive multimod-

Table 2 Postoperative pathologic staging, perioperative complications, and cause of death *n* (%)

	Pre-CRT (<i>n</i> = 80)	Post-CRT (<i>n</i> = 78)	S (<i>n</i> = 80)	<i>n</i> = 238	Statistics χ^2	<i>P</i>
Resection					-	0.011 ¹
R	76 (97.4)	61 (78.2)	64 (80)	201 (84.5)		
P	4 (2.6)	13 (16.7)	13 (16.3)	30 (12.6)		
EB	0 (0)	4 (5.1)	3 (3.8)	7 (2.9)		
Stage					-	0.000 ¹
I	4 (2.6)	0 (0)	0 (0)	4 (1.7)		
II a	22 (27.5)	13 (16.7)	12 (15.4)	47 (19.7)		
II b	29 (36.3)	17 (21.8)	19 (23.8)	65 (27.3)		
III	25 (31.3)	48 (61.5)	49 (61.3)	122 (51.3)		
Complications					-	0.179 ¹
Hemorrhage during Surgery (> 300 mL)	8 (10.0)	2 (2.6)	2 (2.5)	12 (5.0)		
Stomal leakage	1 (1.3)	0 (0)	0 (0)	1 (0.4)		
Stomal stricture	2 (2.5)	3 (3.8)	1 (1.3)	6 (2.5)		
Reflux esophagitis	13 (16.3)	13 (16.7)	15 (18.8)	41 (17.2)		
Acute lung injury	3 (3.8)	0 (0)	0 (0)	3 (1.3)		
Death					-	0.011 ¹
Local recurrence	9 (11.3)	11 (14.1)	28 (35)	48 (20.2)		
Distant metastasis	20 (25)	23 (29.5)	31 (38.8)	74 (31.1)		
Treatment related	3 (3.8)	0 (0)	0 (0)	3 (1.3)		
Not related	1 (1.3)	5 (6.4)	1 (1.3)	7 (2.9)		

¹Fisher's Exact test. R: Radical resection; P: Palliative resection; EB: Esophageal bypass.

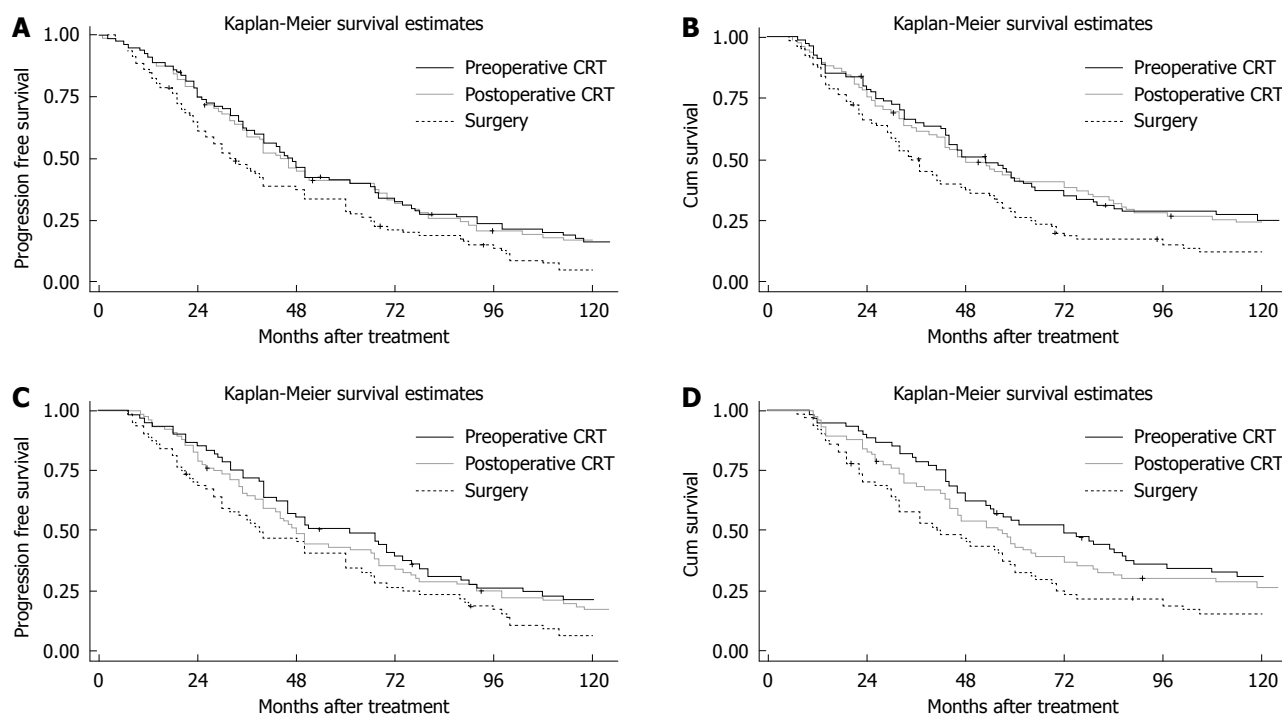


Figure 1 Kaplan-Meier curves. A: Postoperative progression-free survival. Logrank test: $\chi^2 = 8.39$, $P = 0.0151$; "+": Loss to follow-up. Preoperative vs surgery: $\chi^2 = 6.81$, $P = 0.0091$; Postoperative vs surgery: $\chi^2 = 5.38$, $P = 0.0203$; Preoperative vs postoperative: $\chi^2 = 0.14$, $P = 0.7060$; B: Postoperative overall survival. Logrank: $\chi^2 = 8.07$, $P = 0.0176$; "+": Loss to follow-up. Preoperative vs surgery: $\chi^2 = 7.85$, $P = 0.0051$; Postoperative vs surgery: $\chi^2 = 5.33$, $P = 0.0209$; Preoperative vs postoperative: $\chi^2 = 0.46$, $P = 0.4978$; C: Postoperative progression-free survival of radical resection. Logrank: $\chi^2 = 6.82$, $P = 0.0331$; "+": Loss to follow-up. Preoperative vs surgery: $\chi^2 = 6.16$, $P = 0.0130$; Postoperative vs surgery: $\chi^2 = 4.02$, $P = 0.0449$; Preoperative vs postoperative: $\chi^2 = 0.22$, $P = 0.6416$; D: Postoperative overall survival of radical resection. Logrank: $\chi^2 = 8.36$, $P = 0.0153$; "+": Loss to follow-up. Preoperative vs surgery: $\chi^2 = 7.65$, $P = 0.0057$; Postoperative vs surgery: $\chi^2 = 4.78$, $P = 0.0288$; Preoperative vs postoperative: $\chi^2 = 0.16$, $P = 0.6873$.

al treatment to prolong OS^[11,12]. There is still controversy about how to improve prognosis and how to reduce local recurrence and distant metastasis. Both chemotherapy and radiotherapy may be active against different

tumor cell populations, the chemotherapy may be effective against micrometastases while radiation counteracts spatial metastases. In the Western world, preoperative chemotherapy and CRT can increase OS by 4.4% and

6.4%, respectively, however, treatment-related mortality increases by 1.7% with neoadjuvant chemotherapy and by 3.4% with CRT, compared with surgery alone^[13]. There were a few nonrandomized trials on postoperative CRT in treating esophageal carcinoma^[14-19], however, the results had discrepancies. Regretfully, the most optimal sequence of CRT in relation to surgical resection is unclear. Thus our study is a unique randomized controlled study to evaluate the outcome of preoperative CRT in patients with local advanced thoracic ESCC, and it includes a long-term follow-up.

The present study showed a benefit in PFS and OS with neoadjuvant or adjuvant therapy. Meanwhile, our study indicated that preoperative CRT reduced the rate of local recurrence, and the survival benefit resulted from improved local cancer control brought about by the adjuvant arm. Although there was no significant difference found in 1-year PFS and OS, both preoperative CRT and postoperative CRT showed a significant advantage in longtime PFS and OS. A meta-analysis had suggested that preoperative CRT may improve survival and locoregional control but was associated with higher toxicity and increased mortality^[3]. Our results also confirm this point. Rice and coworkers found that 31 patients treated with postoperative adjuvant CRT had improved survival, in a retrospective review^[20]. Together with our present study, it further confirms that postoperative CRT may be an alternative option especially for locally advanced thoracic ESCC. Moreover, the results indicated that postoperative CRT may have almost the same long-term of efficacy as preoperative CRT, although the latter showed a nonsignificant trend of higher survival compared to the former.

To throw further light on the benefit in PFS and OS with neoadjuvant or adjuvant therapy for ESCC, we subsequently analyzed the data in patients who had undergone radical resection. The results also showed the same advantages as the above when including the palliative resection and esophageal bypass patients. The results also showed no significant difference in survival rates when comparing the preoperative CRT and postoperative CRT arms. It further clarifies that adjuvant CRT can provide almost the same long-term of efficacy as neoadjuvant CRT.

In addition, there was no suggestion that treatment-related mortality was increased by the use of postoperative CRT, which was accomplished with manageable toxicity. Most patients had less than grade 2 hematological toxicities. However, our results showed an increase in postoperative deaths and a trend for relatively higher perioperative complications in the neoadjuvant CRT arm. The involved reasons could be as follows: surgeons may undertake a challenging esophagectomy resulting in surgical difficulty and postoperative complications when performed after neoadjuvant CRT. For example, radiation might contribute to the failure of an anastomotic leak and postoperative acute lung injury. The results also showed that preoperative CRT can facilitate complete resection by downstaging tumors when compared to postoperative CRT and surgery alone. Thus, whether

or not the survival benefit of neoadjuvant CRT can be negated by an increase in postoperative deaths should of concern. In addition, loss to follow-up was low (4.2%), with only 3 patients receiving preoperative CRT and 3 patients receiving postoperative CRT and 4 patients receiving surgery alone, thus our study is robust.

In conclusion, long-term survival is maximized by the use of CRT followed by surgery for locally advanced esophageal cancer. However, patients are more likely to develop toxicity. As therapies improve, it is likely that the toxicity may be reduced and neoadjuvant CRT may provide a more marked benefit in esophageal cancer. Meanwhile, postoperative CRT can also be safely administered and considered as the multimodal treatment of choice for locally advanced ESCC. In appropriately selected patients, either pre- or postoperative CRT is a viable strategy. Further comparison of pre- and postoperative CRT in treating esophageal cancer is required for verification through multicenter and large sample randomized clinical trials.

COMMENTS

Background

Esophagectomy is a standard treatment for resectable esophageal carcinoma but relatively few patients are cured. Combined neoadjuvant chemoradiotherapy (CRT) or adjuvant CRT with surgery may improve survival but there is concern about treatment morbidity and the best sequencing of CRT and surgery.

Research frontiers

This study investigated the overall survival and progression-free survival data (up to 10-year survival).

Innovations and breakthroughs

Some studies have demonstrated the effectiveness of neoadjuvant CRT and there are almost no randomized trials comparing adjuvant CRT with surgery alone. As yet no study is available to determine the best sequencing of CRT and surgery. The most optimal sequence of CRT in relation to surgical resection is unclear. This study was a unique randomized controlled study to evaluate the outcome of preoperative and postoperative CRT in patients with local advanced thoracic esophageal squamous cell carcinoma, and it includes a long-term follow-up.

Peer review

The authors present the results of a randomized prospective study which examined the efficacy and safety of 3 treatment regimens in esophageal squamous cell carcinoma stage II and III. The study is of interest to readers of the journal. The discussion is satisfactory and is limited to the topic. Tables depicted essential data and are well constructed.

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Double-balloon enteroscopy for obscure gastrointestinal bleeding: A single center experience in China

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Abstract

AIM: To evaluate the diagnostic value of double-balloon enteroscopy (DBE) for obscure gastrointestinal bleeding (OGIB).

METHODS: The data about 75 OGIB patients who underwent DBE in January 2007-June 2009 in our hospital were retrospectively analyzed.

RESULTS: DBE was successfully performed in all 75 patients without complication. Of the 75 patients, 44 (58.7%) had positive DBE findings, 22 had negative DBE findings but had potential bleeding at surgery and capsule endoscopy, *etc.* These 66 patients were finally

diagnosed as OGIB which was most commonly caused by small bowel tumor (28.0%), angiodysplasia (18.7%) and Crohn's disease (10.7%). Lesions occurred more frequently in proximal small bowel than in distal small bowel (49.3% vs 33.3%, $P = 0.047$).

CONCLUSION: DBE is a safe, effective and accurate procedure for the diagnosis of OGIB.

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Key words: Double-balloon enteroscopy; Capsule endoscopy; Obscure gastrointestinal bleeding; Diagnosis

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INTRODUCTION

Obscure gastrointestinal bleeding (OGIB) is defined as recurrent or persistent gastrointestinal bleeding when gastric and colonic endoscopy is negative^[1]. OGIB accounts for approximately 5% of all gastrointestinal bleeding events^[2]. Most OGIB events are attributable to small bowel diseases.

The detection and management of small bowel bleeding are a challenge in the past due to the length and ana-

tomical position of small bowel. The diagnostic rate of conventional diagnostic strategies including small intestine radiography, abdominal computed tomography (CT), angiography, and red blood cell scan for small intestine disease is low^[3]. Introduction of capsule endoscopy (CE) has significantly revolutionized the study of small bowel as it is a reliable method to evaluate the entire small bowel^[4]. However, application of CE in diagnosis of OGIB is limited by the handling controllability, biopsy, endoscopic treatment, retention of capsule in stenosis intestine^[5].

Etiological diagnosis of OGIB has been markedly improved with the development of double-balloon enteroscopy (DBE) since 2001^[6]. DBE can be performed either through the mouth or through anus, and is thus able to explore a large part of the small bowel. DBE has the advantages including image clarity, handling controllability, biopsy, and endoscopic treatment over CE^[7]. It has been demonstrated that DBE is a safe and useful procedure for the diagnosis of small intestinal disease, especially for OGIB^[8]. In China, very few data are available on the diagnostic value of DBE for OGIB.

In this study, the data about 75 OGIB patients admitted to our hospital from January 2007 to June 2009 were retrospectively analyzed and the diagnostic value of DBE for OGIB was evaluated.

MATERIALS AND METHODS

Patients

DBE was performed in 75 OGIB patients (37 males, 38 females, at a mean age 51.5 ± 16.6 years, range 16-86 years) admitted to our hospital in January 2007-June 2009. Melena, hematemesis, hemafecia, and fecal occult bleeding were detected in the patients enrolled in this study. The duration of symptoms ranged 1 d-over 10 years. The main characteristics of patients are shown in Table 1. All the patients were suspected of small bowel diseases. However, standard gastric and colonic endoscopy for them was negative. Other routine methods such as CT and small intestine radiography showed no exact diagnosis of etiology.

DBE system

OGIB was detected in patients using a Fujinon enteroscope (EN450-P5/20, Fujinon Inc, Saitama, Japan) consisting of a mainframe, an enteroscope, an overtube and an air pump. Two soft latex balloons that can be inflated and deflated are attached to the tip of enteroscope and overtube. The balloons are connected to a pump through an air channel in the endoscope that can automatically modulate the air according to the different balloon pressures. By utilizing the overtube in combination with serial inflation and deflation of the balloons, endoscope can be inserted into the small bowel.

Preoperative preparation

The patients were fasted overnight and 2 boxes of polyethylene glycol electrolyte mixed with 3000 mL water

Table 1 Characteristics of OGIB patients *n* (%)

Characteristics	<i>n</i> = 75
Age (yr)	51.5 ± 16.6 (16-86)
Sex (male/female)	37/38
Causes of OGIB	
Melena	45 (60.0)
Hematemesis and melena	7 (15.6)
Hemafecia	17 (22.7)
Occult bleeding	6 (8.0)
Duration of symptoms (mo)	
< 1	29 (38.7)
1-12	24 (32.0)
> 12	22 (29.3)

OGIB: Obscure gastrointestinal bleeding.

were taken 4-5 h prior to DBE through anus or mouth. At the same time, 5-10 mg of midazolam and 10 mg of scopolamine butylbromide were also injected intramuscularly 10 min before DBE. The patients were anaesthetized with 10 mL of oral 2% lidocaine hydrochloride before DBE through mouth. Oxygen was inhaled with electrocardiography monitored when necessary.

Procedure

DBE through mouth or anus was performed according to the suspected site of lesions. When the site was uncertain, DBE was performed through mouth.

DBE was not performed when the cause of bleeding could be explained, the operation time was too long to be tolerated, and more than half of the small intestine examined was negative.

Statistical analysis

Statistical analysis was performed using SPSS 11.5. Data were expressed as mean \pm SD. Difference was detected by χ^2 test. $P < 0.05$ was considered statistically significant.

RESULTS

General information

DBE was performed 84 times in 75 patients, including 57 times through mouth and 27 through anus. Two patients completed DBE of the entire small bowel through mouth at one time.

All the procedures were successful without anesthesia. No hemorrhage, perforation, acute pancreatitis or other serious complications occurred. Nausea, vomiting, abdominal distension, and abdominal pain occurred in some patients during the procedure. However, these symptoms were transient and tolerable. In general, DBE through anus was more tolerable than through mouth.

DBE findings

Of the 75 patients, 44 (58.7%) had positive DBE, 22 had negative DBE with potential bleeding sites observed at surgery and CE, *etc.* The distribution of OGIB patients

Table 2 DBE findings in OGIB patients

Lesion	Diagnosed by DBE	Diagnosed by other methods	Location			Difference in proximal and distal small bowel
			Stomach and duodenum	Jejunum	Ileum	
Tumor	17	4	3	14	4	66.7% vs 19.0% ($P = 0.002$)
Gastrointestinal stromal tumor	7	1 ²	1	6 ²	1	
Non-hodgkin lymphoma	2	3	0	3	2	
Adenocarcinoma	3	0	1	1	1	64.3% vs 28.6% ($P = 0.128$)
Lipoma	3	0	0	3	0	
Brunner adenoma	1	0	1	-	-	
Angioma	1	0	0	1	0	33.3% vs 66.7% ($P = 0.132$)
Angiodysplasia	7	7	1	9	4	
Crohn's disease	7	1	0	2	6	
Diverticulum	3	2	0	4	1	
Henoch-Schönlein purpura	4 ¹	0	0	2 ¹	2	
Single ulcer	0	4	0	1	3	
Others	7	5	3	3	4	49.3% vs 33.3% ($P = 0.047$)
Un-diagnosed	0	9	-	-	-	
Total	45	32	7	36	24	

¹One case was accompanied with ancylostomiasis; ²One case was accompanied with heterotopic pancreas. DBE: Double-balloon enteroscopy.

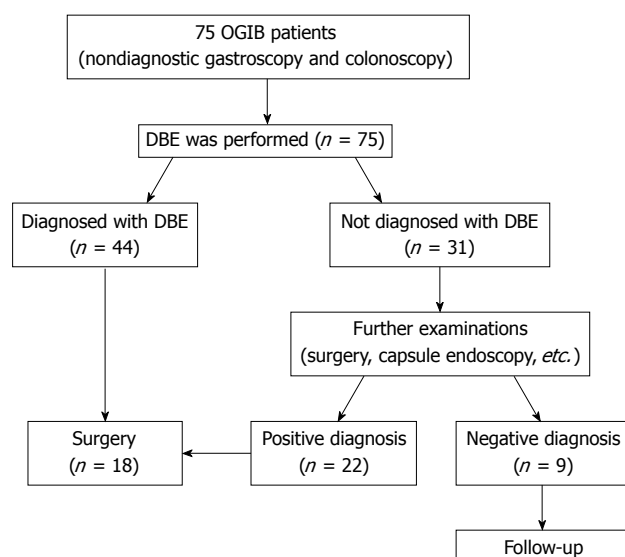


Figure 1 Distribution of obscure gastrointestinal bleeding (OGIB) patients enrolled in this study. DBE: Double-balloon enteroscopy.

enrolled in this study is shown in Figure 1. Among the 66 cases with positive DBE, OGIB was detected in upper digestive tract of 7 cases, in jejunum of 34 cases, in ileum of 24 cases, and at junction of jejunum and ileum of 1 case, respectively. The incidence of OGIB was higher in proximal small bowel (the third and fourth parts of duodenum, jejunum) than in distal small bowel (ileum) (49.3% vs 33.3%, $P = 0.047$). The DBE findings are presented in Table 2.

OGIB was most commonly caused by small bowel tumor (28.0%, 21/75), angiodysplasia (18.7%, 14/75) and Crohn's disease (10.7%, 8/75). Small bowel tumor was detected in duodenum of 3 cases, in jejunum of 14 cases, and in ileum of 4 cases, respectively. The incidence of small bowel tumor was higher in jejunum than in ileum (66.7% vs 19.0%, $P = 0.002$). Histological analysis showed that the tumor was benign in 7 cases (gastrointestinal stro-

mal tumor in 2, lipoma in 3, duodenum adenoma in 1 and angioma in 1) and malignant in 14 cases (gastrointestinal stromal tumor in 6, non-hodgkin lymphoma in 5 and adenocarcinoma in 3) (Figure 1). The detection rate of benign tumor was lower than that of malignant tumor (33.3% vs 66.7%, $P = 0.031$).

Angiodysplasia was detected in jejunum of 9 cases, in ileum of 4 cases, and in dieulafof of gastric fundus of 1 case, respectively, accounting for 18.7% of all the cases with no significant difference between them ($P = 0.128$). Crohn's disease was detected in jejunum and ileum of 2 and 6 cases, respectively, accounting for 10.7% of all the cases with no significant difference ($P = 0.132$). In addition, diverticulum, Henoch-Schönlein purpura, single ulcer, polyp, ancylostomiasis, tuberculosis, and non-specific inflammation were also detected (Figure 2).

DBE detection rate of bleeding and duration of symptoms

Of the 75 cases, 45 presented with melena and 25 (55.6%) with positive DBE. Symptoms of hemafecia were detected in 17 cases with a DBE detection rate of 47.1% (8/17). There was no significant difference between the DBE detection rates of melena and hemafecia (55.6% vs 47.1%, $P = 0.55$). The DBE detection rates of occult bleeding, hematemesis and melena were not compared because of the limited number of cases.

The 75 patients were divided into 3 groups according to their bleeding time. There was no significance between the duration of OGIB symptoms and the DBE detection rates (Table 3).

Operation results

Of the 75 patients, 18 (24.0%) underwent operation. Gastrointestinal stromal tumor, non-hodgkin lymphoma, adenocarcinoma, lipoma and angioma were the most commonly detected tumors. Both gastrointestinal stromal tumor and bleeding from heterotopic pancreas were detected in 1 patient.

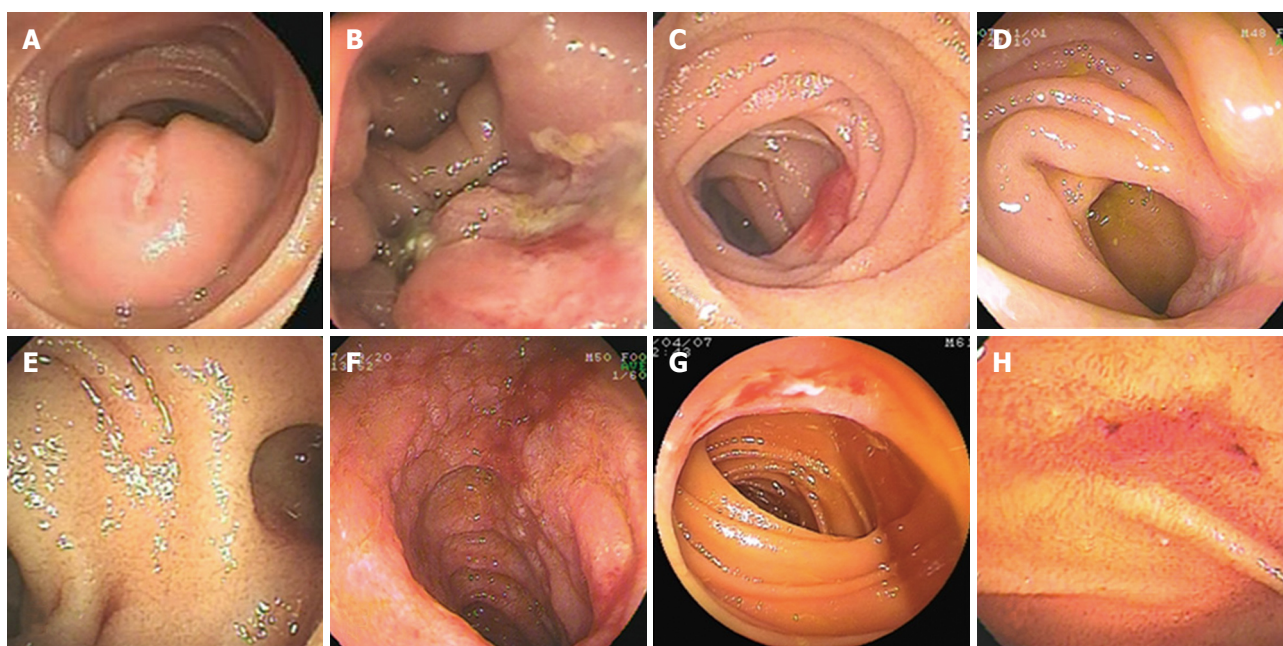


Figure 2 Typical endoscopic imaging. A: Gastrointestinal stromal tumor in jejunum; B: T cell lymphoma in jejunum; C: Angiodysplasia in jejunum; D: Crohn's disease in ileum; E: Diverticulum in jejunum; F: Henoch-Schönlein purpura in jejunum; G: Tuberculosis in ileum; H: Non-specific inflammation in jejunum.

Table 3 DBE detection rate of bleeding and duration of symptoms

	Patients	DBE findings	Detection rate (%)	P
Total	75	44	58.7	-
Causes of OGIB				
Melena	45	25	55.6	0.55 ¹
Hematemesis and melena	7	5	71.4	-
Hemafecia	17	8	47.1	-
Occult bleeding	6	6	100.0	-
Duration of symptoms (mo)				
< 1	29	18	62.1	0.56 ²
1-12	24	13	53.8	0.74 ³
> 12	22	13	54.5	0.83 ⁴

¹Difference between the DBE detection rates of melena and hemafecia;

²Difference between the DBE detection rates of < 1 mo and 1-12 mo;

³Difference between the DBE detection rates of 1-12 mo and > 12 mo;

⁴Difference between the DBE detection rates of < 1 mo and > 12 mo.

DISCUSSION

OGIB is a common problem encountered by gastroenterologists. Its diagnostic rate has been greatly improved due to CE since 2000^[9]. CE has a higher diagnostic rate of OGIB than conventional methods including small bowel barium radiography, push enteroscopy, and cross-sectional imaging^[10]. However, CE may fail to identify lesions such as Meckel's diverticulum, angiodysplasia, and malignancies^[11]. DBE can explore a large part of the small bowel, during which targeted tissue for biopsy can be taken. Moreover, endoscopic treatment procedures, including hemostasis, polypectomy, endoscopic mucosal resection, balloon dilation, and stent placement, can be performed at DBE^[12].

In this study, the diagnostic value of DBE for OGIB

was evaluated. The DBE detection rate of OGIB is consistent with the reported data^[13,14]. No complication occurred in the 75 patients who underwent DBE without anesthesia, suggesting that DBE is a safe, tolerable, and effective procedure for the diagnosis of OGIB.

It was reported that 3%-6% of OGIB events are caused by small bowel tumor^[15,16]. Sun *et al*^[17] showed that the prevalence of gastrointestinal stromal tumor is the highest among different small bowel tumors. DBE can show lesions in about 50%-66% of the small intestine and even in the entire small intestine, thus providing a high diagnostic rate of small bowel tumor^[18].

In this study, angiodysplasia was found to be another common etiology of OGIB, which is also in agreement with the reported data^[19]. The detection rate of lesions was higher in jejunum than in ileum. Since Crohn's disease has been found to be the third commonest etiology of OGIB, and shows a higher incidence in distal intestine, DBE *via* anus is usually recommended^[20].

The selection of DBE is still controversial. For those with no site of lesion indicated, DBE through mouth is preferred because our study and other studies showed that it has a higher diagnostic rate of lesions in proximal small bowel^[21,22] and is relatively easier to perform without twisting the colon, which is also supported by Safatle-Ribeiro *et al*^[23]. However, DBE through anus is also preferred by some endoscopists, since it has a better tolerance^[18].

In summary, DBE is a safe, tolerable, accurate and effective procedure for the diagnosis of OGIB. OGIB is most commonly caused by small bowel tumor and angiodysplasia. Lesions occur more frequently in proximal small bowel and DBE through mouth is recommended as a prior consideration if no evidence indicates the location.

COMMENTS

Background

The diagnosis of obscure gastrointestinal bleeding (OGIB) was rather difficult in the past. Double-balloon enteroscopy (DBE) and capsule endoscopy (CE) have significantly revolutionized the diagnosis of small bowel lesions. Compared with CE, DBE has unique advantages such as handing controllability, biopsy, diagnosis and treatment, *etc.* Few data are available on the diagnostic value of DBE for OGIB.

Research frontiers

The data about 75 OGIB patients were retrospectively analyzed in this article. The DBE detection rate of OGIB and the feasibility of operation were evaluated. The incidence of common diseases in small bowel was compared. The DBE detection rate of lesions in proximal or distal small bowel was different.

Innovations and breakthroughs

Patients could tolerate the whole DBE process with no serious complication. The DBE detection rate of different bleeding events and symptoms of OGIB were compared. DBE through mouth was completed at one time.

Applications

In this study, DBE was proven to be a safe, accurate and effective procedure for the diagnosis of OGIB and can thus be performed in hospital for the diagnosis of OGIB.

Terminology

OGIB is defined as recurrent or persistent gastrointestinal bleeding when gastric and colonic endoscopy is negative. DBE and CE are both new methods enabling diagnostic endoscopy of the entire small intestine, which have their own *pros and cons* in the diagnosis of small bowel diseases.

Peer review

This is an interesting descriptive study concerning a single center experience with DBE for OGIB in China.

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Porcine hepatocyte isolation and reversible immortalization mediated by retroviral transfer and site-specific recombination

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Abstract

AIM: To develop a hepatocyte cell line, we immortalized primary porcine hepatocytes with a retroviral vector SSR#69 containing the Simian Virus 40 T antigen (SV40Tag).

METHODS: We first established a method of porcine hepatocyte isolation with a modified four-step retrograde perfusion technique. Then the porcine hepatocytes were immortalized with retroviral vector SSR#69 expressing SV40T and hygromycin-resistance genes flanked by paired loxP recombination targets. SV40T cDNA in the expanded cells was subsequently excised by Cre/LoxP

site-specific recombination.

RESULTS: The resultant hepatocytes with high viability (97%) were successfully immortalized with retroviral vector SSR#69. One of the immortalized clones showed the typical morphological appearance, TJPH-1, and was selected by clone rings and expanded in culture. After excision of the SV40T gene with Cre-recombinase, cells stopped growing. The population of reverted cells exhibited the characteristics of differentiated hepatocytes.

CONCLUSION: In conclusion, we herein describe a modified method of hepatocyte isolation and subsequently established a porcine hepatocyte cell line mediated by retroviral transfer and site-specific recombination.

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Key words: Hepatocyte isolation; Porcine hepatocytes; Reversible immortalization; Simian virus 40 large T-antigen

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Meng FY, Chen ZS, Han M, Hu XP, He XX, Liu Y, He WT, Huang W, Guo H, Zhou P. Porcine hepatocyte isolation and reversible immortalization mediated by retroviral transfer and site-specific recombination. *World J Gastroenterol* 2010; 16(13): 1660-1664 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v16/i13/1660.htm> DOI: <http://dx.doi.org/10.3748/wjg.v16.i13.1660>

INTRODUCTION

A virtually unlimited supply of hepatocytes for metabolic studies, bio-artificial livers and cell transplantation would be highly desirable^[1,2]. However, the utility of cultured hepatocytes is hampered by difficulties in timely obtained populations of primary cells, which have a limited life span *in vitro*. An attractive alternative source of hepatocytes would be immortalized cells which could make unlimited supplies of cells feasible and exhibit the characteristics of differentiated hepatocytes^[3]. A series of studies have developed the strategy of cell immortalization^[4,5]. However, the oncogene in the immortalized hepatocytes would expose patients to an unacceptable tumorigenic risk. An attractive solution for this problem could be the use of a novel strategy of reversible immortalization by using Cre/loxP site-specific recombination^[6]. It has been reported that Cre/loxP recombination operates efficiently in primary cells^[7]. The procedure of reversible immortalization was devised by retrovirus-mediated transfer of an oncogene that can be subsequently effectively excised by site-specific recombination^[8].

A series of cells can be immortalized by retrovirus-mediated transfer of an immortalizing oncogene (*SV40T*)^[9]. Although the hepatocytes can be transduced with retroviral vectors, the efficiency of transduction is significantly low^[10]. In the process to successfully and effectively transduce retroviral vectors into hepatocytes, establishing an efficient technique of cell isolation seems to be meaningful.

To address these issues, we herein report an efficient procedure for porcine hepatocyte isolation, and subsequent successful immortalization by retrovirus-mediated transfer of *SV40T* which could be subsequently excised by Cre/LoxP-mediated site-specific recombination. The present study expands the strategy of hepatocyte reversible immortalization and represents an important step toward the development of immortalization of hepatocytes.

MATERIALS AND METHODS

Animals

A mini-pig weighing 13 kg was used in the present study. All procedures performed on the pig were approved by Tongji Medical College Animal Care and Use Committee and were within the guidelines for laboratory animals.

Porcine hepatocyte isolation

Porcine hepatocytes were isolated from a surgically resected liver segment with a modified four-step retrograde perfusion technique using dispase and collagenase (Figure 1). Briefly, the resected liver sample (47 g) was cannulated with a suitable pipette into the visible blood vessel on the cut surface (Figure 2A). Then the sample was flushed with a 500 mL calcium-free buffer solution (Step 1), 500 mL EDTA in buffer solution (Step 2) and another 500 mL of buffer solution at 37°C. Continuous recirculating perfusion was then carried out on the tissue using a pre-warmed

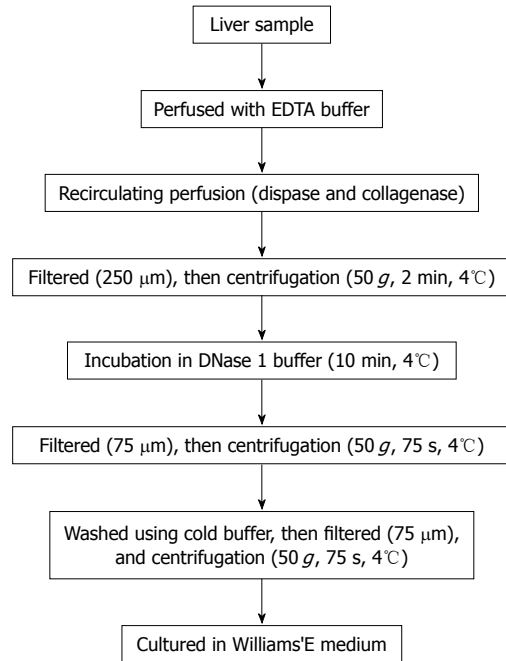


Figure 1 Flow diagram of the preparation of isolated hepatocytes with a modified four-step retrograde perfusion technique.

digestion buffer solution (8.4 g/L dispase II and 0.5 g/L collagenase IV, Sigma, St. Louis, MO, USA) at 37°C (Step 3). When digestion of the parenchyma was visualized, the liver tissue was finally flushed with 500 mL buffer solution (Step 4). Following sufficient digestion, the liver capsule was mechanically disrupted and the emerging cell suspension filtered through 250 μm nylon mesh and centrifuged (50 g, 2 min, 4°C). After that we employed a cell incubation step of 10 min using DNase1 (Sigma, St. Louis, MO, USA) containing buffer solution, during which cell clumps were broken up and damaged cells digested. Then, the resulting suspension was filtered through 75 μm nylon mesh, and the cells were harvested by low speed centrifugation at 50 g for 75 s. This was followed by washes using cold buffer solution and filtration through 75 μm nylon mesh and another centrifugation step (50 g, 75 s, 4°C). The resulting cell clumps were finally resuspended in culture medium (William's medium E, supplemented with 100 mU/mL penicillin, 100 μg/mL streptomycin and 10% fetal bovine serum). Hepatocyte yield and viability was determined using the standard trypan blue exclusion technique. Freshly isolated hepatocytes were seeded at a concentration of 4×10^5 per milliliter in culture flasks. The morphology of the cultured hepatocytes was assessed with a Nikon Diaphot inverted microscope at a magnification of 100 ×.

Porcine hepatocytes immortalization

The retroviral vector SSR#69 (kindly given by Naoya Kobayashi, Okayama University) containing SV40 large TAG and a gene resistant to hygromycin (Figure 3) was cultured in DMEM medium (Sigma, St. Louis, MO, USA) supplemented with 10% newborn calf serum. Freshly isolated primary porcine hepatocytes were transduced with 2 mL of SSR#69 cells supernatant per T25 flask at

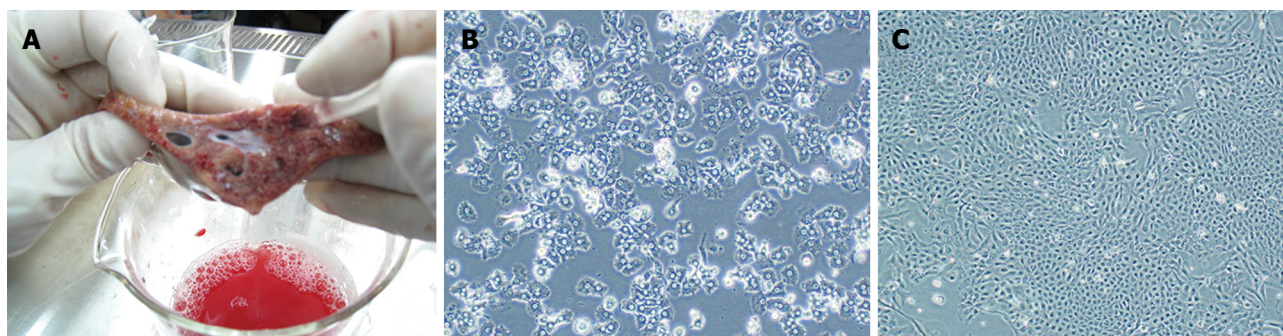


Figure 2 Isolation technique and microscopic appearance of primary and immortalized porcine hepatocytes. A: Perfusion was conducted by inserting a suitable pipette into vessels exposed on a cut surface of the sample; B: Non-immortalized primary porcine hepatocytes grow slowly with low plating efficiency and a short life span; C: Immortalized porcine hepatocytes grow rapidly as islands and had an extended life span. Magnifications: B and C 100 \times .

Retroviral vector SSR#69

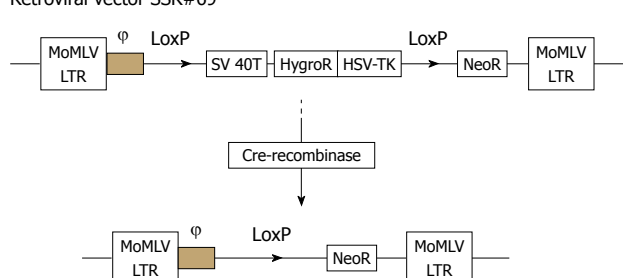


Figure 3 Schematic drawings of the integrating component of retroviral vector SSR#69 before and after Cre-recombination. SSR#69 contains the hygromycin B resistance gene (*Hyg R*) as a positive selectable marker and the herpes simplex virus thymidine kinase gene (*HSV-TK*) as a negative selectable marker. The *SV40T*, *Hyg R* and *HSV-TK* genes are flanked by loxP sites.

37°C for 12 h each day for 5 d. Two days after the final transduction, selection was applied with 100 $\mu\text{g}/\text{mL}$ hygromycin. After two weeks hygromycin selection, colonies of transduced hepatocytes emerged. One of the colonies displayed morphological characteristics of the primary hepatocytes, TJPH-1, and was isolated by cloning rings and expanded in culture. Finally, *SV40T* cDNA in the expanded cells was excised by Cre/loxP site-specific recombination.

Immunofluorescent analysis of SV40T antigen (SV40Tag) in immortalized cells

For detection of *SV40Tag*, indirect immunofluorescent staining was performed, using mouse monoclonal immunoglobulin G antibody to *SV40Tag* (Santa Cruz Biotechnology; Santa Cruz, CA, USA) and the second antibody, rhodamine (TRITC)-conjugated sheep anti-mouse IgG (Sigma, St. Louis, MO, USA). DAPI (4',6-diamidino-2'-phenylindole dihydrochloride, Roche, Cat. No. 10236276001) blue-fluorescent dye was used for staining nuclei (double-stranded DNA).

Gene expression of liver-specific functions in immortalized cells

Total RNA was extracted from immortalized cells and reverse transcription-polymerase chain reaction (RT-PCR) was performed according to the manufacturer's

protocol. Primers used were as follows: *SV40T* (422 base pair, bp), CAGGCATAGAGTGTCTGC (5'primer), CAACAGCCTGTTGGCATATG (3'primer); porcine albumin (324 bp), CTTATTCCAGGGGTCTGTTTC (5'primer) and TCGTTTCTCTCAGGCTCTTCT (3'primer); porcine GAPDH (179 bp), CATCATCCCTGCTTCTACCG (5'primer), CCTGCTTCACCACITTTCTTG (3'primer).

RESULTS

Successful porcine hepatocytes isolation resulted in a cell yield of 1.05×10^7 cells/g liver. The viability of hepatocytes immediately after isolation, using the trypan blue exclusion technique, was 97%. The primary hepatocytes attached to the plates showed typical morphological appearance with a polygonal shape, granular cytoplasm and one or more nuclei (Figure 2B).

After transfection of porcine hepatocytes with *SV40T*, several clones (Figure 2C) grew steadily in the culture medium within 2 wk after 100 $\mu\text{g}/\text{mL}$ hygromycin selection. One of the surviving cell colonies, TJPH-1, isolated by cloning rings displayed morphological characteristics of primary hepatocytes featuring a large round nucleus with a few nucleoli and multiple granules in the cytoplasm. After treatment with Cre recombinaase, the expanded TJPH-1 cells reverted to their pre-immortalized state. The reverted cells lost expression of *SV40T* (Figure 4), resulting in loss of proliferation.

The *SV40Tag* was examined by immunofluorescent staining in the nuclei of all immortalized cells, as shown in Figure 5A. Immortalized and reverted cells expressed the albumin gene shown by RT-PCR, similar to normal porcine hepatocytes (Figure 4).

DISCUSSION

Cultured primary porcine hepatocytes provide a valuable tool for various research strategies and clinical applications, such as studying the regulation of cell growth and of certain differentiated hepatocyte functions and BAL (bio-artificial liver) systems^[11,12]. However, the utility of cultured hepatocytes is hampered by difficulties in obtaining populations of primary cells which cannot be expanded *in vitro*.

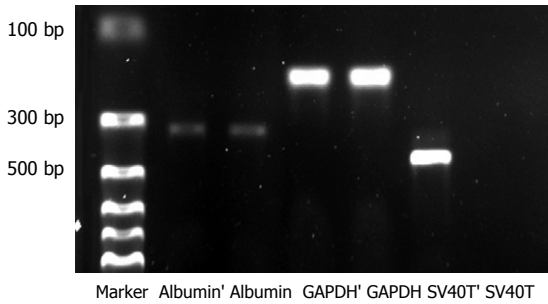


Figure 4 Gene expression of liver-specific functions in immortalized and reverted cells. Lines 1 to 7, from left to right, Marker, Albumin' (immortalized hepatocytes), Albumin (reverted hepatocytes), GAPDH' (immortalized hepatocytes), GAPDH (reverted hepatocytes), SV40T' (immortalized hepatocytes), SV40T (reverted hepatocytes), respectively.

Thus, other alternative sources of hepatocytes have to be explored^[13]. Hepatocytes immortalized with a *SV40Tag* can make unlimited supplies of cells feasible. However, the continued presence of a *SV40Tag* in the immortalized hepatocytes would expose patients to an unacceptable tumorigenic risk. To surmount this hurdle, we herein established reversible immortalized porcine hepatocytes to intentionally control population expansion (Figure 5). Use of the Cre/LoxP-based reversible immortalization strategy represents an important step in the development of a potentially novel strategy for resolving the current limits of primary hepatocytes^[2]. Different from a general gene transfer strategy, the novel strategy of reversible immortalization allows temporary expansion of cell populations by transfer of an oncogene which can be subsequently excised by site-specific recombination. Several studies have confirmed the utility, safety and efficiency of the reversible immortalization procedure^[14]. This novel procedure has extended to several cell types, such as liver endothelial cells^[15], hepatic stellate cells^[16] and rat hepatocytes^[17]. The present study expands the work to primary porcine hepatocytes.

Although porcine hepatocytes can be immortalized by retrovirus-mediated transfer of *SV40T*, the efficiency of transduction was significantly low^[10]. A major challenge of successful transduction is represented by the availability of a sufficient amount of hepatocytes with high post-isolation viability. Thus, in the process to successfully and effectively transduce retroviral vectors into primary hepatocytes, establishing an efficient cell isolation technique would be meaningful.

Hepatocyte isolation started in the mid-1960s, when rat hepatocytes were isolated using a combined mechanical/enzymatic digestion technique^[18]. It was not until 1976, when Seglen^[19] introduced the two-step collagenase perfusion technique, that high-yield preparation of isolated intact hepatocytes was available. After that, different innovative techniques have been introduced to further improve isolation results^[20,21]. In this study, we established a modified four-step collagenase retrograde perfusion technique. The modified four-step method of hepatocyte isolation, compared to the traditional two-step method, can

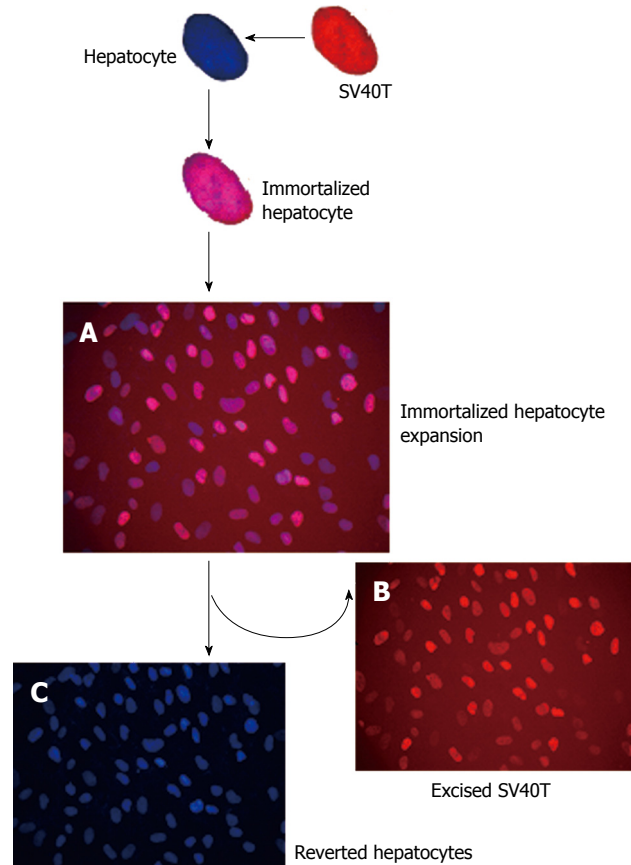


Figure 5 Scheme of reversible immortalization. The primary porcine hepatocytes were immortalized by transfer of an oncogene (*SV40T*). After expansion of the immortalized cells, Cre/loxP recombination was performed to remove the oncogene (*SV40T*) and the cells reverted to their pre-immortalized state. (A-C) Double immunofluorescence of immortalized hepatocytes stained with DAPI (blue), which binds, together with monoclonal antibody anti-*SV40T* revealed with texas red-antibody conjugate (red). Blue and red fluorescence merged as purple. *SV40T* expression is revealed by intense staining of the cell nucleus.

result in an improvement in cell viability and yield^[22]. After cell isolation, we employed an incubation step of 10 min using DNase 1, during which cell clumps were broken up and damaged cells digested. This method resulted in a hepatocyte yield of 1.05×10^7 cells/g liver. The viability of the cells, using the trypan blue exclusion technique, was 97%.

In summary, we propose that the modified four-step technique would improve research results, allowing large-scale production of hepatocytes of high quality. Furthermore, the present study expanded the novel strategy of reversible immortalization to primary porcine hepatocytes. By permitting temporary and controlled expansion of hepatocyte populations, the reversible immortalized cells may therefore be used for various research strategies and clinical applications.

ACKNOWLEDGMENTS

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COMMENTS

Background

The limited lifespan of porcine hepatocytes is a major hurdle to their use. Furthermore, the functional activities of primary porcine hepatocytes decline rapidly after several days in culture. An attractive alternative source of liver cells would be reversible immortalized cells that could make unlimited supplies of cells feasible which would exhibit the characteristics of differentiated hepatocytes. Toward this goal, the authors have focused on reversible immortalization by using Cre/loxP site-specific recombination.

Research frontiers

Cre/loxP site-specific recombination was used to reversibly induce the proliferation of primary cells. A reversible immortalization system using the Cre-loxP site-specific recombination system has been widely used in establishing immortalized cell lines.

Innovations and breakthroughs

The authors described a modified four-step retrograde perfusion technique of hepatocyte isolation and subsequently established a porcine hepatocyte cell line mediated by retroviral transfer and site-specific recombination.

Applications

The modified four-step retrograde perfusion technique would improve research results, allowing large-scale production of hepatocytes of high quality. Furthermore, the present study expanded the novel strategy of reversible immortalization to primary porcine hepatocytes. This reversible immortalized cell line could provide an unlimited supply of cells for research and clinical use.

Terminology

Reversible immortalization: The procedure of reversible immortalization was devised by retrovirus-mediated transfer of an oncogene that can be subsequently effectively excised by site-specific recombination.

Peer review

This manuscript appears to be about two separate, but related topics of hepatocyte isolation and "reversible immortalization". In my opinion, by combining the two topics, it weakens the manuscript. It appears they were able to reproduce techniques described by others, which is good.

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Over-the-scope clip closure of two chronic fistulas after gastric band penetration

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Abstract

Gastrointestinal perforations are conservatively managed at endoscopy by through-the-scope endoclips and covered self expandable stents, according to the size and tissue features of the perforation. This is believed to be the first report of successful closure of two gastrocutaneous fistulas with over-the-scope clips (OTSCs). After laparoscopic gastric banding, a 45-year old woman presented with band erosion and penetration. Despite surgical band removal and gastric wall suturing, external drainage of enteric material persisted for 2 wk, and esophagogastroduodenoscopy demonstrated two adjacent 10-mm and 15-mm fistulous orifices at the esophagogastric junction. After cauterization of the margins, the 10-mm fistulous tract was grasped by the OTSC anchor, invaginated into the applicator cap, and closed by a traumatic OTSC. The other 15-mm fistula was too large to be firmly grasped, and a fully-covered metal stent was temporarily placed. No leak occurred during the following 6 wk. At stent removal: the OTSC

was completely embedded in hyperplastic overgrowth; the 15-mm fistula significantly reduced in diameter, and it was closed by another traumatic OTSC. After the procedure, no external fistula recurred and both OTSCs were lost spontaneously after 4 wk. The use of the anchor and the OTSC seem highly effective for successful closure of small chronic perforations.

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Key words: Gastrointestinal endoscopy; Fistula; Stent; Esophagogastric junction

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INTRODUCTION

Gastrointestinal (GI) perforations require prompt closure and control of extraluminal fluid collections and sepsis. They have been managed traditionally by surgery^[1-3], but a conservative endoscopic approach has recently been preferred with the advantages of negligible morbidity and mortality, a short hospital stay, and the possibility of prompt resumption of an oral diet^[2,4-6].

Routine endoscopic methods to close GI perforations are endoclips and covered self-expandable stents; the choice being related to the width and tissue features of the margins^[7,8]. Endoclips are metallic double-pronged clips loaded on a deployment catheter introduced through the operative channel of the scope [through-the-

scope (TTS) clips^[7]. Although acute small (< 15 mm) perforations can be successfully closed with TTS clips^[9], these devices achieve controversial results when used for larger perforations^[10,11], and seem technically inadequate for chronic perforations, due to poor grasping ability and insufficient strength to close fibrotic margins^[7]. Therefore, TTS clips have rarely closed chronic leaks^[12-17] and are generally considered only as a supplementary technique^[8].

Covered self-expandable metal stents (SEMSs) or self-expandable plastic stents (SEPSs) are an effective method for sealing and healing more than 80% of chronic GI leaks, but have drawbacks: the removal of partially-covered SEMSs may be difficult due to the ingrowth at uncovered ends and the risk of mucosal tears, hemorrhage and subcutaneous emphysema^[4,18,19], whereas fully-covered stents show a migration rate of up to 50% and often require replacement^[6,20,21].

The present case is the first successful closure of two gastro-cutaneous fistulas with the newly designed over-the-scope-clip (OTSC) (Ovesco Endoscopy, Tubingen, Germany).

CASE REPORT

Twenty-six months after laparoscopic gastric banding, a 45-year-old woman presented severe epigastric pain. The diagnosis of band erosion with penetration through two large tears at the posterior wall of the gastric fundus was established by esophagogastroduodenoscopy (EGD), and a subphrenic abscess was demonstrated by computed tomography (CT). Management included surgical band removal, suturing of the gastric wall with two omental patches, and abdominal drainage.

However, external drainage of enteric material persisted for 2 wk and the barium swallow revealed two fistulas at the esophagogastric junction (Figure 1). EGD showed two opposite perforations of 10 and 15 mm (Figure 2), with fistulous tracts partially and fully negotiable by the 9.8-mm endoscope, respectively.

After cauterization of the margins, the proximal segment of the 10-mm fistula was grasped by the OTSC anchor, invaginated into the applicator cap (Figure 3A), and closed by a 9-mm traumatic OTSC (Ovesco Endoscopy GmbH, Tuebingen, Germany) (Figure 3B). On the contrary, the 15-mm fistula was too wide to be firmly grasped by the anchor and therefore it was closed with a fully-covered esophageal self-expandable metal stent (SEMS; Niti-S, Taewoong, South Korea), which also covered the OTSC.

The barium swallow performed after 24 h confirmed the complete sealing of both fistulas (Figure 3C) and the patient was discharged on an oral diet.

After 6 wk, the fully-covered SEMS was easily removed, and the EGD showed that the OTSC was buried into an intense overgrowth reaction (Figure 4). The 15-mm fistula diameter was reduced to 8 mm, and therefore it was sealed by a second 9-mm traumatic OTSC (Figure 5).

During the following 8 wk, no external fistula or



Figure 1 Barium swallow: two fistulas at the esophagogastric junction.

abdominal collection was observed by barium swallow (Figure 6) or the dye leak test with methylene blue. Both OTSCs were lost within the second and fourth weeks of follow-up after SEMS removal.

DISCUSSION

The OTSC has a different conception from TTS clips: (1) the OTSC is in nitinol with a “leghold trap” memory shape, is loaded on a cap placed on the scope tip, and has the capacity to grasp and compress the tissue more widely and with greater strength, without provoking ischemia or cutting the tissue; and (2) the target tissue needs to be caught and pulled into the cap by specifically developed devices that make addressing tangential lesions easier.

Preliminary small case series suggest that the OTSC is effective for hemostasis of non-variceal bleeding^[22,23]. Moreover, results from a few human cases^[22-24] and animal studies^[25-27] have indicated that this device may be appropriate to close acute gastric and colonic perforations that occur during endoscopic mucosal resection, and natural orifice transluminal endoscopic surgery orifices up to 20 mm in size.

This is the first report of successful closure of two inveterate perforations by OTSCs. In our opinion, the success of the procedure should be attributed both to the anchor used to pull the tissue into the cap and to the OTSC itself. The three rigid hooks of the anchor firmly grasp the perforation margins at three equidistant points, permitting to pull the margins circumferentially into the cap. In the present case, the anchor was opened beyond the luminal margins of the fistulous orifice into the proximal segment of the fistulous tract that was partially invaginated into the cap before the release of the OTSC. This mechanical result would not have been achieved using the other OTSC device, the twin grasper, which has two independently movable branches that grasp the perforation margins at two opposite points. This two-point grasping method may be sufficient for acute perforations with normal elastic tissue margins, but not for fibrotic hard chronic ones that would be only partially invaginated into the cap (Figure 7).

Both traumatic OTSCs placed in the present case were lost after a period between the 2nd and 4th week after SEMS removal. Whether traumatic or atraumatic

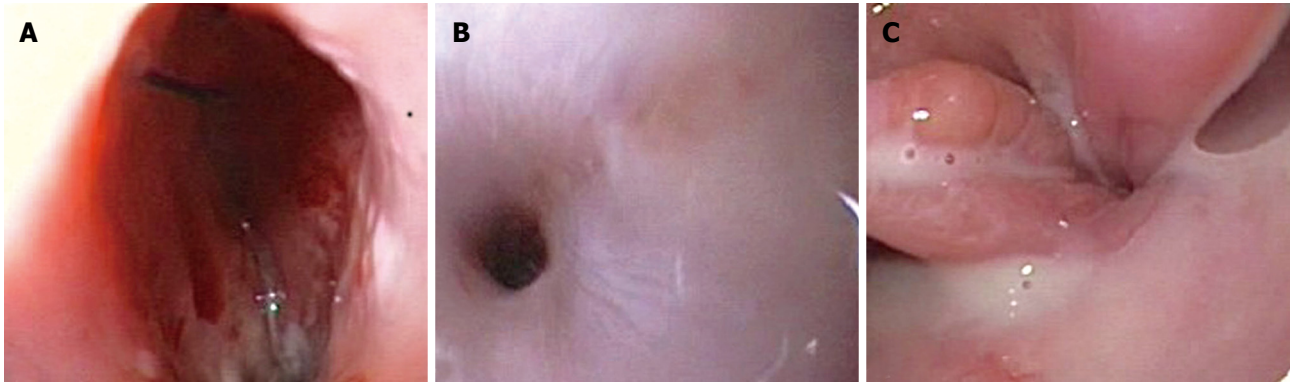


Figure 2 Endoscopic view of the 15-mm (A) and 10-mm (B) fistulous openings on the left and right sides, respectively, above the esophagogastric junction (C).

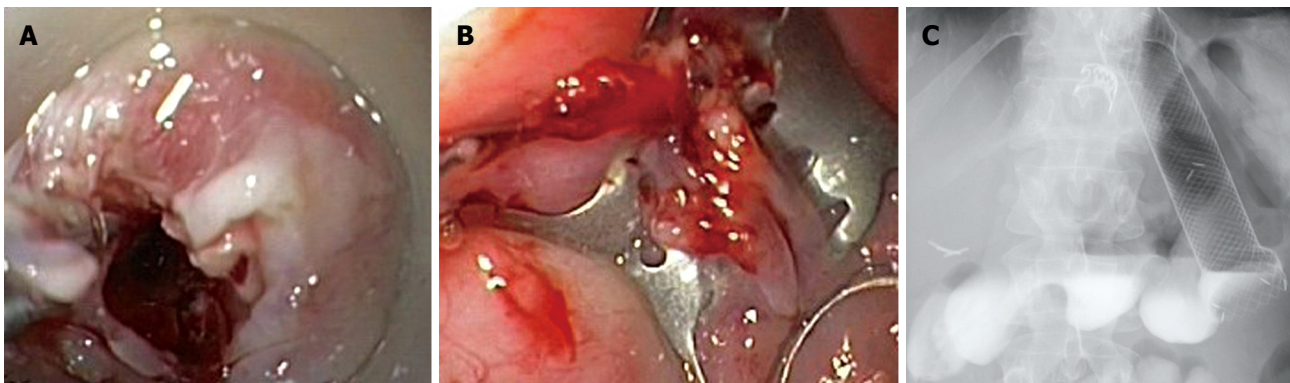


Figure 3 First OTSC placement. A: Invagination of the proximal segment of the 10-mm fistula into the OTSC applicator cap using the anchor; B: Closure of the 10-mm fistula; C: Barium swallow after the first OTSC and stent placement.

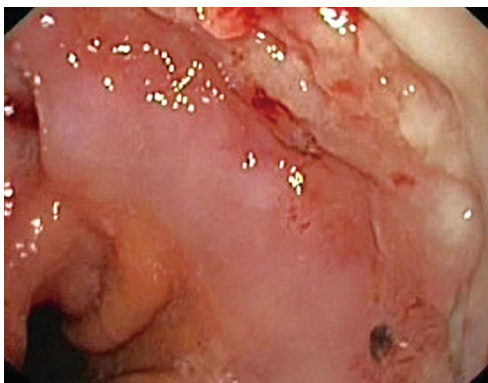


Figure 4 First OTSC completely embedded in hyperplastic overgrowth.

jaws have different “stay-in-place” periods is unknown. In previous studies, acute perforations have been successfully closed independently from the OTSC teeth shape after remaining in place for similar periods of time^[22,26], and only occasionally for > 4 wk^[24,26]. Although the traumatic teeth may reduce the possibility of hard tissue slipping out of the OTSC, prolonging its *in situ* stay, strong tissue compression could be more important to determine long-standing closure. Moreover, the use of oversized OTSCs may increase the capacity to grasp and close fibrotic margins of chronic perforations and its permanence.

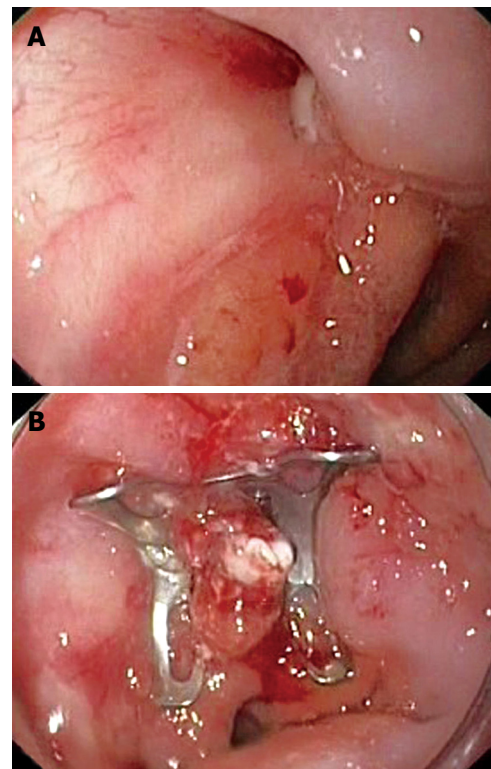


Figure 5 Second OTSC placement. A: Fistula opening reduced in size; B: Fistula closure.

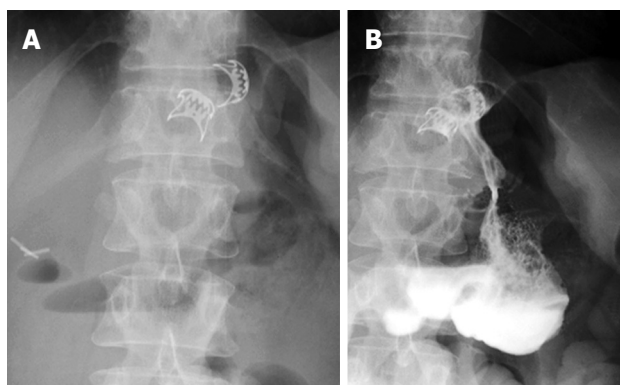


Figure 6 OTSC closure of the two fistulas. A: Two OTSCs in place; B: No leaks from both OTSCs at barium swallow.

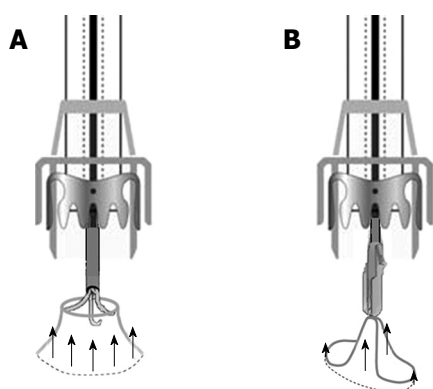


Figure 7 Mechanical results of OTSC devices pulling fibrotic margins of chronic perforations. A: Circumferential invagination using the anchor; B: Two-point invagination with the twin grasper.

Finally, no OTSC-related complications occurred. An intense overgrowth embedded the first OTSC after SEMS removal, but it did not prevent the spontaneous loss of the clip during follow-up. It remains to be established whether the overgrowth reaction would have occurred without the stent. Although endoscopic follow-up was not scheduled in previous studies, OTSC-related overgrowth has not been observed in humans^[22-24], whereas Schurr *et al.*^[26] have observed minimal overgrowth in the colon of some pigs after 1 mo.

In conclusion, the OTSC may be the least invasive method to close chronic small fistulas or leaks, if prospective large comparative studies with fully-covered stents will confirm our observation.

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Therapeutic angiography for giant bleeding gastro-duodenal artery pseudoaneurysm

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INTRODUCTION

Upper gastrointestinal bleeding (UGIB) is one of the leading causes of admission to the emergency department. While the vast majority of these bleeding episodes resolve spontaneously and most of the remaining cases are successfully managed endoscopically, a small percentage of patients require treatment using more invasive procedures such as therapeutic angiography and surgery. UGIB can be caused by a number of various pathologies. Gastro-duodenal artery (GDA) pseudoaneurysm is a rare but potentially fatal complication of surgery and, hence, early recognition and management of this complication is critical^[1]. Although open surgery has traditionally been the treatment of choice, therapeutic angiography is emerging as an effective treatment modality for selected cases^[2]. In this article we present such a case as well as remarkable images of the giant GDA aneurysm. Additionally, we discuss the pathophysiology of the condition, the treatment options and our rationale for opting for angiographic intervention over surgery.

CASE REPORT

An 18-year-old female was transferred to our surgery department due to unsuccessful treatment of acute upper gastrointestinal bleeding. Seven months prior to this admission she was diagnosed with Crohn's disease of the ileo-cecal region. She was treated with oral steroids and

Abstract

We present the case of an 18-year-old female transferred to our center from an outside hospital due to persistent gastrointestinal bleeding. Two weeks prior to her transfer she underwent duodenal omentopexy for a perforated duodenal peptic ulcer. The patient underwent a computed tomography angiogram which identified the source of bleeding as a giant gastro-duodenal artery (GDA) pseudoaneurysm. The patient was taken to interventional radiology where successful microcoil embolization was performed. We present this rare case of a giant GDA pseudoaneurysm together with imaging and a review of the medical literature regarding prevalence, etiology and treatment options for visceral arterial aneurysms.

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Key words: Gastro-duodenal artery; Gastrointestinal; Bleeding; Angiography; Pseudoaneurysm



Figure 1 Computed tomography angiography (CTA). A: CTA arterial late phase showing the giant GDA pseudoaneurysm (arrow); B: CTA eight days post the procedure which shows the reduction in size of the pseudoaneurysm (long arrow), angiographic coils (short arrow) and no perfusion.

mesalamine (Pentasa) with gradual improvement of the disease symptoms. Five months later she was admitted to a local community hospital due to acute abdominal pain. She was taken immediately to the operating theatre. At that time, a perforated duodenal ulcer was identified and treated with duodenal omentopexy (Graham's patch). On post-operative day 14, the patient began bleeding from the surgical wound and experiencing episodes of UGIB. She was transfused with multiple units of packed blood cells and was transferred to a second hospital where she underwent an upper endoscopy (Figure 1A). The endoscopy showed erosive gastritis and blood clots without active bleeding. Due to unsatisfactory explanation for the source of the hemorrhage she underwent computed tomography angiography (CTA) which demonstrated a giant pseudoaneurysm of the GDA, surrounded by fresh clots of blood. The patient was transfused with an additional five units of packed blood cells and transferred to our institution as the initial hospital did not have an interventional radiology unit. Upon arrival at the emergency department, the patient's pulse was 80 bpm and blood pressure was 110/70 mmHg. A pulsatile abdominal mass was palpated at the right upper quadrant. Blood tests showed hemoglobin level of 11.6 mg/dL and normal coagulation studies. A naso-gastric tube was inserted which drained multiple clots of blood, at which time she was treated with intravenous omeprazole and taken to the angiography suite. After cannulation of the celiac trunk and the superior mesenteric artery *via* a right transfemoral approach, the giant pseudoaneurysm emerging from the GDA was demonstrated. Arterial embolization was performed by placement of several detachable micro-coils until flow-arrest was obtained. The procedure was performed without complication. Eight days following the procedure she underwent CTA which showed the angiographic coils surrounded by a small collection of fluid and no signs of perfusion in the GDA (Figure 1B). She was discharged on the 10th d of hospitalization. After two years of follow up, the patient is asymptomatic and has not been hospitalized due to recurrent UGIB.

DISCUSSION

Visceral artery pseudoaneurysms are considered rare

pathologies. Nevertheless, pseudoaneurysm rupture and bleeding can lead to significant morbidity and mortality. The visceral arteries which most commonly develop pseudoaneurysm are the splenic artery, hepatic arteries, gastric and gastroepiploic arteries, gastro-duodenal artery and branches of the mesenteric arteries^[3]. The most common artery involved is the splenic. GDA aneurysms are considered to be extremely rare. Although pseudoaneurysms and aneurysms of splanchnic arteries have traditionally been considered uncommon clinical entities, the prevalence discovered during autopsy study was surprisingly high (10.4%)^[1]. More recent, higher rates of detection are likely related to increased frequency of imaging studies such as ultrasonography and computed tomography. There are multiple etiologies for development of pseudoaneurysms including: atherosclerosis, trauma, surgery, pancreatitis, infection, collagen vascular disease and congenital abnormalities. Infection and trauma are probably the most common causes. Infection can occur as a direct process around the vessel or as lymphatic spread from a primary focus such as *Mycobacterium tuberculosis* located away from the vessel^[4]. Patients with pseudoaneurysm may be asymptomatic or present with symptoms of gastrointestinal bleeding, intraperitoneal hemorrhage, obstructive jaundice due to external pressure by the pseudoaneurysm, hematemesis and rupture into the portal vein.

A review of visceral aneurysms conducted by Moore *et al.*^[5] concluded that 35% of GDA aneurysms are ruptured at presentation, carrying a mortality rate of 21%. In the past, these pseudoaneurysms were treated surgically. However, in the last two decades, radiographic intervention has emerged as an attractive method for treating these patients. Surgical treatments include resection of the aneurysm and placing an interposition graft or performing aneurysmectomy with or without patching using a great saphenous vein. Endovascular treatments used are embolization or stent graft repair of the aneurysm. A laparoscopic procedure has also been reported for treating splenic artery aneurysm^[6]. The potential complications of angiography, aside from those of standard femoral cannulation, include infarction of viscera and abscess

formation. Fortunately, the complication rate regarding the former is relatively low due to rich collateral blood supply. Saltzberg *et al*^[7] retrospectively reviewed the outcome of 65 patients diagnosed with visceral aneurysm, 18 of whom were treated by angiography and 9 by surgery. He reported that the initial technical success rate of the endovascular procedures was 94.4% (17/18). Major complications occurred in 22.2% (4/18). However, all four of these were in patients who were treated for splenic artery aneurysms. They concluded from their experience that endovascular management of visceral artery aneurysms is a reasonable alternative to open surgical repair, except for patients with splenic artery aneurysm. Carrafiello *et al*^[8] published a case report of a patient with an evolving asymptomatic GDA pseudoaneurysm who underwent a combination of percutaneous, ultrasound-guided thrombin injection directly into the pseudoaneurysm sac and microcoil embolization of the gastro-duodenal artery. They concluded that thrombin injection may reduce the pseudoaneurysm's wall tension and probably diminishes the risk for rupture.

Our patient presumably developed the pseudoaneurysm due to a leak occurring after the omentopexy repair for her perforated duodenal ulcer which initiated an infectious/inflammatory process in the region of the GDA area, causing damage to the vessel wall. Bleeding into the peritoneal space or the gastrointestinal tract beginning several days following an operation should be a cause for suspicion for the possibility of a ruptured pseudoaneurysm. Any operation which involves handling of an infected space or organ is a risk factor for infecting the peritoneal cavity and developing the pseudoaneurysm. Angioembolic occlusion of this giant aneurysm provided an effective treatment with minimal morbidity risk. Sur-

gical repair in this case would have been particularly challenging and possibly morbid as it would have required re-exploring a previously operated space. We conclude that angiographic management is not only a feasible but possibly preferred treatment modality in selected cases of visceral artery aneurysms.

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A geriatric patient with diffuse idiopathic skeletal hyperostosis

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Abstract

The most frequent health problems seen in senility are chronic and degenerative diseases. A 75-year-old male patient with the complaints of weight loss and difficulty in swallowing was admitted to our hospital from a nursing home. Upper system fiber-optic gastrointestinal endoscopy was performed and a mass at the junction of the hypopharynx and esophagus just below recessus piriformis obstructing almost the whole of the lumen and blocking the distal passage was detected. Computed tomography revealed marked narrowing secondary to osseous hypertrophy in the air column of the hypopharynx and proximal esophagus. Diffuse idiopathic skeletal hyperostosis or Forestier's disease is an idiopathic disease characterized by the ossification of the anterior longitudinal ligament of vertebra and some of the extraspinal ligaments. In the present case we aim to discuss an elderly patient who suffered from dysphagia and weight loss and the diagnostic stages.

INTRODUCTION

Diffuse idiopathic skeletal hyperostosis (DISH) is a common but often unrecognized systemic disorder observed mainly in elderly people^[1]. All papers related to DISH demonstrate a consistent and marked increase of the disease with advancing age^[2]. Various local structural lesions such as oropharyngeal tumors, vascular pathologies, retropharyngeal abscesses, and anterior cervical osteophytes may lead to mechanical esophageal dysphagia^[3-5]. The prevalence of DISH in adults over the age of 50 years is around 25% for men and 15% for women, with an increased incidence in patients with obesity, gout and diabetes^[6].

Although most patients with cervical spine involvement are either asymptomatic or have limited cervical spine movement and unspecified pain, possible complications described in the literature are dysphagia^[7], stridor^[8], dyspnea^[9], ossification of the posterior longitudinal ligament and/or myelopathy^[10] and hoarseness^[11].

CASE REPORT

A 75-year-old male patient was found drunk and unconsci-

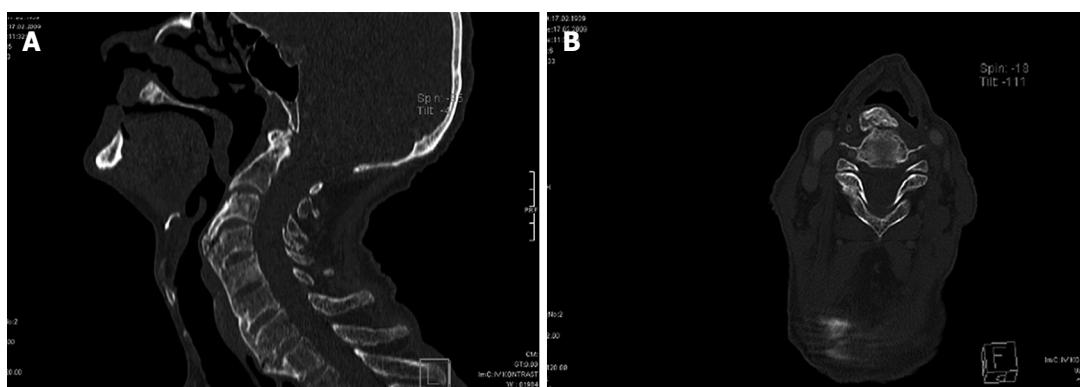


Figure 1 Cervical CT. A: Sagittal section revealed hypertrophic bridging alterations of anterior corpus parts at the level of C3-T1 vertebra and restriction of the esophagus due to osteophytic sprue formations; B: Axial section revealed osteophytic formations on the anterior parts of vertebral corpus.

ous on the street in November 9, 2008 and was transferred to the nearest state hospital. After an operation for intertrochanteric femur fracture he was sent to a nursing home. Because of progressive weight loss and swallowing difficulty, some blood tests were done and Hb and erythrocyte sedimentation rate (ESR) were found to be 9 g/dL and 75 mm/h, respectively. The patient was then transferred to our hospital on 28 January, 2009 with the preliminary diagnosis of malignancy. His height was 160 cm, weight 41 kg and body mass index was 16 kg/m². Physical examination revealed only cachectic appearance and limited neck movements; the other findings were normal, and the thyroid gland was nonpalpable. Due to detected anemia two packs of erythrocyte suspensions were transfused. In the biochemical analyses hepatic and renal functions were normal; he didn't have any electrolyte imbalance or systemic disease (fasting plasma glucose: 79 mg/dL, blood urea nitrogen: 22 mg/dL, creatinine: 1.0 mg/dL, uric acid: 4.8 mg/dL, aspartate aminotransferase: 8 U/L, alanine aminotransferase: 3 U/L, potassium: 4.3 mmol/L, iron: 41 µg/dL total iron binding capacity: 173 µg/L). His vitamin B12 and folic acid levels were 57 pg/mL and 1.46 ng/mL respectively. Total protein value was 5.7 g/dL and albumin was 2.3 g/dL. Evaluation of cognitive functions revealed his Mini Mental State Examination score was 12. There was not any evidence for malignancy in his thoracic and abdominal CT scans. Upper system fiber-optic gastro-intestinal endoscopy was performed and a mass at the junction of the hypopharynx and esophagus just below recessus piriformis obstructing almost whole of the lumen and blocking the distal passage was detected. Biopsy couldn't be performed because of the risk of bleeding and aspiration. As a result of ear-nose-throat clinic consultation, and after laryngoscopic examination, anesthesia confirmation and cervical CT was performed for taking a biopsy from the mass thought to have originated from the pharyngeal area. Cervical CT revealed hypertrophic bridging alterations of anterior corpus parts at the level of C3-T1 vertebra (Figure 1A and B). At the hypopharynx and proximal parts of esophagus restriction secondary to osseous hypertrophy was demonstrated and this aspect was evaluated as consistent with DISH or Forrester disease. As a result of

multidisciplinary evaluation, an operation wasn't planned because of the patient's poor general condition. Patient's nutrition was regulated with fluid foods. No problem was established during nutrition with fluids. The patient was sent back to nursing home after recovery of general status.

DISCUSSION

Although mortality decreases and life expectancy gets longer with developing technology and medicine, chronic diseases remain common problems of elderly patients. In the differential diagnosis for dysphagia, larynx, lung and mediastinal tumors, esophagus motility disorders, esophagitis, restriction of esophagus, spinal tumors, vascular abnormalities, Zencker diverticle, Plummer-Vinson syndrome, gastroesophageal reflux and globus histericus should be kept in mind. For differentiating these disorders barium esophagography has a great importance^[12].

A medical history should be taken in detail and questions about anatomical structure and functions of the oral cavity, pharynx, larynx, and esophagus should be examined when a patient complaining of dysphagia applies to ear-nose and throat clinics. Physical examination of the oral cavity, pharynx, larynx and neck should be done for determining the possible pathologies. Esophageal cancer, web, achalasia, diffuse esophageal spasm and esophagitis are common causes of esophageal dysphagia. Rarely, mediastinal tumors pressing the esophagus, vertebral bone processes and impressions due to the aorta can be the causes^[13]. In our case, the patient's complaints were anemia, weight loss and difficulty in swallowing and initially esophageal malignancy was thought to be the cause of these complaints and upper gastrointestinal system endoscopy was planned. As a result of these evaluations, hypertrophic bridging alterations of anterior corpus parts at the level of C3-T1 vertebra were revealed. It was reported that perforation during esophagoscopy improves because of compression due to cervical osteophytes^[14].

Treatment for DISH is primarily conservative, non-steroidal anti-inflammatory drugs and myorelaxants are used. But in refractory cases there are two surgical approaches to remove osteophytes by lateral cervical or

peroral transpharyngeal ways^[15]. In our case, we initially preferred a medical approach and started treatment with anti-inflammatory drugs and nutrition with liquid foods, after recovery of oral intake we maintained with high calorie formulas.

In our case, the patient hadn't applied to any hospital in the period that he lived alone but after he began to stay in the nursing home for elderly these complaints and problems were established and he was transferred to our hospital. At the present time, it is emphasized that without increasing the quality of the life, longer life will have no meaning and health expectancy is more important than life expectancy. Consequently, while treatment is the target in a young population, in a geriatric population the main target is the preservation of quality of life. We avoided the complications of surgical management and anesthesia, we tried nutrition with liquids and facilitated oral intake. Eventually, recovery in our patient's status and weight gain started. Gastrostomy may be planned if necessary and the patient is followed at particular intervals. During the evaluation of elderly patients, social status, cognitive functions and exhaustive physical examination should be performed definitely.

In conclusion, DISH should be considered an important, although rare, cause of dysphagia among older adults. However, it should not be accepted as the cause of dysphagia until all other causes have been ruled out.

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Peeling a giant ileal lipoma with endoscopic unroofing and submucosal dissection

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abdominal CT immediately after removal of the lesion showed a small amount of free air, conservative treatment was successfully carried out for the perforation. Histologically, the removed lesion was a lipoma.

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Key words: Lipoma; Endoscopic submucosal dissection; Endoscopic unroofing; Perforation

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Abstract

Lipoma is relatively common in the colon but is less often in the small intestine. Most lipomas are incidentally detected at endoscopy and are usually small and asymptomatic. However, some of them can present with obstruction and/or intussusceptions. Surgical resection is commonly recommended to remove such significant lipomas with a limited pedicle and larger than 2 cm in size, as endoscopic resection may result in unfavorable complications such as intestinal perforations. We report a case of 62-year-old man presenting with hematochezia. Colonoscopy showed a submucosal tumor, about 50 mm in size, in the terminal ileum. A clinical diagnosis of lipoma was established based on the findings of colonoscopy and abdominal computed tomography (CT). As the patient complained of hematochezia and mild iron deficiency anemia associated with repeated tumor prolapse, we decided to remove his lipoma. Consequently, the lesion was completely removed *en bloc*. Although

INTRODUCTION

Lipoma is relatively common in the colon (65%-75%) but is less often in the small intestine (20%-25%). Most lipomas are incidentally detected at endoscopy and are usually small (less than 2 cm) and asymptomatic. However, some of them can present with obstruction and/or intussusceptions^[1]. Surgical resection is commonly recommended to remove such lipomas with a limited pedicle and larger than 2 cm in size, as endoscopic resection may result in unfavorable complications such as intestinal perforations^[2]. We herein report a case of a large lipoma, 50 mm in diameter, arising from the end of ileum, which was completely removed with the so-called endoscopic

unroofing technique in combination with endoscopic submucosal dissection (ESD)^[3,4].

CASE REPORT

A 62-year-old man presented with hematochezia. Colonoscopy showed a pedunculated lesion, about 50 mm in size, originating from the ileum end. With peristalsis, the lesion prolapsed through the ileocecal valve into the cecum and ascending colon (Figure 1). The surface was regularly covered with normal mucosa, except for a part of nodular change suggesting hyperplastic mucosa due to repeated prolapse, and “Cushion-sign” was positive^[5]. Moreover, computed tomography (CT) showed a uniform mass with a very low-density in the ileum end corresponding to the above detected lesion (Figure 2A and B). A clinical diagnosis of ileal lipoma was established based on the above findings. Although there was not any symptom of intestinal intussusception, multiple erosions were seen in the ascending colon perhaps caused by the tumor prolapsing with peristalsis. As oozing of blood was also seen, the ileal lipoma was considered as the bleeding source of his hematochezia. Therefore, local resection was decided for treatment. At first, conventional endoscopic mucosal resection (EMR, lift and cut technique) was attempted under CO₂ insufflation. However, the tumor was too large for the largest commercially available snare with a maximum diameter of 33 mm in our department to capture the whole lesion after injection of glycerol at the base of the lesion (Figure 3A). Alternatively, endoscopic submucosal resection (ESD) was conducted with an IT-knife for removal. Unfortunately, the muscle layer was lacerated and serosal membrane was observed during dissection (Figure 3B). Thus, we used the snare to break the mucosal layer on the top of the lipoma, and then dissection from the defect on the top rather than from the base of the lesion was continued. Capsulated fat tissue extruding through the overlying mucosa during dissection (naked fat sign) was seen (Figure 3C)^[5,6]. Finally, the lesion was completely removed *en bloc* (Figure 3D). The vital signs were stable and no abdominal distension or discomfort was found during the whole procedure. The lacerated muscle layer could not be completely but only partially sutured with metallic clips due to the narrow lumen in the terminal ileum. Abdominal CT immediately after removal, however, showed only a small amount of free air around the ileum end without ascites. Conservative treatment including withholding of oral intake, intravenous administration of antibiotics and hyperalimentation was started after consultation with the surgeons. The vital signs were stable except for fever (at the highest degree of 38°C) for 2 d. No sign of peritoneal irritation suggesting diffuse peritonitis was seen on close physical examination. Oral intake was restarted on the fifth day after ESD, and the patient was discharged uneventfully 2 d thereafter. Histologically, the removed lesion was a lipoma.

DISCUSSION

This lipoma could be correctly diagnosed before resec-

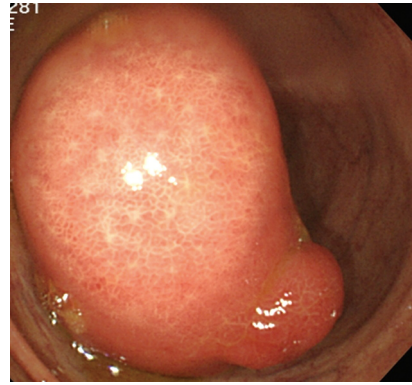


Figure 1 Colonoscopy showing a large pedunculated tumor, 50 mm in size, originating from the ileum end.

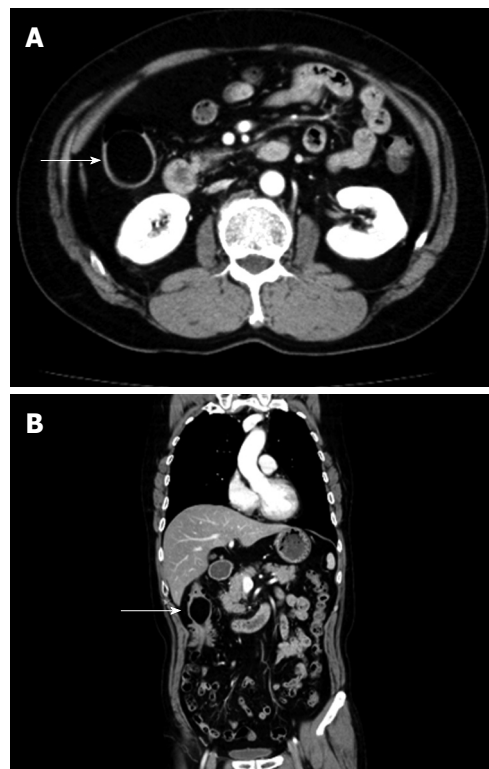


Figure 2 Abdominal CT showing a round, smooth and well-demarcated tumor at the end of the ileum (A) with a fat attenuation coefficient of -116 Hounsfield units (arrows) (B).

tion, as endoscopically it was a submucosal tumor with a positive “Cushion sign”, and moreover computed tomography disclosed a round and smooth, well-demarcated tumor with a fat attenuation^[7,8]. Although, lipohyperplasia, frequently discovered at the right-sided colon (especially in the ileocecal valve), should be included in the differential diagnosis, the encapsulated yellowish adipose tissue detected during endoscopic dissection finally supported the endoscopic diagnosis of a lipoma rather than lipohyperplasia before histological evaluation. We decided to treat this ileal lipoma, as the patient complained of hematochezia and mild iron deficiency anemia associated with erosions caused by repeated tumor prolapse. Various techniques including endoscopic

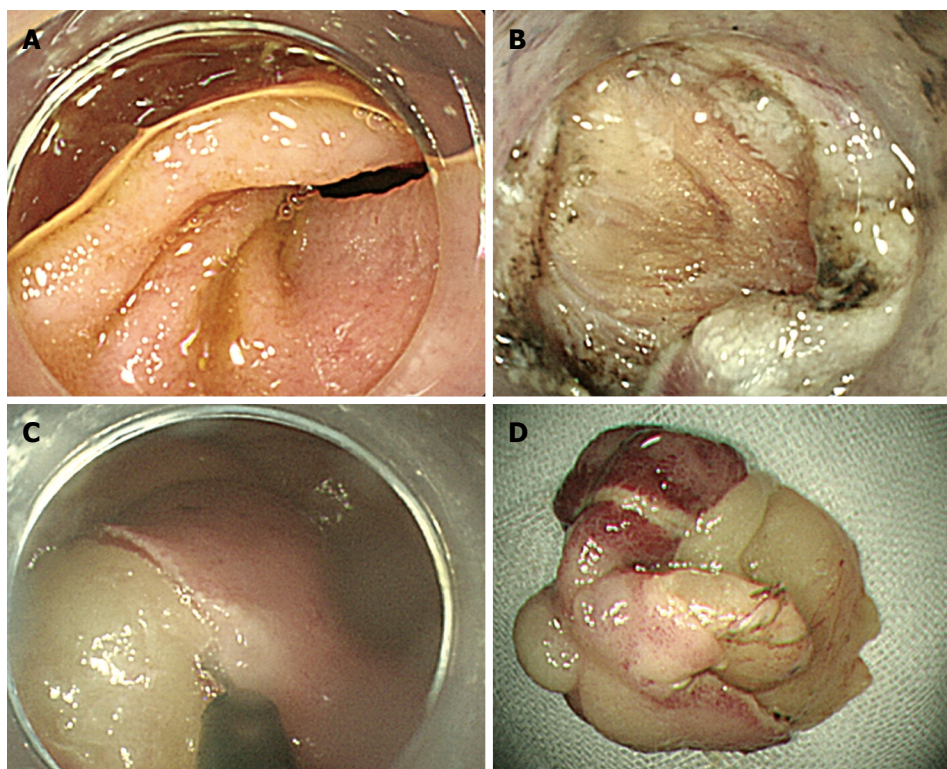


Figure 3 Whole captured lesion after injection of glycerol at the base of the lesion. The base of the lipoma (A), lacerated muscle layer (B), dissected overlying mucosa and capsule (C), and completely removed lipoma (D).

removal and surgical resection have been reported^[9,10]. We firstly attempted conventional EMR. However, unfortunately the tumor could not be completely captured with the largest snare. Piecemeal resection was also tried but failed to electrosurgically resect the tumor with the snaring technique. We therefore used the ESD technique to remove this large lesion, as this newly developed technique can remove this kind of large lesions *en bloc*^[11].

During dissection, since laceration of the muscle layer and thereafter the serosa were unfortunately endoscopically identified, we modified the ESD technique for removal. We enrolled the reported unroofing technique in combination with ESD for endoscopic resection^[3,4,12]. At first, the overlying mucosa and capsule of the lipoma were peeled off with the tip of the snare and IT knife from the top to the bottom of the tumor. The underlying adipose tissue was thereafter seen like a peeling banana. This modified technique finally enabled us to completely remove the lipoma *en bloc*. Different from the submucosal direct dissection at the base, we dissected this lipoma from the top to the bottom rather than from the base. We believe that this modified technique can remove large lipomas more safely and quickly than ESD^[11].

Perhaps, perforation is one of the most serious complications associated with endoscopic resection. We did not close the lacerated muscle layer immediately, as the clips in the narrow lumen of ileum would disturb continuing the endoscopic procedure. Fortunately, we could complete the endoscopic removal, as the patient did not develop pneumoperitoneum and the lumen was not collapsed. Furthermore, we could not close the perforation completely with metallic clips. However, conservative treatment was carried out successfully. Our case was

treated successfully without laparotomy. However, the choice of conservative or surgical treatment for iatrogenic colonic perforation remains controversial. In this case, non-surgical treatment was selected because the vital signs of the patient were stable, the abdominal pain was mild and localized, no unexplained peritoneal fluid was found in the abdominal CT, and the colonic preparation was extremely good. We also believe that CO₂ insufflation plays an important role in this successful conservative treatment, as room air insufflation would lead to pneumoperitoneum collapsing the lumen and vital signs (abdominal compartment syndrome)^[13-16].

In conclusion, large ileal lipoma can be completely removed with the modified ESD procedure in combination with endoscopic unroofing technique (from the top to the bottom like peeling a banana).

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Upper gastrointestinal hemorrhage caused by superwarfarin poisoning

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INTRODUCTION

Superwarfarins are anticoagulants similar to warfarin. As substitutes for acute rodenticides, superwarfarins are usually the first choice of rodenticide, especially in rural areas. Esophagogastroduodenoscopy (EGD) is a common tool for diagnostic and therapeutic goals. Significant bleeding after cold mucosal biopsy is seldom seen. Here, we report a woman with severe upper gastrointestinal hemorrhage after gastroscopic mucosal biopsy, which is a rare and previously unreported complication of superwarfarin poisoning.

CASE REPORT

A 55-year-old woman was referred to our hospital for epigastric pain and abdominal distention. Her medical history and physical examination were unremarkable. She had no history of anticoagulation therapy or use of non-steroid anti-inflammatory drugs. She came to the outpatient department for further evaluation.

Routine EGD revealed an uneven granular mucosa and several erosion lesions scattering in the stomach, covered with fresh blood crusts (Figure 1). Five mucosal biopsies of the stomach, two from the gastric angle and three from the lesser curvature side of gastric antrum, were performed for pathological analysis and *Helicobacter pylori* (*H. pylori*) detection. Gastric biopsy specimens revealed atrophic gastritis accompanying intestinal metaplasia. Rapid urease test was negative for *H. pylori* infection. No active bleeding was seen immediately after endoscopic biopsy and throughout the whole endoscopic procedure. However, she was admitted to the emergency department of our hospital because of hematemesis and melena the next day after EGD examination.

Abstract

Superwarfarins are a class of rodenticides. Gastrointestinal hemorrhage is a fatal complication of superwarfarin poisoning, requiring immediate treatment. Here, we report a 55-year-old woman with tardive upper gastrointestinal hemorrhage caused by superwarfarin poisoning after endoscopic cold mucosal biopsy.

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Key words: Esophagogastroduodenoscopy; Upper gastrointestinal hemorrhage; Superwarfarin poisoning

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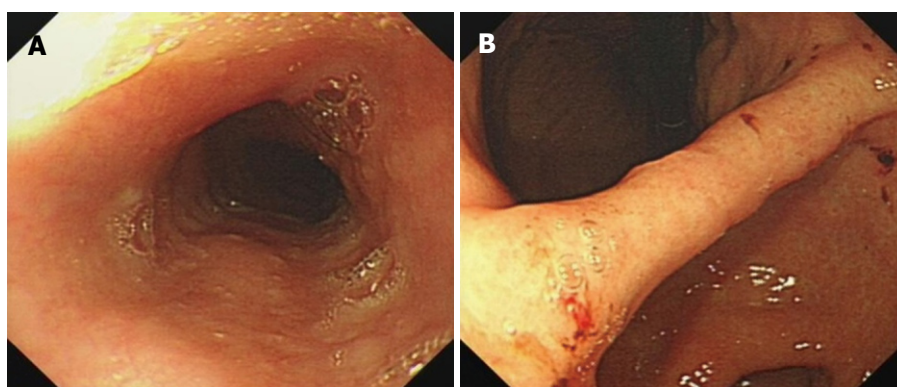


Figure 1 Initial esophagogastroduodenoscopy (EGD) demonstrating normal esophageal mucosa (A) and erosion lesions scattering in the stomach (B).

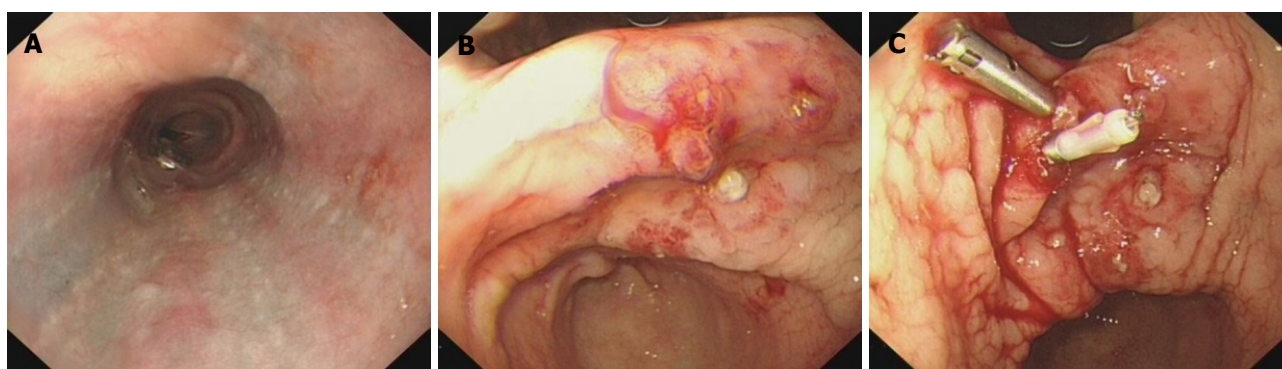


Figure 2 Second EGD showing submucosal congestion in the esophagus (A), active bleeding in the original biopsy sites (B), and titanium clips used for hemostasis (C).

Table 1 Findings in hematologic and coagulation laboratory tests

Variables	Normal range	Day 2	Day 4	Day 8
RBC ($10^{12}/L$)	3.5-5.5	1.7	2.25	2.72
Hemoglobin (g/L)	110-160	55	74	89
Prothrombin time (s)	11.0-14.0	> 180	16.8	13.8
Activated partial thromboplastin time (s)	35.0-55.0	94.3	30.5	26.9

Routine blood test showed 1.7×10^{12} erythrocytes per liter (normal 3.5-4.5 erythrocytes/liter) and 56 g hemoglobin per liter (Table 1). Coagulation function test revealed a prolonged prothrombin time (PT > 180 s) and an activated partial thromboplastin time (APTT = 94.3 s) (Table 1), both of which could be corrected when mixed with normal plasma at a 1:1 ratio. Other blood tests, such as platelet count, fibrinogen, renal function, and liver function, were normal (data not shown).

Superwarfarin poisoning was taken into consideration. The patient's serum and urine specimens were sent to Affiliated 307 Hospital of Academy of Military Sciences of China for toxicology analysis. Laboratory evaluation for some common superwarfarins was performed with the help of reverse-phase high performance enzyme-linked chromatography, showing that the level of brodifacoum and bromadiolone in serum was 1665 ng/mL and 132 ng/mL, respectively, and 216 ng/mL and 15 ng/mL in urine, respectively. The diagnosis of superwarfarin

poisoning was confirmed. The patient was also allowed to have intentionally ingested rodenticides to commit suicide 10 d before the routine EGD examination because of severe depression.

Emergency EGD showed submucosal congestion of the esophagus (Figure 2A), and five hemorrhagic sites in the stomach, which were still bleeding during the endoscopic procedure (Figure 2B). All the bleeding sites were closed with 5 titanium clips to achieve hemostasis (Figure 2C).

The patient was treated with concentrated red blood cells, fresh frozen plasma and intravenous vitamin K (30 mg/d) for 8 d until her coagulation parameters returned to normal, RBC count and hemoglobin concentration were greatly improved (Table 1). She began to have oral vitamin K1 (30 mg/d) from day 9 after admission. Toxicology analysis on day 7 showed a brodifacoum concentration of 986 ng/mL and a bromadiolone concentration of 36 ng/mL in serum. Brodifacoum (7 ng/mL) could only be detected in the urine specimen. On day 10, EGD showed that the submucosal congestion of esophagus was absorbed with no sign of bleeding in the stomach, and titanium clips in good condition (Figure 3). Plain radiography and CT scanning revealed 5 titanium clips in the stomach (Figure 4).

Two days after the last EGD examination, the patient was discharged from our hospital and oral vitamin K1 was prescribed (10 mg/d) for 1 mo. Weekly PT, prothrombin activity and international normalized ratio measurements

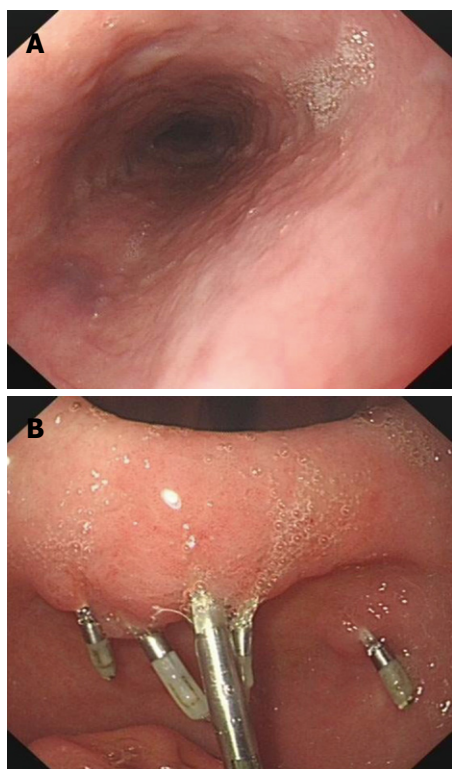


Figure 3 Final EGD showing the absorbed esophageal submucosal congestion (A) and bleeding in the stomach with titanium clips in good condition (B).

were also advised. During the 1-mo following-up after discharge, her coagulation parameters remained normal with no recurrent bleeding.

DISCUSSION

Superwarfarins, a class of rodenticides with brodifacoum and bromadiolone as their representative, are long acting anticoagulants^[1,2] and are 100 times as potent as warfarin. The half life of brodifacoum and bromadiolone can be as long as 24 d^[3] or 30 d^[1] and 31 d^[2], respectively.

Superwarfarins are supposed as the first-line rodenticides all over the world. However, superwarfarin poisoning cases are often reported, especially in rural areas. The number of superwarfarin poisoning cases has also increased in the USA^[4,5]. The causes of superwarfarin poisoning include accidental exposure, suicide intention and occupational exposure. In addition to oral intake, inhalation and skin absorption can also result in superwarfarin poisoning.

Gastrointestinal hemorrhage is a fatal complication of superwarfarin poisoning, requiring immediate treatment. Treatment modalities include use of antidotes, such as vitamin K1, often requiring a high dose (20-125 mg/d) and a prolonged time because of the long half-life of superwarfarins^[2].

Endoscopic treatment for hemostasis is somewhat

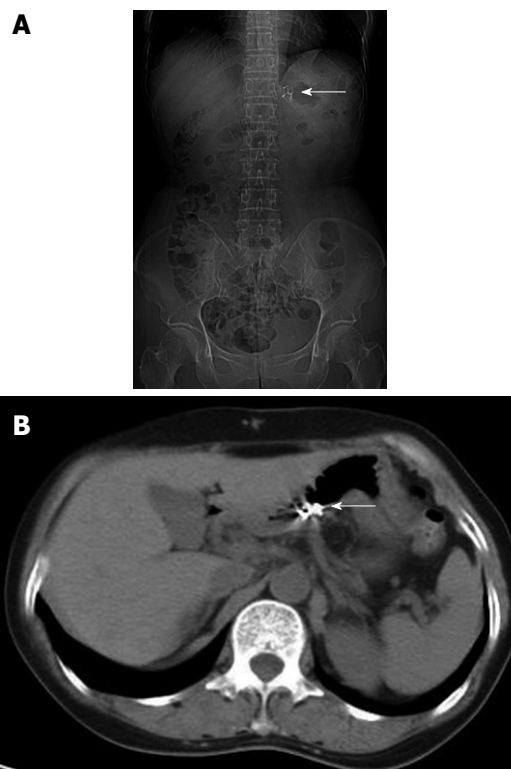


Figure 4 Plain radiography (A) and CT scanning (B) revealing 5 titanium clips in the stomach (arrows).

effective. Due to the extensive bleeding and hemodynamic instability of our patient, blood transfusion and fluid infusion should also be applied to correct the blood volume before emergency endoscopy. In this case, hemostasis was achieved by placing five titanium tips to clip the bleeding sites with the help of an EGD.

In conclusion, with the help of antidotes against superwarfarins and EGD, upper gastrointestinal hemorrhage caused by superwarfarin poisoning after endoscopic cold mucosal biopsy can be successfully and safely controlled.

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Conference

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The Caribbean

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March 05-07
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Meeting

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- 3 **Tian D**, Araki H, Stahl E, Bergelson J, Kreitman M. Signature of balancing selection in Arabidopsis. *Proc Natl Acad Sci USA* 2006; In press

Organization as author

- 4 **Diabetes Prevention Program Research Group**. Hypertension, insulin, and proinsulin in participants with impaired glucose tolerance. *Hypertension* 2002; **40**: 679-686 [PMID: 12411462 PMCID:2516377 DOI:10.1161/01.HYP.0000035706.28494.09]

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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]

No author given

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

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- 7 **Geraud G**, Spierings EL, Keywood C. Tolerability and safety of frovatriptan with short- and long-term use for treatment of migraine and in comparison with sumatriptan. *Headache* 2002; **42** Suppl 2: S93-99 [PMID: 12028325 DOI:10.1046/j.1526-4610.42.s2.7.x]

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- 9 Outreach: Bringing HIV-positive individuals into care. *HRS-A Careaction* 2002; 1-6 [PMID: 12154804]

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- 10 **Sherlock S**, Dooley J. Diseases of the liver and biliary system. 9th ed. Oxford: Blackwell Sci Pub, 1993: 258-296

Chapter in a book (list all authors)

- 11 **Lam SK**. Academic investigator's perspectives of medical treatment for peptic ulcer. In: Swabb EA, Azabo S. Ulcer disease: investigation and basis for therapy. New York: Marcel Dekker, 1991: 431-450

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- 12 **Breedlove GK**, Schorfheide AM. Adolescent pregnancy. 2nd ed. Wiczorek RR, editor. White Plains (NY): March of Dimes Education Services, 2001: 20-34

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- 13 **Harnden P**, Joffe JK, Jones WG, editors. Germ cell tumours V. Proceedings of the 5th Germ cell tumours Conference; 2001 Sep 13-15; Leeds, UK. New York: Springer, 2002: 30-56

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- 14 **Christensen S**, Oppacher F. An analysis of Koza's computational effort statistic for genetic programming. In: Foster JA, Lutton E, Miller J, Ryan C, Tettamanzi AG, editors. Genetic programming. EuroGP 2002: Proceedings of the 5th European Conference on Genetic Programming; 2002 Apr 3-5; Kinsdale, Ireland. Berlin: Springer, 2002: 182-191

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- 15 Morse SS. Factors in the emergence of infectious diseases. Emerg Infect Dis serial online, 1995-01-03, cited 1996-06-05; 1(1): 24 screens. Available from: URL: <http://www.cdc.gov/ncidod/EID/eid.htm>

Patent (list all authors)

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

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Write as mean \pm SD or mean \pm SE.

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Controlling postoperative ileus by vagal activation

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intervention to activate this neuroimmune pathway is enteral administration of lipid-rich nutrition. Perioperative administration of lipid-rich nutrition reduced manipulation-induced local inflammation of the intestine and accelerated recovery of bowel movement. The application of safe and easy to use antiinflammatory interventions, together with the current multimodal approach, could reduce postoperative ileus to an absolute minimum and shorten hospital stay.

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Key words: Postoperative ileus; Inflammation; Vagus; Nutritional antiinflammatory pathway

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Abstract

Postoperative ileus is a frequently occurring surgical complication, leading to increased morbidity and hospital stay. Abdominal surgical interventions are known to result in a protracted cessation of bowel movement. Activation of inhibitory neural pathways by nociceptive stimuli leads to an inhibition of propulsive activity, which resolves shortly after closure of the abdomen. The subsequent formation of an inflammatory infiltrate in the muscular layers of the intestine results in a more prolonged phase of ileus. Over the last decade, clinical strategies focusing on reduction of surgical stress and promoting postoperative recovery have improved the course of postoperative ileus. Additionally, recent experimental evidence implicated antiinflammatory interventions, such as vagal stimulation, as potential targets to treat postoperative ileus and reduce the period of intestinal hypomotility. Activation of nicotinic receptors on inflammatory cells by vagal input attenuates inflammation and promotes gastrointestinal motility in experimental models of ileus. A novel physiological

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INTRODUCTION

Postoperative ileus is a common pathological condition in the surgical ward and presents as an inability to tolerate enteral nutrition, nausea, abdominal distension, and lack of flatus and defecation. Although all surgical patients are at risk of developing postoperative ileus, the condition is mostly observed after abdominal surgery with manipulation of the gastrointestinal tract^[1]. Cessation of bowel movement and delayed gastric emptying, which can be up to 5 d after colorectal surgery, results in increased morbidity and a prolonged hospital stay^[2-4]. The duration of postoperative ileus has a major financial impact, adding an average of 6.300 US\$ to hospital costs

per patient who develops ileus^[5]. The additional health care costs in the US have been estimated to be 1.5 billion US\$ annually^[6]. Increased insight into the pathophysiology and discovery of novel treatment options could diminish the length of postoperative ileus, decrease patient morbidity, and reduce hospital costs.

PATHOPHYSIOLOGY OF POSTOPERATIVE ILEUS

The pathophysiology underlying postoperative ileus is complex and multifactorial, consisting of endogenous and pharmacological characteristics. Recent experimental studies have demonstrated that the pathogenesis of the endogenous component of postoperative ileus can be grossly divided in two distinct phases^[1]. The first phase, or neural phase, results from activation of mechanoreceptors and nociceptors by stimuli, such as incision of the skin and, more importantly, by direct manipulation of the intestine^[7]. Activation of these receptors initiates a neural reflex, which is dependent on release of mediators, such as α -calcitonin gene-related peptide and substance P, which inhibit gastrointestinal motility and result in generalized intestinal hypomotility^[8-10]. The neural phase of postoperative ileus lasts minutes to hours and resolves after closure of the wound when the noxious stimuli have ceased^[9,11,12]. The motility of the colon in particular depends heavily on input from the autonomic nervous system, which might explain colonic susceptibility to isolated and prolonged ileus^[13].

The second, more protracted, inflammatory phase is caused by formation of an inflammatory infiltrate in the muscular layers of the intestine^[7,14,15]. Manipulation of the intestine initiates an inflammatory cascade starting with activation and degranulation of mast cells^[16-18]. Subsequently, resident macrophages are activated either *via* mast cell-derived mediators or by luminal antigens^[17,19,20]. These activated macrophages produce cytokines and chemokines, which attract neutrophils to the muscular layer of the intestine. Invaded neutrophils directly impair intestinal smooth muscle cell contractility *via* release of nitric oxide and prostaglandins^[21,22]. The formation of an inflammatory infiltrate not only impairs motility in the manipulated areas, but also leads to generalized hypomotility of the gastrointestinal tract *via* activation of inhibitory adrenergic neural pathways. There is emerging evidence that inflammation also plays a vital role in postoperative ileus in humans, therefore a major focus of current research has been directed at the development of antiinflammatory treatments^[18,23,24]. In experimental models of intestinal manipulation, it was demonstrated that administration of antiinflammatory agents, such as mast cell stabilizers^[17], non-steroidal antiinflammatory drugs^[25,26], and interleukin (IL)-10^[27], prevent development of postoperative ileus. In addition, it was recently shown in patients undergoing major abdominal surgery that an intervention with the mast cell stabilizer, Ketotifen, reduced gastroparesis^[24].

CLINICAL STRATEGIES TO TREAT POSTOPERATIVE ILEUS

A number of strategies for preventing postoperative ileus are combined in the so-called fast-track program. The goals of fast-track surgery are reduction of perioperative surgical stress and promotion of postoperative recovery. Adequate pain relief, minimal invasive surgery and early enteral nutrition are important to achieve these goals^[28]. Adequate pain relief can attenuate postoperative ileus in two important ways. First, intraoperative spinal anesthesia and postoperative epidural analgesia with local anesthetics during abdominal surgery reduce the neural phase of ileus by interruption of neural transmission. Second, local anesthetic interventions minimize the use of opioid-derivatives^[29,30]. Both endogenous opioids, released in response to noxious stimuli, and exogenous opioids are notorious for their inhibitory effect on gastrointestinal motility, thereby aggravating postoperative ileus^[31]. Blocking the μ -opioid receptor with Alvimopan, a selective, peripherally active antagonist, has been demonstrated to accelerate recovery of bowel function and decrease hospital stay, without affecting the analgesic effects of opioids^[32,33]. In addition, non-steroidal anti-inflammatory drugs seem promising for their opioid-sparing and antiinflammatory effects^[26,34]. However, caution should be taken as the use of cyclo-oxygenase-2 inhibitors after colonic surgery has been associated with increased anastomotic leakage^[35].

Surgical trauma and direct manipulation of the intestine are major factors in the occurrence of postoperative ileus. The degree of gastrointestinal hypomotility correlates with the degree of manipulation and intestinal inflammation^[19]. The introduction of minimally invasive techniques, such as laparoscopy, significantly reduced the duration of postoperative ileus and length of hospital stay^[36]. This improvement is probably due to minimization of trauma, resulting in less pain and a diminished release of neurotransmitters and inflammatory mediators^[18,28,37].

Finally, enteral nutrition is found to be essential for enhanced recovery after surgery. Ingestion of nutrients elicits various reflexes and releases several neuropeptides that promote gastrointestinal motility^[38,39]. Traditionally however, a nil-by-mouth regime is often enforced starting from several hours before surgery until days postoperatively. Recent studies have demonstrated that early enteral nutrition is safe and well tolerated after abdominal surgery. In addition, early enteral nutrition reduces postoperative ileus and length of hospital stay^[40,41]. Unfortunately, studies investigating the effect of early enteral nutrition on postoperative ileus remain difficult to interpret, as the studies often lack essential information on the type of analgesia that was used^[2]. Enteral nutrition is a promising intervention to treat ileus; however, future well-designed studies are needed to evaluate the effect of early enteral nutrition on intestinal motility. When implementing early enteral nutrition routinely,

caution should be taken, as there is a small chance that enteral nutrition could lead to intestinal ischemia in the circulatory compromised patient^[42,43].

The implementation of fast-track regimes in the surgical field has improved the course of postoperative ileus. However, despite these efforts, it still remains an important clinical challenge. Inhibition of the inflammatory phase, by targeting the cellular and molecular changes underlying postoperative ileus is another focus of treatment.

EXPERIMENTAL STRATEGIES TO CONTROL POSTOPERATIVE ILEUS

The inflammatory phase dominates the course of postoperative ileus. Novel experimental interventions aimed at preventing the activation of inflammatory cells, such as administration carbon monoxide^[44,45], pretreatment with blocking antibodies to intracellular adhesion molecule-1 and lymphocyte function-associated antigen-1^[7,46], inactivating macrophages^[47], and preventing mast cell activation^[17], have displayed promising results in reducing gastrointestinal hypomotility. Borovikova *et al.*^[48] described a novel approach for modulating the inflammatory response; electrical stimulation of the vagus nerve attenuates systemic inflammation in a murine endotoxin model. Stimulation of the vagus nerve modulates inflammation *via* release of acetylcholine that binds to nicotinic receptors on inflammatory cells, hence the term “cholinergic anti-inflammatory pathway”^[49,50]. In addition, the vagus nerve has recently been identified as an important modulator of intestinal health; loss of vagal integrity aggravates intestinal inflammation and augments loss of gut barrier function^[51,52].

In a murine model of intestinal manipulation, electrical stimulation of the vagus nerve ameliorates postoperative gastrointestinal hypomotility *via* inhibition of local intestinal inflammation. Vagal stimulation activates the $\alpha 7$ nicotinic acetylcholine receptor on intestinal macrophages and attenuates release of pro-inflammatory cytokines *via* the Jak2-Stat3 signaling pathway^[53]. Furthermore, administration of the selective $\alpha 7$ receptor agonist, AR-R17779, prevented postoperative ileus in mice^[54]. Although very effective in preventing postoperative ileus in animal models, caution should be taken when implementing electric vagus stimulation and pharmacologic interventions in patients. Electrical stimulation remains an invasive procedure, while pharmacologic stimulation of nicotinic receptors might cause unwanted stimulation of different cell types and organs^[55,56].

A more physiological way to activate the vagal anti-inflammatory pathway is by administration of enteral nutrition enriched with lipids. Administration of lipid-rich nutrition prior to, or following, hemorrhagic shock attenuates systemic inflammation and preserves intestinal integrity^[57,58]. These positive effects of lipid-rich nutrition on gut barrier function and systemic inflammation are specific for the amount of lipids in the nutrition, as a low-lipid control feeding did not exert these protec-

tive effects. The enteral presence of lipids activates the autonomic nervous system *via* cholecystokinin (CCK) receptors. Subsequently, inflammation is inhibited through activation of nicotinic receptors on inflammatory cells *via* the efferent vagus^[59]. Enteral administration of lipid-rich nutrition was demonstrated to reduce postoperative ileus in a rodent model of intestinal manipulation^[16]. Enteral nutrition enriched with lipids prevented degranulation of mast cells, inhibited release of macrophage-derived tumor necrosis factor- α and IL-6, and prevented influx of neutrophils into the intestinal muscularis to a greater extent than the control, low-lipid, nutrition. More importantly, the beneficial effect of lipid-rich nutrition on manipulation-induced local inflammation promoted gastrointestinal transit in a CCK-receptor-dependent manner^[16]. These findings indicate that lipid-rich nutrition reduces postoperative ileus *via* activation of the nutritional antiinflammatory pathway. Luminal lipids are known to activate the autonomic nervous system *via* CCK-mediated stimulation of peripheral CCK-1 receptors on afferent vagal fibers, resulting in several regulatory digestive functions, such as satiety^[60]. Therefore, the antiinflammatory potential of lipid-rich enteral nutrition could rely on activation of a nutritional CCK-dependent vagovagal reflex.

Interestingly, sham feeding is another physiological technique that activates the cephalic vagal axis by mimicking food intake, thereby stimulating bowel motility^[38,39]. Furthermore, activation of the cephalic phase elicits digestive functions *via* vagovagal cholinergic reflexes^[61]. Sham feeding by chewing gum has been shown to improve bowel movement and reduce time to first flatus and first defecation after open gastrointestinal surgery, and demonstrates a trend towards a reduced hospital stay^[62,63]. However, the exact mode of action remains to be investigated.

CONCLUSION

Surgical interventions, and abdominal surgery in particular, are frequently accompanied by the occurrence of postoperative ileus. Postoperative ileus is a multifactorial surgical complication that requires a multifactorial treatment approach. Minimal invasive surgery to reduce surgical stress, epidural analgesia to block inhibitory reflexes, minimizing opioid use, and attenuation of intestinal inflammation by antiinflammatory interventions should reduce postoperative ileus to a minimum. The development of safe and easy-to-use treatments to prevent intestinal inflammation will play a key role in controlling postoperative ileus and deserves further investigation. Stimulation of the vagal antiinflammatory pathway, by interventions such as enteral administration of lipids, is one of the promising interventions contributing to a further reduction of postoperative ileus.

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Safe procedure in endoscopic submucosal dissection for colorectal tumors focused on preventing complications

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Abstract

Endoscopic submucosal dissection (ESD) is efficient for *en bloc* resection of large colorectal tumors. However, it has several technical difficulties, because the wall of the colon is thin and due to the winding nature of the colon. The main complications of ESD comprise postoperative perforation and hemorrhage, similar to endoscopic mucosal resection (EMR). In particular, the rate of perforation in ESD is higher than that in EMR. Perforation of the colon can cause fatal peritonitis. Endoscopic clipping is reported to be an efficient therapy for perforation. Most cases with perforation are treated conservatively without urgent surgical intervention. However, the rate of postoperative hemorrhage in ESD is similar to that in EMR. Endoscopic therapy including endoscopic clipping is performed and most of the cases are treated conservatively without blood transfusion. In blood examination, some degree of inflammation is detected after ESD. For the standardization of ESD, it is most important to decrease the rate of perforation. Adopting a safe strategy for ESD and a suitable choice of knife are both important ways

of preventing perforation. Moreover, appropriate training and increasing experience can improve the endoscopic technique and can decrease the rate of perforation. In this review, we describe safe procedures in ESD to prevent complications, the complications of ESD and their management.

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Key words: Endoscopic submucosal dissection; Colorectal tumor; Perforation; Complication; Safe procedure

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INTRODUCTION

Endoscopic mucosal resection (EMR) has been generally performed for colorectal tumors worldwide, including Japan. It is difficult to perform *en bloc* resection by EMR for a colorectal tumor whose size is larger than 20 mm^[1-3]. The rate of *en bloc* resection by EMR for tumors with a diameter of more than 20 mm was reported to be approximately 30%^[2,3]. Piecemeal EMR enables us to remove large colorectal tumors. However, colorectal cancer has a high rate of local recurrence^[2-5]. Moreover, precise histopathological diagnosis is difficult when using separate resected specimens of piecemeal EMR^[6]. Thus, laparoscopic-assisted colectomy (LAC) has been regarded as a standard therapy for large colorectal tumors throughout the world^[7]. However, LAC is more invasive than endoscopic treatment. Endoscopic submucosal

dissection (ESD) has a high rate of *en bloc* resection for large colorectal tumors, and it is less invasive than LAC. The rate of *en bloc* resection for large colorectal tumors has been reported to be 84.0%-98.9%^[8-17]. However, the procedure has not been standardized because of its associated technical difficulties. The colon is winding in nature, and the colonic wall is thinner than the gastric wall. Moreover, there are many folds in the colorectum. Therefore, the rate of perforation in ESD is reported to be higher than that in EMR. A safe strategy, suitable knife, and adoption of other equipment are necessary while performing ESD in order to prevent its associated complications, including perforation.

In Japan, a special working group consisting of experts in ESD suggested specific indications for ESD^[6]. Briefly, ESD is suitable for a tumor when it is difficult to use a snare EMR for *en bloc* resection. ESD should be performed for tumors that are diagnosed as carcinomas with intramucosal to shallow submucosal invasion. Moreover, ESD is performed for lesions with submucosal fibrosis that cannot be removed by conventional EMR even if the size of the lesion is less than 20 mm. However, tumors in a location where the endoscope will not be able to be operated smoothly should not be removed by ESD.

METHOD OF SAFE ESD

Preparation and equipment

ESD is performed using a general lower gastrointestinal endoscope with a single channel. In our institution, EC 590 MP (Fuji Film Medical, Tokyo, Japan) or PCF Q260AI (Olympus Medical Systems Co., Tokyo, Japan) are used. ESD requires a high-frequency generator with an automatically controlled system. In our institution, VIO300D or ICC200 (Erbe Elektromedizin Ltd., Tübingen, Germany) are used. An upper gastrointestinal endoscope is adopted in some institutions because it is slim and can be used in the retroflexed position^[10]. A transparent short hood (Olympus Medical Systems Co., Tokyo, Japan) is fitted at the tip of the endoscope. A mixture of 1% hyaluronic acid solution (Mucoup; Johnson & Johnson K.K., Tokyo, Japan) and 10% glycerin solution (Glyceol; Chugai Pharmaceutical Co., Tokyo, Japan) is used as the injection liquid to induce a higher elevation of the submucosa and to lengthen the duration of the continuous elevation of the submucosa^[18,19].

Before ESD, residual feces and liquid are removed from the entire colon even if the tumor is located at the rectum. The ESD procedure should be abandoned if the residual feces can not be removed enough. Residual feces prevent smooth submucosal dissection. Moreover, it is essential to remove residual feces in order to prevent the outflow of feces into the abdomen in the case of perforation.

Various knives are used in ESD for colorectal tumors (Figure 1A-F). Among the obtuse short-tipped types are the Flush knife (Fujifilm Medical, Tokyo, Japan), Dual knife (Olympus Medical Systems Co., Tokyo, Japan), B-knife (Zeon Medical, Tokyo, Japan), and Splash needle

(Pentax Co., Tokyo, Japan)^[9,15,20]. The Flush knife and Splash needle are capable of injecting substances into the submucosa. They enable us to omit switching between the knife and the injection needle^[13,15]. A Dual knife has a ball disk at the tip of the knife, enabling us to hook the submucosa. The B-knife and Flush knife both have a new type of ball tip. The insulated tipped (IT) knife (Olympus Medical Systems Co., Tokyo, Japan), whose efficacy has been reported to be satisfactory in ESD for gastric tumors, is used in certain institutions^[21]. Speedy dissection can be performed with the IT knife, but it may cause large perforations due to its long blade. A Hook knife (Olympus Medical Systems Co., Tokyo, Japan) is used particularly when the dissection of the submucosa is difficult due to poor elevation of the submucosa^[12]. The B-knife is the only bipolar knife; burning of the muscularis propria layer is considered to be less with this knife than with other monopolar knives. A grasping-type scissor forceps has been reported as an original knife^[22]. In our institution, the Flush knife is mainly used because it can be effectively used to administer local injections, while the Hook knife is used when the risk of perforation is high due to the poor elevation of the submucosa^[12,23].

Mucosal incision (initial complete circumferential incision and partial circumferential incision)

The border of the tumor is observed carefully by applying indigo carmine dye. It is generally unnecessary to make placement of borders by coagulation because in the majority of cases, the borders of the tumor are clearly visible. Injection into the submucosa for its elevation is performed with a 23-25 gauge needle (TOP Co., Tokyo, Japan) after observation of the border of the tumor. Then, a mucosal incision is taken. An initial complete circumferential incision or a partial circumferential incision is made according to the institution's procedure and the lesion's characteristics, as reported previously (Figure 2A and B)^[8,12]. In initial complete circumferential incision, injection of hyaluronic acid solution into the submucosa is performed from the oral edge of the tumor. A mucosal incision is made after adequate elevation of the submucosa is obtained. Simultaneously, an incision up to the deep submucosa is made. Then the solution is injected into the anal edge of the tumor and the mucosal incision is made. Thus, a mucosal incision is made all around the tumor. On the other hand, in partial circumferential incision, the anal side of the tumor is the first to be incised after the injection of hyaluronic acid solution for submucosal elevation. Both types of mucosal incisions are performed with the endocut mode (e.g. Output 40W, effect 2 in ICC200; or endocut I, effect 2, duration 2, interval 1 in VIO300D). However, each incision has its own merits and demerits.

In initial complete circumferential incision, leakage of injection fluid can easily occur following which submucosal elevation cannot be obtained. Moreover, injection of the fluid into the oral side of the tumor causes the position of the tumor to be perpendicular to the endoscope (Figure 3A and B). This makes submucosal dissection difficult. In addition, the uncut residual mucosa on the oral

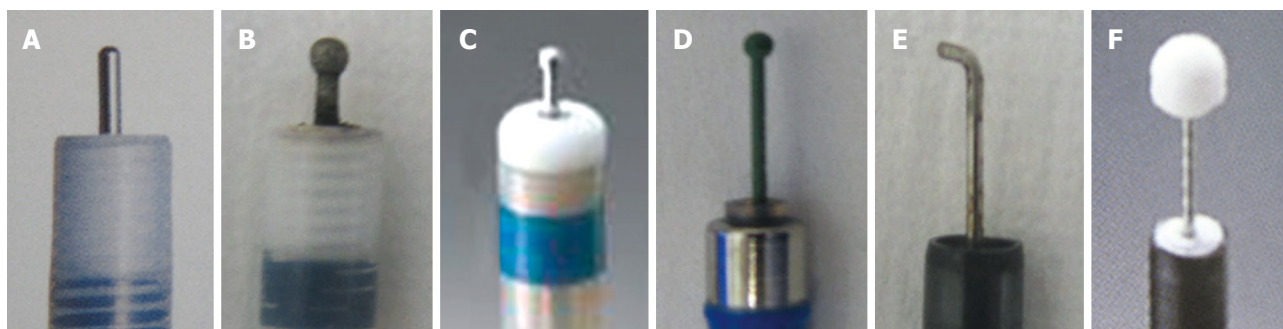


Figure 1 Various knives used in endoscopic submucosal dissection (ESD) for colorectal tumors. A: Flush knife; B: Flush knife with ball tip; C: Dual knife; D: B-knife with ball tip; E: Hook knife; F: Insulated tipped (IT) knife.

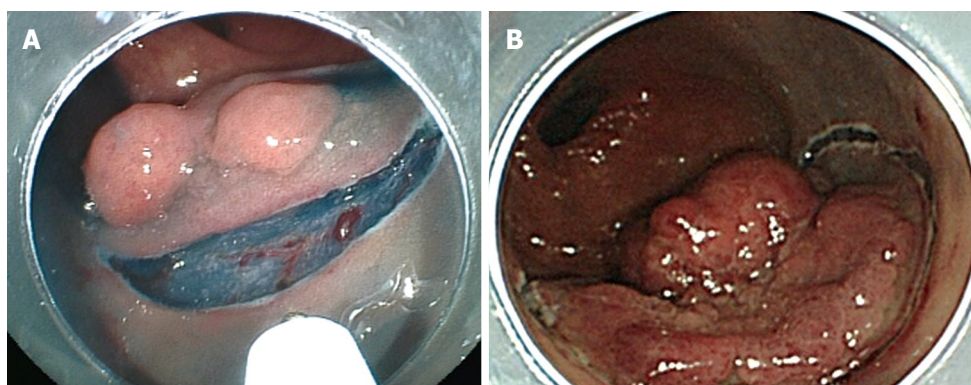


Figure 2 Mucosal incision. A: Partial circumferential incision; B: Initial complete circumferential incision.

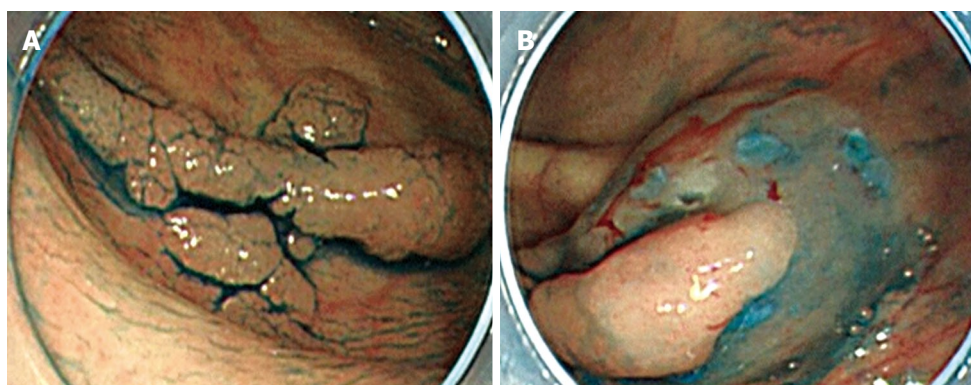


Figure 3 A case of the tumor perpendicular to the endoscope. A: Colonic tumor, 0-IIa 20 mm in the ascending colon; B: The position of the tumor became vertical with respect to the endoscope following injection into the proximal side.

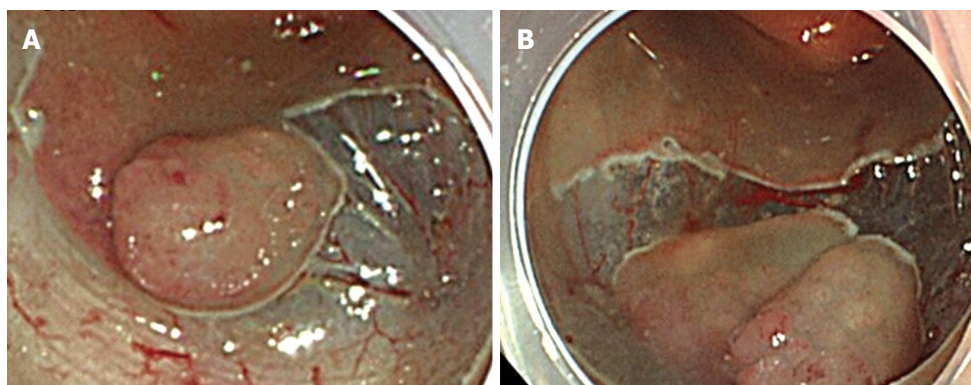


Figure 4 Another case of the tumor perpendicular to the endoscope. A: Residual mucosa on the oral side pulled the tumor upward; B: The substance caused by the residual mucosa was lost during full mucosal resection, and the position of the tumor became vertical with respect to the endoscope.

side pulls the tumor upward (Figure 4A); however, this substance caused by the residual mucosa is lost in initial complete circumferential incision, and the tumor becomes perpendicular to the endoscope (Figure 4B). These fac-

tors are experienced frequently in tumors whose size is less than 50 mm. In partial circumferential incision, higher elevation of the submucosa can be maintained because the uncut residual mucosa on the oral side of the tumor

prevents the leakage of injected fluid. However, in partial circumferential mucosal incision, it is sometimes difficult to resect the residual mucosa on the oral side owing to the presence of the partially resected tumor. Thus, each type of mucosal incision has its own merits and demerits; the type of incision to be used should be decided according to the tumor size, location of the tumor, and types of knives being used. In our institution, partial circumferential incision is performed for tumors measuring less than 50 mm or for which fluid injection into the tumor's oral side would likely negatively influence the position of the tumor.

Dissection of the submucosa below the tumor

After mucosal and submucosal incisions are made around the tumor, the submucosa below the tumor is dissected from the anal side of the tumor. Dissection of the submucosa is performed using the Endocut (e.g. Output 40W, effect 2 in ICC200; endocut I, effect 2, duration 2, interval 1 in VIO300D) or the coagulation mode (e.g. Forced coagulation, Output 40W in ICC200 or Forced coagulation, Output 40W, effect 3 in VIO300D). To achieve submucosal elevation, the glycerin solution or the mixture of hyaluronic acid solution and glycerin solution is injected with the injection needle or knife with the function of injection, as appropriate. Then continuing to dissect with prevention of perforation and hemorrhage, *en bloc* resection of the tumor is performed.

COMPLICATIONS

Perforation

Perforation following ESD for colorectal tumors can be fatal because peritonitis caused by colorectal bacteria and feces is known to be more severe than peritonitis occurring after gastric perforation.

The rate of perforation has been reported to be 1.4%-10.4% (Table 1). In our experience with perforations, there were no statistical differences regarding the location of the tumor, i.e. in the colon or in the rectum^[23]. Another report has revealed that perforation is associated with large tumor size (> 30 mm) and the presence of fibrosis^[17]. The rate of perforation of ESD is dramatically high when compared with that observed for EMR^[1-3]. One of the reasons for the high rate of perforations is the thinness of the colorectal wall as compared to the gastric wall. Knife coagulation is the most common cause of perforation^[23]. The paradoxical movement of the endoscope during ESD due to the winding nature of the colorectum causes coagulation in the muscularis propria. A longer operation time increases the amount of air in the abdomen, causing greater paradoxical movement of the endoscope. This situation is experienced specifically in tumors located in the cecum up to the descending colon. Obtuse short-tipped knives such as the Dual knife and the Flush knife can easily cause this type of perforation. In contrast, it is difficult to cause perforations while using the Hook knife because it enables us to hook and separate the submucosa from the muscularis propria and thereby cut safely. Rare

Table 1 The rate of perforation and postoperative hemorrhage in ESD for colorectal tumors

Author	Country	n	Perforation rate (%)	Postoperative hemorrhage rate (%)
Fujishiro <i>et al</i> ^[10]	Japan	200	10.4	1.0
Hurlstone <i>et al</i> ^[16]	UK	42	2.3	2.3
Tanaka <i>et al</i> ^[8]	Japan	70	10.0	1.4
Tamegai <i>et al</i> ^[11]	Japan	71	1.4	0.0
Toyonaga <i>et al</i> ^[15]	Japan	468	1.5	1.5
Yoshida <i>et al</i> ^[12]	Japan	119	7.5	1.6
Zhou <i>et al</i> ^[14]	China	74	8.1	1.3
Takeuchi <i>et al</i> ^[13]	Japan	50	2.0	12.0 ¹
Isomoto <i>et al</i> ^[17]	Japan	292	8.2	0.7

¹This study included mild hemorrhage cases which did not need endoscopic treatment. ESD: Endoscopic submucosal dissection.

reasons for perforation include resection by using a snare, coagulation by special hemostat forceps with soft coagulation, and endoscopic clipping onto coagulated submucosa^[23]. Further, endoscopic clipping is also performed when perforation is detected. Multiple endoscopic clipping is performed to close the perforation depending upon its size. Small perforations can be closed by endoscopic clipping^[24,25]. If abdominal distention due to air leakage is severe, decompression of the pneumoperitoneum must be performed using a 20-gauge puncture needle^[14]. The majority of cases with perforation are treated conservatively without emergency surgery. Recently, it has been shown that large perforations can be closed using a new closure device consisting of a clip with loop^[26]. On the other hand, there are cases in which perforation is not detected by endoscopy, but free air is detected by computed tomography (CT). The possible reasons for this are that small perforations cannot be detected during ESD or that very small perforations may occur during deep injection by the injection needle. However, these cases are generally not clinically serious because they can be successfully treated by withholding oral intake; no abdominal pain is typically detected after ESD.

Severe abdominal tympanic fullness, emphysema, and severe abdominal pain are possible symptoms of perforation. A high index of suspicion should be maintained when these symptoms are observed; moreover, the patient, nurse and physician should all watch out for these symptoms.

On the other hand, delayed perforation has been reported as a serious complication after ESD^[10]. The rate of delayed perforation is reported to be 0.3%-0.7%^[10,17,27]. The reasons for delayed perforation are unknown, but it is reported to be related to excessive coagulation in the muscularis propria. It has been reported that delayed perforations are typically large in size and require treatment by emergency surgery^[10,17,27].

The rate of perforation is reported to be decreased with the increased experience of the endoscopist^[8,9]. It is important to actively prevent perforations when the endoscopist does not have much experience with ESD. The indication of ESD according to endoscopist's skill, appro-

priate strategy of ESD and the choice of a suitable knife in each case is important in preventing perforations^[8].

Hemorrhage during ESD and postoperative hemorrhage

To prevent hemorrhage during ESD, when a vessel less than 2 mm in diameter is detected in the submucosa it is cut with a knife in the coagulation mode (e.g. Forced coagulation, Output 40W in ICC or Forced coagulation Output 40W, effect 3 in VIO300D). When a vessel more than 2 mm in diameter is detected, special hemostat forceps (e.g. Coagrasper; FD-410LR, Olympus Optical Co, Tokyo, Japan) are used in the soft coagulation mode (e.g. Output 50W in ICC; Output 60W, effect 5 in VIO300D) to prevent hemorrhage during ESD^[12]. These forceps can be rotated and they are used to gently catch and lift the vessels upward from the muscularis propria. In our institution, a unique use of the hemostat forceps has been adopted for resecting vessels. In brief, a vessel is coagulated using hemostatic forceps in the soft coagulation mode and then resected with the forceps in the endocut mode. Moreover, the coagulated submucosa surrounding the vessel is also resected with the forceps. Removing the coagulated vessel and the surrounding submucosa ensure that the subsequent submucosal dissection is safer and easier than otherwise (Figure 5A-D). When massive bleeding that cannot be stopped by the knife occurs during ESD, special hemostat forceps are used in the soft coagulation mode as described above. Endoscopic clipping is performed when bleeding cannot be controlled with the special forceps.

The rate of postoperative hemorrhage in ESD is reported to be 0%-12.0% (Table 1)^[8-17]. This rate is comparable to that reported for EMR^[1-3]. A study has reported the rate of postoperative hemorrhage to be 12.0%, including mild cases^[13]. Most cases of postoperative hemorrhage are treated only by endoscopic clipping and withholding oral intake without emergency surgery or blood transfusion.

Restlessness

Severe restlessness of the patient owing to abdominal fullness and pain have rendered submucosal dissection impossible in some cases. Conscious sedation is effective for some patients for the prevention of restlessness. Carbon dioxide insufflations have been reported to be effective for the prevention of abdominal fullness^[28]. In our institution, conscious sedation is performed with midazolam (Dormicum; Astellas Pharma Inc., Tokyo, Japan) and pentazocine (Pentajin; Daiichi Sankyo Co., Tokyo, Japan) with monitoring using an automatic blood pressure monitor. In our ESD study that included 105 cases, there were 22 patients for whom the operation time exceeded 2.5 h, and patient restlessness occurred in 15 out of these 22 cases (68.1%) despite conscious sedation. In contrast, in cases with an operation time less than 2.5 h, patient restlessness occurred in only 10 out of 83 cases (12.0%). Thus, restlessness due to abdominal fullness and pain occurs frequently in cases with an operation time exceeding 2.5 h. Therefore, according to our experience, ESD is indicated when the operation time is expected to be less than 2.5 h. On the other hand,

the use of propofol for conscious sedation extends the possibility of longer operation times than 2.5 h without causing restlessness and discomfort^[14]. However, this drug requires further examination before its standardized use in ESD procedures.

Other complications

Inflammation has been reported to a certain degree in some cases. In our previous report, the mean amount of C-reactive protein 2 d after ESD was 5.82 ± 12.10 mg/L in cases with perforation and 1.27 ± 2.00 mg/L in cases without perforation^[29]. Fever and abdominal pain were also reported without perforation. A rare complication was acute colon obstruction after ESD of a colonic tumor located at the cecal base^[30].

CASE PRESENTATION

We present here the case of a 71-year-old male with a tumor graded 0-II a, measuring 20 mm, and located in the ascending colon (Figure 6A). The surface of the tumor was slightly depressed and the deformity of the colonic wall was detected with indigo carmine dye. Magnifying endoscopy revealed a V₁ pit pattern^[31]. The tumor was diagnosed as early colonic cancer with invasion up to the mucosa, and ESD was performed. Injection was first performed from the anal side of the tumor. However, the elevation of the tumor by injection was poor on the oral side of the tumor (Figure 6B). Therefore, either severe fibrosis or submucosal invasion was suspected. A mucosal incision was made and submucosal dissection was performed below the tumor using a Flush knife from the anal side of the tumor (Figure 6C). After that, a mucosal incision was made on the oral side of the tumor. Severe fibrosis was detected at the oral side of the tumor (Figure 6D). Then submucosal dissection was then performed with a Hook knife. However, owing to a thin submucosa, perforation was caused while hooking the submucosa (Figure 6E). Endoscopic clipping was performed minimally as the clipping did not prevent resection of the tumor, and the tumor was immediately resected using a snare. Then several endoscopic clippings were performed (Figure 6G). A small amount of free air was detected by abdominal CT after ESD. The patient had neither abdominal pain nor severe inflammation following surgery and was discharged 5 d after ESD. The resected specimen was fixed and the tumor was diagnosed by histopathological examination as early colonic cancer. The macroscopic tumor type was 0-II a and the tumor was 20 mm in diameter. Invasion of the tumor was limited to the mucosa. Lymphatic and venous invasion was not detected. Lateral and vertical margins of the tumor were histopathologically free of the tumor (Figure 6F).

THE STANDARDIZATION OF ESD FOR COLORECTAL TUMORS

ESD is a feasible endoscopic treatment because of its

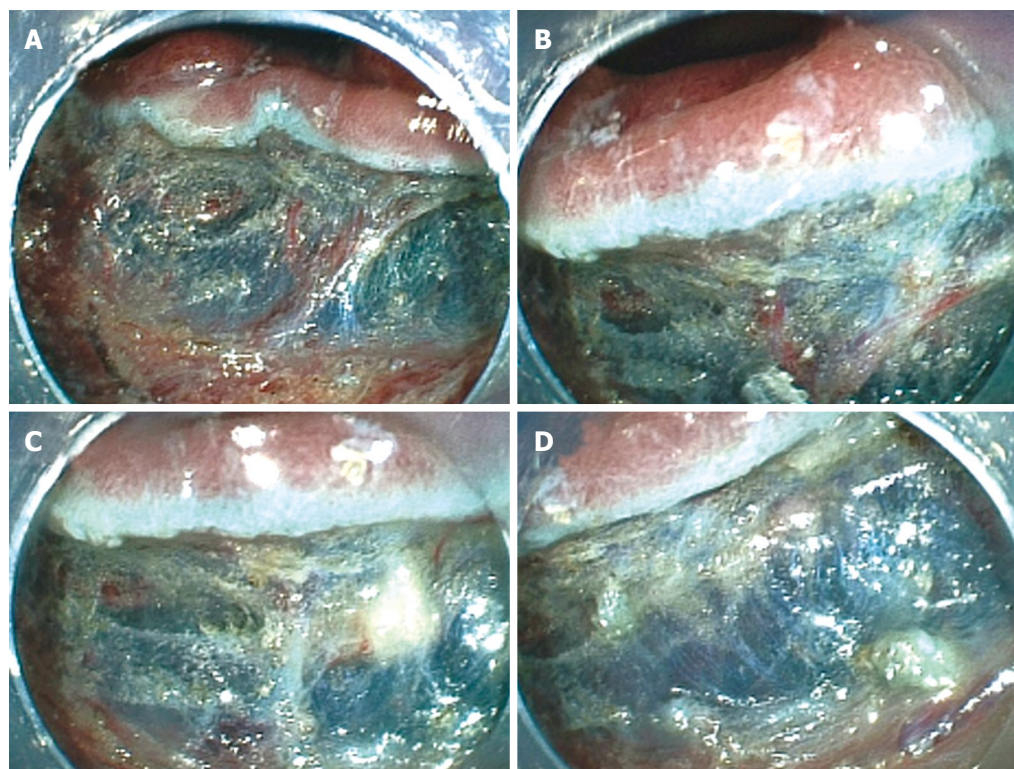


Figure 5 A unique use of hemostat forceps. A: Thick vessels were detected in the submucosa; B: The vessels were grasped by the hemostat forceps; C: The vessels and surrounding submucosa were coagulated and became whitish. The view of the submucosa was obscured; D: The coagulated vessels and submucosa were resected using the hemostat forceps, following which the view was restored.

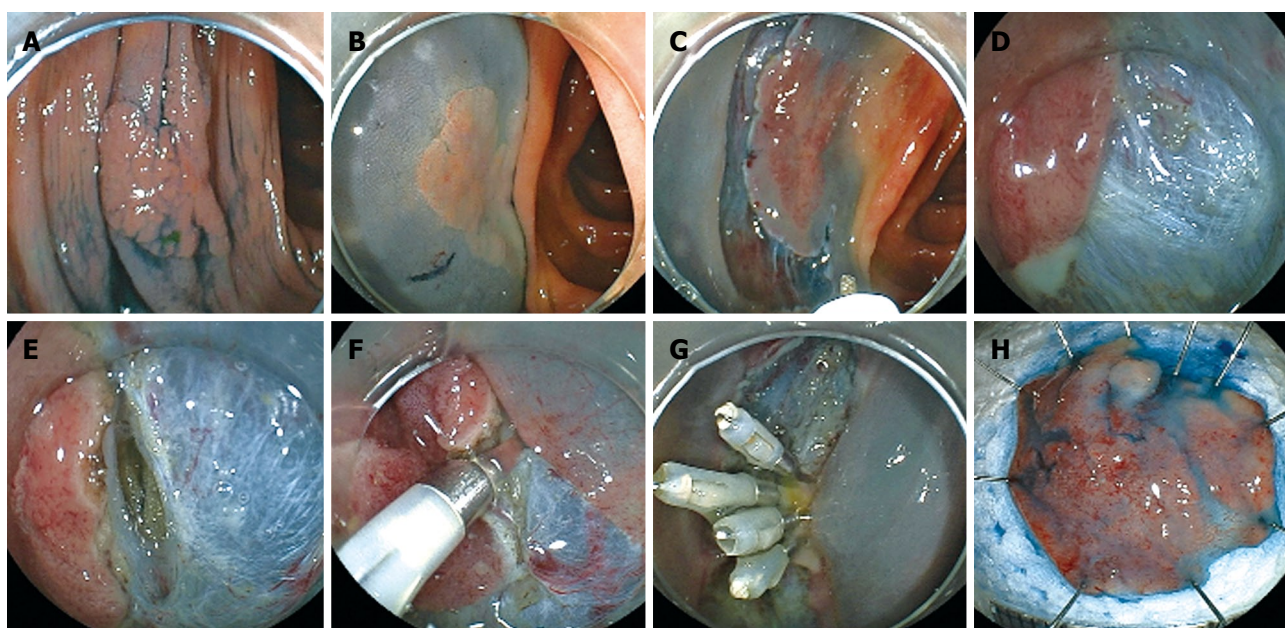


Figure 6 A case with perforation. A: The tumor in this patient was graded 0-II a, measured 20 mm, and was located in the ascending colon. The surface of the tumor was slightly depressed and the deformity of the colonic wall was detected with indigo carmine dye; B: Injection was performed on the anal side of the tumor. However, the elevation of the tumor following the injection was poor on the oral side of the tumor; C: Mucosal incision and submucosal dissection below the tumor were performed with the Flush knife from the anal side of the tumor; D: Mucosal incision on the oral side of the tumor was performed. Severe fibrosis was detected on the oral side of the tumor; E: Submucosal dissection was then performed with the Hook knife. However, owing to the thin submucosa, perforation occurred during hooking; F: Endoscopic clipping was performed minimally as clipping did not prevent the resection of the tumor; G: Several endoscopic clippings were performed after resection of the tumor; H: The resected specimen was fixed and the tumor was diagnosed by histopathological examination as early colonic cancer. The tumor was 20 mm in diameter. Tumor invasion was limited to the mucosa. Lateral and vertical margins of the tumor were histopathologically free of the tumor.

high rate of *en bloc* resection for large colorectal tumors. Hospitalization after ESD is less than that after LAC^[8,9,12]. However, ESD has disadvantages, with longer operation times and the possibility of perforation^[32]. In-

creased experience in the procedure can, however, solve these problems^[8,9].

Visits to other institutions with ESD experts and observation of such experts at work is an important compo-

ment of training in performing ESD procedures. Adequate practice at performing ESD may be obtained by using animal models. Systematic training systems for use of ESD in colorectal tumors are essential for the prevention of perforation and long procedure times. Acquiring experience in gastric ESD prior to attempting colorectal ESD is reported to be a safer way to prevent perforation^[8]. In our institution, EMR with circumferential mucosal incision has been used as training for ESD^[15]. Moreover, better devices that are particularly suitable for ESD, such as knives, endoscopes, and other new equipment, need to be designed for shortening the operation times and to prevent perforation^[33-36]. The indication of ESD should be decided according to the technique of the endoscopists in each institution. ESD for colorectal tumors is now improving, and a standardized method is expected to be developed in the near future. However, it is extremely important to diagnose the colorectal tumor correctly with suitable modalities, such as magnifying endoscopy; further, based on the diagnosis, the most appropriate methods of therapy should be considered, such as ESD, piecemeal EMR, and LAC.

CONCLUSION

In this review, we have assessed technical aspects and complications of ESD for colorectal tumors. For the standardization of ESD, it is most important to decrease the rate of perforation. Adopting a safe strategy of ESD and a suitable choice of knife are both efficient in the prevention of perforation. Moreover, appropriate training and increasing experience can improve the endoscopic technique and decrease the rate of perforation. We hope that standardization of ESD will be established in the near future, and that ESD will be performed in US and Europe where ESD is not widely adopted.

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Recent trends in the treatment of well-differentiated endocrine carcinoma of the small bowel

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Abstract

Well-differentiated endocrine carcinomas of the small bowel are fairly rare neoplasms that present many clinical challenges. They secrete peptides and neuroamines that may cause carcinoid syndrome. However, many are clinically silent until late presentation with major effects. Initial treatment aims to control carcinoid syndrome with somatostatin analogs. Even if there is metastatic spread, surgical resection of the primitive tumor should be discussed in cases of retractile mesenteritis, small bowel ischemia or subocclusive syndrome in order to avoid any acute complication, in particular at the beginning of somatostatin analog treatment. The choice of treatment depends on the symptoms, general health of the patient, tumor burden, degree of uptake of radionuclide, histological features of the tumor, and tumor growth. Management strategies include surgery for cure (which is rarely achieved) or for cytoreduction, radiological interventions (transarterial embolization or radiofrequency ablation), and chemotherapy (interferon and somatostatin analogs). New biological agent and radionuclide targeted therapies are under investigation. Diffuse and non-evolving lesions should also be simply

monitored. Finally, it has to be emphasized that it is of the utmost importance to enroll these patients with a rare disease in prospective clinical trials assessing new therapeutic strategies.

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Key words: Gastrointestinal neoplasms; Neuroendocrine carcinoma; Carcinoid tumor; Somatostatin analogs; Therapeutic chemoembolization; Surgery

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INTRODUCTION

Neuroendocrines tumors (NETs) of the small bowel account for 23%-28% of all gastroenteropancreatic (GEP) NETs and about 2% of all gastrointestinal (GI) tumors. Incidence rates of 0.28 to 0.8 per 100 000 population per year have been reported^[1,2]. Their prevalence has increased in the last 3 decades as awareness and diagnostic techniques have improved^[3]. A recent study showed a significant increase in their reported annual age-adjusted incidence from 1973 (1.09/100 000) to 2004 (5.25/100 000) in the US population^[4]. Most of these tumors are well-differentiated and have an indolent course. As a consequence of the long delay between onset of symptoms and final diagnosis, many patients have advanced disease at the time

of diagnosis. Jejunio-ileal (JI) NETs originate from the diffuse endocrine system (enterochromaffin cells), located in the GI tract that may produce serotonin. Clinical and biological features are heterogeneous^[2]. This explains that classification is a critical point and is evolving. The recent World Health Organization (WHO) classification is based on clinical pathological criteria^[5]. The WHO classification subdivides JI NETs, similar to other GEP endocrine tumors, into 3 general categories^[5]: (1) well-differentiated endocrine tumor (carcinoid) of benign behavior (confined to the mucosasubmucosa, non-angioinvasive, < 1 cm in size) or uncertain behavior (non-functioning, confined to mucosasubmucosa, > 1 cm in size, or angioinvasive); (2) well-differentiated endocrine carcinoma (malignant carcinoma) with low-grade malignant behavior, deeply invasive (muscularis propria or beyond) or with metastases; and (3) poorly-differentiated endocrine carcinoma (small-cell carcinoma), high-grade malignant. However, because of its prognostic limits, a need for development of a TNM classification has been identified and such work is underway^[6]. A proposal for a TNM classification for tumors of the lower jejunum and ileum, where T1-4 describes the size of the tumor (with T2 tumors > 1 cm) and the depth of involvement of the intestinal wall, was discussed. Stage I comprises T1 tumors with limited growth. Stage II identifies tumors that are larger in size or more invasive, i.e. T2 or T3, but without metastases. Stage III encompasses tumors invading surrounding structures (IIIA), T4, or the presence of regional lymph node metastases (IIIB), whereas stage IV indicates distant metastases. In the proposal, a grading system is also included, in which the G1 tumor category has a mitotic count < 2 or Ki-67 < 2%, G2 tumors a mitotic count 2-20 or Ki-67 2%-20%, and G3 tumors mitotic count > 20 or Ki-67 > 20%. Clinical features of JI NETs are heterogeneous: they can either remain asymptomatic for years, or occur with obstructive symptoms, or with liver metastases diagnosed by abdominal computed tomography (CT) or magnetic resonance imaging (MRI) for other purposes, or as incidental terminal ileum tumors by colonoscopy, or less frequently occur with symptoms related to hormonal hypersecretion ("functioning tumors"). The term "carcinoid" should be reserved for a characteristic clinical syndrome that results from the intermittent release of serotonin and other substances, such as tachykinins, prostaglandins and bradykinins, into the systemic circulation that occurs in about 10%-20% of patient with JI NETs^[2]. Signs and symptoms of the carcinoid syndrome can include one or any of the following: flushing, diarrhea, carcinoid heart disease (CHD), and intermittent bronchoconstriction. These tumors are commonly metastases to the liver. Tumor size is an unreliable predictor of metastatic potential, and metastases can occur with primary tumors that are smaller than 1 cm in diameter^[7].

Survival of JI NETs correlates closely with the stage of the disease at presentation with a 5-year survival of 65% in patients with localized or regional disease and 36% in those with distant metastases^[8,9]. Pape *et al*^[10] reported

on 75 JI NETs, with more than 90% metastatic and 97% low-grade malignant; the 5-year survival rate was 90%. In addition, recent studies demonstrated the prognosis relevance of the WHO classification and the newly proposed TNM classification system for NETs^[10-13].

Treatment should be highly individualized based on the diverse range of symptoms, general health of the patient, tumor burden, degree of uptake of radionuclide, histological features, and tumor growth. Critical assessment of every treatment option is difficult because of the complexity, heterogeneity, and rarity of GEP NETs. There is a paucity of relevant randomized trials. However, some different groups (Nordic NE Tumour Group^[14,15], European Neuroendocrine Tumour Society^[9,16], National Comprehensive Cancer Network guidelines in the United States, Canadian National Carcinoid Expert Group^[17], United Kingdom NETwork^[18]) have recently published guidelines for the management of NETs. The treatment of carcinoid syndrome by somatostatin analogs is the first priority. Then the minimally required imaging studies and biochemical tests to decide any treatment include chromogranin A and urinary 5-hydroxyindoleacetic acid (5-HIAA)^[9]. Imaging studies are essential to localize the primary tumor as well as to guide management by staging, to monitor tumor growth, and for follow-up evaluation after therapy. The standard imaging procedures for JI NETs include abdominal ultrasonography, contrast-enhanced helical CT or MRI of the abdomen, pelvis and thorax, somatostatin receptor scintigraphy (SSRS), endoscopy, echocardiography, and bone scan or spine MRI to prove bone metastases if SSRS is negative^[9]. Capsule endoscopy and double balloon enteroscopy can be useful to detect the intestinal primary tumors^[9].

Management strategies include surgery for cure (which is rarely achieved) or cytoreduction, radiological interventions (by chemoembolization or radiofrequency ablation), chemotherapy and new biological agents, interferon, somatostatin analogs and peptide-receptor radionuclide therapy. Tumor growth is evaluated from clinical symptoms, repeated biological tests and imaging studies (every 3 to 6 mo), allowing decision-making between a curative treatment including aggressive treatment of metastatic disease, a palliative or symptomatic treatment, or a simple follow-up.

DIAGNOSIS AND TREATMENT OF CARCINOID SYNDROME

Presence of carcinoid syndrome, occurring in about 10%-20% of patients with JI NETs^[2], must be treated as a priority. Urinary 5-HIAA has a sensitivity of 73% and a specificity of 100% in predicting the presence of a carcinoid syndrome in the midgut area^[19]. 5-HIAA should be collected with strict dietary restrictions to avoid false positive levels. Somatostatin analogs remain the mainstay of symptomatic treatment for JI NETs. They could be started immediately in patients with inoperable disease or preoperatively in patients who have operable

disease (liver resection with or without resection of the primary tumor). They are administered subcutaneously every 6-12 h. Long-acting formulations require infrequent administration and have contributed to an improved quality of life for patients. To date, the most effective formulations include long-acting octreotide (10, 20 or 30 mg) and lanreotide autogel (60, 90 or 120 mg), which are widely accepted as effective in controlling tumor-related symptoms in about 50%-80% of patients and in reducing serum concentration of tumor markers by 40%-60%^[20]. These drugs are well tolerated and safe, with mild adverse effects and high tolerability after sustained use. However, tachyphylaxis and resistance to octreotide or lanreotide are known to occur. Toumpanakis *et al.*^[21] reported that in 17% of patients with loss of symptomatic response with the initial dose, symptoms were controlled by just an increase of somatostatin analogs dose, whilst the other patients required additional treatment (interferon, or transarterial hepatic embolization, *etc.*). Also, for all patients with a functioning carcinoid tumor, a potential carcinoid crisis should be prevented by prophylactic administration of octreotide, given by constant intravenous infusion at a dose of 50 mg/h for 12 h before and at least 48 h after surgery or other stress arising from invasive treatment (e.g. embolization, radiofrequency ablation)^[22]. A phase II trial, reported in 2005, aimed to examine the efficacy of a novel somatostatin analog, pasireotide (SOM-230), in 45 patients with carcinoid syndrome who were refractory to octreotide therapy^[23]. SOM-230 is a hexapeptide compound with 30 to 40 times higher binding affinity than octreotide to somatostatin receptors (SSR) subtype 1 and 5 and has a similar binding affinity to SSR 2^[24,25]. Treatment with SOM-230 was well tolerated except for a few episodes of hyperglycemia. This trial established a 27% rate of symptom improvement in patients who switched from octreotide to SOM-230. A 2-stage, randomized *vs* placebo, multicenter study of SOM-230 followed by SOM-230 LAR in patients with malignant carcinoid tumors whose disease-related symptoms are inadequately controlled by somatostatin analogs is currently underway (www.clinicaltrials.gov).

In addition to the presence of SSR, the expression of dopamine D2 receptors in GEP NETs has recently been studied^[26,27]. BIM23A760 is a new chimeric compound that selectively interacts with these receptors. The development of this drug is ongoing for the control of pituitary adenomas and Cushing's syndrome^[28,29] and a phase II study to assess the efficacy of this drug in patients with carcinoid syndrome is currently underway (www.clinicaltrials.gov). Another novel approach for the management of the carcinoid syndrome is LX1032, produced by Lexicon Pharmaceuticals. LX1032 is an orally bioavailable small molecule designed to inhibit peripheral serotonin synthesis^[30]. Its use in a phase 1 clinical study of 87 subjects has been reported by the European Neuroendocrine Tumors Society^[31]. A dose-dependent reduction in urinary 5-HIAA levels and whole blood serotonin concentration was observed. The development

of LX1032 continues in a phase II clinical trial (www.clinicaltrials.gov). Patients not responding to somatostatin analogs may also be candidates for other therapeutic measures, such as debulking surgery, hepatic embolization, and radiofrequency ablation^[32]. Other agents, such as loperamide or diphenoxylate for diarrhea and H1 or H2 blockers (or both) for histamine-secreting tumors may be administered as required.

Finally, patients with carcinoid syndrome should have an echocardiogram at diagnosis, permitting detection of cardiac involvement, which occurs in more than 50% of cases. The use of somatostatin analogs, titrated to manage symptoms or to normalize 5-HIAA levels, can help to prevent or minimize CHD, but CHD may continue to progress even if 5-HIAA is carefully controlled^[33]. If CHD develops, heart failure rather than metastatic disease may be the cause of death. Medical therapy for heart failure should be introduced when necessary. Cardiac surgery with valvular replacement should be considered for patients with symptomatic CHD, which can significantly increase survival^[34,35]. Cardiac surgery should be performed before major liver surgery or liver embolization.

ANTITUMOR TREATMENT

The aim of treatment should be curative whenever possible but is palliative in the majority of cases. These patients often maintain a good quality of life for a long period of time despite having metastases. Although the rates of growth and malignancy are variable, the aim should always be to maintain a good quality of life for as long as possible.

SURGICAL TREATMENT

The treatment of non-metastatic cases (stage I-III of the TNM classification) is based on a complete surgical resection to obtain a microscopic healthy margin (R0), the only way to significantly improve the 5-year survival rate^[3]. However, in cases of retractile mesenteritis, metastatic disease or peritoneal carcinomatosis, removal of the primary tumor should still be considered as this might prevent subsequent local complications of small-bowel obstruction or mesenteric ischemia^[9,36,37]. During laparotomy, a careful exploration of the entire abdominal cavity including the entire small bowel must be performed in order not to miss a second localization, which occurs in 30% of cases^[3]. This surgery requires a lymphadenectomy as wide as possible toward the mesenteric artery origin, associated with a prophylactic cholecystectomy because of possible future treatment with somatostatin analogs or future arterial embolization of hepatic metastases. No positive phase III studies in an adjuvant situation are available. Given the rarity of these tumors and the poor results of anti-tumoral treatment in metastatic disease (chemotherapy, interferon), these tumors only necessitate forward monitoring, except in cases of trial inclusion.

Only 15% of patients had unilobar or bilobar liver metastases without extrahepatic spread. These patients should be assessed for the possibility of aggressive approaches with curative surgical resection. The 5-year survival rate is around 60% in patients who undergo resection of liver metastases compared with 30%-40% in patients with unresected liver metastases^[38,39]. However, the recurrence risk is high, increasing from 55% to 84% within 5 years. Hepatic metastasis resection should be proposed for fit patients who have no extra-hepatic metastasis or tricuspid valve deficiency, and when complete resection of the primary tumor is possible. Concerning synchronous metastases, surgical management should be discussed according to several parameters including general health, number, size, and localization of liver metastases. Radiofrequency ablation can also be performed either before or during surgery^[40]. A 2-stage hepatectomy can be proposed in patients with liver metastases in both lobes. The first step associates the resection of the primary tumor with the resection of the left liver metastases (+/- radiofrequency ablation) and the ligation of the right branch of the portal vein. The second step consists of a right hepatectomy 4 or 6 wk later, when left liver hypertrophy is obtained: the hepatectomy can be extended to segment I and IV^[41]. As liver metastatic recurrences are nearly systematic in the course of the disease, major hepatectomy must be weighted against medical or isotopic therapy. In rare individuals such as young patients with no extra-hepatic metastasis and low Ki67, a liver transplantation may be proposed. The 5-year survival rate is about 45%, with a 5-year survival rate without recurrence of around 25%^[42].

Palliative resection should also be considered in patients who remain symptomatic despite the use of medical therapy if more than 90% of the tumor load can be removed, as this can achieve survival benefit and good symptom control^[16,38]. Liver metastasis cytoreduction can also be required in the case of a voluminous compressive mass (gastric compression).

RADIOLOGICAL TREATMENT

Hepatic artery embolization and transarterial chemoembolization

Many patients with JI NETs have liver metastases at the time of diagnosis, most of them being hypervascular. Selective hepatic trans-catheter arterial embolization (TAE) or chemoembolization (TACE) may be used to treat liver metastases in patients where surgery is not feasible. These modalities are effective in the control of symptoms and tumor growth. The intra-arterial injection of a cytotoxic drug, such as doxorubicin, streptozotocin, cisplatin or mitomycin C, is administered together with non-polar contrast^[43-47]. Embolization with gelatine sponge particles or microspheres is used until evidence of a marked decrease in blood flow. Although TACE has been used for 20 years, no current evidence exists that TACE is superior to TAE. TACE has been proved to be effective

in symptom relief in 63%-100% of patients^[43,44,48-51]. Long-term palliation can be achieved with repeated TACE sessions^[43]. Objective tumor response rates are noted in 33%-86% of patients^[43,44,48-51]. Median time to progression is about 15 mo; 5-year survival is about 50%^[40]. Contraindications for embolization are complete portal vein obstruction, liver insufficiency, and previous biliary reconstruction. Concomitant antibiotics and somatostatin analogs are used to avert a carcinoid crisis and diminish the possibility of hepatic abscess. Adverse events include a post-embolization syndrome (nausea, fever, elevated liver enzymes, and abdominal pain) which occurs in 90% of patients. Major side effects, including acute liver or renal failure, carcinoid crisis, cholecystitis, or bleeding peptic ulcers, occur in about 10% of patients. Treatment-related deaths are very rare. More recently, the radiocontrast agent lipiodol has become available with ¹³¹I instead of cold iodine, permitting embolization with radioactive material. Radioembolization using resin ⁹⁰Y-microspheres is also currently increasingly used. It can deliver high doses of radiation preferentially to hepatic metastases of NETs. In comparison to published reports of other local treatments of liver metastases from NETs, radioembolization shows a similar safety profile and improvement in debulking of tumor and survival^[52,53]. Another interesting modality of TACE is to use drug-eluting beads (DEBs). de Baere *et al.*^[54] reported in 20 patients that TACE plus DEBs loaded with 100 mg doxorubicin were well tolerated and appeared effective. This new procedure may increase the necrosis and reduce side effects from chemotherapy. Comparative studies with standard TAE, TACE, radioembolization and TACE with DEBs are warranted to define the best protocol for transarterial treatment of NET liver metastases. In addition, as not all patients are responsive to TAE or TACE, better selection criteria are still needed to obtain better responses to the procedure.

Radiofrequency ablation

Radiofrequency ablation has been used percutaneously or laparoscopically^[55], with some effect in reducing tumor size, but randomized trials are lacking. Response rates from 80% to 95% are reported. It may be indicated in patients with inoperable bilobar metastases in whom hepatic artery embolization has failed^[56]. Radiofrequency ablation can be used to reduce hormone secretion if at least 90% of the visible tumor can be destroyed or to reduce tumor burden. The main limitation for radiofrequency ablation is the size (only tumors < 3-5 cm in diameter should be treated) and number of tumors (less than 3-5 tumors should be treated per session), which are often small, and numerous in NETs. Radiofrequency ablation morbidity is low.

MEDICAL TREATMENT

Somatostatin analogs

Although the inhibitory effects of octreotide on hormonal secretion are well established, the effects on cell

proliferation and tumor growth are somewhat controversial. However octreotide has not yet been registered in any country as an antitumor agent. Their effects on tumor growth are limited: less than 5% of patients have objective radiological tumor regression, although 35%-70% of patients have stabilization of tumor size^[57-65]. The first placebo-controlled, double-blind, phase III study on the effect of octreotide LAR (long-acting release) in the control of tumor growth in patients with well-differentiated metastatic midgut NETs has been reported recently^[66]. The median time to tumor progression in the octreotide LAR and placebo groups was 15.6 and 5.9 mo (hazard ratio = 0.34, $P = 0.000072$), respectively. However, only a limited group of patients—those with less than 10% tumor mass in the liver along with resected primary tumors—responded to treatment^[67]. Therefore the effectiveness of octreotide LAR for control of growth in patients with a larger tumor burden is unproven. Also, we did not know whether patients had progressive disease at the beginning of the treatment. Another similar multicenter, placebo-controlled European Phase III study is currently underway to assess whether lanreotide autogel prolongs time to disease progression in patients with non functioning GEP-NETs (www.clinicaltrials.gov).

Interferon

Interferon- α is given for the same indications as somatostatin analogs, with the exception of carcinoid crisis. A biochemical response and symptomatic improvement could be noted in 40%-60% and 49%-70% of patients, respectively, whereas partial tumor size responses could be demonstrated in 10%-15%^[9,68,69]. The duration of response was 12 to 36 mo. Because of more pronounced side effects, interferon is generally used as second-line therapy for symptomatic control^[9]. Interferon, usually recombinant interferon- α , is given subcutaneously at 3-5 MU 3-5 times per week. A pegylated formulation, given once a week is available but not yet registered^[70]. Minor side effects include flu-like symptoms, weight loss and fatigue. Major side effects include autoimmune reactions, depression and mental disturbances. Bone marrow toxicity is usually mild as is hepatotoxicity, which can be managed by dose adjustments. There is no evidence that addition of interferon to somatostatin analogs increases the tumor response^[71,72]. However patients progressing on monotherapy of either drug may benefit from the addition of the other^[9].

Peptide receptor radionuclide therapy

Most JI NETs express SSR, especially subtype 2. Targeting these receptors with radiolabeled somatostatin analogs may not only be used for imaging, but also for radiotherapy. Since the early 1990s, different radiolabeled analogs have been used for tumor-targeted therapy. For metastatic disease with evidence of avid uptake on OctreoscanTM, radionuclide targeted therapies (for example, ¹³¹I metaiodobenzylguanidine, ⁹⁰Y or ¹⁷⁷Lu labeled soma-

tostatin analogs for peptide receptor targeted therapy) show promising results^[73]. Randomized studies of peptide receptor radionuclide therapy (PRRT) are lacking, making comparison of published data difficult. A symptomatic response in up to 80% of patients, a partial tumor response in up to 35% of patients and a disease stabilization in up to 56% of patients have been reported. Side effects are limited as long as radiation dose to the kidney and bone marrow are kept within dose limits; the use of kidney protection by co-infusion of amino acids (lysine and arginine) allows the administration of higher doses of the radiopharmaceuticals^[9]. The most recent data indicate a partial or complete response in 28% of patients with a median time to progression of more than 36 mo for Lutate^[74,75]. These radiopharmaceuticals are only available in a few centers. PRRT is recommended in SSRS-positive tumors in symptomatic patients refractory to medical treatment with inoperable disease.

External radiation therapy

Carcinoid tumors have often been regarded as being radioresistant. However, external beam radiotherapy is recommended for some brain and symptomatic bone metastases.

Traditional chemotherapy

Although pancreatic NETs seem to be moderately sensitive to systemic cytotoxic chemotherapy, well-differentiated JI NETs are generally considered to be chemoresistant. Single agents [5-fluorouracil (5-FU), doxorubicin, DTIC] and combinations (streptozotocin + doxorubicin or 5-FU) generally produce responses in less than 15% of patients^[76-79]. Dahan *et al.*^[80] reported no significant difference in progression-free survival and overall survival between interferon and 5-FU plus streptozotocin (even a trend in favor of interferon) in a phase III randomized trial in patients with metastatic carcinoid tumors. Based on the activity of dacarbazine and 5-FU in NETs^[81,82], there is a rationale for the study of the oral agents temozolomide, which is converted as dacarbazine into the active alkylating agent MTIC^[83-85] and capecitabine (an oral pro-drug for 5-FU)^[86]. Kulke *et al.*^[84] reported on the combination of temozolomide with thalidomide in a variety of NETs, documenting an objective response rate of 45% in pancreatic NETs *vs* only 7% in carcinoid tumors. Interestingly, the response to temozolomide-based therapy appeared to be correlated with O6-methylguanine DNA methyltransferase deficiency^[87]. Combining capecitabine and temozolomide may be an interesting association in patients with NETs^[88]. Oxaliplatin seems to be also an interesting drug in NETs^[86,89]. Even if some new drugs are coming, there is a general consensus that chemotherapy with agents available today is not recommended in patients with well-differentiated JI NETs^[9,36].

Angiogenesis inhibitors and other novel targets

Currently, there are a number of new drugs undergoing

evaluation in the clinic. These could be divided into 3 groups as follows: (1) drugs targeting vascular endothelial growth factor (VEGF), such as the VEGF monoclonal antibody bevacizumab and a more recent related compound, VEGF-trap; (2) small molecules that inhibit the intracellular tyrosine kinase domain of vascular endothelial growth factor receptor or other growth factor receptors, such as sunitinib, sorafenib, imatinib and valatinib; and (3) other compounds inhibiting different signaling pathway components such as epithelial growth factor receptor, insulin-like growth factor 1 receptor, phosphoinositide-3-kinase, RAC- α serine/threonine-protein kinase (AKT), and mammalian target of rapamycin (mTOR).

The first reported phase II trial of bevacizumab in NETs was performed in 44 patients with advanced carcinoid tumors. Patients were randomly assigned to 18 wk of treatment with either octreotide plus bevacizumab or octreotide plus pegylated interferon^[90]. At week 18, the progression-free survival rate was 96% in the group receiving bevacizumab *vs* only 68% in the group receiving pegylated interferon. Moreover, a rapid reduction in blood tumor perfusion measured by functional CT scan was demonstrated in the bevacizumab-treated patients. Based on the promising results in the bevacizumab study, a confirmatory randomized phase III trial is underway by the Southwest Oncology Group, comparing octreotide plus bevacizumab or interferon in advanced carcinoid tumors, with progression-free survival as the primary endpoint. The experience with bevacizumab in other solid tumors such as colorectal cancer has shown that its addition to chemotherapy can significantly improve outcome, whereas it has very little clinical activity as a single agent. The combination of temozolomide and bevacizumab has recently been reported in a small phase II trial with 0% of objective response rate in carcinoid tumors *vs* 24% in pancreatic NETs^[91]. Currently, different combination are ongoing: a phase I - II trial of FOLFOX plus bevacizumab in refractory carcinoid and pancreatic endocrine tumors in the USA; a phase II trial of capecitabine plus bevacizumab in chemotherapy-naïve patients with carcinoid tumors in France; a phase II trial of bevacizumab plus 2-methoxyestradiol (Panzem) in patients with locally advanced or metastatic carcinoid tumors (www.clinicaltrials.gov).

Similarly, a multi-institutional phase II study of sunitinib, a novel tyrosine kinase inhibitor with activity against VEGFR-1 to 3, PDGFR, FLT-3, c-Kit and RET, was also conducted among patients who had advanced NETs^[92]. In this trial on 66 patients with pancreatic NETs and 41 patients with carcinoid tumors^[92], the treatment was well tolerated and a partial response occurred in 11 (17%) of pancreatic NETs and 1 (2%) of carcinoid tumors. Rates of stabilized disease were high in both groups (68% in pancreatic NETs and 83% in carcinoid tumors). Raymond *et al*^[93] reported the first phase III randomized, double-blind study, with the efficacy of sunitinib given daily as a continuous dose *vs* placebo in patients with advanced islet

cell tumors. The median disease-free survival was 11.1 mo in the sunitinib group *vs* 5.5 mo in the placebo group. No similar phase III trial is ongoing for carcinoid tumors. In addition, a pending phase II trial will investigate the use of sunitinib after TAE in the treatment of NETs with liver-predominant metastases. The purpose of the trial will be to assess whether angiogenesis inhibition assists in prolonging the time to disease progression following liver embolization. Previously, use of sorafenib, an oral and multitarget agent with potent activities against VEGF-R-3, PDGFR-b, FLT-3, c-Kit and fibroblast growth factor receptor-1, has also been reported in 50 carcinoid tumors, but with more than 40% of grade 3-4 toxicity^[94]. Other trials testing different tyrosine kinase inhibitors are currently underway (e.g. valatinib).

The third approach for new drugs has focused on inhibiting different signaling pathway components. For example, mTOR is an intracellular serine/threonine kinase that acts as a central regulator of multiple signaling pathways (IGF- I , EGF, VEGF) that participates in the regulation of apoptosis, angiogenesis, proliferation and cell growth through modulation of cell cycle progression^[95]. Two rapamycin derivatives have recently been evaluated in NETs: temsirolimus^[96] and everolimus^[97,98]. The results of a first phase II study combining everolimus (RAD001) at 5 or 10 mg orally daily and depot octreotide at 30 mg intramuscularly every 28 d in low-grade NETs have been recently reported^[97]. Of the 60 patients, there were 30 with carcinoids and 30 with islet cell carcinomas. Toxicities were mild to moderate, including those expected from everolimus such as stomatitis and myelosuppression. Tumor response rates were higher in the group with RAD001 at the 10 mg dose level *vs* the 5 mg dose level, and in the islet cell carcinoma (27%) *vs* the carcinoid tumor (17%) group^[97]. Tumor response was low, but control of disease was very promising (97% in the carcinoid tumor group). Based on these encouraging results, 3 large trials were sponsored by Novartis (RADIANT-1,-2,-3). RADIANT-1 is an open-label, stratified, single-arm phase II study of RAD001 in patients with advanced pancreatic NETs after failure of cytotoxic chemotherapy^[98]. Two further phase III, placebo-controlled, randomized trials in patients receiving depot octreotide recently closed: RADIANT-2 in patients with advanced carcinoid tumors and RADIANT-3 in patients with advanced pancreatic islet cell tumors. In contrast, interest in EGF receptor inhibitors, such as gefitinib, on carcinoid tumors seems to be poor^[99].

ALGORITHMS OF TREATMENT IN PATIENTS WITH JI WELL- DIFFERENTIATED ENDOCRINE CARCINOMAS

Recently, management algorithms have been proposed by several experts^[16-18,37,100]. Treating these tumors needs collaboration of different types of physicians including

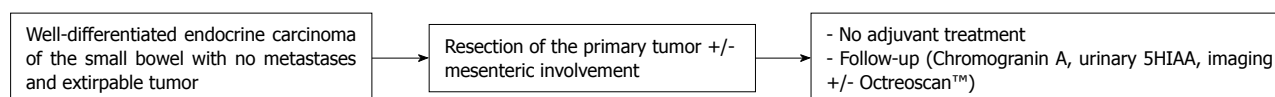


Figure 1 Management algorithm for patients with non metastatic well-differentiated endocrine carcinoma of the small bowel (stage I -III of TNM classification). 5HIAA: 5-hydroxyindoleacetic acid.

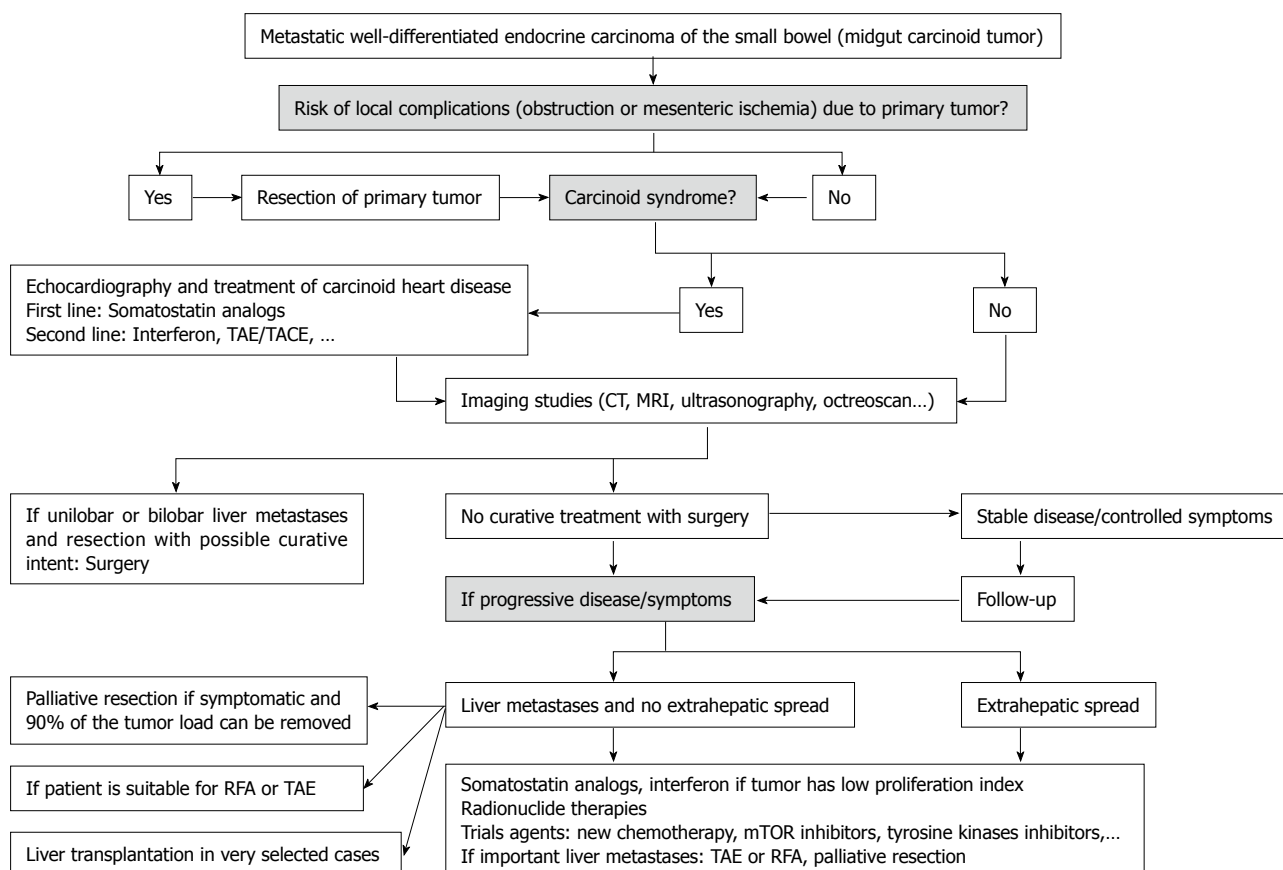


Figure 2 Management algorithm for patients with metastatic well-differentiated endocrine carcinoma of the small bowel (stage IV of TNM classification). TAE: Transarterial embolization; TACE: Transarterial chemoembolization; RFA: Radiofrequency ablation; mTOR: Mammalian target of rapamycin; CT: Computed tomography; MRI: Magnetic resonance imaging.

endocrinologists, gastroenterologists, oncologists, interventional radiologists, pathologist, and surgeons. Only a complete surgical resection permits cure of localized well-differentiated endocrine carcinoma of the small bowel (stage I -III of TNM classification). After curative surgery, there is no indication for medical therapy other than pre- and peri-operative somatostatin analogs to avoid a carcinoid crisis (Figure 1). The strategy for treatment of metastatic tumors (stage IV of TNM classification) is more complicated and varies depending on tumor spread, general health of the patient, predominant symptoms, and tumor growth (Figure 2). Somatostatin analogs remain the primary treatment for carcinoid syndrome. Since the PROMID study, they may be also considered for asymptomatic patients with low hepatic tumor load. TAE, TACE, radioembolization or TACE with DEBs are indicated for patients with non-resectable multiple metastases and persistence of carcinoid syndrome despite treatment with somatostatin analogs,

with the intention of reducing tumor size and hormone output. In patients with disabling carcinoid syndrome who are still refractory, interferon is frequently used in second-line treatment; the novel somatostatin analog SOM-230 could be useful in the near future for palliation of carcinoid syndrome^[101]. Perhaps the most promising avenue of research is the field of PRRT. Moreover, it is very probable that angiogenesis inhibitors and therapy targeting mTOR will become a new standard treatment for these vascular malignancies; however, as in pancreatic NETs with sunitinib, randomized phase III trials will be needed to demonstrate improvements in time to progression or overall survival.

CONCLUSION

Finally, the development of centers of excellence and NET clinical teams to coordinate multicenter studies, extend clinical and tissue databases, and ultimately develop

molecularly targeted therapeutics are needed to advance treatment and survival for patients with GEP NETs.

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Walled-off pancreatic necrosis

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ing results have recently been reported in the medical literature, rendering these techniques invaluable in the treatment of WOPN. Applying the recommended therapeutic strategy, which comprises early treatment with antibiotics combined with restricted surgical intervention, fewer patients with ANP undergo surgery and interventions are ideally performed later in the course of the disease, when necrosis has become well demarcated.

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Key words: Walled-off pancreatic necrosis; Infected pseudocyst; Severe pancreatitis; Acute necrotizing pancreatitis; Pancreas; Inflammation; Alcoholism

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Abstract

Walled-off pancreatic necrosis (WOPN), formerly known as pancreatic abscess is a late complication of acute pancreatitis. It can be lethal, even though it is rare. This critical review provides an overview of the continually expanding knowledge about WOPN, by review of current data from references identified in Medline and PubMed, to September 2009, using key words, such as WOPN, infected pseudocyst, severe pancreatitis, pancreatic abscess, acute necrotizing pancreatitis (ANP), pancreas, inflammation and alcoholism. WOPN comprises a later and local complication of ANP, occurring more than 4 wk after the initial attack, usually following development of pseudocysts and other pancreatic fluid collections. The mortality rate associated with WOPN is generally less than that of infected pancreatic necrosis. Surgical intervention had been the mainstay of treatment for infected peripancreatic fluid collection and abscesses for decades. Increasingly, percutaneous catheter drainage and endoscopic retrograde cholangiopancreatography have been used, and encourag-

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INTRODUCTION

Since the first interdisciplinary symposium on acute pancreatitis in Marseille in 1964 remarkable progress has been gained in creating a uniform classification system for the variable clinical features of this disease. The most important and frequent definitions in terms of pancreatic infection are as follows: (1) Pancreatic infection: the presence of microbes including bacteria or fungi overgrowing the pancreas or the peripancreatic space and causing pathologic changes. Pancreatic infection usually occurs secondary and time-dependently to acute pancreatitis. This definition includes infected necrosis, pancreatic

abscesses and infected pancreatic pseudocysts; (2) Infected necrosis: intrapancreatic or extrapancreatic necrosis with a positive smear or culture for bacteria or fungi. Usually no major collection of pus is present; (3) Pancreatic abscess: a localized collection of purulent material with little or no necrosis in the region of the pancreas, which is delineated by a wall of collagen and granulation tissue; and (4) Infected pancreatic pseudocyst: a localized collection of infected fluid in the region of the pancreas and, like an abscess, also walled off by a membrane of collagen and granulation tissue. Usually the presence of bacteria or fungi is of no clinical significance and represents contamination only. There may be communication with the pancreatic ductal system; pus or necrosis is generally not found^[1]. The term “Walled-off pancreatic necrosis (WOPN)” was first introduced by Connor *et al*^[2] in 2005. This term was officially established later, on the 2006 Digestive Disease Week during the American Gastroenterological Association Clinical Symposium, “Problems and Pitfalls of Atlanta Classification for acute pancreatitis: American Gastroenterological Association, American Pancreatic Association and International Association of Pancreatology to revisit,” chaired by Dr. Peter Banks^[3]. WOPN, formerly known as pancreatic abscess is uncommon and usually occurs in the setting of pancreatitis, usually in complicated cases of pseudocysts or sterile pancreatic necrosis. Infections outside this setting are extremely uncommon but they have been reported to occur with perforation of the bowel into the pancreas^[4] or splenic parenchymal involvement^[5]. It embodies a quite late complication of pancreatitis, chronic or acute, commonly, after the formation of pseudocysts. In pancreatitis, enzymes can be walled off by granulation tissue, by developing pseudocysts or *via* bacterial seeding of pancreatic or peripancreatic tissue, leading to development of WOPN^[6]. Even if WOPN constitutes 1%-9% of all acute pancreatitis complications, it still remains a lethal surgical entity, which can be alleviated through early detection and application of the indicated therapeutic measures^[7]. The aim of this article is to review WOPN, its etiology, epidemiology, clinical features, diagnosis and new clues, as they are presented, in the latest articles of the literature.

EPIDEMIOLOGY

In the US, the incidence of pancreatitis is approximately 185 000 cases per year. At least 80% of cases are due to alcohol and cholelithiasis, and all the rest are associated with other triggering conditions, as illustrated in Table 1. Acute necrotizing pancreatitis (ANP) is reported by some to occur in approximately 20% of all episodes of pancreatitis. Although sterile necrosis may occur, a variable percentage develops infection of the necrotic tissue. Bacterial contamination of the necrotic pancreas occurs in as many as 70% of cases, and the mortality rate approaches 100% if surgical intervention and drainage are not undertaken for WOPN. A difference in the rate of WOPN formation between men and women has not been clearly demonstrated^[8,9]. WOPN

Table 1 Etiology of pancreatitis

Biliary tract disease	Obstruction of the pancreatic duct at the level of the ampulla of Vater
Excessive alcohol consumption	Direct toxic effect
Hyperlipidemia	Restricted blood flow (atherosclerotic emboli, hypoperfusion, vasculitis) results in ischemic disturbance to the acinar structures and an increasingly acidic environment
Hypercalcemia	Induces pancreatic injury <i>via</i> a secretory block, accumulation of secretory proteins and possibly activation of proteases
Hereditary	Cationic trypsinogen mutations
Trauma	Acute release of toxic factors (resulting from inflammatory response) into the systemic circulation
Ischemia	Similar to hyperlipidemia
Pancreatic duct obstruction	Similar to biliary tract disease
Viral infections	Mumps or cytomegalovirus
Scorpion venom	Direct toxicity
Idiopathic	Develops without readily identifiable cause
Drugs	Frequently 6-Mercaptopurine Azathioprine Corticosteroids Synthetic estrogens Furosemide Mesalamine Methyldopa Sulphonamides Tamoxifen Occasionally Asparaginase Chlorothiazide Cisplatin Hydrochlorothiazide Interferon- α Sulindac Tetracycline Valproic acid

occurs in 1%-9% of the cases of acute pancreatitis, usually 4 to 6 wk after the initial episode and is heralded by pain, fever and chills^[10]. The mortality rate of ANP (about 25% of acute pancreatitis) has been reported to be between 10% and 30%^[7,11].

ETIOLOGY-PATHOPHYSIOLOGY

ANP is the most severe end of a spectrum of inflammation associated with pancreatitis. During the past decade, significant progress has been achieved in our understanding of the inflammatory response in pancreatitis^[12], see also Table 1^[13-24]. By contrast, very little is known about the mechanisms mediating another major pathologic response in pancreatitis, the parenchymal cell death. In experimental models of acute pancreatitis, acinar cells have been shown to die through both necrosis and apoptosis^[25]. The apoptosis/necrosis ratio varies in different experimental models of pancreatitis. Of note, the severity of experimental pancreatitis directly correlates with the extent of necrosis and inversely with that of

apoptosis^[26]. Inflammation causes cell death with resultant devitalized tissue, which is likely to become infected. The amount of necrotic tissue is the strongest predictor of mortality in ANP. After pancreatic necrosis occurs, 3 potential outcomes exist, resolution, pseudocyst, or WOPN. Pseudocysts may result in prolonged abdominal pain, rupture leading to acute peritonitis, fistula formation, and erosion into vessels with acute hemorrhage^[27,28]. Pancreatic ascites or pleural effusion may be developed. Pseudocysts or WOPN may also cause hollow viscus obstruction by compression of surrounding structures, including the colon, stomach, duodenum and the common bile duct. The role of proinflammatory cytokines in this process is being vigorously examined^[27]. WOPN forms through various mechanisms, including fibrous wall formation around fluid collections, penetrating peptic ulcers, and secondary infection of pseudocysts. A pseudocyst arises as a local complication of ANP. Over a period of 3–4 wk, sequestration of necrotic tissue occurs, forming a fibrous capsule without an epithelial lining. At any point after the initial injury in ANP, infection of necrotic tissue may occur, leading to WOPN development. When this occurs prior to the formation of the fibrous wall, it is termed infected necrosis. WOPN can be located in single or multiple locations and vary greatly in size^[3]. Of note, pseudocyst formation is directly related to the degree of necrosis present. Approximately 3% of patients with acute pancreatitis develop WOPN. Balthazar and Ranson's radiographic staging criteria predict the formation of pseudocysts and, therefore, WOPN development. Grade A: normal pancreas; Grade B: focal or diffuse enlargement; Grade C: mild peripancreatic inflammatory changes; Grade D: single fluid collection; Grade E: two or more fluid collections or gas within the pancreas or within peripancreatic inflammation. In grade A, B, C, or D, the probability of WOPN development is less than 2%. With grade E disease (2 or more collections of peripancreatic fluid), the probability rises to 57%^[29]. WOPN should be distinguished from infected pancreatic necrosis in that little or no necrotic material is present. WOPN also typically appears later in the course of pancreatitis, often 4 wk or more after the start of the attack^[30].

PRESENTATION

Diagnosed pancreatitis with an unexpectedly prolonged course, hemodynamic instability, fever, failure of medical therapy, or the presence of fluid collections on a computed tomography (CT) scan all point to the possibility of necrosis and, potentially, WOPN later in the course. Abdominal pain with or without a mass on palpation of the epigastrium is suggestive of parietal peritoneal irritation. Classic physical examination findings, such as Grey-Turner sign or Cullen sign, are supposedly characteristic of pancreatitis but rarely are noted in clinical practice. Other physical findings are nonspecific and include abnormal vital signs consistent with sepsis, abdominal

guarding, and rebound tenderness^[31]. Peripancreatic fluid encased in a fibrinous capsule defines pseudocysts. Superinfection of pseudocysts is one way that WOPN may be developed, even if pseudocysts are not a prerequisite. Evidence suggests that colonic translocation of bacterial flora accounts for many cases of local infected fluid formations^[32]. The most typical organisms isolated from infected necrosis and abscesses are gut flora-associated and *Candida spp*^[33].

DIAGNOSIS

No specific hematologic studies define WOPN. A persistently elevated white blood cell count with a left shift and positive blood cultures is suggestive of this diagnosis. The degree of pancreatic enzyme elevation does not directly indicate the degree of necrosis^[34]. The presence of air in necrotic tissue in a pseudocyst on imaging studies is also specific for infection. Abdominal CT scan with IV contrast; ultrasound, either endoscopic or transabdominal; and magnetic resonance imaging (MRI) (with gadolinium) are potential modes for imaging pancreatic necrosis or abscess^[35]. MRI is becoming the imaging study of choice because of concerns regarding the use of iodinated contrast, which is said, by some, to devitalize marginal tissue, increasing the burden of necrotic tissue. The current criterion standard for initial evaluation is contrast-enhanced CT scan, which may reveal ischemic pancreatic tissue as evidenced by the lack of uptake of contrast. Contrast-enhanced CT scan (and in particular a contrast-enhanced thin-section multidetector-row CT scan) is the best imaging technique to exclude conditions that masquerade as acute pancreatitis, to diagnose the severity of acute pancreatitis, and to identify complications of pancreatitis^[35–37]. MRI may be of some additional benefit in the acute evaluation of ANP; gadolinium does not cause a worsening of ischemia in experimental models^[38]. Demonstrable necrotic tissue in the pseudocyst may exist. Typically, this develops more than 3 wk after the initial bout of pancreatitis^[39]. The presence of either bacterial or fungal flora in pancreatic fluid collections usually aspirated *via* CT-guided needle biopsy is the sine qua non of WOPN. The presence of organisms either on Gram stain or culture is essential for WOPN.

TREATMENT

Generally, medical care is supportive, with attention paid to blood pressure and volume status. Patients frequently are transiently bacteremic, so antibiotics are routinely administered. The choice of antibiotics is thoroughly guided by the likely flora and degree of antibiotic penetration into the locations of WOPN and the other necrotic tissue. The most commonly isolated bacteria in WOPN are gut flora, by means of translocation^[40]. The most common pathogens are *Escherichia coli*, *Klebsiella pneumoniae*, *Enterococcus faecalis*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Proteus mirabilis* and *Streptococcus spp*^[41]. In

several trials, imipenem^[42] has been shown to have good penetration into pancreatic tissue, and it has a good activity against all likely pathogens^[43]. Other antibiotics that have been shown to be efficacious in ANP include cefuroxime^[44] and a regimen of ceftazidime, amikacin and metronidazole^[45]. It must be emphasized that the ultimate treatment for WOPN is complete removal by surgical resection or drainage^[46]. Primary drainage is the treatment of choice for WOPN. Cases of WOPN have been reported of treatment, in which death has not resulted; however, the standard of care is drainage involving an open procedure^[47]. Case series have been reported of WOPN patients, who have been treated with CT-guided drainage tube placement, but these seemed to show inferior results to open drainage^[48]. Recent advances in endoscopic treatment using endoscopic ultrasound (EUS) have made guided transgastric treatment of the complications of ANP possible^[49]. EUS-guided necrosectomy has been promising to date. The introduction of formal widespread pancreatic and peripancreatic necrosectomy with a following procedure to manage the peripancreatic space, allowing continued drainage and debridement, has markedly decreased the mortality associated with infected necrosis. In specialized centers, this is rapidly becoming the treatment of choice^[50]. CT-guided drainage has a role in some patients, who cannot tolerate an open procedure. EUS with trans-gastric drainage is another option. Consideration can be given to medical management of nonsurgical candidates until their clinical status improves^[51]. Other forms of aspiration, including endoscopic drainage of WOPN, are currently being investigated, with controversial results^[3,52,53]. Surgical drainage of WOPN is the procedure for cure. Placement of indwelling drains after the initial procedure may be necessary for complete resolution^[54,55]. Nil *per os* or a jejunal feeding tube is initially recommended for ANP; however, no contraindication for enteral feeding exists if the pancreatitis has resolved. If the course is prolonged, the institution of total parenteral nutrition can be of benefit^[56]. Antibiotics are the primary medical therapy in WOPN, used for the control of bacteremia and sepsis. Supportive care with fluids is needed, and the use of vasopressors may be required, in hemodynamically unstable patients. Overall, it can be presumed that WOPN is an entity that can lead to several complications such as fistula formation, recurrent pancreatitis, bowel obstruction or death. Studies, including the present review based on retrospective observations, have demonstrated that non-operative management has been associated with a favorable outcome in patients with this dreaded complication^[57]. If bacteremia and sepsis can be restrained with prolonged antibiotic therapy along with antifungal agents, the need for necrosectomy associated with high morbidity and mortality in these patients can be avoided. Applying the recommended therapeutic strategy, which comprises early application of antibiotics combined with restricted indication for surgical intervention, fewer patients with ANP undergo

surgery, and the interventions are performed later in the course of the disease, ideally when necrosis has become well demarcated^[58]. Endoscopic sphincterotomy plays a role in patients with a dilated common bile duct from an impacted stone at risk of impending cholangitis. Surgery in acute pancreatitis is used for cholecystectomy in the patient with gallstone pancreatitis, as well as for infected pancreatic necrosis, WOPN, pseudocysts and traumatic pancreatitis with a ruptured duct system^[7,59].

CONCLUSION

Today, treatment of acute pancreatitis is mainly conservative and surgery is on the retreat. Infection of pancreatic necrosis is still the main risk factor of morbidity and mortality in the course of necrotizing disease. A prophylactic treatment with antibiotics can reduce both infectious complications and mortality. Thus, antibiotics should be administered in severe pancreatitis. If pancreatic infection is suspected, fine needle aspiration should be performed. Today, WOPN is a well accepted indication for open surgery, when minimal invasive treatment is not efficient^[60]. Natural orifice transluminal endoscopic surgery (NOTES) has emerged as an innovation in endoscopic access that allows incisionless surgery. Integral techniques used in NOTES have developed in part through advances in endoscopic retroperitoneal access to pancreatic pathology over the past 2 decades. In 2004, Kalloo *et al*^[61] described peroral endoscopic access to the peritoneal cavity for a liver biopsy, reporting a new access technique for surgical procedures. In actuality, transluminal endoscopic access by Kozarek *et al*^[62] was well documented as early as 1985 for the treatment of pancreatic pseudocysts. A study^[23] reviewing the senior author's (Gary C Vitale) 15-year experience with natural orifice transluminal endoscopic retroperitoneal access for the drainage of pancreatic abscesses showed that the optimal approach to pancreatic abscess drainage has yet to be studied by a randomized controlled trial, but the literature demonstrates that endoscopic therapy has greatly reduced morbidity and mortality rates compared with surgery.

The natural orifice transluminal approach to pancreatic fluid collections has evolved to address complicated cases that previously would have necessitated surgery^[23]. The development of EUS has expanded the safety and efficacy of this modality by allowing one to access and drain more challenging fluid collections^[7]. The aim of the surgical procedure is to remove the septic focus by debridement of the infected pancreatic and peripancreatic necrosis^[3]. The optimal timepoint for the surgical intervention is when necrotic tissue is well demarcated. Therefore bleeding complications and removal of vital tissue can be avoided. Today, surgical procedures should combine the necrosectomy with a postoperative method to continuously remove necrosis and debris. This is the case with the following 2 techniques: postoperative continuous lavage and closed packing. Fulminant acute pancreatitis is a rare subgroup of acute pancreatitis,

characterized by a rapidly progressive multiple organ failure in the first days following the onset of the disease with a high probability of death despite intensive care unit therapy. There is a poor outcome with both, surgical and conservative therapies. Thus, surgery should only be performed as a last resort^[63]. If left untreated, WOPN precipitates to sepsis and death. Adequate drainage of a pancreatic pseudocyst may help prevent some cases of WOPN. However, in many cases the disorder is not preventable^[64]. Until now, there are no official established guidelines for the treatment of WOPN. That is a major consideration for the future.

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Pathologic research update of colorectal neuroendocrine tumors

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Abstract

Colorectal neuroendocrine tumors (NETs) originate from neuroendocrine cells in the intestinal tract, and represent a small area within oncology, but one which has provided increasing new data during the past years. Although the World Health Organization has determined clinical and histological features to predict prognosis for such tumors, they may not be valid on an individual basis. We aim to give an overview of the recent findings with regard to pathology, molecular genetics and diagnosis of NETs.

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Key words: Neuroendocrine tumors; Carcinoid; Colorectal; World Health Organization classification; Tumor-node-metastases

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INTRODUCTION

Neuroendocrine (NE) cells are distributed throughout the human body, including the gastrointestinal (GI) tract, pancreas, lung, thyroid, adrenal gland and many other organs^[1-4]. The GI tract has the largest population of NE cells^[5]. However, neuroendocrine tumors (NETs) of the colon and rectum are rare. Results from analyses of the Surveillance, Epidemiology and End Results database demonstrated that the age-adjusted incidence of carcinoids of the small intestine and digestive system has increased by 460% and 720%, respectively, in the past 30 years due to, at least in part, the improvements in diagnostic technology such as endoscopy, as well as doctors' increased awareness^[6]. In 2003, Modlin *et al*^[7] reported that the incidence of NETs in the GI tract was 2.5-5 cases per 100 000 population annually. Males are more often affected than females, with a proportion of 1.8:1^[8]. In the large intestine, NETs are more commonly found in the rectum (54%), then in the cecum (20%), sigmoid colon (7.5%), rectosigmoid colon (5.5%) and ascending colon (5%)^[9].

Colon NETs usually appear as large tumors and often already have regional lymph nodes or liver metastasis at the time of diagnosis, therefore the prognosis is poor. On the other hand, NETs occurring in the rectum are often diagnosed incidentally during colonoscopy, therefore they are typically small, localized, non-functioning tumors with rare metastasis, probably as a result of early detection^[6,8,10,11].

CLASSIFICATION

Colorectal NETs are traditionally classified as typical

Table 1 Classification of NETs of the colon and rectum^[14,15]

Well-differentiated neuroendocrine tumor (carcinoid)
Benign: Non-functioning, confined to mucosa-submucosa, non-angioinvasive, < 1 cm in size (ileum) or ≤ 2 cm colon and rectum
Serotonin-producing tumor
Enteroglucagon-producing tumor
Benign or low-grade malignant (uncertain malignant potential): non-functioning, confined to mucosa-submucosa, angioinvasion, or < 1 cm in size (ileum) or ≤ 2 cm colon and rectum
Serotonin-producing tumor
Enteroglucagon-producing tumor
Well-differentiated neuroendocrine carcinoma (malignant carcinoid)
Low-grade malignant: invasion of the muscularis propria and beyond or metastases
Non-functioning or functioning serotonin-producing carcinoma (with carcinoid syndrome)
Non-functioning enteroglucagon-producing carcinoma
Poorly-differentiated neuroendocrine carcinoma
High-grade malignant

NETs: Neuroendocrine tumors.

carcinoid, atypical carcinoid and undifferentiated cancer according to the differentiation of the disease^[12,13]. The term carcinoid can no longer characterize the entire morphologic and biologic spectrum of neoplasms of the disseminated NE cell system. The updated World Health Organization (WHO) classification of 2000 adopted the neutral and inclusive terms, tumor and carcinoma. In this classification, it is clearly explained how to characterize well-differentiated NETs (benign behavior or uncertain malignant potential), well-differentiated NE carcinoma (low-grade malignancy), and poorly-differentiated NE carcinoma of high-grade malignancy [small cell carcinomas (SCCs)]^[14,15]. The morphologic/biologic criteria including tumor size, angioinvasion, proliferative activity, histological differentiation, metastasis, invasion, and hormonal activity (association with clinical syndromes or diseases) have also been added (Table 1).

Well-differentiated NE tumor was referred to as typical carcinoid historically, and well-differentiated NE carcinomas were termed atypical carcinoid. Although this framework is helpful, lesions of uncertain behavior are poorly defined. In the WHO classification, SCC is synonymous with the term “poorly-differentiated NE carcinoma”, but there is no clear differentiation between large cell NE carcinomas (LCNECs) and mixed endocrine-glandular neoplasms. No coincident repeat diagnosis could be made by different pathologists or by different institutions, although the WHO classification of colorectal NETs has been used for several years. The main reason is that the WHO classification has not been closely adhered to^[1]. Pathological recognition of these tumors is critical, as the treatment strategy, particularly chemotherapy for a particular subtype, largely depends on the underlying pathology^[16,17].

Well-differentiated NE tumor-carcinoid

Colorectal carcinoids account for approximately 6% of

all GI NETs, often accompanied by chronic inflammatory disease such as ulcerative colitis or Crohn's disease. The tumors are usually multiple when complicated by these diseases^[18]. In Crohn's disease, the incidence of carcinoids may increase, by a recent estimation of 15-fold^[19,20]. In most instances, colorectal carcinoids present without obvious signs or symptoms and remain undetectable for years^[8]. Most of the diagnoses are made incidentally at the time of surgery for other abdominal disorders. Their relatively high incidence in large autopsy series has provided evidence for this observation^[21].

The tumors with NE differentiation have classical histological architecture of trabecular, insular, or ribbon-like cell clusters, and have no or minimal cellular pleomorphism and sparse mitoses^[8,22,23]. Colorectal carcinoids display moderate neurofilament staining. They stain positive for chromogranin A (CgA) in more than 70% of cases, positive for neuron specific enolase (NSE) in more than 50% of cases, and prostatic acid phosphatase is expressed in 80%-100% of cases. However, the staining pattern is variable^[8]. Other markers such as synaptophysin (Syn), somatostatin, 5-HT, or CD56 may be present as well. The Ki-67 index is higher in carcinoids of a size more than 5 mm, as compared to those sized less than 5 mm in diameter^[24].

Rectal carcinoids are usually discovered incidentally during colonoscopy examinations. The tumors are usually small, non-functional and without regional or distant metastasis^[25,26]. The 5-year overall survival rate of patients with rectal carcinoids is 88%. However, the prognosis of colon carcinoids is worse than that of its rectal counterpart^[8,10,26,27]. There are data showing that there is a high frequency of combined adenocarcinoma and NETs in the proximal colon^[28,29]. Perhaps this is one of the reasons explaining the worse prognosis in colon carcinoids than for their rectal counterpart.

Well-differentiated NE carcinoma-malignant carcinoid

Malignant carcinoid was termed as atypical carcinoid, historically. Only a few cases have been reported as malignant carcinoid of the large intestine due to uncertainty of its definition.

Well-differentiated NE carcinomas have histological features and a biological behavior that fall between well-differentiated NE tumors and poorly-differentiated NE carcinomas^[8]. These are aggressive lesions and represent forms poorly differentiated from carcinoid with increased mitotic activity and presence of necrosis. Mitoses range from 1 to 10/10 high power field (hpf). The presence of necrosis serves as an important character of malignant carcinoid^[8,30,31]. The cells range in appearance from uniform, large, polygonal, or fusiform types with abundant eosinophilic granular cytoplasm and round to oval nuclei similar to the cells seen in typical carcinoid, to pleomorphic cells with scanty cytoplasm and hyperchromatic variably sized and shaped nuclei^[32]. The tumors are nonargentaaffinic but strongly argyrophilic and they are

stained with the usual immunohistochemical markers of NE cell differentiation.

There are few reports of the clinical outcome of colorectal malignant carcinoids. It has been reported, however, that a patient with an atypical carcinoid of the lung has a significantly worse prognosis than those with typical carcinoids^[33,34].

Poorly-differentiated NE carcinoma (small cell carcinoma)

Colorectal SCCs constitute 0.2%-0.8% of all colorectal tumors, mostly located in the right colon^[1,35]. They are clinically aggressive; therefore have extremely poor prognosis, even when diagnosed at an early stage. Most patients present with overt distant metastases. Sometimes they are discovered in the background of colon inflammatory diseases, or in the background of NE cell proliferation^[36,37].

Colorectal SCCs are identical to lung SCCs morphologically. These small blue cell tumors present the character of densely packed, small, and oval-, spindle-, fusiform-shaped anaplastic cells with minimal amounts of cytoplasm and granular nuclear chromatin. The size of the nuclei measures approximately twice the size of mature lymphocytes^[38]. Solid sheets, nests, and rosettes as well as ribbon-like structures composed of small cells and intermediate cells form, which all exhibit more than 10/10 hpf mitoses^[1].

In cases with classic histological features, it is unnecessary to demonstrate NE differentiation to establish the diagnosis. However, further analysis may be necessary in puzzling cases. SCCs typically express CgA, Syn, NSE, and CD56 by immunohistochemistry. Pan cytokeratin (AE1/AE3) and low molecular weight keratin are also reported to be positive in all SCCs^[1,39]. Shida *et al.*^[40] reported that 70% of GI SCCs expressed human achaete-scute homologue gene-1 protein (hASH1), which is usually absent in normal GI NE cells, carcinoid and adenocarcinoma. The sensibility and specificity of hASH1 is better than other classic markers, such as CgA and Syn, and this protein may serve as a new biomarker for GI SCC diagnosis. There are also some reports demonstrating that cytokeratin is positive in these tumors and the Ki-67 index beyond 75% is often seen in SCCs^[1].

As mentioned above, the prognosis of patients with GI SCCs is usually extremely poor. Most patients present with regional or overt distant metastases. Up to 80% of patients already have regional lymph nodes and/or distant metastases at the time of diagnosis^[41]. When tumor size is larger than 5 cm, the median survival time for patients is usually less than 4 mo, and the median survival of those with smaller tumor is around 12 mo^[1].

LCNEC

LCNECs are rare, poorly-differentiated NE carcinomas. LCNECs are amongst the worst studied group of colorectal NETs and their diagnostic criteria are briefly described in many standard texts^[14]. The features of these tumors are similar to their counterpart in the

lung^[42]. They are malignant neoplasms composed of large cells structured in organoid, nested, trabecular, rosette-like, and palisading patterns that suggest NE differentiation. Compared to SCCs, LCNECs have more cytoplasm, nuclei are more vesicular, and nucleoli are prominent^[42]. Focal necrosis is often observed. These tumors exist alone or associated with adjacent adenomas or conventional adenocarcinomas^[43]. LCNECs are defined through both NE morphology and immunohistochemical positivity of NE markers.

CK20 is expressed in colorectal LCNECs; a generally acknowledged marker in adenocarcinoma which is rarely found in other NETs^[44,45]. This phenomenon may indicate that there is some relationship between LCNECs and adenocarcinomas.

LCNECs have a similar prognosis to colorectal SCCs. Kumarasinghe *et al.*^[46] suggested that LCNECs should be included in the poorly-differentiated NE carcinoma category based on their poor prognosis.

Mixed endocrine-glandular neoplasm

Mixed endocrine-glandular neoplasms constitute a heterogeneous group of rare neoplasms. These tumors are composed of at least two distinct tumor populations, with the NE part containing at least 30% of obviously endocrine cells^[14,47,48]. The degree of NE cell differentiation varies between tumors and between different tumor areas. The most common component is carcinoid, while poorly-differentiated NE type also exists, and another component can be adenocarcinoma or squamous carcinoma^[14,15,49]. The NE component is usually well-differentiated, and easily recognized by its suggestive histological features; the NE nature of the tumor cells can be verified by the immunodetection of specific NE markers (such as CgA and Syn). Especially when the NE component is poorly differentiated, the demonstration of NE markers to confirm the diagnosis is needed^[50]. More often, the diagnosis cannot be made until the tumors are stained with NE cell markers when the endocrine cells are inconspicuous and the quantity present is not obvious^[1].

Because of their rarity and unusual presentation, the optimal strategy of management of mixed endocrine tumors is largely unknown. The more aggressive component of the mixed endocrine tumors must be taken into account when considering their treatment. Mixed tumors containing a well-differentiated NE component and an adenocarcinoma component are suggested to be treated as adenocarcinoma. Mixed tumors containing a poorly-differentiated NE component must be treated as poorly-differentiated NE carcinomas^[50]. Some researchers report mixed NETs as having worse prognosis, thus the ascertainment of the NE component is important to predict the prognosis^[48].

MOLECULAR GENETICS OF COLORECTAL NETs

The molecular mechanisms of NET tumorigenesis are

Table 2 Proposed staging system for rectal and colon NETs^[62,63]

	Rectal	Colon
Depth of invasion and size		
T1	Up to and into muscularis propria, ≤ 1 cm	Any depth of invasion, ≤ 1 cm
T2	Up to muscularis propria, > 1 to ≤ 2 cm Beyond muscularis propria, ≤ 1 cm Into muscularis propria, > 1 to ≤ 2 cm Up to and into muscularis propria, > 2 cm	Up to or including muscularis propria, > 1 to ≤ 4 cm Beyond muscularis propria, > 1 to ≤ 4 cm Up to or including muscularis propria, > 4 cm
T3	Invasion beyond muscularis propria, > 1 cm	Beyond muscularis propria, > 4 cm
Lymph node		
N0	No lymph node metastasis	
N1	Regional lymph node metastasis	
Distant metastasis		
M0	No distant metastasis	
M1	Distant metastasis	
Stage		
I	T1; N0; M0	
II	T1; N1; M0 or T2; Any N; M0	
III	T3; N0; M0 or T3; N1; M0	
IV	Any T; Any N; M1	

unclear; however, these aspects have been focused on in many recent reports^[51]. Increased knowledge of the molecular background for the development of NETs may improve the management of these tumors in the future. A number of genetic syndromes including multiple endocrine neoplasia syndrome-type 1 (MEN1), von Hippel-Lindau syndrome, and neurofibromatosis-type 1 may be associated with intestinal NETs^[8,52]. Some studies have demonstrated neither classic oncogenes (*src*, *ras*, *myc*, *fos*, *jun*) nor suppressor genes (*P53*, *RB*) present in NETs^[52,53]. However, there are reports of other gene site deletions. For example, *PDCD4* was found to be absent in NETs; this is a gene which is interrelated with cell proliferation, located at 11q13 close to *MEN1*^[54].

The microsatellite instability (MSI) caused by mismatch repair (MMR) damage is an important cause of tumorigenesis in hereditary nonpolyposis colorectal cancer (HNPCC)^[55-58]. Stelow *et al.*^[59] discovered the loss of *MMR* genes in colorectal SCCs, but not along with HNPCC. The chromosome instability caused by the loss of *MMR* genes possibly follows a different pathway from MSI in SCC tumorigenesis. Arnold *et al.*^[60] studied 34 poorly-differentiated colorectal NETs, 38 well-differentiated benign or malignant fore-/midgut NETs, and 150 sporadic colorectal cancers with known MSI status and found that 20/34 (59%) colorectal NETs *vs* 11/38 (29%) fore-/midgut NETs were CpG island methylator phenotype (CIMP) positive. The Ki-67 index was significantly higher in poorly-differentiated colorectal NETs compared with the less malignant fore-/mid-gut NETs. However the CIMP status did not correlate with survival.

Research in NET molecular genetics should target early detection, prognosis predicting, or treatment selection. Currently, new technologies should be used to find new sensitive and specific biochemical and tissue markers for colorectal NETs.

PROPOSED TNM STAGE AND RISK FACTORS

NETs are a challenging group of diseases, and the lack of a widely accepted staging system limits the clinician's ability to provide meaningful prognostic information to the patients. In October 2009, the 7th edition of the AJCC for the first time gave a detailed TNM description for NETs, classifying primary tumor (T), regional lymph nodes (N), and distant metastasis (M), respectively. However, no clarified TNM staging has been established for colon and rectal NETs^[61].

In 2008, Landry *et al.*^[62,63] proposed a different staging system from that of conventional colorectal adenocarcinomas in the study of 4710 rectal NETs and 2459 colon NETs (Table 2). In their report^[62], it was revealed that rectal NETs were the only primary malignancy in 82% of patients, 17% patients had one additional malignancy and 1% had two or more additional malignancies. The mean and median sizes of primary tumor were 1 and 0.6 cm, respectively. About 4.1% had regional lymph node metastases, and 2.4% presented with distant metastases at the time of diagnosis. The 5-year survival rates for patients with stages I and II disease were 97% and 84%, respectively. The 5-year survival rate for stage III patients was 27% with a median survival time of 45 mo. The 5-year survival rate for stage IV patients was 20% with a median survival time of 31 mo. The 10-year survival rates of the disease for stages I through IV were 91%, 56%, 14%, and 2.5%, respectively.

In another study^[63] of colon NETs, the researchers demonstrated that there were regional lymph node metastases in 48% of patients, and distant metastases in 24% of patients at the time of diagnosis. The 5-year survival rates for stages I and II were 97% and 69%, respectively. Similarly, the 5-year survival rate for stage III disease was 21%, with a median survival time of 27 mo.

The 5-year survival rate for stage IV disease was 17%, with a median survival time of 20 mo. The 10-year survival rates for stages I through IV were 92%, 47%, 15%, and 5.4%, respectively.

Some studies have suggested some risk factors of colorectal NETs related to poor outcome, such as tumor size, depth of wall penetration, presence of lymphatic or venous invasion, and mitotic rate of metastasis in colorectal NETs^[64-69]. The tumor size is related to prognosis. Most rectal NETs are less than 1 cm at the time of diagnosis^[70,71]. The malignancy rate increases when the tumor grows larger than 1 cm. However, for small-sized tumors less than 1 cm at detection with predominant submucosal invasion there is usually a relatively high incidence of distant metastasis, about 1.7%-3.4%^[71,72]. Some reports have shown that cancer-specific survival of patients with colorectal NETs without metastasis was better than that of those with adenocarcinomas. However, the survival rate is similar between carcinoid and adenocarcinoma if the tumors have lymph node or distant metastases^[65]. On the other hand, colorectal NE carcinomas behave aggressively and are associated with a worse prognosis than that of conventional colorectal adenocarcinomas of the same stage^[16,39].

CONCLUSION

The complexity, heterogeneity and scarcity of NETs, rare colorectal neoplasia, account for almost no improvement of survival rates in the past 30 years. The diagnosis for colorectal NETs mostly depends on pathology with the aid of immunohistochemistry. As we have summarized here, the 2000 WHO classification of colorectal NETs provides a solid basis for localization, biology and prognosis of individual NETs, but there are still some types of tumors not properly described. More studies will be needed with regard to diagnosis, treatment and prognosis.

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Real-time sono-elastography in the diagnosis of diffuse liver diseases

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RESULTS: Due to the limitations of the method, we obtained high-quality elastography information in only 73.48% of the patients. The κ -means clustering method was applied to assess the inter-observer diagnosis variability, which showed good variability values in accordance with the experience of ultrasound examination of every observer. Cohen's κ test indicated a moderate agreement between the study observers ($\kappa = 0.4728$). Furthermore, we compared the way the two observers clustered the patients, using the test for comparing two proportions (t value, two-sided test). There was no statistically significant difference between the two physicians, regardless of the patients' real status.

CONCLUSION: Transabdominal real-time elastography is certainly a very useful method in depicting liver hardness, although it is incompletely tested in large multi-center studies.

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Key words: Elastography; Ultrasonography; Liver steatosis; Viral hepatitis; Liver cirrhosis

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Abstract

AIM: To analyze whether computer-enhanced dynamic analysis of elastography movies is able to better characterize and differentiate between different degrees of liver fibrosis.

METHODS: The study design was prospective. A total of 132 consecutive patients with chronic liver diseases and healthy volunteers were examined by transabdominal ultrasound elastography. All examinations were done by two doctors.

INTRODUCTION

Palpation continues to be of great value in modern medicine, both practiced by doctors and as a technique

for self-examination. However, palpation is limited to a few accessible organs, and the interpretation of the information sensed by the fingers is highly subjective. Recently, elastography has emerged as an option in several commercial ultrasound systems, and is starting to prove clinically valuable in many areas, particularly for example in assisting breast cancer diagnosis^[1,2], or in guiding minimally invasive treatment of prostate cancer^[3,4]. The technique reveals the physical properties of the tissue by characterizing the difference in hardness between diseased and surrounding tissue^[5,6]. The method measures mechanically induced deformation (strain) of structures in the B mode image to quantify the elasticity of the tissue. By measuring the tissue strain induced by compression, it is possible to estimate the tissue hardness. The region of interest for the elastography calculations is selected manually and should include the targeted area and the surrounding tissues^[7].

Elasticity measurements have been reported to be useful for the diagnosis and differentiation of many tumors, which are usually harder than normal surrounding tissues^[8-10]. Furthermore, different solid tumors situated near the gastrointestinal tract might be also visualized by endoscopic ultrasound (EUS) elastography and potentially characterized by this technique. EUS elastography has been used in several studies for the characterization and differentiation of benign and malignant lymph nodes, with variable sensitivity, specificity, and accuracy, with better results than those obtained by conventional EUS criteria^[11-13]. Furthermore, the feasibility of EUS elastography has been tested in pancreatic diseases, with very good results^[14,15].

Recently, transabdominal real-time elastography was proposed as a new method for noninvasive staging of liver fibrosis^[16-18]. For many years, liver biopsy was the only method to evaluate liver fibrosis and it has traditionally been considered as the gold standard^[19]. However, it is a painful invasive method associated with poor patient compliance, discomfort and, in very rare cases, with serious complications^[16,20]. High inter-observer variability has been reported between two pathologists when analyzing the same biopsy sample^[19,21]. Therefore, recent research has been focused on the evaluation of noninvasive methods for the assessment of liver fibrosis, both by biochemical tests as well as imaging methods^[22], as an alternative to liver biopsy.

The aim of this study was to analyze whether computer-enhanced dynamic analysis of elastography movies is better able to characterize and differentiate between different degrees of liver fibrosis. We previously have reported that analysis of selected elastography images is operator-dependent, therefore, our approach was to use a dynamic analysis of several frames of elastography movies that might reduce possible selection bias or artifacts.

MATERIALS AND METHODS

Patients

The study design was prospective. A total of 132 conse-

cutive patients with chronic liver diseases and healthy volunteers were examined by transabdominal ultrasound elastography during an 18-mo period in the Research Center of Gastroenterology and Hepatology, University of Medicine and Pharmacy Craiova, Romania. Patients (73 men and 59 women) were 26-74 years old. As a result of the limitations of the method, only 97 patients were considered for further elastography analysis as follows: healthy volunteers ($n = 27$), chronic viral B and C hepatitis ($n = 26$), and liver cirrhosis ($n = 29$), fatty alcoholic liver disease ($n = 21$). Chronic viral hepatitis was proven by the presence of hepatitis C virus antibodies or hepatitis B surface antigen in serum and the persistence of liver inflammation or liver parameter alterations for > 6 mo. In all the patients with chronic viral hepatitis, liver biopsy was performed 1-3 d before real-time elastography. Liver cirrhosis was proven by clear demonstration of portal hypertension signs (including esophageal and/or gastric varices) in a clinical suggestive setting. Patients with ascites were excluded from the study. Fatty alcoholic liver disease was diagnosed by ultrasound aspects of the liver and excessive alcohol intake (> 30 g daily), in the absence of viral infection and any doubt of cirrhosis. The control group consisted of healthy adult volunteers [normal levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST); negative tests for blood viral markers] who did not have a history of relevant concomitant illness or cancer. None of the healthy volunteers had an excessive daily alcohol intake (< 10 g daily).

Examination protocol

EUS elastography equipment includes a Hitachi EUB 8500 ultrasound system with an embedded elastography module (Hitachi Medical Systems Europe Holding AG, Zug, Switzerland) and a 6.5-MHz linear probe. The same conditions of brightness, contrast, intensity, and gain of the ultrasound system were used in all examinations. However, because the numeric elastography information is displayed using a rainbow color-coded scale, with values from 1 to 256, changes in the system settings did not affect the subsequent post-processing analysis.

All examinations were done by two doctors with different degrees of experience in ultrasound, a proficient physician (SA) and a physician beginner in ultrasound technique (GDI), in a typical clinical setting with previous knowledge of the patient's underlying disease. The ultrasound probe was placed in a convenient right intercostal space and elastography information was gathered during breath holding at the end-expiration phase. Three 10-s movies were recorded by each examiner for every patient, with a region of interest set to include the liver and surrounding tissues (Figure 1A-C). The movies were stored uncompressed at maximum quality for further accurate computer-enhanced analysis.

Liver biopsy was performed through the right intercostal space in the right liver lobe after transabdominal ultrasound found the most useful area. After betadine disinfection and local anesthesia, liver biopsy was per-

formed at the previously marked site. Liver fibrosis stages were evaluated semi-quantitatively according to the Metavir scoring system^[23-25]. Liver fibrosis was staged on an F0-F4 scale: F0-no fibrosis, F1-portal fibrosis without septa, F2-portal fibrosis with few septa, F3-numerous septa without cirrhosis, and F4-cirrhosis.

Computer-enhanced elastography movies analysis

The elasticity of tissue was reconstructed within the region of interest and translated into a color signal that overlay the grey scale image. To visualize tissue elasticity patterns, different elasticity values were marked with different colors (on a scale of 1 to 256). The system was set-up to use a hue color map (red/green/blue), where hard tissue areas were marked with dark blue, medium hard tissue areas with cyan, intermediate tissue areas with green, medium soft tissue areas with yellow, and soft tissue areas with red. The complete spectrum from blue to red was applied to every elastography record and indicated the graduation of relative elasticity within the region of interest. The quality of tissue compression was indicated by a numeric scale from 1 to 7 within the image.

Each recorded elastography movie was subjected to a computer-enhanced dynamic analysis using a public domain Java-based image processing tool (Image J)^[26] with a special plug-in developed by the IT Department of the University of Medicine and Pharmacy, Craiova. The plug-in was used to compute and dynamically analyze the individual hue histograms of each frame from an elastography movie; all programmers and statisticians being blinded to the clinical and pathological information to minimize the human bias. Shortly, every color frame was transformed into numerical form, and characterized by a single average hue histogram vector. Each individual value of the vector corresponded to the number of pixels of each color, in other words, to the number of pixels that corresponded to each elasticity level, from 1 to 256. The numeric values displayed by computer were offered to the statistical team and analyzed.

Statistical analysis

A prospective cross-sectional study was carried out on 97 samples of elastography records from chronic hepatitis patients and healthy volunteers recorded by two doctors. Using all clinical and laboratory test data, expert doctors (Săftoiu A and Gheonea DI) diagnosed the patients into four types (normal, liver steatosis, chronic hepatitis, and cirrhosis). First, an a priori power analysis determined the acceptability of the patient record sample size. Second, the κ -means clustering algorithm was applied to assess the inter-observer diagnosis variability. Basically, we used the κ -means algorithm to cluster automatically the 97 patients into four groups that corresponded to the four types of diseases; furthermore we compared these clusters with the final diagnosis. Finally, the analysis of variance, comparison of the proportions of well-diagnosed patients, Cohen's κ statistics, the proportion of agreement between the two examiners, and

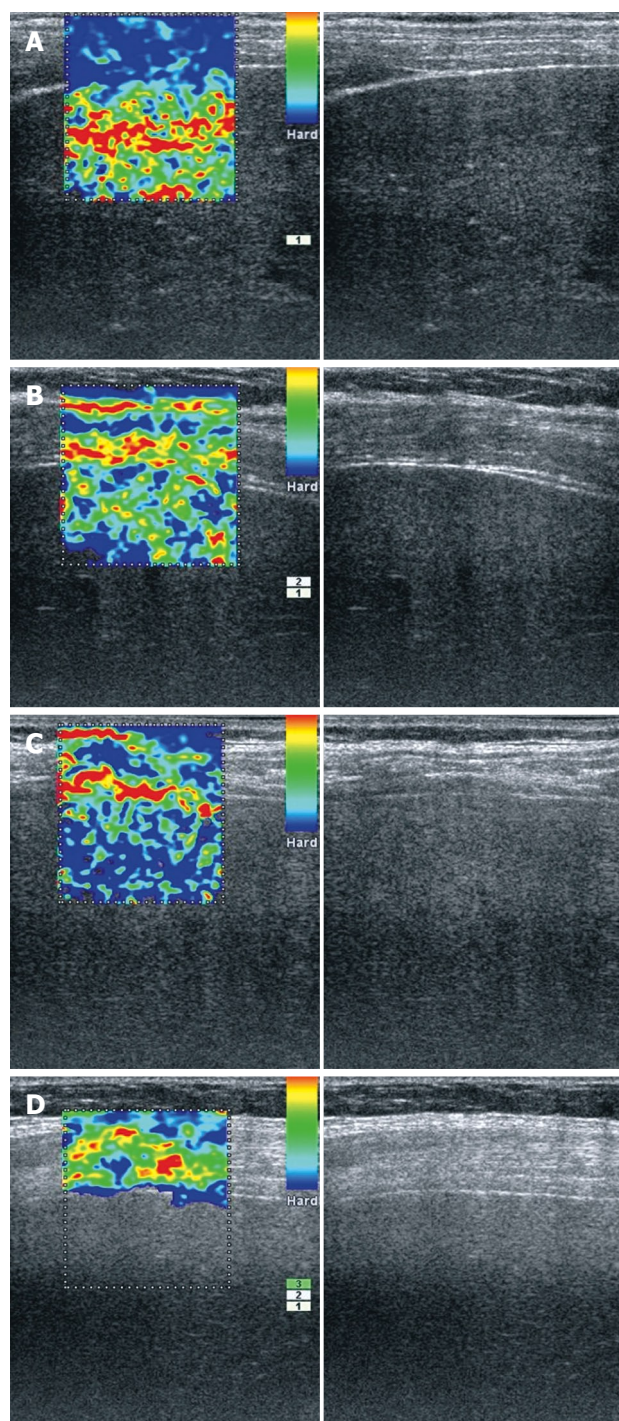


Figure 1 Real-time elastography images of right liver lobe. A: 58-year-old patient with alcoholic liver steatosis - a very soft liver parenchyma (red/yellow/green) in contrast with hard intercostal muscles and diaphragm (blue); B: 56-year-old patient with chronic hepatitis C - parenchyma with mixed appearance (green/blue) indicative of elasticity; C: 57-year-old patient with alcoholic cirrhosis - very hard liver parenchyma (predominantly blue); D: 67-year-old obese woman with chronic hepatitis. The elastography software was not able to characterize elasticity inside liver.

Stuart-Maxwell's statistics were performed to evaluate the appropriateness of each doctor's diagnosis and the inter-observer reliability between expert doctors.

Furthermore, statistical analysis concerning the liver biopsy Metavir scores (F1, F2, F3) compared with the

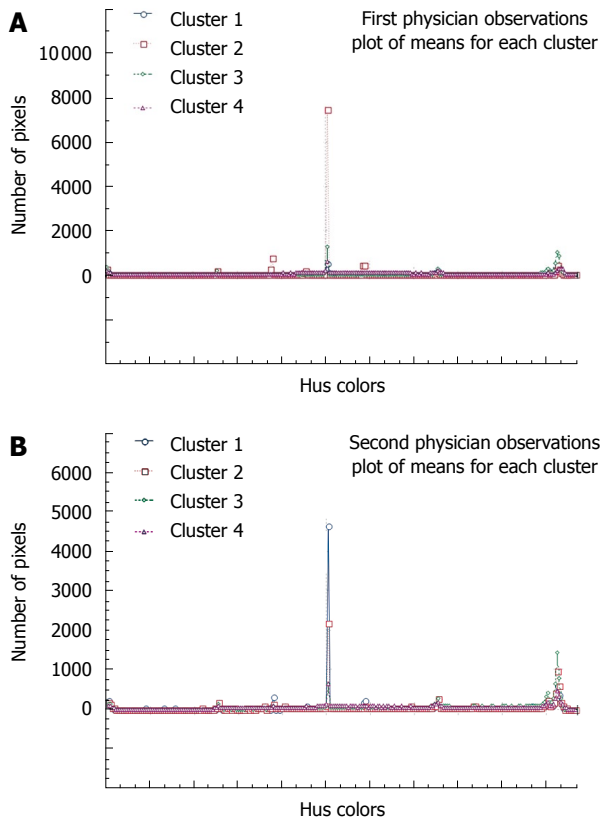


Figure 2 Graphs showing the way the first (A) and the second investigator (B) clustered the patients.

elasticity value (determined by computer-enhanced elastography movies assessment) was performed using Spearman's correlation coefficient.

RESULTS

A total of 132 consecutive patients were examined by real-time elastography using the right intercostal space as an acoustic window for gathering elasticity information about the liver. Due to the limitations of the method concerning the low penetrability of elastography into the tissues (Figure 1D), we obtained high-quality elastography information in only 97 patients (73.48%). The two examiners categorized the study patients into four groups (cirrhosis, chronic hepatitis, alcoholic steatosis, healthy volunteers) in accordance with the determined clinical status. Furthermore, the results of computer-enhanced analysis of the elastography movies were compared statistically.

First, we performed a power analysis to determine the acceptability of the patient record sample size, because one of the main goals of this study was to investigate the appropriateness of each observer examination and the inter-observer reliability between expert doctors. Accordingly, a sample of 97 records were considered, which provided a statistical power of 95% (type I error rate $\alpha = 0.10$).

Second, the κ -means clustering method was applied to assess the inter-observer diagnosis variability. It aimed to distinguish between different groups of patients and

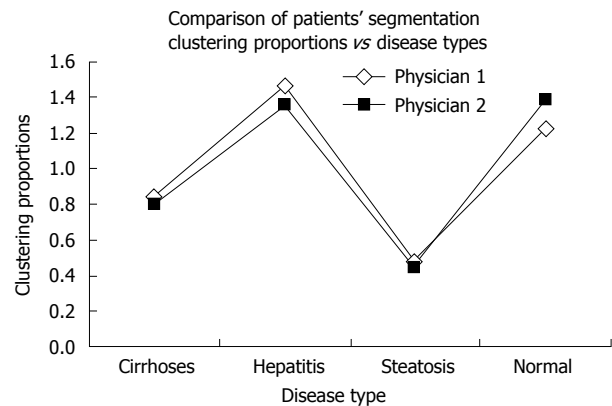


Figure 3 Patient segmentation across the four types of status, showing a similar manner of clustering the patients, irrespective of examiner.

can therefore be used to enhance knowledge of diseases and make automatic diagnosis predictions. Technically, based on each observer film computer analysis, we have used the κ -means algorithm to cluster automatically the 97 patients into four groups corresponding to the four types of status. Next, we compared the results of the two segmentations of patients, which corresponded to each doctor's diagnosis, to investigate the possible differences between them. To evaluate the appropriateness of each examination, we used the well-known analysis of variance, which compared the within-cluster variability (small if the classification was good) to the between-cluster variability (large if the classification was good). Thus, we obtained a very good classification for Săftoiu A ($P = 0.014$) and a poorer classification for Gheonea DI ($P = 0.15$), which meant that the first decision was better than the second one (more homogeneous clusters and more different from each other). This implied that the first observer was much more experienced than the second in performing elastography. We also considered the two graphs of the means across clusters, which corresponded to each examiner (useful for visually summarizing the differences in means between clusters). As we saw from these two graphs (Figure 2A and B), there was no significant visual difference between the two doctors' ways of clustering the patients, even if the previous analysis of variance showed a difference between them. Moreover, we considered the patients' segmentation across the four types, which showed a similar way of clustering the patients, irrespective of examiner (Figure 3). Besides the above approaches used to assess the inter-observer variability, we applied the test for comparing two proportions (t value, two-sided test). We found that there was no statistically significant difference between the two physicians' computer-enhanced movie analysis, regardless of disease type: cirrhosis ($P = 0.54$), chronic hepatitis ($P = 0.85$), steatosis ($P = 0.81$), and healthy subjects ($P = 0.78$).

Another way of analyzing the agreement between the two observers was application of Cohen's κ test as a measure of association between the two measurements (categorical variables), which consisted of the real-time elastography examinations performed by the two doctors.

Table 1 Cohen's κ test as a measure of association between the two measurements showed moderate agreement ($\kappa = 0.4728$) for the two study examiners

Observed κ	Standard error	95% CI	
		Lower limit	Upper limit
0.4728	0.0762	0.3235	0.6221

We considered for each examiner only one examination per patient, which gave a sample of 97 records. Each examiner classified each patient into one of the following categories: steatosis, normal, hepatitis, cirrhosis, thus, the 97 records referred to the above four categories. The four categories were nominal, therefore, Cohen's simple unweighted coefficient κ was the only form that could meaningfully be used (Table 1). According to Landis and Koch^[27], $\kappa = 0.4728$ indicated a moderate agreement between the two doctors. Thus, we conclude that, of all the decisions that we would have expected to be non-concordant if nothing more than coincidence were operating, 47.28% of the decisions were in fact concordant.

Independently of the Cohen's κ value, it is also possible to measure the proportion of agreement between the two observers^[28] within each of the four categories separately (confidence intervals for proportions are calculated according to the Wilson efficient-score method, corrected for continuity). Table 2 shows that the greatest agreement concerned normal patients (84.85%), while the smallest was for patients with cirrhosis (33.33%). In addition, we used Stuart-Maxwell's test for the four categories as a measure of the overall disagreement between the two doctors. The null hypothesis H_0 was that the distribution of diagnosis type among the four categories was the same for the two observers. Using the corresponding χ^2 statistics with 4-1 (3) degrees of freedom, the corresponding significance level ($P = 0.001$) showed that we could reject hypothesis H_0 , that is, there seemed to be a significant overall disagreement between the two doctors.

Although all the correlation methods applied for determining the correspondence between real status of the examined patients (normal, liver steatosis, chronic hepatitis, cirrhosis) and elasticity assessed by real-time elastography were positive, we could not establish a good correlation between the Metavir score (chronic hepatitis patients) and the results of elastography movies analysis (results not shown).

DISCUSSION

Recently, elastography has been presented as a new ultrasound-associated technology for the assessment of tissue elasticity. Computer-enhanced dynamic analysis of liver elastography movies was the objective of our current study. Furthermore, we studied the inter-observer variability and the correspondence between elastography and clinical (final) diagnosis. This approach would also eliminate the selection bias induced by analysis of static

Table 2 Wilson efficient-score method, corrected for continuity to measure the proportion of agreement between the two observers

Decision	Proportions of agreement			95% CI	
	Maximum possible	Chance expected	Observed	Lower limit	Upper limit
Normal	0.8485	0.2169	0.5250	0.3634	0.6818
Steatosis	0.5789	0.1961	0.3953	0.2537	0.5555
Hepatitis	0.5517	0.1381	0.5000	0.3168	0.6832
Cirrhosis	0.3333	0.0089	0.3333	0.0177	0.8747
Composite	0.7882	0.3084	0.6353	0.5232	0.7350

The greatest agreement concerned normal patients (84.85%), while the smallest concerned cirrhosis patients (33.33%).

images^[7], because it takes into account the information contained in several frames of a liver elastography movie.

The first goal of our statistical analysis was to see if percutaneous elastography of the liver was able to diagnose correctly the real status of the patients. We analyzed three independent cine-loop elastography examinations recorded by two separate examiners who were blinded to each other. For each patient, we thus recorded six real-time cine-loop elastography examinations, which were further analyzed through hue histogram analysis, with averaged values for a 10-s cine loop. Although the aspects of the images suggested the correct diagnosis (Figure 1A-C), we automatically analyzed every frame of the movies recorded by the two investigators. To evaluate the appropriateness of each examination, the κ -means clustering method was performed, which showed agreement between the clinical diagnosis and the results of computer analysis of the recorded movies. The results were very good, especially for the first investigator (Săftoiu A), thus proving that experience in performing ultrasound is important in obtaining quality and accurate recordings of the study patients.

As we previously suggested^[7], the region of interest for all the movies was set to include also the surrounding liver tissue (fatty tissue, intercostals muscles, diaphragm, peritoneum), which were considered to have the same elasticity in all patients despite their subsequent disease. We do not believe that inclusion of hepatic vein branches^[29] would be very useful as a reference structure, because the presence of vessels induces clear artifacts in the elastography images (Figure 4A), similar to the presence of ascites (Figure 4B). This is easily understandable if we look at the colors displayed by the real-time elastography software in the region of interest. In the presence of a very highly elastic structure such as the hepatic vein or ascites, the rest of the liver would be depicted as hard, irrespective of its elasticity. However, the development of ascites is a strong indicator for the presence of cirrhosis, which makes noninvasive staging of fibrosis unnecessary.

Liver biopsy is an important diagnostic tool and helps therapeutic decision making in chronic liver diseases patients^[30]. Histopathological examination was considered the most appropriate method in chronic hepatitis for assessing changes after antiviral therapy^[31], and is considered

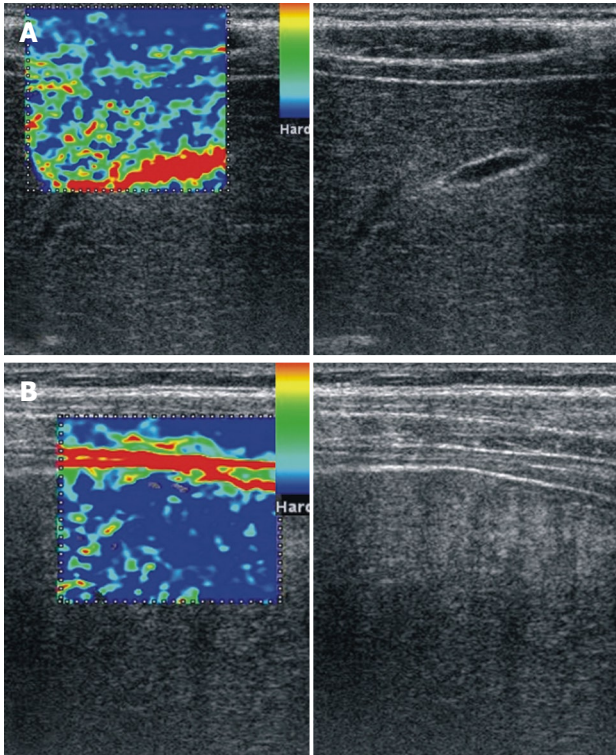


Figure 4 Real-time elastography images of right liver lobe. A: 45-year-old patient with alcoholic liver steatosis. Region of interest include a large vessel, therefore, the liver parenchyma had a hard appearance (blue/green) in contrast with the extremely compressed (red) vessel; B: 52-year-old patient with liver cirrhosis and small amount of ascites surrounding the liver. There was very compressive and elastic fluid (red), and the liver parenchyma was depicted as very hard (blue). Consequently, other types of ascites might possibly induce similar artifacts, even in the presence of normal liver tissue.

mandatory for grading and staging in most patients. The question is whether liver biopsy can be regarded as the gold standard for the staging and grading^[32] of diffuse liver diseases when risks of biopsy, inadequate sampling, and intra-observer and inter-observer error are taken into account^[30]. Our elastography statistical results showed a very good inter-observer variability analyzed by all three methods presented above. Therefore, computer-aided diagnosis of elastography calculations can be a very useful and reproducible method in depicting the hardness of the liver.

Even so, we were not able to distinguish between intermediate degrees of liver fibrosis (F1, F2, F3) in the chronic hepatitis patients subgroup in which we performed liver biopsy. To the best of our knowledge, there is only one published study^[16] that has succeeded partially in correlating the degree of fibrosis with real-time elastography calculation, especially in patients with $F \geq 2$. Unfortunately, the obtained values were calculated as means of static images selected by the examiner, which could have had a significant influence on the results. Furthermore, it is not clear if the region of interest was set to include also the surrounding tissues of the liver as a reference area.

One important limitation of our approach was the examination of the liver with a linear transducer of 6.5 MHz, which might be too high to examine the right

liver lobe correctly and consistently. We did not select only patients with a normal body mass index, therefore, in only 73.48% of the examinations did we obtain constant and high quality elastography information from the area of interest. The penetration of real-time elastography is limited to 3–4 cm, therefore, it is difficult to record useful elastography information inside the liver if the thoracic wall is thicker than 2–3 cm. A better option might be represented by the use of a lower frequency linear transducer, or another means of performing elastography of the liver, for example, by using EUS with the transducer placed in the stomach near the left liver lobe. Development of a pressure gauge is certainly necessary because manual application of pressure cannot be standardized. Usually, a small deformation ($< 2\%$) of the tissues is needed, and this is very difficult to obtain, even by experienced doctors.

In conclusion, transabdominal real-time elastography is certainly a very useful method in depicting liver hardness, although it has been tested incompletely in large multicenter studies and should be compared with other noninvasive methods (blood markers, transient elastography). We also suggest an improvement of the examination methodology, which should take into account previous observations made by different authors (better transducers, improved elastography software) to establish real-time elastography as a new revolutionary method that can replace liver biopsy for assessment of different stages of fibrosis in patients with chronic hepatitis.

COMMENTS

Background

Chronic liver diseases are marked by the gradual destruction of liver tissue over time, which eventually causes liver cirrhosis. Cirrhosis is the seventh leading cause of death in the United States, according to the National Institute of Diabetes and Digestive and Kidney Diseases. Liver biopsy is still the gold standard in many centers for quantifying liver fibrosis.

Research frontiers

Recently, research has focused on the evaluation of noninvasive methods for the assessment of liver fibrosis: routine hematological and biochemical tests, surrogate fibrosis markers in the blood and their algorithms, glycomics, proteomics, transient elastography, and real-time elastography.

Innovations and breakthroughs

Elasticity measurements have been reported to be useful for the diagnosis and differentiation of different diseases. Recently, transabdominal real-time elastography was proposed as a new method for noninvasive staging of liver fibrosis. The presents study clearly demonstrated that computer-enhanced dynamic analysis of elastography movies is better able to characterize and differentiate between different degrees of liver fibrosis.

Applications

Transabdominal real-time elastography is certainly a very useful method in depicting liver hardness and allows the replacement of other invasive methods, such as liver biopsy, which are associated with patient discomfort and mortality in some cases.

Terminology

Transabdominal real-time elastography is an imaging technique, completely noninvasive, that visualizes the tissue strain during compression that characterizes the difference in hardness between diseased and normal tissues. Tissue compression produces strain within the tissue. The strain is smaller in harder compared with softer structures.

Peer review

In the present study, the authors aimed to analyze whether computer-enhanced

dynamic analysis of elastography movies is better able to characterize and differentiate between different degrees of liver fibrosis. The topic is interesting, and novel.

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Effectiveness of narrow-band imaging magnification for invasion depth in early colorectal cancer

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[odds ratio (OR) = 402.5, 95% confidence interval (CI): 12.4-13133.1] and vessel regularity: negative (OR = 15.9, 95% CI: 1.2-219.1). Both of these findings when combined were an indicator of sm-d invasion with sensitivity, specificity and accuracy of 81.4%, 100% and 92.9%, respectively. Pit pattern diagnosis sensitivity, specificity and accuracy, meanwhile, were 86.0%, 98.6% and 93.8%, respectively, thus, the NBI with magnification findings of non-dense vessel density and negative vessel regularity when combined together were comparable to pit pattern diagnosis.

CONCLUSION: Non-dense vessel density and/or negative vessel regularity observed by NBI with magnification could be indicators of ECC sm-d invasion.

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Key words: Colorectal neoplasms; Narrow-band imaging; Microvasculature

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Abstract

AIM: To evaluate the surface microvascular patterns of early colorectal cancer (ECC) using narrow-band imaging (NBI) with magnification and its effectiveness for invasion depth diagnosis.

METHODS: We studied 112 ECC lesions [mucosal/submucosal superficial (m/sm-s), 69; sm-deep (sm-d), 43] ≥ 10 mm that subsequently underwent endoscopic or surgical treatment at our hospital. We compared microvascular architecture revealed by NBI with magnification to histological findings and then to magnification colonoscopy pit pattern diagnosis.

RESULTS: Univariate analysis indicated vessel density: non-dense ($P < 0.0001$); vessel regularity: negative ($P < 0.0001$); caliber regularity: negative ($P < 0.0001$); vessel length: short ($P < 0.0001$); and vessel meandering: positive ($P = 0.002$) occurred significantly more often with sm-d invasion than m/sm-s invasion. Multivariate analysis showed sm-d invasion was independently associated with vessel density: non-dense

INTRODUCTION

Magnified colonoscopy and the development of pit pattern diagnosis^[1] not only permits us to distinguish neoplastic from non-neoplastic colorectal lesions^[2-5], but also helps to assess the invasion depth of early colorectal cancers (ECC)^[6-9]. Similarly, vascular findings on the surface of gastric lesions have also been observed by

magnification endoscopy, and the usefulness in predicting the histological nature of such lesions and assessing their invasion depth has also been reported in the upper gastrointestinal (GI) tract^[10-12].

The recently developed narrow-band imaging (NBI) system is a noninvasive optical technique that uses reflected light that provides clearer images of surface microvascular architecture than the conventional observation modality^[13]. To date, the use of magnification endoscopy with the NBI system has been studied in the upper GI tract^[14-20] and the suitability of this new modality for differentiating neoplastic from non-neoplastic lesions and its potential for pit pattern diagnosis have also been reported for the lower GI tract^[21-30].

As previously indicated, colorectal lesions with mucosal (m) or submucosal (sm) superficial invasion $< 1000\ \mu\text{m}$ (sm-s) have an extremely low risk of lymph-node metastasis and are good candidates for endoscopic treatment^[31]. It is helpful therefore, to differentiate endoscopically between m/sm-s and deeper sm invasion (sm-d $\geq 1000\ \mu\text{m}$) lesions. There have been only a few reports concerning invasion depth diagnosis using NBI with magnification in a large series of cases, however, a number of questions remain regarding the comparative effectiveness of a diagnosis based on NBI observation and one using pit pattern analysis by dye chromoendoscopy for determining invasion depth.

Using magnification colonoscopy with the NBI system, we evaluated the characteristics of the surface microvascular architecture of ECC and investigated the effectiveness of this new optical modality for the diagnosis of invasion depth. In addition, we evaluated the comparative relationship between NBI with magnification and pit pattern diagnoses.

MATERIALS AND METHODS

NBI system

NBI is a novel technique that uses spectral narrow-band optical filters instead of the full spectrum of white light. It is based on the phenomenon that the depth of light penetration depends on its wavelength, with a short wavelength penetrating only superficially and a longer wavelength penetrating into deeper layers. In the NBI mode, optical filters that allow narrow-band light to pass at wavelengths of 415 and 540 nm are mechanically inserted between a xenon arc lamp and a red/green/blue rotation filter. Thin blood vessels such as capillaries on the mucosal surface can be seen most clearly at 415 nm, which is the wavelength that corresponds to the hemoglobin absorption band, while thick vessels located in the deep layer of the mucosa can be observed at 540 nm. Current NBI technology limits mucosal surface light penetration, thereby enhancing visualization of the fine capillary vessel structure on the surface layer.

Patients and evaluation methods

We studied a total of 112 ECC lesions $\geq 10\ \text{mm}$ analyzed with NBI with magnification colonoscopy examination, which then underwent endoscopic or surgical treatment at the National Cancer Center Hospital between January 2006 and February 2007. All colonoscopies were per-

formed with a PCF-Q240ZI or CF-H260AZI endoscope (Olympus Optical Co. Ltd., Tokyo, Japan) by three experienced endoscopists (MF, YS, TM) each of whom had annually performed more than 1000 magnifying chromoendoscopy examinations and at least 500 NBI examinations per year. Endoscopic images of each lesion were taken in the following order: conventional colonoscopy, NBI with magnification, chromoendoscopy and magnification chromoendoscopy. When a lesion was detected by conventional colonoscopy, its surface was washed with proteinase to remove excess mucus. Magnification NBI views of the microvascular architecture concentrated on those portions of the lesion where invasion seemed to have permeated the deepest regions, such as depressed areas and large nodules^[32,33].

After completion of NBI with magnification, the pit pattern of each lesion was assessed with magnification chromoendoscopy performed using 0.4% indigo-carmin (IC) dye spraying. When high magnification observation with IC dye did not permit us to determine adequately the surface structure for pit pattern analysis, 0.05% crystal violet was applied for staining^[7]. The visible pit pattern was then assessed during the course of the examination by the endoscopist conducting the procedure. All lesions were resected subsequently endoscopically or surgically and histological diagnosis was performed by three experienced pathologists based on the Vienna classification^[34,35]. The depth of sm invasion was determined as being either sm-s $< 1000\ \mu\text{m}$ or sm-d $\geq 1000\ \mu\text{m}$ ^[31]. After pathological diagnosis was completed on all resected lesions, three endoscopists (Fukuzawa M, Saito Y and Matsuda T) who performed the examination individually reviewed the endoscopic images of the NBI findings that were taken prior to treatment. All endoscopic images were chosen by one of these endoscopists. Their evaluation of the NBI images of the m/sm-s and sm-d lesions focused on the suspected areas, respectively, of higher grade dysplasia and deepest suspected invasion. Each characteristic of microvascular architecture was finally determined based on the agreement of at least two of the three reviewing endoscopists. Microvascular findings with a high frequency of sm-d were assessed as to whether those were significant sm-d indicators by univariate and multivariate analysis. In addition, sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) and diagnostic accuracy were calculated for each microvascular architectural feature observed during NBI, as well as every pit pattern diagnosis determined by magnification chromoendoscopy. We then compared the various types of microvascular architecture characteristics revealed by NBI with magnification to the chromoendoscopy pit pattern diagnoses.

The protocol for this study was approved by our institutional review board and all patients gave written informed consent.

Chromoendoscopy with magnification

Our pit pattern evaluation method relied on the clinical classification system proposed by Fujii *et al.*^[7] and Matsuda *et al.*^[8], with reference to the Kudo Classification System. Lesions were categorized into noninvasive and invasive

patterns. The noninvasive pattern included regular crypts with or without a demarcated area (e.g. depression, large nodule, or reddened area) and irregular pits without a demarcated area, and are usually observed in Kudo's types III_s, III_L, IV and V_I without demarcated areas (e.g. adenomatous polyps, m and sm-s cancers), with endoscopic resection being the appropriate treatment. The invasive pattern was characterized by irregular and distorted crypts in a demarcated area, as observed in Kudo's type V_N and V_I with a demarcated area (e.g. sm-d), and should be treated by surgical resection. As indicated, Kudo's type V_I can be observed in either noninvasive or invasive patterns. Those differences are dependent on the presence or absence of a demarcated area.

Microvascular architecture of ECC

Microvascular architectural images taken during magnification colonoscopy with NBI were reviewed retrospectively by three endoscopists who referenced the microvascular architectural features of superficial esophageal carcinoma^[15], and included the following characteristics: (1) caliber, narrow or wide; (2) caliber regularity, positive or negative; (3) meandering, positive or negative; (4) vessel regularity, positive or negative; (5) vessel length, short or long; and (6) vessel density, non-dense or dense. These characteristics were evaluated by comparing the NBI with magnification images to representative photographs of model examples (Figure 1).

Statistical analysis

We compared microvascular architecture as revealed by NBI with magnification to histological findings using the χ^2 test of independence or Fisher's exact test for univariate analysis. Variables with a *P* value of < 0.05 in our univariate analysis were subsequently included in a logistic regression multivariate analysis. The StatView program, version 5.0 (SAS Institute, Cary, NC, USA), was used for data analysis and *P* < 0.05 was considered to be statistically significant.

RESULTS

Clinicopathological features of patients and lesions

The clinicopathological details of the patients and colorectal lesions involved in this study are shown in Table 1.

Univariate analysis

Univariate analysis indicated characteristics involving vessel density: non-dense (*P* < 0.0001); vessel regularity: negative (*P* < 0.0001); caliber regularity: negative (*P* < 0.0001); vessel length: short (*P* < 0.0001); and vessel meandering: positive (*P* = 0.002) occurred significantly more often with sm-d invasion than m/sm-s invasion (Table 2).

Multivariate analysis

Multivariate analysis demonstrated that sm-d invasion was independently associated with vessel density: non-dense [odds ratio (OR) = 402.5, 95% confidence interval (CI): 12.4-1313.1]; and vessel regularity: negative (OR = 15.9, 95% CI: 1.2-219.1) (Table 2). The sensitivity, speci-

Table 1 Clinicopathological features of evaluated colorectal lesions

	m/sm-s	sm-d
Lesions (n = 112)	69	43
Gender (male/female)	42/27	24/19
Age (range, yr)	63.2 (37-79)	62.5 (32-80)
Location		
Right colon	29	15
Left colon	18	12
Rectum	22	16
Morphology ¹		
Ip/Is/Isp	21	18
IIa/IIa + IIc/IIc	10	16
LST-G	20	5
LST-NG	18	4
Mean size (range, mm)	32.3 (10-100)	24.4 (10-90)

¹Update on the Paris classification of superficial neoplastic lesion in the digestive tract^[36]. LST-G: Laterally spreading tumor-granular type; LST-NG: Laterally spreading tumour-non granular type; m/sm-s: Mucosal/submucosal superficial; sm-d: Submucosal-deep.

ficity, PPV, NPV and diagnostic accuracy rate for each characteristic are shown in Table 3. The two vascular findings that were confirmed by multivariate analysis had the highest values for specificity, PPV and accuracy (non-dense vessel density: specificity 0.99, PPV 0.95, accuracy 90.2%; negative vessel regularity: specificity 0.99, PPV 0.95, accuracy 90.2%).

Pit pattern diagnosis

The pit patterns of 21 m/sm-s lesions were evaluated following IC dye spraying, whereas the pit patterns of the other 48 m/sm-s lesions and all 43 sm-d lesions were assessed after crystal violet staining. We subsequently calculated the sensitivity, specificity, PPV, NPV and accuracy in differentiating m/sm-s from sm-d for: (1) the pit patterns that were diagnosed as being invasive; and (2) the NBI with magnification characteristic findings of (a) non-dense vessel density and/or negative vessel regularity and (b) non-dense vessel density and negative vessel regularity, which were both considered to be indicators for sm-d invasion. Pit pattern analysis sensitivity, specificity, PPV, NPV and diagnostic accuracy were 0.86 (95% CI: 0.72-0.95), 0.99 (0.92-0.99), 0.97 (0.86-0.99), 0.92 (0.83-0.97) and 93.8%, respectively. The NBI with magnification characteristic findings of non-dense vessel density and negative vessel regularity were comparable to pit pattern diagnosis results [0.81 (0.67-0.92), 1.00 (0.95-1.00), 1.00 (0.90-1.00), 0.90 (0.81-0.95), 92.9%] (Table 4). Seven of the lesions in this study were incorrectly diagnosed using pit pattern analysis including six sm-d lesions mistakenly diagnosed as m/sm-s invasion depth. In two of these cases, however, both non-dense vessel density and negative vessel regularity had also been observed by magnification NBI, which suggests its potential use as a supplementary diagnostic tool to pit pattern diagnosis (Figures 2 and 3).

DISCUSSION

It has been reported previously that observation of intra-

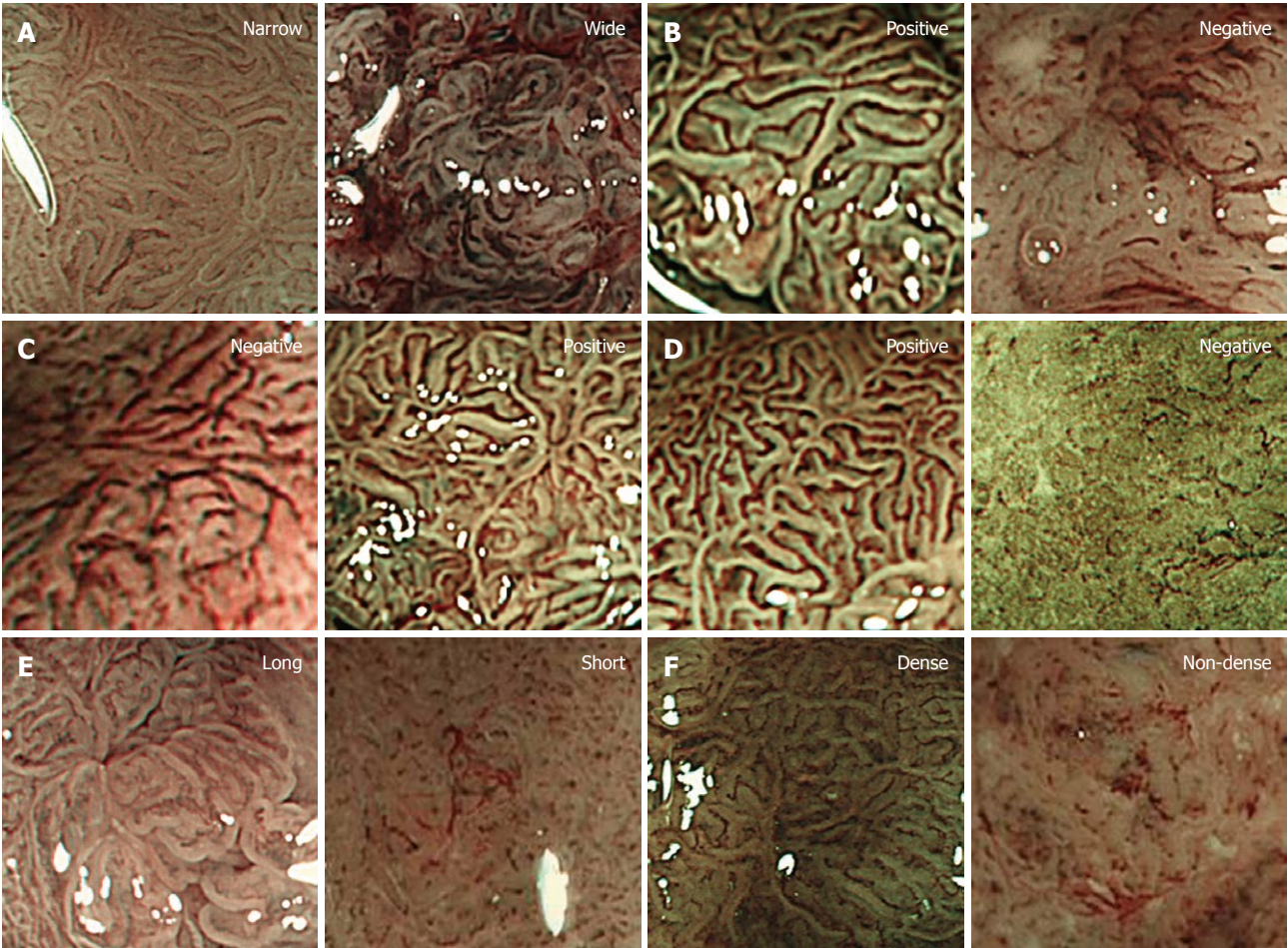


Figure 1 Microvascular architecture. A: Caliber, narrow: Capillaries are narrow diameter. Caliber, wide: Capillaries are wide diameter; B: Caliber regularity, positive: Capillaries are uniform thickness. Caliber regularity, negative: Capillaries are unequal thickness; C: Meandering, negative: Capillaries are linear. Meandering, positive: Capillaries are meandering; D: Vessel regularity, positive: Capillaries surround mucosal glands regularly. Vessel regularity, negative: Capillaries irregularly branching; E: Vessel length, long: Long capillaries. Vessel length, short: Short capillaries; F: Vessel density, dense: Dense capillaries. Vessel density, non-dense: Sparse capillaries.

Table 2 Microvascular architecture & invasion depth						
Variables			Univariate analysis <i>P</i> -value ¹	Multivariate analysis		
				<i>P</i> -value ¹	Odds ratio	95% CI
Vessel density	m/sm-s sm-d	Non-dense/dense	< 0.001	0.001	402.5	12.4-13133.1
		1/68 33/10				
Vessel regularity	m/sm-s sm-d	Negative/positive	< 0.001	0.038	15.9	1.2-219.1
		8/61 38/5				
Caliber regularity	m/sm-s sm-d	Negative/positive	< 0.001	0.056	17.3	0.9-323.4
		44/25 42/1				
Vessel length	m/sm-s sm-d	Short/long	< 0.001	0.161	0.2	0.01-2.10
		20/49 37/6				
Meandering	m/sm-s sm-d	Positive/negative	0.002	0.110	0.1	0.01-1.60
		49/20 41/2				
Caliber	m/sm-s sm-d	Wide/narrow	NS			
		62/7 41/2				

¹ χ^2 or Fisher's test. 95% CI: 95% confidence interval; NS: Not significant.

papillary capillary loops by magnification endoscopy is useful in the diagnosis of invasion depth of superficial

esophageal cancer^[10,11]. The intra-papillary capillary loops can be seen in the normal esophageal mucosa by mag-

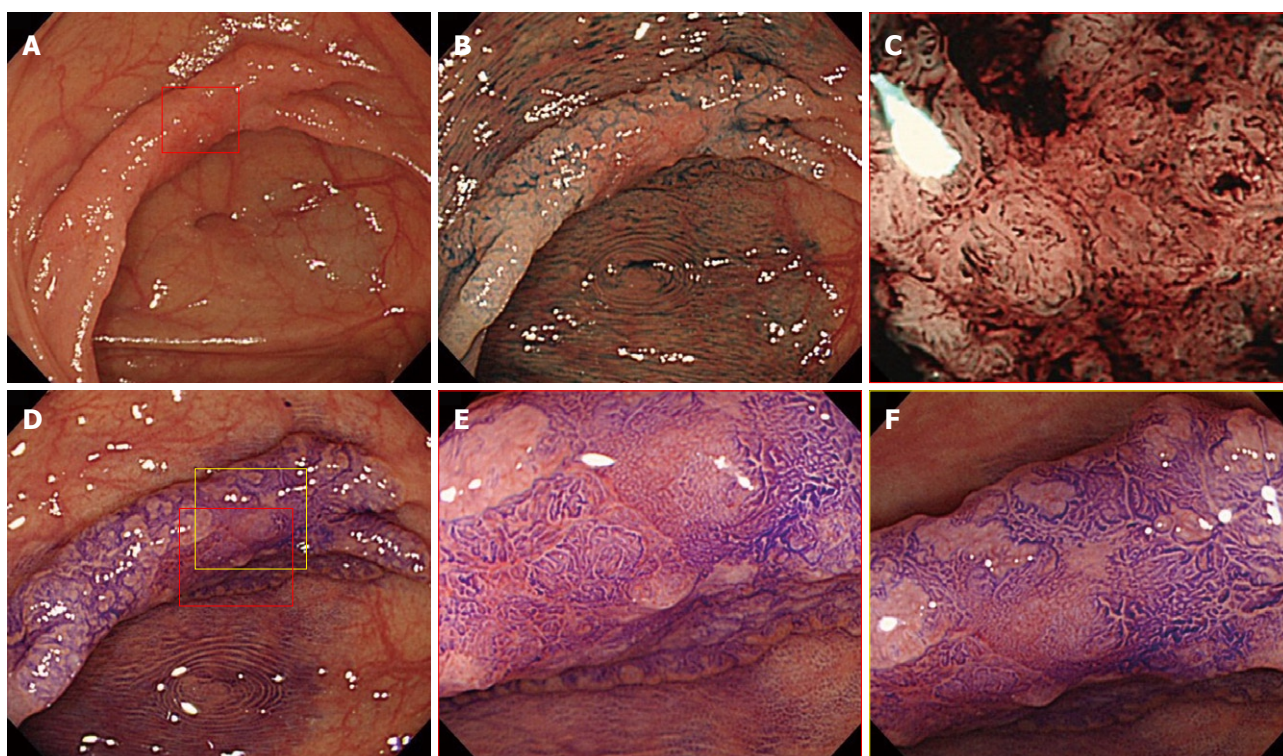


Figure 2 35 mm laterally spreading tumor, non-granular (LST-NG) type, located in the ascending colon. A: Conventional colonoscopy image; B: Conventional colonoscopy image following 0.4% IC dye spraying; C: Narrow-band imaging (NBI) with magnification image at center of the lesion enclosed by the red box in A. Microvascular architecture consisted of non-dense vessel density and negative vessel regularity; D: Crystal violet staining image; E: Magnification view of the portion enclosed by the red box in D revealed a noninvasive pattern; F: Magnification view of the portion enclosed by the yellow box in D also revealed a noninvasive pattern, such the estimated depth was intramucosal and this LST-NG lesion was treated by endoscopic submucosal dissection.

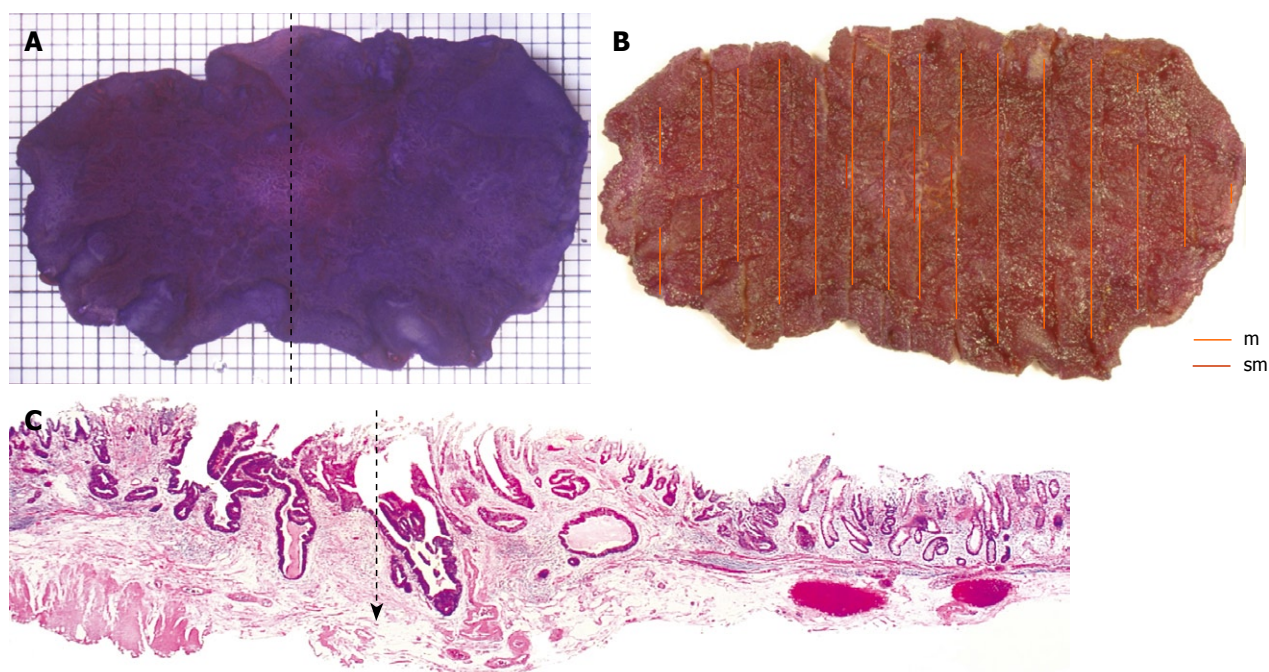


Figure 3 Stereomicroscopic view and histological images. A: Stereomicroscopic view; B: Red lines indicate submucosal penetration of the tumor; C: Histological diagnosis at dotted line in A was a well-differentiated adenocarcinoma and depth of invasion was sm (1300 mm) shown with the arrow. Invasion depth diagnosis using NBI with magnification was correct, based on findings of non-dense vessels and negative vessel regularity, but pit pattern diagnosis of this lesion was inaccurate.

nifying endoscopy. In cancerous lesions, characteristic changes of the intrapapillary capillary loops can be seen in the superficial mucosa according to the depth of tumor

invasion. There have been few studies to assess invasion depth in cancerous lesions from microvascular architecture. However, the NBI system enabled observation of

Table 3 Assessment of the carcinomatous invasion depth based on microvascular architecture

Microvascular architecture	Sensitivity (95% CI)	Specificity (95% CI)	PPV (95% CI)	NPV (95% CI)	Accuracy (%)
Vessel density	33/43	68/69	0.97 (0.85-0.99)	0.87 (0.78-0.94)	90.2
Non-dense	0.77 (0.61-0.88)	0.99 (0.92-0.99)			
Vessel regularity	38/43	61/69	0.83 (0.69-0.92)	0.92 (0.83-0.97)	88.4
Negative	0.88 (0.75-0.96)	0.88 (0.78-0.95)			
Caliber regularity	42/43	25/69	0.49 (0.38-0.60)	0.96 (0.80-0.99)	59.8
Negative	0.98 (0.88-0.99)	0.36 (0.25-0.49)			
Vessel length	37/43	49/69	0.65 (0.51-0.77)	0.89 (0.78-0.96)	76.8
Short	0.86 (0.84-0.99)	0.71 (0.59-0.81)			
Meandering	41/43	20/69	0.46 (0.35-0.56)	0.91 (0.71-0.99)	54.5
Positive	0.95 (0.84-0.99)	0.29 (0.19-0.41)			
Caliber	41/43	7/69	0.40 (0.30-0.50)	0.78 (0.40-0.97)	42.9
Wide	0.95 (0.84-0.99)	0.10 (0.04-0.20)			

PPV: Positive predictive value; NPV: Negative predictive value.

Table 4 Assessment of the carcinomatous invasion depth: comparison between microvascular architecture & pit pattern analysis

Microvascular architecture	Sensitivity (95% CI)	Specificity (95% CI)	PPV (95% CI)	NPV (95% CI)	Accuracy (%)
Non-dense vessel density	38/43	60/69	0.81 (0.67-0.91)	0.92 (0.83-0.97)	87.5
and/or negative vessel regularity	0.88 (0.75-0.96)	0.87 (0.77-0.94)			
Non-dense vessel density	35/43	69/69	1.00 (0.90-1.00)	0.90 (0.81-0.95)	92.9
and negative vessel regularity	0.81 (0.67-0.92)	1.00 (0.95-1.00)			
Pit pattern	37/43	68/69	0.97 (0.86-0.99)	0.92 (0.83-0.97)	93.8
(Invasive pattern)	0.86 (0.72-0.95)	0.99 (0.92-0.99)			

microvascular architecture of the tumor surface in the GI tract. In a similar fashion, we used NBI with magnification to investigate whether or not quantitative ECC invasion depth diagnosis was possible based on analysis of capillary vessel patterns instead of pit patterns. Based on our results, it appeared that non-dense vessel density and negative vessel regularity, as observed by NBI with magnification, could be diagnostic indicators of sm-d invasion, as effectively as pit pattern analysis.

Regular hexagonal or honeycomb-like capillary patterns are formed around the crypts of normal colorectal mucosa. In contrast, it has been reported that these capillaries are larger in tumor adenomas, whereas vascular disruption, caliber irregularity and dense vessels have been observed in severe atypical cases^[37]. In addition, vascular changes do not generally occur in non-neoplastic lesions such as hyperplastic polyps, with the exception of inflammatory polyps^[38]. The NBI technique provides clearer observation of microvascular architectural characteristics, therefore, it has been reported that differentiation of neoplastic from non-neoplastic lesions on the basis of different vascular patterns is equally possible using NBI or chromoendoscopy^[21-30], and pit pattern diagnosis has likewise been explored using NBI^[21,24,27,28]. Previous studies have shown that the accuracy of pit pattern diagnosis of invasion depth by magnification endoscopy was 98.8%^[8], whereas such diagnostic accuracy in this study was 93.8%.

The area surrounding crypts in the superficial layer of the mucosa is covered with capillaries and has previously been recognized as a pit using the NBI technique. Machida *et al.*^[21] have reported that NBI pit pattern diag-

nosis is significantly more useful ($P < 0.001$) than conventional observation, but inferior to chromoendoscopy ($P < 0.05$). Hirata *et al.*^[24] have reported that overall diagnostic consistency in pit patterns between magnification NBI and dye-spraying observations was 84%, but even higher for types II, III, IV and V_N pit patterns, although somewhat lower at 78%, for the type V_I pit pattern. In addition, Tischendorf *et al.*^[27] have reported that there is no significant difference in the PPV for neoplastic lesions as determined by pit pattern and vascular findings using NBI. There was a discrepancy, however, between two endoscopists in their NBI pit pattern diagnosis of types III-V neoplastic lesions^[27]. This may have been because the actual pit structure was not observed using the NBI technique, unlike the results from the contrast and staining methods; or, it could have been caused by the NBI pit pattern diagnosis of types III-V lesions, which are considered particularly important in determining the most suitable method of treatment, not having been performed accurately.

More recently, Katagiri *et al.*^[30] have reported that capillary patterns observed by NBI with magnification are highly accurate in distinguishing between low-grade and high-grade dysplasia/invasive cancer, and thus could be used to predict the histopathological features of colorectal neoplasia. In addition, Hirata *et al.*^[25] have reported vascular findings of significant sm-d invasion based on their NBI observation of thick blood vessels with irregularity on the surface of tumors. This differs somewhat from the results of our investigation, but the difference could be caused by a number of factors, such as variations in our respective definitions of vascular findings, and the macroscopic types of lesions involved in the two studies.

Magnification observation with dye spraying and staining, in particular crystal violet staining, however, can be time-consuming. Patient symptoms including abdominal discomfort and peristalsis are more likely to appear in longer duration colonoscopy examinations, which may render detailed observation more problematic. In contrast, the press of a single button on the handle of the endoscope with the NBI system can almost immediately change from NBI to the conventional view and back again, thereby shortening examination times and reducing the burden on patients and endoscopists alike. A mucous attachment on the endoscope can also interfere with diagnosis, and washing the surface of a lesion with pronase solution takes additional time during pit pattern diagnosis by magnification colonoscopy with IC dye spraying or crystal violet staining. Hirata *et al.*^[24] have further reported that NBI observation results in more accurate pit pattern diagnosis than dye spraying observation in cases with mucous attachment.

Our study suffered from some limitations. First, the NBI assessments were made on still images by three endoscopists, whereas the pit pattern diagnosis was done in real time after initial inspection with NBI, which could account for some further bias. Second, the different NBI features of the microvasculature are not independent: the endoscopist is not blinded to one feature if he scores the other. In addition, lesions that were diagnosed histologically as cancer had a diameter of at least 10 mm, thus lesions < 10 mm in diameter were not assessed in this study. Accordingly, future prospective studies will require that relevant data be accumulated and analyzed on a more objective basis.

In conclusion, the results of this study indicated that two microvascular architectural characteristics, non-dense vessel density and negative vessel regularity, observed using NBI with magnification during colonoscopy examinations could be reliable indicators of ECC sm-d invasion.

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COMMENTS

Background

The intra-papillary capillary loops can be seen in the normal esophageal mucosa by magnifying endoscopy. In cancerous lesions, characteristic changes of the intrapapillary capillary loops can be seen in the superficial mucosa according to the depth of tumor invasion. Narrow-band imaging (NBI) enables detailed observation of microvascular architecture of the tumor surface.

Research frontiers

NBI provides clearer observation of microvascular architectural characteristics, and it has been reported that differentiation of neoplastic from non-neoplastic lesions on the basis of different vascular patterns is equally possible using NBI or chromoendoscopy. However, there have been only a few reports concerning invasion depth diagnosis using NBI with magnification in a large series of cases. This study clarifies the efficiency of NBI with magnification colonoscopy for invasion depth diagnosis of early colorectal cancer (ECC).

Innovations and breakthroughs

Some studies have already reported the clinical usefulness of pit pattern

diagnosis using magnifying chromoendoscopy for predicting the depth of invasion of ECC. The authors' results indicate that NBI with magnification findings were comparable to pit pattern diagnosis results.

Applications

Magnification observation with dye spraying and staining, in particular crystal violet staining, however, can be time-consuming. In contrast, the press of a single button on the handle of the endoscope with the NBI system can almost immediately change from NBI to the conventional view and back again, thereby shortening examination times and reducing the burden on patients and endoscopists alike.

Peer review

The authors present a trial analyzing the impact of NBI colonoscopy on assessing the invasion depth in ECC. Overall, 112 patients were included; additionally pit pattern analysis was performed in 64 patients. The study investigated interesting questions.

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Expression of nesfatin-1/NUCB2 in rodent digestive system

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RESULTS: Immunohistochemical staining showed that the nesfatin-1/NUCB2 IR cells were localized in the central part of the pancreatic islets, the lower third and middle portion of the gastric mucosal gland, and the submucous layer of the duodenum in SD rats and ICR mice. HE staining revealed that the morphological features of nesfatin-1/NUCB2 IR cells were mainly islet cells in the pancreas, endocrine cells in the stomach, and Brunner's glands in the duodenum. Western blotting revealed that NUCB2 protein expression was higher in the pancreas, stomach and duodenum than in the esophagus, liver, small intestine and colon ($P = 0.000$).

CONCLUSION: Nesfatin-1/NUCB2 IR cells are expressed in the pancreas, stomach and duodenum in rodents. These cells may play an important role in the physiological regulation of carbohydrate metabolism, gastrointestinal function and nutrient absorption.

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Key words: Nesfatin-1; Nucleobindin-2; Pancreas; Stomach; Duodenum, Brunner glands

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Abstract

AIM: To observe the regional distributions and morphological features of nesfatin-1/nucleobindin-2 (NUCB2) immunoreactive (IR) cells in the rodent digestive system.

METHODS: Paraffin-embedded sections of seven organs (pancreas, stomach, duodenum, esophagus, liver, small intestine and colon) dissected from sprague-dawley (SD) rats and institute of Cancer Research (ICR) mice were prepared. The regional distributions of nesfatin-1/NUCB2 IR cells were observed by immunohistochemical staining. The morphological features of the nesfatin-1/NUCB2 IR cells were evaluated by hematoxylin and eosin (HE) staining. Fresh tissues of the seven organs were prepared for Western blotting to analyze the relative protein levels of NUCB2 in each organ.

Zhang AQ, Li XL, Jiang CY, Lin L, Shi RH, Chen JD, Oomura Y. Expression of nesfatin-1/NUCB2 in rodent digestive system. *World J Gastroenterol* 2010; 16(14): 1735-1741 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v16/i14/1735.htm> DOI: <http://dx.doi.org/10.3748/wjg.v16.i14.1735>

INTRODUCTION

Nesfatin-1, an anorexigenic peptide derived from nucleobindin-2 (NUCB2), is the latest addition to the surprisingly large number of chemical messengers telling the body that the "tank (appetite) is full"^[1]. Nesfatin-1

injected directly into the brain of rats promotes anorexia, whereas an antibody specific to nesfatin-1 injected into the brain potentially stimulates feeding^[1]. Previous studies have shown that nesfatin-1/NUCB2 immunoreactive (IR) cells are present in a number of discrete neuronal populations or nuclei, including the hypothalamic arcuate nucleus (ARC), paraventricular nucleus (PVN), supra-optic nucleus, lateral hypothalamic area, and the dorsal motor nucleus of the vagus and nucleus tractus solitarius in the brain stem^[1-5]. Energy homeostasis-regulating circuits are found within and connect these nuclei. NUCB2 gene expression and the nesfatin-1 concentration in the PVN were reduced after a fast of 24 h^[1]. Nesfatin-1 alters the membrane potential of different subpopulations of neurons within the PVN^[6]. In the ARC, nesfatin-1 exerts its anorexigenic effects by inhibiting the orexigenic neuropeptide Y neurons^[7]. In mice, 5-hydroxytryptamine (5-HT) systems, *via* 5-HT_{2c} receptors, upregulate the expression of hypothalamic NUCB2 and induce anorexia *via* a leptin-independent pathway^[8]. These observations indicate a physiological role of central nesfatin-1 in the regulation of food intake.

Two different groups have demonstrated that peripherally injected nesfatin-1 crosses the blood-brain barrier in a non-saturable way to reach brain tissues^[9,10]. Central and peripheral administration of nesfatin-1 resulted in a reduced food intake^[1,11]. These observations, and the fact that various centrally active regulatory neuropeptides are also produced in the periphery, particularly the digestive system^[12], raise the question as to whether peripheral sites also express nesfatin-1/NUCB2.

Therefore, in the present study, we investigated the regional distributions and morphological features of nesfatin-1/NUCB2 IR cells in various organs of the digestive system of sprague-dawley (SD) rats and institute of Cancer Research (ICR) mice, to lay a foundation for the further investigation of its functions in the digestive system.

MATERIALS AND METHODS

Ethics

All procedures strictly adhered to the guidelines of the Institution Council of Animal Care and were approved by the Ethics Committee of Nanjing Medical University.

Materials

Adult male SD rats, weighing 220-250 g, and adult ICR mice, weighing 22-25 g, were obtained from the Experimental Animal Center of Nanjing Medical University (China). Animals were housed in groups of three per cage under controlled illumination (12:12 h light/dark cycle, lights on/off: 6 h/18 h), humidity (60%) and temperature (22 ± 2°C). Animals were fed a standard rodent diet and tap water *ad libitum*.

Antibodies

Mouse anti-nesfatin-1 monoclonal antibody [1:40 000, diluted in 0.3% Triton X-100 in phosphate-buffered

saline (PBS) for immunohistochemistry and 1:1000, diluted in Tris-buffered saline (TBS) in 5% (g/L) nonfat milk for Western blotting], was purchased from Alexis Biochemicals Company, USA. The SP Eivision™ plus kit used for immunohistochemistry, was purchased from Maixin_Bio Fuzhou, Co., Ltd., China. Mouse anti-β-actin monoclonal antibody [1:5000, diluted in TBS/5% nonfat milk for Western blotting], was purchased from Sigma-Aldrich, Co., Inc., USA. Horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG antibody [1:5000, diluted in TBS/5% nonfat milk for Western blotting], was purchased from KPL Gaithersburg, MD, USA.

Immunohistochemistry

Animals (fed *ad libitum*) were euthanized at 09:00 am by intraperitoneal injection of 10% chloral hydrate anesthetic and the pancreas, stomach, duodenum, esophagus, liver, small intestine and colon were quickly resected and transferred to 4% paraformaldehyde and fixed at 4°C for 24 h. After being dehydrated through an ethanol-xylene series, the specimens were embedded in paraffin. Sections were cut (5 μm thick) and mounted on polylysine-coated slides.

Each representative section was immunostained using the streptavidin-peroxidase method^[13]. Sections were de-waxed in xylene and dehydrated with a gradient alcohol series, then incubated for 20 min at 100°C in citrate buffer (pH 6.0) for antigen retrieval^[14]. Endogenous peroxidase was blocked by incubating the sections with 0.3% hydrogen peroxide for 15 min and non-specific binding was reduced by pretreatment with 3% normal goat serum for 20 min. The sections were then incubated with primary antibody (mouse anti-nesfatin-1 monoclonal antibody) overnight at 4°C. After washing in PBS, the sections were incubated for 20 min at room temperature (RT) with polymer enhancer and then incubated with the secondary antibody (polymerized HRP-conjugated anti-mouse IgG antibody) supplied with the SP Eivision™ plus kit for 30 min at RT. The peroxidase reaction was carried out in 3,3'-diaminobenzidine tetrachloride solution (MaiXin_Bio, Fuzhou, Co., Ltd., China) for 2 min and regularly checked under a light microscope. After being lightly counterstained with Mayer's hematoxylin for 1 min, the section was mounted on a slide, dehydrated with absolute alcohol followed by xylene, air-dried and coverslipped with Permount. Sections were observed and photomicrographed under a confocal microscope (Zeiss, AxioCam MRc5, Germany).

To investigate the specificity of the nesfatin-1 antibody under our conditions, the same protocol was applied for immunostaining after pre-absorption of the anti-nesfatin-1-antibody. Recombinant rat nesfatin-1 (50 μg ALX-522-116 Alexis Biochemical, USA) was incubated with mouse monoclonal anti-nesfatin-1 antibody at 1:40 000 in 0.3% Triton X in PBS for 2 h at RT followed overnight at 4°C. The solution was centrifuged for 15 min at 12 000 × g and the supernatant was used for staining as described above, as a negative control. For the positive

control, we used tissue sections obtained from the hypothalamus of three *ad libitum*-fed male SD rats, which had been confirmed to express the peptides of interest^[1]. Cells expressing these peptides were colored brown.

Hematoxylin and eosin staining

The paraffin specimens used for immunohistochemical staining were also used for hematoxylin and eosin (HE) staining. Each section was de-waxed in xylene and dehydrated with a graded alcohol series. After being washed with distilled water for 3 min, the sections were counter-stained with Mayer's hematoxylin for 15 min, washed in distilled water for 3 min and incubated in the eosin staining solution for 3 min. Finally, the sections were mounted on a slide, dehydrated with absolute alcohol followed by xylene, air-dried and coverslipped with Permount. All specimens were observed and photomicrographed under a confocal microscope.

Sodium dodecylsulfate polyacrylamide gel electrophoresis and Western blotting analysis

Tissue specimens from seven digestive organs of SD rats and ICR mice were kept on ice and ultrasonicated in the presence of one tablet of protease split cocktail [20 mmol/L Tris buffer pH 6.8, 4 mmol/L ethylene diamine tetraacetic acid pH 8.0 and 2% sodium dodecylsulfate (SDS)]. The crude protein fractions were obtained by centrifugation of the homogenates in a Sorvall centrifuge at $12000 \times g$ for 20 min at 4°C to remove cell debris and nuclei. The supernatant was used as the protein fraction. The final protein concentrations of the protein fractions were determined using a BCA protein assay (Pierce Biotechnology, Rockford, IL, USA).

Gel samples were prepared by mixing protein samples with gel sample buffer [10% SDS, 0.05% bromophenol blue (g/L), 25% glycerol (g/L), 10% mercaptoethanol (mL/L) in 0.1 mmol/L Tris buffer pH 6.8]. The samples were boiled at 100°C for 5 min before gel electrophoresis. Forty micrograms of each protein sample was loaded on a 5%-10% SDS polyacrylamide gel and run in a running buffer [25 mmol/L Tris-HCl, 192 mmol/L glycine, 0.1% SDS (g/L)]. After SDS-polyacrylamide gel electrophoresis, the proteins were transferred by electrophoresis to polyvinylidene difluoride membranes (Roche Diagnostics Indianapolis, IN, USA) in a transfer buffer [48 mmol/L Tris-HCl, 39 mmol/L glycine, 0.037% SDS] for 60 min on ice. The membranes were washed twice with TBS [10 mmol/L Tris-HCl, 150 mmol/L NaCl, 0.05% Tween-20 (mL/L)]. Non-specific binding sites were blocked by incubation in TBS containing 5% nonfat milk (Guangming, Co., Ltd., Shanghai, China) for 1 h at RT. The membranes were then incubated with the primary antibodies [mouse anti-nesfatin-1 monoclonal antibody (1:1000) and mouse anti- β -actin monoclonal antibody (1:5000)] overnight at 4°C. β -actin, a house-keeping protein, served as an internal control for the Western blotting. The next day, the membranes were washed three times with TBS and then incubated with the secondary

antibody [HRP-conjugated goat anti-mouse IgG antibody (1:5000)] for 2 h at 37°C. After the membranes were washed three times in TBS, enhanced chemiluminescence detection of the target protein was performed using the ECL plus Western blotting detection system (Pierce Biotechnology, Rockford, IL, USA) and exposed using a Kodak autoradiography system (Kodak, Image Station 2000 mm, USA). Densitometry was performed using Kodak Molecular Imaging software. The relative protein levels of NUCB2 in each organ were represented as the density ratio *vs* β -actin (NUCB2/ β -actin). All Western blotting analyses were repeated at least three times.

Statistical analysis

Statistical analysis was performed using SPSS 11.5 software (SPSS Inc., Chicago, IL, United States). Data were expressed as means \pm SD. Comparisons between groups were made using one-way analysis of variance (ANOVA) followed by Student-Newman-Keuls (SNK) *post hoc* tests for multiple comparisons. *P* values less than 0.05 were considered statistically significant.

RESULTS

Nesfatin-1/NUCB2 IR cells in pancreas, stomach and duodenum detected by immunohistochemical and HE staining

Nesfatin-1/NUCB2 IR, visualized as brown granules, was exclusively localized in the cytoplasm, but not in the nucleus or cell membrane. No brown granules were detected in the negative control sections stained with pre-absorbed anti-nesfatin-1-antibody, indicating that the immunostaining was specific for the target protein.

In the SD rats, nesfatin-1/NUCB2 IR cells were detected in the center of the pancreatic islets, but no immunopositive cells were detected in the pancreatic exocrine cells (Figure 1A). Nesfatin-1/NUCB2 IR cells were also found in the lower third and the middle portion of the gastric oxyntic glands (Figure 1B) and the submucosa of the duodenum (Figure 1C).

In the ICR mice, nesfatin-1/NUCB2 IR cells were also detected in the center of the pancreatic islets (Figure 1D). However, in the stomach, they were only localized in the lower third of the gastric oxyntic glands (Figure 1E). In the duodenum, nesfatin-1/NUCB2 IR cells were also expressed in the submucosal layer glands (Figure 1F). No nesfatin-1/NUCB2 IR cells were detected in the esophagus, liver, small intestine or colon in either the SD rats or in the ICR mice (data not shown).

We further observed the features of nesfatin-1/NUCB2 IR cells in the SD rats (Figure 2A-C) and the ICR mice (Figure 2D-F) using HE staining. The IR cells in the pancreas were islet cells. In histological sections of the pancreas, the islets were seen as relatively pale-staining groups of cells embedded in a region of darker-staining exocrine tissues (Figure 2A and D). In the stomach, they were endocrine cells. These cells were round, oval, triangular, spindle-, shuttle- or flask-like in shape and

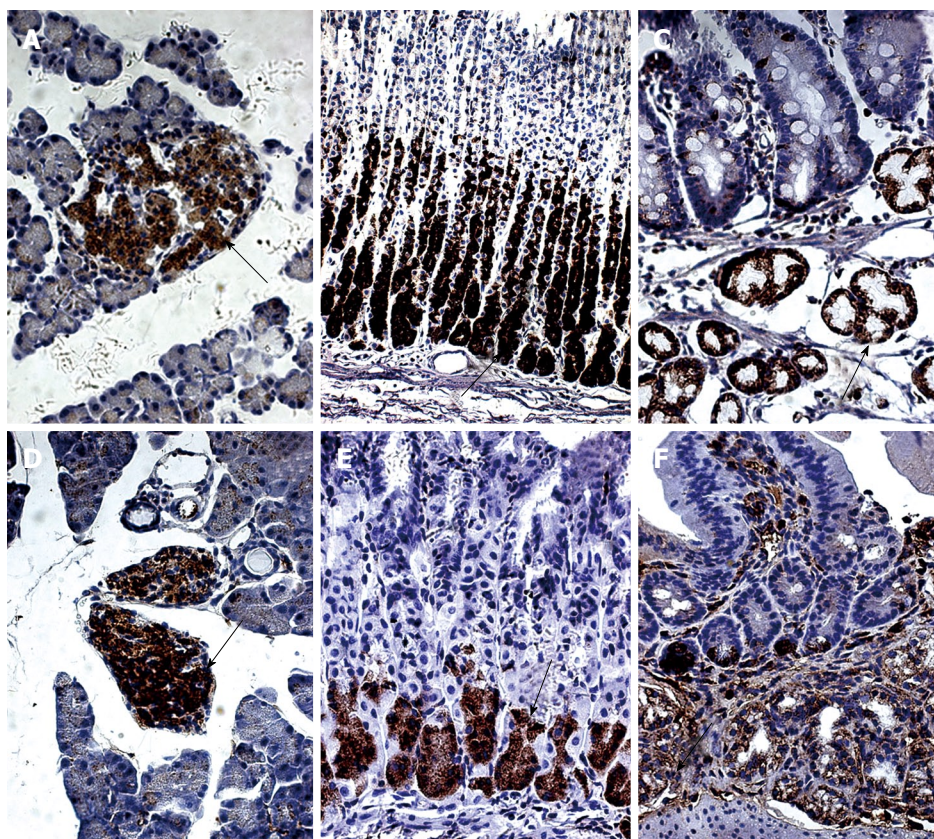
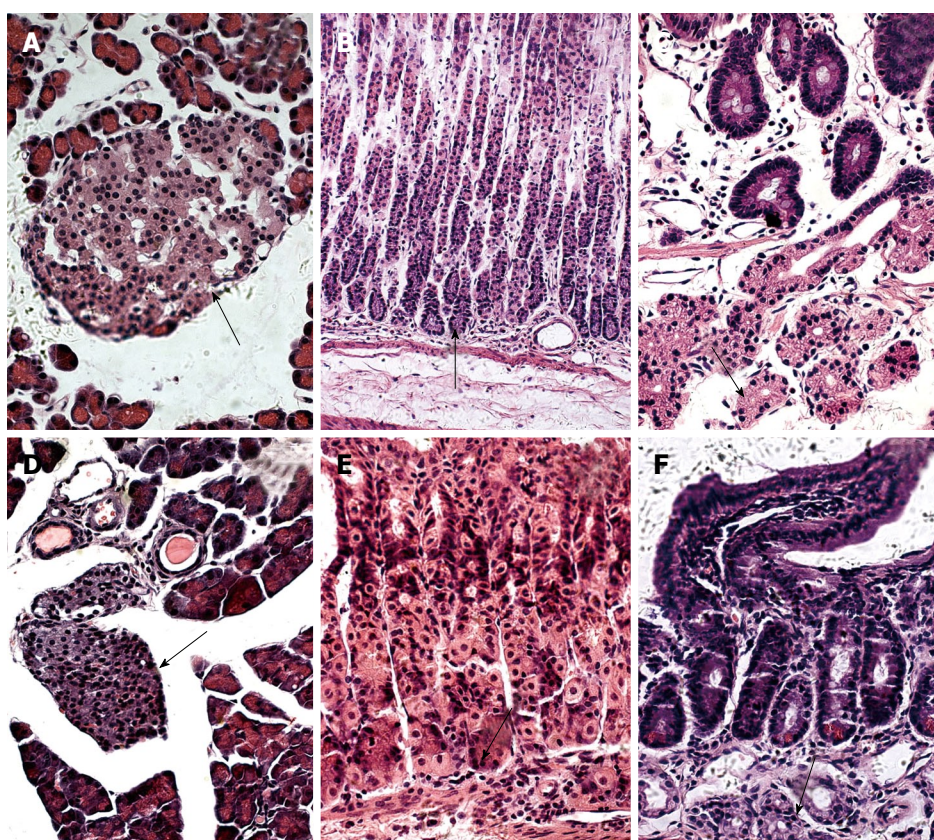


Figure 1 Immunolocalization of nesfatin-1/nucleobindin-2 (NUCB2) in the pancreas, stomach and duodenum in sprague-dawley (SD) rats (A-C) and institute of Cancer Research (ICR) mice (D-F). A and D: Pancreas; B and E: Stomach; C and F: Duodenum. Tissues were subjected to immunohistochemistry using antibodies to nesfatin-1. Arrows denote areas of positivity (A, C-F: $\times 40$; B: $\times 20$).



located between the glandular cells and the basement membrane at the basal portion of the gastric oxyntic glands (Figure 2B and E). The submucosal glands in the

duodenum were Brunner's glands, which were compound tubular submucosal glands consisting of epithelial tubules with frequent distal branches (Figure 2C and F).

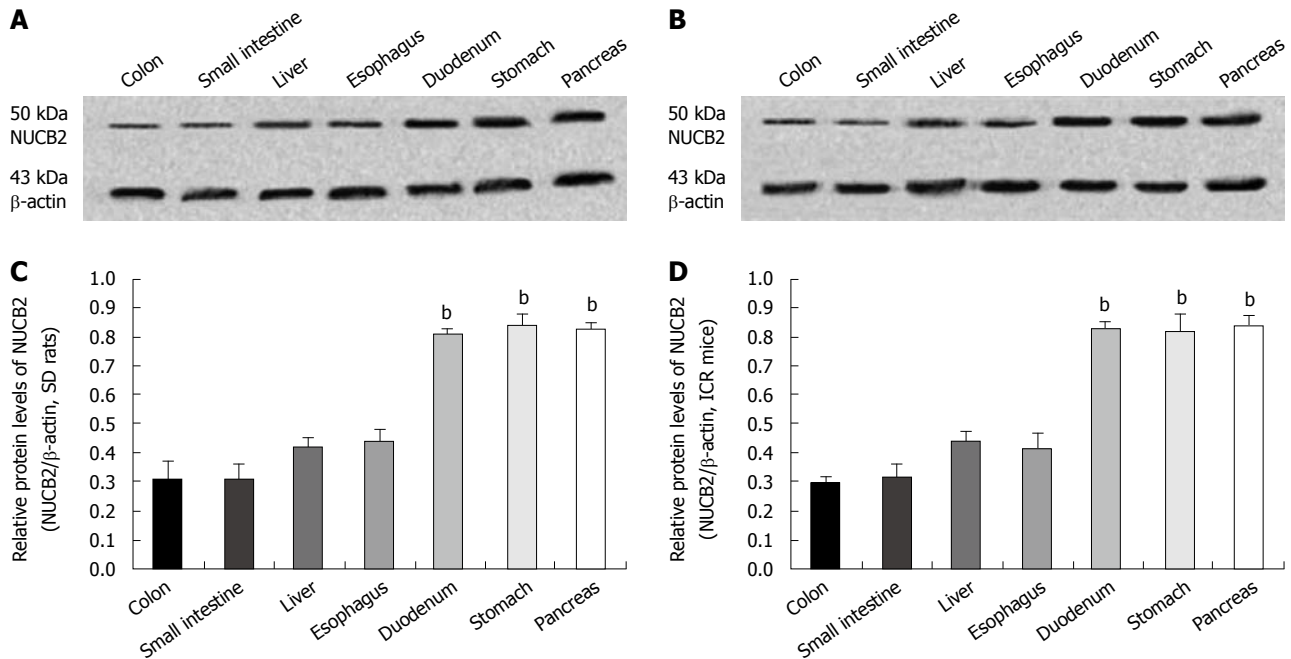


Figure 3 Western blotting for NUCB2 protein in the digestive system of the SD rats (A, C) and ICR mice (B, D). A, B: The predicted band for full-length NUCB2 protein (50 kDa) and β -actin (43 kDa) were observed in all organs; C, D: The relative protein levels of NUCB2 were significantly higher in the pancreas, stomach and duodenum than in the esophagus, liver, small intestine and colon. ^a $P < 0.001$. Data are mean \pm SD. analysis of variance (ANOVA) followed by Student-Newman-Keuls (SNK) test.

Protein levels of NUCB2 in the seven digestive organs

Western blotting of crude proteins of the seven digestive organs with the mouse anti-nesfatin-1 monoclonal antibody, which targets nesfatin-1 and its precursor NUCB2, showed a band corresponding to full-length NUCB2 protein (50 kDa). By contrast, no band corresponding to nesfatin-1 (9.7 kDa) was detected in either SD rats (Figure 3A) or the ICR mice (Figure 3B). In SD rats ($n = 6$), NUCB2 protein was highly expressed in the pancreas (relative expression; 0.82 ± 0.03), stomach (0.84 ± 0.04) and duodenum (0.80 ± 0.02), with lower expression in the esophagus (0.43 ± 0.05), liver (0.42 ± 0.03), small intestine (0.31 ± 0.05) and colon (0.31 ± 0.06) (Figure 3C). A similar pattern of expression was found in the ICR mice ($n = 6$), with high expression of NUCB2 in the pancreas (0.84 ± 0.03), stomach (0.82 ± 0.06) and duodenum (0.83 ± 0.02), and lower expression in the esophagus (0.42 ± 0.05), liver (0.43 ± 0.04), small intestine (0.31 ± 0.05) and colon (0.29 ± 0.02) (Figure 3D). The relative protein levels of NUCB2 in each immunopositive organ (pancreas, stomach and duodenum) were significantly higher than that in the immunonegative organs (esophagus, liver, small intestine and colon) in the SD rats and ICR mice ($P = 0.000$; ANOVA followed by SNK test).

DISCUSSION

Although the function of nesfatin-1/NUCB2 remains largely unknown, its sequence is highly conserved from rats and mice to humans, indicating an important biological role^[1]. NUCB2 is proteolytically processed by prohormone convertase to nesfatin-1 (residues 1-82), nesfatin-2 (residues 85-163) and nesfatin-3 (residues 166-396).

However, of these, only nesfatin-1 was effective in the reduction of food intake^[1]. The initial reports by several researchers have described a wide distribution of nesfatin-1 in the central nervous system of rats. In the present study, our findings expand these reports to the digestive system of SD rats and ICR mice. We found an abundant distribution of nesfatin-1/NUCB2 IR cells in the pancreatic islets, gastric endocrine cells and the duodenal Brunner's glands of the rodents. In particular, our results have revealed that nesfatin-1/NUCB2 IR cells are located in the submucosal Brunner's glands of the duodenum, which has not been previously reported in rats^[15]. Because the number of Brunner's glands in the duodenum is relatively small, which are restricted to a small region of the duodenum, they may have been overlooked in the earlier studies. Thus, to the best of our knowledge, this is the first report to show the presence of nesfatin-1/NUCB2 IR cells in the Brunner's glands of the duodenum. The Brunner's glands are primarily confined to the duodenal bulb and their number gradually decreases to absent in the duodenojejunal flexure^[16]. The main function of Brunner's glands is to produce an alkaline secretion containing bicarbonate and mucus to: (1) protect the duodenum from the acidic content of chyme, which is introduced into the duodenum from the stomach; (2) provide an alkaline condition for the intestinal enzymes to be active, thus enabling absorption to take place; and (3) lubricate the intestinal walls^[17]. Although the functional role of nesfatin-1/NUCB2 in the duodenum is currently unknown, the abundant expression of nesfatin-1/NUCB2 IR cells in the Brunner's glands found in this study suggests that nesfatin-1/NUCB2 may be involved in the regulation of intestinal

enzyme activation, nutrition absorption and preservation of the intestinal walls. To verify this hypothesis, further studies should focus on the molecular mechanism underlying nesfatin-1/NUCB2 IR cells in the Brunner's glands to gain a basic understanding of their pathobiology.

The immunohistochemical analysis also showed abundant nesfatin-1/NUCB2 IR cells in the center of the pancreatic islets, but none in the pancreatic exocrine cells. The islets of Langerhans in the pancreas regulate blood glucose levels. Islets consist of four types of secretory cells: glucagon-producing α -cells, insulin-producing β -cells, somatostatin-producing δ -cells, and PP cells, which contain pancreatic polypeptide. Interestingly, the different cell types within an islet are not randomly distributed. β -cells occupy the central portion of the islet and are surrounded by a layer of α - and δ -cells^[18]. In our experiment, we found that the nesfatin-1/NUCB2 IR cells were principally concentrated in the central region of the islets. Therefore, based on this localization, we speculated that they were β -cells. This is consistent with a recent study, which demonstrated that insulin-producing β -cells in mice and rats co-express nesfatin-1 immunoreactivity^[19]. These findings suggest possible roles of nesfatin-1/NUCB2 in carbohydrate metabolism.

A recent study has shown the presence of nesfatin-1/NUCB2 IR cells in the stomach of rats^[15]. Furthermore, the expression of NUCB2 mRNA in gastric endocrine cells is significantly down-regulated after a 24-h fast^[15]. That study suggested a regulatory anorexigenic role of peripheral nesfatin-1/NUCB2 in energy homeostasis. Similarly, in the present study, we found abundant nesfatin-1/NUCB2 IR cells in the lower third and middle portion of the gastric oxyntic glands in SD rats but only in the lower third of the gastric oxyntic glands in ICR mice. These results suggest fine distinctions in species-specific expression patterns of nesfatin-1/NUCB2 in the rodent stomach. The morphological features of these cells were endocrine cells. Therefore, it is assumed that nesfatin-1/NUCB2 in the stomach may play an important role in the regulation of gastric functions.

In the present study, the 50-kDa NUCB2 protein was detected ubiquitously in all digestive organs, but the protein was only detected in appreciable amounts in the pancreas, stomach and duodenum by immunohistochemistry, which is consistent with the higher levels of NUCB2 protein detected in these three tissues by Western blotting. Nesfatin-1 consists of 82 amino acids and has a predicted molecular weight of 9.7 kDa^[1]. Although the antibody we used in the Western blotting is targeted against the nesfatin-1 and its precursor NUCB2, the Western blottings of all tissues studied here only detected a single band of approximately 50 kDa representing NUCB2, but failed to show a band at 9.7 kDa. Therefore, as described by Stengel *et al.*^[15], the protein identified in this study was called nesfatin-1/NUCB2.

Taken together, the findings of this study demonstrate the presence of nesfatin-1/NUCB2 IR cells in the pancreas, stomach and duodenum but not in the esophagus, liver,

small intestine or colon of the SD rats and the ICR mice and the relative protein levels of NUCB2 were significantly higher in pancreas, stomach and duodenum. Like many other feeding behavior regulatory peptides, nesfatin-1 was expressed both centrally and in the periphery. The broad distributions of nesfatin-1/NUCB2 in the digestive system suggest a possible regulatory role of peripheral nesfatin-1/NUCB2 in carbohydrate metabolism, gastrointestinal function and nutrition absorption. Going forward, it will be crucial to understand the site and mechanism of action for the anorectic signal mediated by nesfatin-1. Identifying a receptor or non-classical binding partner for nesfatin-1 will be an important first step in this journey.

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COMMENTS

Background

In 2006, Oh-I and his colleagues discovered a new feeding inhibitory molecule, and named it nesfatin-1. They initially found the novel anorexigenic peptide expressed in a number of discrete neuronal populations or nuclei, which are involved in feeding behavior. Subsequently, several researchers have expanded the distributions of nesfatin-1 in the central nervous system. It is well known that various centrally active food regulatory neuropeptides are also produced in the periphery, particularly the digestive system. The authors investigated the regional distributions of nesfatin-1/ nucleobindin-2 (NUCB2) in various organs of the digestive system of sprague-dawley (SD) rats and institute of Cancer Research (ICR) mice.

Research frontiers

Although the function of nesfatin-1/NUCB2 remains largely unknown, its sequence is highly conserved from rats and mice to humans, indicating an important biological role. The nesfatin-1 signaling pathway might be associated with the melanocortin signaling pathway in the hypothalamus. The hypothalamic leptin signaling pathway does not exist downstream of the pathway by which nesfatin-1 causes anorexia. Nesfatin-1 can cross the blood-brain barrier without saturation. Nesfatin/NUCB2-immunoreactivity was recently identified in the rat gastric mucosa. Systemic or local administration of nesfatin-1-related drugs may improve metabolic disorders by reducing the body weight of patients with obesity and metabolic syndrome.

Innovations and breakthroughs

This is the first study to report the expression of nesfatin-1/NUCB2 in the duodenal Brunner's glands. Furthermore, a quantitative analysis with Western blotting studies showed higher protein levels of NUCB2 in the immunopositive organs than in the immunonegative organs.

Applications

By understanding where nesfatin-1/NUCB2 is expressed and by quantitatively analyzing its expression in the digestive system, this study may lay a foundation for the further investigation of its functions in the digestive system. In addition, this may help understand the relationship between the peripheral and the central nervous system.

Peer review

The authors examined the expression of nesfatin-1/NUCB2 in seven organs of the digestive system in SD rats and ICR mice. The study not only confirmed that nesfatin-1/NUCB2 is expressed in the pancreas and stomach, but is also for the first time to reveal that nesfatin-1/NUCB2 is expressed in the duodenal Brunner's gland. The authors conducted a quantitative analysis to determine the protein levels of NUCB2 in the seven organs. The results are interesting and may lay a foundation for the further investigations of its functions in the digestive system.

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Current surgical treatment of diverticular disease in the Netherlands

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Abstract

AIM: To evaluate the development of diagnostic tools, indications for surgery and treatment modalities concerning diverticular disease (DD) in the Netherlands.

METHODS: Data were collected from 100 patients who underwent surgery for DD in three Dutch hospitals. All hospitals used the same standardized database. The collected data included patient demographics, patient history, type of surgery and complications. Patients were divided into two groups, one undergoing elective surgery (elective group) and the other undergoing acute surgery (acute group).

RESULTS: Two hundred and ninety-nine patients were admitted between 2000 and 2007. One hundred

and seventy-eight patients underwent acute surgery and 121 patients received elective operations. The median age of the 121 patients was 69 years (range: 28-94 years), significantly higher in acute patients ($P = 0.010$). Laparoscopic resection was performed in 31% of elective patients. In the acute setting, 61% underwent a Hartmann procedure. The overall morbidity and mortality were 51% and 10%, and 60% and 16% in the acute group, which were significantly higher than in the elective group (36% and 1%). Only 35% of the temporary ostomies were restored.

CONCLUSION: This study gives a picture of current surgical practice for DD in the Netherlands. New developments are implemented in daily practice, resulting in acceptable morbidity and mortality rates.

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Key words: Diverticulitis; Surgery; Diverticular disease

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INTRODUCTION

Diverticular disease (DD) accounts for 14 000 hospital admissions annually in the Netherlands. The incidence of DD is rising, mainly among younger patients^[1]. The treatment depends on the severity of the disease, vary-

ing from light symptomatic diverticulosis to perforated diverticulitis. For years it was thought that the risk of perforation and other complications increased after each recurrence. Therefore, the American Association of Colorectal Surgeons suggested to perform an elective sigmoid resection after two episodes of acute diverticulitis, after a single episode in young patients or when complications, such as stenosis or fistulae, occur^[2].

However, new insights in the natural course of DD resulted in a more conservative approach. Severe complicated diverticulitis, leading to acute surgical intervention, is most often the primary presentation of the disease^[3]. More recent studies highlight the benign course of recurrent episodes of diverticulitis with a low complication rate in patients treated conservatively for an acute episode of diverticulitis^[4-6]. It is suggested that elective, prophylactic sigmoid resections based on the number of episodes is not always indicated.

New diagnostic tools and therapeutic techniques have improved the treatment of DD. For instance, the ability to treat large abscesses by computed tomography (CT)-guided percutaneous drainage prevents the emergency surgery and can form a bridge to an elective resection when indicated^[7]. The rise of laparoscopic surgery since the 90s resulted in decreased morbidity and mortality rates, making it the preferred approach in elective sigmoid resections^[8].

Whether a laparoscopic approach can also be applied to patients with perforated diverticulitis and generalized peritonitis remains to be confirmed. Laparoscopic sigmoid resection cannot be accomplished completely because of extensive pericolic infiltration and faecal or purulent contamination. Hartmann's procedure is the treatment of first choice for most surgeons. However, several recent studies showed that a primary anastomosis with or without a deviating ileostomy could be performed safely under these circumstances^[9,10]. Even laparoscopic lavage can be a safe alternative for Hartmann's procedure in case of perforated purulent diverticulitis^[11].

The aim of this study is to evaluate development of diagnostic tools, indications for surgery and treatment modalities concerning DD in the Netherlands. We analyzed the results of 299 patients with DD treated in three Dutch hospitals. Since the patients treated for acute diverticulitis have a worse prognosis compared with electively treated patients, we analyzed them in separate groups.

MATERIALS AND METHODS

In January 2008, we collected and pooled the data in the last 100 patients with DD treated surgically in three Dutch hospitals. The hospitals were the VU University Medical Center in Amsterdam, the Deventer Ziekenhuis in Deventer and the Reinier de Graaf Gasthuis in Delft, evenly distributed over the country. Patients were selected using national coding systems (i.e. ICD-9) and the national pathology database (PALGA).

The following data were collected: gender, age, ASA-grade (American Society of Anesthesiologists), type of admission (elective or acute), previous episodes of diverticulitis, results of radiological tests (CT-scan, ultrasound and contrast enemas), operative technique, duration of operation, type of surgeon [gastrointestinal (GI)-surgeon, resident or general surgeon], Hinchey classification^[12], intensive care unit (ICU) admission, complications, creation/restoration of ostomies and mortality. The data were collected in a standardized database by retrospective analysis of the medical records.

Minor complications include urinary tract infections, conservatively treated ileus, small wound infection and/or postoperative delirium. Anastomotic leakages, evisceration, necrotic ostomy, re-operation for other reasons and/or cardiopulmonary distress were considered to be major complications. Mortality was defined as death within 30 d after operation or during initial hospital admission.

Statistical analysis

Data of the three hospitals were combined into one database. Patients were divided into two groups: one undergoing elective surgery and the other undergoing acute surgery. Statistical analysis was performed using SPSS 15.0.1 (SPSS Inc., Chicago, IL, USA). Values were expressed as median and range for continuous variables. Distributions of dichotomous data were given in percentages. Continuous variables with normal distribution were compared using Student's *t* test. Wilcoxon *W* test was employed for continuous variables. Pearson χ^2 test was used for the analysis of discrete variables.

RESULTS

Population

The last 100 patients with DD treated surgically in the three hospitals were included, all were admitted between April 2000 and December 2007. After combining the datasets, one patient was excluded because the indication for surgery was revised. Two hundred nineteen patients with complicated DD were analyzed. Patient characteristics are shown in Table 1. The median age was 69 years (range: 28-94 years), the patients in the acutely admitted group were 8 years older ($P = 0.010$) than the elective group. Only 5% of patients were younger than 40 years and 46% over 70 years of age. The male to female ratio was 42% to 58%. No differences were found in ASA-grade between groups. Sixty percent was of ASA-grade two.

Patient history and diagnostic procedures

Data on history and diagnosis are depicted in Table 1. Over half of the patients (52%) with an acute indication for surgery, had no history of diverticulitis. In the elective group, 54% of patients underwent a sigmoid resection after one episode of diverticulitis, the others had a history of two or more episodes. Conservative treatment

Table 1 Patient characteristics *n* (%)

	Acute (<i>n</i> = 178)	Elective (<i>n</i> = 121)	<i>P</i> value
Age (yr)			
median (range)	74 (28-94)	64 (28-88)	0.010
< 40	9 (5)	5 (4)	0.710
> 70	101 (57)	37 (31)	0.000
Gender			
Male	84 (47)	41 (34)	0.022
ASA grade			0.157
I	25 (14)	22 (18)	
II	102 (57)	78 (65)	
III	44 (25)	18 (15)	
IV	7 (4)	3 (2)	
Previous episodes			0.000
0	92 (52)	0 (0)	
1	68 (38)	65 (54)	
2	10 (6)	23 (19)	
≥ 3	8 (4)	33 (27)	
Imaging			
Ultrasound	32 (18)	22 (18)	0.964
CT-scan	103 (58)	98 (81)	0.000
Barium enema	2 (1)	57 (47)	0.000
Colonoscopy	16 (9)	76 (63)	0.000
Hinchey			0.000
I	56 (32)	48 (65)	
II	24 (14)	26 (35)	
III	75 (43)	0 (0)	
IV	19 (11)	0 (0)	
Percutaneous drainage	7 (29)	3 (12)	0.578

CT: Computed tomography.

of a mild episode of diverticulitis usually consisted of dietary advice and sometimes antibiotic therapy. Percutaneous drainage of abscesses was only reported in 20% of Hinchey II patients.

CT-scan seemed the most valuable diagnostic tool in an acute setting, being performed in 58% of patients. In the preoperative work-up for an elective intervention, CT-scan (81%) and colonoscopy (63%) were performed. Severity of the disease was classified by the Hinchey classification, knowing that Hinchey III and IV perforated diverticulitis can only be distinguished during surgery; and 65% of elective patients were Hinchey I and 35% Hinchey II. Indications for elective sigmoid resection were: recurrent episodes of acute diverticulitis, persistent complaints, colovesical or colovaginal fistulae and symptomatic stenosis of the sigmoid colon.

Perforated diverticulitis with generalized peritonitis was the main indication for an acute intervention (Hinchey III 43% and Hinchey IV 11%). Other acute indications were total bowel obstruction, very large or persisting abscesses and failure of conservative treatment. In 54% of the acute patients, surgery was performed on the day of admission, and in 15% the following day.

Operation

Operative data are shown in Table 2. Laparoscopic sigmoid resection was performed in 31% of the elective patients, with a conversion rate of 15%. A deviating ileostomy was created in 12%. There was a wide inter-hospital variety in the preferred elective approach.

Table 2 Surgical data *n* (%)

	Acute (<i>n</i> = 178)	Elective (<i>n</i> = 121)	<i>P</i> value
Time to acute intervention			
Same day	96 (54)		
1 d	26 (15)		
> 1 d	54 (30)		
Unknown	2 (1)		
Operating time (min)			
mean (range)	125 (30-295)	160 (48-305)	0.000
Operating surgeon			0.000
GI-surgeon	32 (18)	78 (65)	
General surgeon	75 (42)	5 (4)	
Resident	71 (40)	38 (31)	
Surgical approach			0.000
Laparoscopy	8 (4)	38 (31)	
Laparotomy	170 (96)	83 (69)	
Conversion rate	5 (63)	7 (15)	0.000
Surgical procedure			0.000
Resection with primary anastomosis	69 (39)	116 (96)	
Protective ileostomy	10 (14)	9 (8)	
Hartmann's procedure	109 (61)	5 (4)	
ICU admission			0.000
None	48 (27)	86 (71)	
1-2 d	67 (38)	29 (23)	
3-4 d	25 (14)	3 (3)	
≥ 5 d	38 (21)	3 (3)	
Morbidity	107 (60)	44 (36)	0.000
Minor complications	36 (20)	20 (17)	0.177
Wound infection	28 (16)	14 (12)	
Urinary tract infection	8 (4)	6 (5)	
Major complications	71 (40)	24 (20)	0.000
Anastomotic leakage	2 (1)	8 (7)	
Necrotic ostomy	4 (2)	2 (2)	
Cardiopulmonary	29 (16)	5 (4)	
Intraabdominal abscess	8 (5)	1 (1)	
Evisceration	5 (3)	3 (2)	
Other	23 (13)	5 (4)	
Mortality	28 (16)	1 (1)	0.000

ICU: Intensive care unit; GI: Gastrointestinal.

In the acute setting, Hartmann's procedure was performed in 61% of patients. The other patients were treated by means of resection and primary anastomosis, 14% of them received a deviating ileostomy. Laparoscopic approach was attempted in 4% of the patients, with a high conversion rate (63%).

Median operating time in acute operations was significantly shorter than in an elective setting (125 min *vs* 160 min, *P* = 0.000). Results, complications and operating time may largely depended on the operating surgeons, i.e. GI-surgeon, general surgeon or resident. This distribution was determined by the setting in which an operation was performed (acute or elective). Most of the elective surgeries were performed by GI-surgeons (65%), whereas general surgeons and residents (under supervision) performed most of the acute operations (82%). In total, 36% of all interventions for DD were performed by residents, 37% by GI-surgeons and 27% by general surgeons.

Complications and follow-up

The overall morbidity and mortality of the total cohort

were 51% and 10%, respectively, in which elective surgery was associated with significantly better outcomes (Table 2). Following elective surgery, 36% of patients had postoperative complications, 16% having minor and 20% having major complications. After acute operation, the morbidity was 60%, including 20% minor and 40% major complications. The mortality in the acute group was 16% in contrast with 1% in elective group.

Twenty-nine percent of elective patients were admitted to the ICU after operation, 79% of these patients returned to the normal surgical ward within 1 d after operation. In the acute setting, the ICU admission rate was 73%, 48% of the patients stayed in ICU for more than 2 d.

A total of 133 ostomies were created, 86% of which used Hartmann's procedure. Only 35% of these ostomies, 50% of the ileostomies and 33% of Hartmann's procedures were actually reversed. The median interval until reversal was 29 wk (6-213 wk).

DISCUSSION

This study describes the current surgical practice in DD in the Netherlands. DD is associated with substantial postoperative morbidity (51%) and mortality (10%). A morbidity rate of 60% and a mortality rate of 16% are especially high in the acute setting. These numbers are comparable to other recent series, and little improvement has been seen over the past years^[9,13,14]. This could have been expected because acute interventions remained the same for decades, especially the Hartmann's procedure. No apparent reduction of these adverse outcomes has been achieved by improvement in peri-operative care, better patient selection and enhanced guarding on the ICU when needed.

Significantly more complications were seen in the acute group, mostly major complications. Usually a prominent share of morbidity and mortality is accounted for by anastomotic leakages although in this series a low percentage of 5% was found. None of these anastomotic leakages resulted from a primary anastomosis in Hinchey III or IV patients. Moreover, the majority of anastomotic leakages occurred in elective operations (7%) and not in acute interventions (3%). As can be expected, the total number of primary anastomosis was higher in the elective group than in the acute group. Perhaps primary anastomosis in the acute setting was only considered under favorable conditions, resulting in a positive selection bias.

Elective sigmoid resections are progressively approached laparoscopically. It is not only a safe alternative for open techniques, several advantages have been demonstrated in recent trials^[8]. Postoperative pain is reduced, duration of hospital stay is shortened and morbidity rates are decreased. Surprisingly, only 31% of all elective patients in this study underwent a laparoscopic sigmoid resection. This might be explained by differences in laparoscopic experience among different hospitals. In

one clinic, a laparoscopic sigmoid resection is always attempted, whereas the other center has a preference for the open technique. In this context, it has to be realized that the beneficial effects of laparoscopic surgery are exclusively generated in high-volume centers by experienced laparoscopic surgeons. Patients may benefit more from an open sigmoid resection than a laparoscopic approach when the surgeon is at the beginning of his 'learning curve'^[15,16].

More recently, alternatives for Hartmann's procedure have been proposed. The technique of laparoscopic lavage is being evaluated in a prospectively randomized study in the Netherlands and no patient in this study was treated by this promising technique. Furthermore, laparoscopic resection was only attempted in 4% of the acute patients, resulting in a considerable conversion rate (63%). The number of primary anastomoses in the acute setting is increasing, with or without deviating ileostomy. In this series, 39% of the acute patients received a primary anastomosis, which is a substantial rise when compared to 27% in another Dutch study on 291 patients between 1995 and 2005^[17].

New insights in the natural history of DD have resulted in an increasingly conservative approach to this disease. It seems that uncomplicated, conservatively treated diverticulitis has a good prognosis, with a low recurrence rate and a rather benign course^[3-6]. Nevertheless in this series, 48% of the acute patients had an earlier episode of diverticulitis. When considering more than 5 d ICU stay in 21% of the acute patients and a mortality rate of 16%, some of these patients may have benefited from early elective sigmoid resection. Further research of this substantial group might reveal high-risk patients to be associated with a more hazardous course of DD, and certain comorbid conditions (auto-immune diseases and chronic renal failure), medication (steroids and non-steroidal anti-inflammatory drugs) or younger age are suggested factors^[18-20].

In a retrospective multi-center study, bias is unavoidable, and data collection is dependent on the individual search strategies and interpretation of different researchers. In this study, patient data were collected in a standardized fashion in all three hospitals by means of retrospective analysis of the medical records. Because of the deliberate choice to invite three different types of hospitals (university and teaching) in different parts of the Netherlands to participate in the study, extrapolation of the results to a broader perspective seems possible. These results are likely to reflect the national policy on DD.

It seems that Dutch hospitals are up-to-date, new developments are implemented in daily practice, resulting in acceptable morbidity and mortality rates. Indications for elective surgery are based on complaints and complications of DD, and not so much on the number of episodes. Furthermore, when elective surgery is indicated, the laparoscopic approach has been adopted in some hospitals, dependent on the experience of the surgeons.

In the acute setting, Hartmann's procedure is no longer the only option in generalized peritonitis. For further enhancement of the treatment of DD, national audits and prospective trials are needed. We can conclude that DD is a common disease which necessitates surgery in acute as well as in elective settings. Currently, no directives are available concerning best treatment strategies for acute and elective DD. Different treatment strategies are applied leading to an acceptable morbidity and mortality rate.

COMMENTS

Background

Diverticular disease (DD) accounts for 14 000 hospital admissions in the Netherlands annually. The incidence of DD is rising, mainly among younger patients.

Research frontiers

The aim of this study is to evaluate developments in diagnostic tools, indications for surgery and treatment modalities concerning DD in the Netherlands. Therefore, the authors analyzed the results of 299 patients treated for DD in three Dutch hospitals.

Innovations and breakthroughs

Recent studies highlight the usually benign course of recurrent episodes of diverticulitis with low complication rates, in patients treated conservatively for an acute episode of diverticulitis. It is suggested that planned, prophylactic sigmoid resections based on the number of episodes is not always indicated. New diagnostic tools and therapeutic techniques have improved the treatment of DD.

Applications

This study gives a picture of current surgical practice of DD in the Netherlands. New developments seem to be implemented in daily practice, resulting in acceptable morbidity and mortality rates.

Peer review

This article shows the current surgical treatment for DD in the Netherlands. The aims of this work are well delineated; the patients and methods are clearly described and appropriate statistical measures are indicated. The results are well reported and support the aims of the work. Discussion section is concise and well organized.

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Successful antiviral therapy is associated with a decrease of serum prohepcidin in chronic hepatitis C

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Abstract

AIM: To assess serum concentrations of prohepcidin in chronic hepatitis C individuals and evaluate their associations with disease activity and efficacy of pegylated interferon (PEG-IFN)/ribavirin (RBV) therapy.

METHODS: Prohepcidin was measured in sera of 53 chronic hepatitis C patients. Concentrations of prohepcidin and other iron metabolism markers were analyzed at 9 time points before, during and after the end of antiviral therapy.

RESULTS: In hepatitis C virus (HCV) genotype 1-infected individuals, a gradual decrease of prohepcidin during antiviral therapy was observed in responders (88.8 ± 14.7 ng/mL before therapy vs 60.6 ± 0.3 ng/mL in the 48th wk, $P = 0.04$). In contrast, no decrease was observed in non-responders. A similar association was

observed in HCV genotype 3a individuals, with a statistically significant decline in serum prohepcidin only in the responder group (99.5 ± 5.2 ng/mL at baseline vs 72.7 ± 6.1 ng/mL in the 24th wk, $P = 0.01$). Moreover, HCV-RNA at week 12 of therapy was positively correlated with baseline ($R = 0.63$, $P < 0.005$) and week 12 ($R = 0.60$, $P = 0.01$) serum prohepcidin concentrations in HCV genotype 1 infection.

CONCLUSION: Successful PEG-IFN/RBV therapy results in a decline of serum prohepcidin concentration in chronic hepatitis C, which may suggest a direct effect of HCV on iron metabolism at the prohormonal level of hepcidin.

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Key words: Iron metabolism; Hepcidin; Hepatitis C virus; Interferon; Sustained viral response

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INTRODUCTION

Liver iron overload is a well described, but not completely understood, feature of hepatitis C virus (HCV) infection. Several lines of evidence have suggested its negative influence on chronic hepatitis C outcome. Recent reports have emphasized the strong association between hepatic iron storage markers and oxidative DNA damage in

persistent HCV infection. In the study by Fujita *et al*^[1], phlebotomy resulted in a significant decrease of oxidatively generated DNA damage markers with concomitant reduction in serum transaminases as well as iron-related markers in chronic hepatitis C. Iron accumulation has also been linked with insulin resistance and liver steatosis in HCV-infected patients^[2,3]. Furthermore, experimental data have shown that iron deposits may trigger hepatic stellate cell activation and thus induce liver fibrosis^[4]. Among other potential links between iron metabolism and chronic hepatitis C, the possibility of enhancing HCV replication by serum iron has been postulated in experimental settings^[5].

Fujita *et al*^[1] found that total iron score correlated positively with transaminase activity, histological grading and staging in chronic hepatitis C subjects. Interestingly, in this study baseline iron metabolism alterations were more pronounced in non-sustained viral response (non-SVR) than in SVR to interferon (IFN)/ribavirin (RBV) treatment, therefore suggesting the association between iron deposition and resistance to anti-HCV therapy. This observation has been further confirmed in a recent study^[6] showing inadequate hepcidin expression in chronic HCV, which could be restored by eradication of the virus.

The mechanisms of iron metabolism deregulation in chronic hepatitis C are not fully elucidated. Takeo *et al*^[7] showed significantly higher liver mRNA expression of transferrin receptor 2 (TfR2) and ferroportin in chronic hepatitis C than in hepatitis B virus (HBV)-infected patients, which additionally correlated with the total hepatic iron score during the HCV infection. Authors have suggested that upregulation of hepatic iron transporters may contribute to the hepatic iron accumulation. Recently discovered iron regulatory hormone, hepcidin, synthesized predominantly in the liver, has changed the understanding of iron metabolism regulation^[8]. Hepcidin has been found to suppress intestinal absorption of iron through the binding to ferroportin, which is robustly expressed by enterocytes and liver macrophages^[9]. Synthesis of hepcidin is increased by iron overload and decreased by anemia, hypoxia-inducible factors and reactive oxygen species. Moreover, it may be induced by infection and inflammatory responses, which result in rapid plasma iron decrease^[10-12]. Recent results have shown hepcidin deregulation in chronic hepatitis C and have suggested the pivotal role of this hormone in the pathogenesis of iron overload^[1,6,13].

In the current study, levels of prohepcidin, a hepcidin precursor protein predominantly synthesized in the liver, were measured. In the hepatocytes, prohepcidin undergoes two cleavages and is rapidly secreted from cells as the mature, 25 amino acid peptide, hepcidin. It has been shown that, to some extent, prohepcidin is also secreted by hepatocytes^[14]. Recently, we have suggested the association between serum prohepcidin levels and liver function impairment in liver cirrhosis patients^[15]. The aim of the study was to assess the serum concentration of this hepcidin prohormone in chronic hepatitis C and evaluate its possible association with the

disease activity as well as the efficacy of pegylated IFN (PEG-IFN)/RBV therapy.

MATERIALS AND METHODS

Patients

Prohepcidin levels were measured in sera of 53 chronic hepatitis C patients (15 females and 38 males; median age: 46.5 years, min: 20 years, max: 67 years) before and during PEG-IFN- α /RBV therapy. Twenty-nine of them were infected with HCV genotype 1 and 24 with HCV genotype 3a. All patients had chronic hepatitis C proven through the presence of anti-HCV antibodies and HCV-RNA in sera for at least 6 mo. Quantitative HCV-RNA and HCV genotyping were performed by two-step real-time quantitative RT-PCR using TaqMan. Disease activity was evaluated by liver biopsy (Hepafix System, Braun, Melsungen, Germany) performed before the start of anti-HCV therapy. Paraffin-embedded biopsy specimens were stained and evaluated using Scheuer's scoring system^[16]. None of the included patients had liver cirrhosis diagnosed, nor HBV, hepatitis delta virus or human immunodeficiency virus co-infections. Clinical characteristics of the studied population are presented in Table 1.

Patients received a combination therapy with a weekly dose of PEG-IFN- α 2a administered subcutaneously and RBV administered orally at daily doses of 1000 or 1200 mg/d based on body weight (< 75 or \geq 75 kg, respectively) for genotype 1 and 800 mg/d for genotype 3a. The total duration of treatment was 48 wk for genotype 1 and 24 wk for genotype 3a. Patients were divided into two groups: responders, defined as undetectable HCV-RNA at week 24 after the end of therapy, and non-responders, defined as HCV-RNA positive at week 24 after the end of therapy.

Prohepcidin serum concentrations were measured at baseline, in the 4th, 12th, 24th and 48th (genotype 1) wk of antiviral therapy and in the 24th wk after termination of the treatment (week 72). During the same visits, liver function tests and the serum iron metabolism markers, serum iron, total iron binding capacity (TIBC), transferrin saturation, and ferritin, were measured. Moreover, serum iron saturation was calculated using the following formula: (serum Fe/TIBC) \times 100%. Serum prohepcidin values were compared with those collected from 15 healthy volunteers (7 females and 8 males, median age: 40 years).

Hepcidin prohormone measurement

Venous blood was collected on ice using vacutainer tubes and centrifuged at $2500 \times g$ at 4°C within 30 min of collection. Serum samples were assayed for prohepcidin with the quantitative sandwich enzyme immunoassay kit (DRG Instrument GmbH, Marburg, Germany) according to manufacturer instructions. The employed antibody detects both the pro-region and pro-hepcidin (aa 25-84). The sensitivity of assay is 3.95 ng/mL, intra-

Table 1 Baseline characteristics of studied population (mean \pm SE)

	Control group (n = 15)	Genotype 1		Genotype 3a	
		R (n = 13)	NR (n = 16)	R (n = 21)	NR (n = 3)
Age [median (min-max), yr]	40 (26-54)	47 (21-67)	48 (23-58)	47 (25-64) ^a	24 (20-26)
Sex (M/F)	7/8	11/2	13/3	12/9	2/1
HCV-RNA log ₁₀ (cps/mL)	-	6.01 \pm 5.74	5.86 \pm 5.73	3.95 \pm 3.65	2.95 \pm 2.30
ALT (U/L)	-	110.4 \pm 29.3	75.1 \pm 8.7	111.5 \pm 23.6	133.0 \pm 25.2
Bilirubin (mg/dL)	-	1.1 \pm 0.1	1.2 \pm 0.1	1.0 \pm 0.1	1.1 \pm 0.1
Liver inflammatory activity [median (min-max), pts]	-	3 (1-3)	2 (1-4)	2 (1-3)	2.5 (2-3)
Liver fibrosis [median (min-max), pts]	-	1 (1-3)	1 (1-3)	1 (1-2)	1.5 (1-2)
Fe (μ g/dL)	-	138.9 \pm 18.3	162.2 \pm 19.9	127.3 \pm 10.9	165.0 \pm 29.0
TIBC (μ g/dL)	-	352.3 \pm 20.7	316.5 \pm 21.1	345.5 \pm 13.5	361.3 \pm 25.2
Transferrin saturation (%)	-	42.5 \pm 6.9	39.1 \pm 5.9	38.0 \pm 4.1	43.7 \pm 10.1
Ferritin (ng/mL)	-	255.4 \pm 50.2	204.5 \pm 35.1	193.4 \pm 58.1	134.8 \pm 61.8
Hemoglobin (g/dL)	-	15.0 \pm 0.32	14.5 \pm 0.4	14.6 \pm 0.3	15.4 \pm 0.9
Prohepcidin (ng/mL)	84.1 \pm 7.8	88.8 \pm 14.7	88.3 \pm 11.4	99.5 \pm 5.2	94.1 \pm 7.9

^a $P < 0.05$ in comparison to non-responders, all of the other differences are non-significant. R: Responders; NR: Non-responders; HCV: Hepatitis C virus; ALT: Alanine aminotransferase; TIBC: Total iron binding capacity.

assay variation coefficient of variation (CV) 4.69% and inter-assay variation CV 4.82%.

Statistical analysis

Values were expressed as mean \pm SE of mean. The significance of differences was calculated by non-parametric Mann-Whitney *U*, Kruskal-Wallis and Friedman ANOVA tests. For correlation analysis, the Spearman non-parametric correlation was used. A $P < 0.05$ value was considered as statistically significant. Statistical analyses were performed with Statistica 7.0 for Windows software (Statsoft Inc., Tulsa, USA).

Informed consent was obtained from each patient. The procedures followed were in accordance with the Helsinki Declaration of 1975, as revised in 1983. The study was approved by the Bioethical Committee of the Medical University of Białystok.

RESULTS

Serum concentration of prohepcidin measured before PEG-IFN/RBV therapy in chronic hepatitis C individuals slightly, but not significantly, exceeded values observed in healthy controls (93.2 ± 5.3 ng/mL *vs* 84.1 ± 7.8 ng/mL, respectively, $P = 0.46$). Interestingly, we observed a higher concentration of serum prohepcidin in HCV genotype 3a compared to genotype 1 (98.8 ± 4.7 ng/mL *vs* 88.5 ± 8.9 ng/mL, respectively, $P = 0.02$). In the latter group the values were also significantly higher than in controls ($P = 0.04$) (Figure 1).

Baseline serum prohepcidin concentration showed a positive correlation with serum ferritin in chronic hepatitis C ($R = 0.50$, $P = 0.02$) (Table 2). In HCV genotype 1 the positive correlation between prohepcidin and ferritin was even stronger ($R = 0.63$, $P = 0.001$). Furthermore, an association with alanine aminotransferase (ALT) activity ($R = 0.38$, $P = 0.04$) was observed. We did not notice correlations between serum prohepcidin and either age,

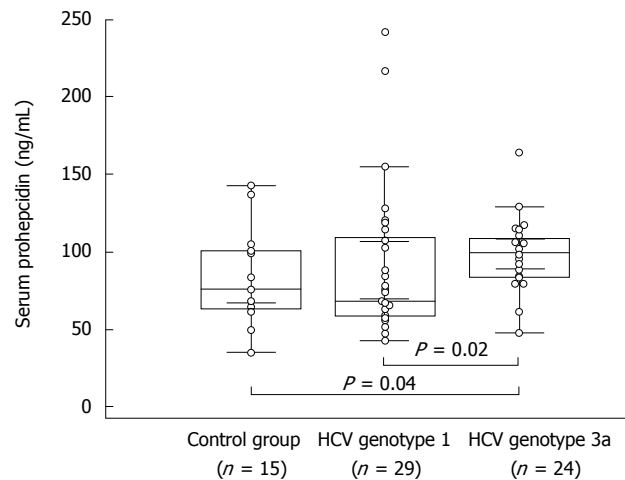


Figure 1 Baseline concentration of prohepcidin in healthy individuals as well as in hepatitis C virus (HCV) genotype 1 and genotype 3 patients. Dots indicate individual values in studied groups, boxes depict mean and standard error of mean, bars show standard deviations.

sex, liver histology, baseline HCV viral load, serum iron or TIBC.

Serum prohepcidin concentrations showed an association with PEG-IFN/RBV therapy effectiveness. In the first 7 d after the introduction of PEG-IFN/RBV, a gradual and reversible decrease in serum prohepcidin was observed regardless of HCV genotype (Tables 3 and 4). In the following phase, a further decrease of prohepcidin during antiviral therapy was noted in the HCV genotype 1 responder group, from 88.8 ± 14.7 ng/mL before therapy to 60.6 ± 2.3 ng/mL in the 48th wk (Friedman ANOVA test, $\chi^2 = 9.6$, $P = 0.04$). Such an association was not revealed in non-responders, with serum prohepcidin showing stable values (88.3 ± 11.4 ng/mL at baseline *vs* 82.3 ± 4.4 ng/mL in 48th wk) (Table 3 and Figure 2). A similar relationship was observed in genotype 3a individuals, with a statistically significant decrease in serum prohepcidin only in responders (99.5 ± 5.2 ng/mL

Table 2 Correlations between baseline serum prohepcidin (ng/mL), ALT activity, HCV-RNA, and iron metabolism parameters in studied population

	Genotype 1		Genotype 3a	
	R	P	R	P
HCV-RNA (cp/mL)	-0.09	0.620	0.08	0.750
ALT (U/L)	0.38	0.040 ¹	-0.06	0.810
Fe (µg/dL)	-0.06	0.510	-0.48	0.040 ¹
Hgb (g/dL)	-0.03	0.850	0.18	0.420
TIBC (µg/dL)	0.07	0.730	-0.11	0.640
Ferritin (ng/mL)	0.63	0.001 ¹	0.25	0.330

¹Statistical significance calculated by use of spearman rank test.

Table 3 Serum prohepcidin concentrations in genotype 1 HCV-infected patients during antiviral therapy with respect to SVR (mean ± SE)

	Prohepcidin (ng/mL)		P
	Responders (n = 13)	Non-responders (n = 16)	
Baseline	88.8 ± 14.7	88.3 ± 11.4	0.980
6 h	75.2 ± 10.2	79.5 ± 7.2	0.620
24 h	72.6 ± 6.8	68.4 ± 4.6	1.000
48 h	67.9 ± 7.6	64.8 ± 4.3	0.930
7th d	87.9 ± 8.6	85.9 ± 9.6	0.690
4th wk	77.7 ± 9.8	76.0 ± 7.8	0.920
12th wk	71.6 ± 6.7	76.1 ± 5.2	0.350
24th wk	63.9 ± 4.8	82.4 ± 4.3	0.004
48th wk	60.6 ± 2.3	82.3 ± 4.4	< 0.001
ANOVA ¹	χ ² = 9.6, P = 0.040	χ ² = 2.8, P = 0.590	

¹Calculated from data collected at baseline and weeks 4, 12, 24, 48. SVR: Sustained viral response.

at baseline *vs* 72.7 ± 6.1 ng/mL in the 24th wk, P = 0.01) (Table 4 and Figure 2).

The above mentioned observations were further confirmed by the significant association between serum prohepcidin and HCV viral load during the treatment. HCV-RNA at the 12th wk was positively correlated with baseline (R = 0.63, P < 0.005) and 12th wk (R = 0.60, P = 0.01) serum prohepcidin concentrations in genotype 1 HCV-infected individuals. A similar analysis was not possible for genotype 3a since HCV-RNA was detectable only in 6 of 24 patients at the 12th wk.

To assess the possibility of the interference of RBV-induced anemia with prohepcidin synthesis we performed a correlation analysis between hemoglobin concentration and prohepcidin. We found no association between these parameters at baseline (Table 1) or at different time points during the PEG-IFN/RBV therapy (data not shown).

DISCUSSION

Recent years have brought to light new facts concerning the pathogenesis of iron metabolism alterations in chronic liver diseases. The discovery of hepcidin has linked inflammatory disorders and iron turnover disturbances^[8].

Table 4 Serum prohepcidin concentrations in genotype 3a HCV-infected patients during antiviral therapy with respect to SVR (mean ± SE)

	Prohepcidin (ng/mL)		P
	Responders (n = 21)	Non-responders (n = 3)	
Baseline	99.5 ± 5.2	94.1 ± 7.9	0.620
6 h	87.6 ± 5.1	83.1 ± 4.7	0.760
24 h	84.3 ± 4.0	78.6 ± 6.5	0.730
48 h	76.8 ± 7.0	97.3 ± 22.1	0.630
7th d	99.6 ± 6.1	96.2 ± 6.3	0.940
4th wk	97.5 ± 6.3	84.9 ± 18.8	0.640
12th wk	83.7 ± 4.7	90.1 ± 13.3	0.750
24th wk	72.7 ± 6.1	103.5 ± 12.4	0.080
ANOVA ¹	χ ² = 14.8, P = 0.010	-	

¹Calculated from data collected at baseline and weeks 4, 12, 24. ANOVA was not calculated for non-responders because of insufficient number of patients.

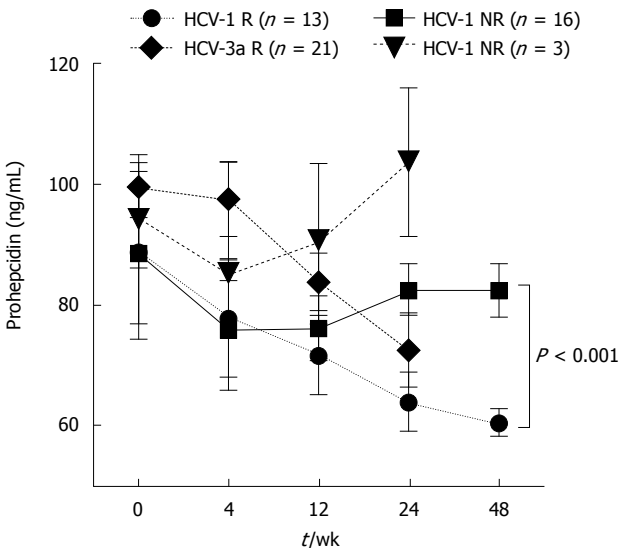


Figure 2 Serum prohepcidin concentrations in chronic hepatitis C during antiviral therapy with respect to the treatment response. R: Responders [HCV-RNA undetectable 6 mo after the end of pegylated interferon (PEG-IFN) + ribavirin (RBV) therapy]; NR: Non-responders (HCV-RNA positive 6 mo after the end of PEG-IFN + RBV therapy).

The name of hepcidin originated from its antimicrobial properties and the predominant production of this protein in the liver. Following infectious, inflammatory stimuli or iron-overload its production increases, resulting in the decrease of plasma iron by negative regulation of iron uptake by duodenal enterocytes and sequestration by macrophages^[12,17]. In contrast, down-regulation of hepcidin is an important factor facilitating iron deposition in parenchymal organs, especially in the liver. The regulation of hepcidin production is multilateral and still not fully understood. Main pathways involved include iron-storage, hypoxic and inflammatory-related^[18]. Hemochromatosis protein (HFE) seems to maintain the basal expression of hepcidin, probably *via* Tfr2 and hemojuvelin, acting as a body iron store sensor^[19]. In contrast, inflammatory-related

hepcidin induction appears to be independent of HFE, Tfr2 and mainly relies on interleukin-6 (IL-6) *via* STAT-3 activation^[20,21].

It is only in the last years that some insights into the role of hepcidin in chronic hepatitis C have been presented. Nagashima *et al*^[13] found significantly lower concentrations of serum prohepcidin in chronic hepatic C compared to chronic HBV infection and healthy individuals, suggesting that failure of prohepcidin regulation may be induced by HCV infection. More recently, Fujita *et al*^[22] evaluated hepatic hepcidin mRNA expression in chronic hepatitis B and C individuals. They showed significant positive correlations between hepatic hepcidin expression, serum iron, and ferritin, as well as liver total iron score. However, liver hepcidin mRNA expression was comparable in chronic hepatitis B and C in this study. Only after adjustment for serum ferritin were hepcidin indices significantly lower in HCV infection. The same group^[23] found a positive correlation between hepatic oxidatively generated DNA damage and serum ferritin, total iron score and liver hepcidin mRNA in chronic hepatitis C. Interestingly, they also showed more prominent baseline hepatic oxidative stress markers in non-SVR to IFN/ribavirin therapy.

We investigated the association between PEG-IFN/ribavirin therapy and iron metabolism parameters, especially hepcidin prohormone, prohepcidin, concentrations in chronic hepatitis C. We found comparable levels of serum prohepcidin in chronic hepatitis C and healthy individuals. Interestingly enough, baseline prohepcidin was significantly higher in HCV genotype 3a than in HCV-1. This new finding could be possibly explained by differences in iron metabolism in patients with genotype 3. Sebastiani *et al*^[3] showed that hepatic iron deposits were significantly more frequent in HCV-3-infected individuals and strongly associated with viral-induced hepatic steatosis.

Baseline serum prohepcidin in this current study showed a strong positive correlation with serum ferritin. Moreover, an association between baseline serum prohepcidin and ALT activity was found. These observations are in accordance with other reports^[22] and probably reflect above mentioned pathways of hepcidin regulation. The new finding of this study is the association between serum prohepcidin and PEG-IFN/ribavirin efficacy in chronic hepatitis C. We showed a statistically significant, gradual decrease of serum prohepcidin during successful antiviral treatment in HCV-1 and 3a individuals. The reduction in prohepcidin concentration for genotype 1 was 31% in the 48th wk of treatment (Table 3) and 27% in the 24th wk for HCV-3a (Table 4) in the SVR groups. In contrast, in non-SVR the prohepcidin serum concentrations did not change significantly compared to baseline values. This finding seems to contradict findings of a recent clinical study performed by Fujita *et al*^[6], however it has to be underlined that the current study measured a hepcidin precursor, not an active hormone. In the aforementioned work^[6], serum hepcidin was measured by mass spectrometry in 73 untreated chronic hepatitis C patients

and in 27 patients during PEG-IFN + RBV therapy. The baseline serum hepcidin was higher in chronic hepatitis C compared to healthy individuals but only in those with hyperferritinemia. On the other hand, HCV-infected individuals with normal ferritinemia had comparable serum prohepcidin with the control group. Interestingly, the most recent analysis by Girelli *et al*^[24] showed that in a rigorously selected population with HFE genotyping performed, serum hepcidin was significantly reduced in chronic hepatitis C in comparison to healthy controls and strongly associated with body iron deposits, regardless of IL-6 concentrations.

The current study applied a different approach. The assay used detected a hepcidin prohormone, not a mature peptide. It has been shown that serum assessment of prohepcidin does not reflect the levels of biologically active mature hepcidin peptide^[14]. On the contrary, it may give some insight into the mechanisms of hepcidin maturation. Hepcidin is almost exclusively synthesized in the liver as a 84 amino acid prepropeptide, which is further processed in hepatocytes to the mature form^[25]. Recent evidence showed that the proteolytic cleavage of prohepcidin to hepcidin is regulated by the hepatic prohormone convertase furin^[14]. The mechanism involved in the transport of hepcidin to the extracellular zone is not fully understood. Valore *et al*^[14] showed that a larger hepcidin precursor protein undergoes two cleavages (the signal sequence, then the pro-region) and is rapidly secreted from the cell. Moreover, in primary human hepatocytes, prohepcidin was transiently detected in culture supernatant immediately after labeling but it appeared to be present at low levels compared to mature hepcidin. Of interest, the inhibition of furin activity prevented the conversion of prohepcidin to hepcidin but also appeared to stabilize the prohepcidin peptide both in the cell and the media. In situations of liver function impairment the prohepcidin synthesis as well as activity or expression of converting enzymes might be altered and affect circulating prohepcidin concentrations. This finding could also suggest that HCV interference with hepcidin synthesis may occur at the step of prohormone synthesis or maturation in the liver.

In conclusion, we found an association between successful PEG-IFN/ribavirin therapy and serum prohepcidin concentration decrease in chronic hepatitis C patients. This may suggest a direct link between HCV and hepcidin maturation in the liver.

COMMENTS

Background

Iron accumulation in the liver has a negative influence on chronic hepatitis C outcome. It has been linked with oxidative DNA damage, insulin resistance and liver steatosis, but may also trigger hepatic stellate cell activation and thus induce liver fibrosis. Recently, a key iron regulatory hormone, hepcidin, was discovered. This hormone has been found to suppress intestinal absorption of iron through its binding to ferroportin. Hepcidin is synthesized in the liver from its precursor protein, prohepcidin.

Research frontiers

The mechanisms of iron metabolism deregulation in chronic hepatitis C are

not fully elucidated. Hecpcidin, a versatile regulator of iron homeostasis is likely engaged in this process. Nevertheless, the factors influencing maturation of hepcidin in the setting of chronic liver injury are unclear. Recently, the authors have suggested the association between serum prohepcidin levels and liver function impairment in liver cirrhosis patients. The aim of the current study was to assess the serum concentration of hepcidin prohormone in chronic hepatitis C and evaluate its possible association with disease activity as well as with the efficacy of pegylated interferon (PEG-IFN)/ribavirin therapy.

Innovations and breakthroughs

In the present study, comparable levels of serum prohepcidin in chronic hepatitis C and healthy individuals were noted. Interestingly, baseline prohepcidin was significantly higher in hepatitis C virus (HCV) genotype 3a than in HCV genotype 1. The new finding of the study is the association between serum prohepcidin and PEG-IFN/ribavirin efficacy in chronic hepatitis C. A statistically significant, gradual decrease of serum prohepcidin during successful antiviral treatment in HCV-1 and 3a individuals was shown, which was not the case in patients who did not respond to anti-HCV therapy.

Applications

The results of this study may suggest that in situations of liver function impairment prohepcidin synthesis, as well as activity or expression of converting enzymes, might be altered and affect circulating prohepcidin concentrations. This finding could also suggest that HCV interference with hepcidin synthesis may occur at the step of prohormone synthesis or maturation in the liver.

Peer review

This study was conducted to elucidate the effect of HCV and the influence of PEG-IFN/ribavirin combination therapy on the iron metabolism in patients with chronic hepatitis C. The manuscript was well prepared. The data analysis was appropriate. The authors obtained a reasonable result and the discussion was good.

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***NOD2/CARD15*, *ATG16L1* and *IL23R* gene polymorphisms and childhood-onset of Crohn's disease**

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Abstract

AIM: To assess whether the polymorphisms of *NOD2/CARD15*, autophagy-related 16-like 1 (*ATG16L1*), and interleukin-23 receptor (*IL23R*) genes play a more critical role in the susceptibility of childhood-onset than in adult-onset Crohn's disease (CD).

METHODS: Polymorphisms R702W, G908R, and 3020insC of *NOD2/CARD15*; rs2241880 A/G of *ATG16L1*, and rs11209026 (R381Q) of *IL23R* gene were assessed in 110 childhood-onset CD, 364 adult-onset CD, and 539 healthy individuals. Analysis of polymorphisms R702W, G908R, and 3020insC of *NOD2/CARD15* genotyping was performed by allele specific polymerase chain reaction (PCR) or by PCR-restriction fragment length polymor-

phism analysis. The polymorphisms rs2241880 A/G of the *ATG16L1*, and rs11209026 (R381Q) of the *IL23R* gene in the children's cohort were genotyped by PCR and melting curve analysis whereas adult group genotyping was performed using the Affymetrix Genome-Wide Human SNP Array 5.0 (500K).

RESULTS: The 3020insC allele in *NOD2/CARD15* was significantly higher in childhood than in adult-onset CD ($P = 0.0067$). Association with at least 1 *NOD2/CARD15* variant was specific for ileal disease (with or without colonic involvement). Even if the frequency of G allele of the rs2241880 *ATG16L1* polymorphism was increased in both paediatric and adult CD patients compared to controls ($P = 0.017$ and $P = 0.001$, respectively), no difference was observed between the childhood and the adult cohort. The rare Q allele of *IL23R* rs11209026 polymorphism was underrepresented in both paediatric and adult CD cases ($P = 0.0018$ and $P = 0.04$, respectively) and no difference was observed between the childhood and the adult cohort. The presence of the rs2241880 *ATG16L1* and rs11209026 *IL23R* polymorphisms did not influence disease phenotype.

CONCLUSION: Polymorphism 3020insC in *NOD2/CARD15* occurs statistically significantly more often in patients with childhood-onset CD than in patients with adult-onset CD. The *ATG16L1* and *IL23R* variants are associated with susceptibility to CD, but not early-onset disease.

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Key words: Genetics; Childhood-onset; Inflammatory bowel disease; Crohn's disease; Genetic susceptibility; *NOD2/CARD15*; *ATG16L1*; *IL23R*; Polymorphisms

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INTRODUCTION

Inflammatory bowel disease (IBD), Crohn's disease (CD) and ulcerative colitis (UC) are characterized by chronic relapsing inflammation of the digestive tract. As a multifactorial disorder, IBD is caused by a complex interaction of genetic, microbial, and immunological factors. Approximately 10%-20% of all IBD will present either in childhood or adolescence^[1].

Although age of onset seems to be a random event, recent data have shown that a subgroup of patients with early-onset IBD may have specific phenotypes that differs from adult onset IBD, suggesting that the pathogenesis of pediatric IBD and adult IBD may differ^[2-4]. A compelling speculation is that pediatric-onset IBD is more likely to be influenced by genetics compared to late onset, as there is less time for environmental modifiers to influence the onset of the disease. Adult-onset IBD is more likely to be confounded by abundant environmental exposure compared to childhood-onset IBD populations^[5].

Genetic risk factors for IBD have been extensively studied during the last year. *NOD2/CARD15* polymorphisms R702W, G908R, and 3020insC are independently associated with an increased risk of developing CD^[6-8]. Existing data remain conflicting as to whether *NOD2/CARD15* polymorphisms are associated with the age of onset of IBD, with some studies showing an effect toward a younger age of onset^[9,10], and others showing no effect^[11,12].

Recently, genome wide association studies (GWAS), in addition to offering further confirmation of the importance of the *NOD2/CARD15* gene, provided evidence for several determinants, including genes encoding autophagy-related 16-like 1 (*ATG16L1*) and interleukin-23 receptor (*IL23R*)^[13]. Moreover, Kugathasan *et al*^[14], by employing GWAS in a cohort of individuals with pediatric-onset IBD, provide further insights into disease pathogenesis.

Hampe *et al*^[15] were the first group to implicate the autophagy pathway in CD. The association of the Ala197Thr (rs2241880 A/G) variant of the *ATG16L1* gene with susceptibility to CD, has now been replicated in several independent cohorts^[16,17]. Recent studies have explored genotype associations in adult-onset IBD, and to a more limited extent in pediatric disease. Prescott *et al*^[18] suggested an association between the Ala197Thr variant allele and early-onset CD, as well as an effect of *ATG16L1* genotype on age at diagnosis. Baldassano *et al*^[19] replicated this association in a pediatric cohort. In contrast, Van Limbergen *et al*^[20] reported that the *ATG16L1* variant is not associated with early-onset IBD in a pediatric population in Scotland.

The *IL23R* gene is located on chromosome 1p31 and its corresponding ligand IL23 is a key component of the immunoregulatory pathway. The identification of an association between the R381Q variant of *IL23R* and CD is thus an important step toward the delineation of pathways related to inflammation to chronic inflammatory cascade characteristics of CD. Recent studies suggest that the R381Q variant in *IL23R* is associated with pediatric-onset CD^[21,22].

In view of these discrepant data regarding the association of key regulatory genes with CD susceptibility, the purpose of our study was investigate whether the known DNA polymorphisms in the *NOD2/CARD15*, *ATG16L1*, and *IL23R* genes determine susceptibility for CD in Greek children, and to compare these data with the frequency of these gene polymorphisms in adult-onset CD.

MATERIALS AND METHODS

Patients and controls

We examined 110 Greek children with CD, diagnosed before the age of 17, who attended the First Department of Pediatrics of Athens University, "Aghia Sophia" Children's Hospital between January 2007 and December 2008. The diagnosis of CD was based on standard clinical, endoscopic, radiologic and histopathologic criteria^[23]. Cases of UC and indeterminate colitis were excluded. Also excluded were children who had concomitant immune-mediated diseases such as asthma, diabetes type 1, juvenile diabetes, or juvenile arthritis. Blood samples from 364 adult CD patients were collected at the Inflammatory Bowel Disease (IBD) Outpatient Clinic of the Evagelismos Hospital. Most of them had already been used for genotype studies on *NOD2/CARD15*^[24]. The main clinical characteristics of IBD patients are detailed in Table 1. This cohort was compared to 539 healthy controls (94 children and 445 adults). Before commencement of the study, the Ethics Committee at the participating centers approved the recruitment protocols. All participants were informed of the study.

Genotyping

DNA was isolated from blood with the NucleoSpin blood kit (Macherey-Nagel, Germany). Patients were genotyped for the 3 common *NOD2/CARD15* polymorphisms i.e. R702W, G908R, and 3020insC using previously described methods^[8,24]. Polymorphisms rs2241880 A/G of the *ATG16L1*, and rs11209026 (R381Q) of the *IL23R* gene in the children's cohort were genotyped by PCR and melting curve analysis, using a pair of fluorescence resonance energy transfer (FRET) probes in LightCycler® 2.0 Instrument (Roche Diagnostics, Mannheim, Germany) as previously described^[25,26]. The adult group genotyping was performed using the Affymetrix Genome-Wide Human SNP Array 5.0 (500K)^[27].

Statistical analysis

The sample size and the power of the present sample size

Table 1 Demographics and clinical features of patients with CD (mean \pm SD) *n* (%)

	Childhood-onset CD (<i>n</i> = 110)	Adult-onset CD (<i>n</i> = 364)
Sex (male/female)	60/50	178/186
Age of diagnosis (yr)	11.5 \pm 4.8	28.99 \pm 14.22
Family history in first-degree relative	8 (7.27)	12 (3.29)
Smoking habit		
Never		146 (40.11)
Ex-smoker		32 (8.79)
Current		186 (51.10)
Localization of disease		
Ileitis	21 (19.09)	121 (33.24)
Colitis	29 (26.36)	38 (10.50)
Ileocolitis	48 (43.64)	187 (51.38)
Upper gastrointestinal	12 (10.91)	18 (4.94)
Disease features		
Inflammatory	78 (70.91)	223 (61.26)
Stricturing	20 (18.18)	99 (27.20)
Fistulizing	12 (10.91)	42 (11.54)
Extraintestinal manifestations	21 (19.09)	58 (15.93)

CD: Crohn's disease.

(90%) were calculated using the <http://sampsizes.sourceforge.net/iface/s3.html#cc> software. Statistical analysis was done using the software package GraphPad v. 3.00 (GraphPad Software, San Diego, CA). The Hardy-Weinberg equilibrium was tested by comparing the expected and observed genotypes in 2×3 chi-square tables. All the samples were in Hardy-Weinberg equilibrium ($P > 0.1$). For comparison between categorical variables, the Fisher's exact test or χ^2 test was used where appropriate. Single marker allelic tests were performed with Fisher's exact test. Odds ratios (OR) were calculated for the minor allele at each polymorphisms.

RESULTS

Genotype and allele frequencies of the *NOD2/CARD15* polymorphisms R702W, G908R, and 3020insC are detailed in Table 2.

The R702W polymorphism frequency did not differ significantly between child- and adult-onset CD. However, a statistically significant association was found between the R702W polymorphism and adult-onset CD only. The T allele frequency, leading to heterozygous and homozygous R702W polymorphisms, was increased in the adult CD population (9.1%) compared to controls (5.47%) ($P = 0.004$). In pediatric-onset CD, T allele frequency was also increased (8.18%), but no statistical significant difference was observed when compared to controls.

The C allele frequency, encoding the G908R variant, was also found significantly elevated (10%) in adult patients ($P = 0.014$), but was slightly reduced (9.54%) in childhood CD patients and in controls (6.77%).

For the 3020insC polymorphic allele, a significantly increased prevalence was found both in paediatric (16.36%), and in adult (9.47%) CD patients as compared, with the controls ($P < 0.0001$ in both cases). The

Table 2 Genotype and allele frequencies of *NOD2/CARD15* polymorphisms in childhood-onset CD patients, adult-onset CD, and controls *n* (%)

	Childhood-onset CD (<i>n</i> = 110)	Adult-onset CD (<i>n</i> = 364)	Controls (<i>n</i> = 539)
R702W			
Genotype			
CC	94 (85.45)	300 (82.40)	482 (89.40)
CT	14 (12.72)	62 (17.00)	55 (10.20)
TT	2 (1.81)	2 (0.55)	2 (0.37)
<i>P</i> value ¹	NS		
<i>P</i> value ²	NS		
<i>P</i> value ³		0.0260	
T allele	18 (8.18)	66 (9.10)	59 (5.47)
<i>P</i> value ¹ , OR (95% CI)	NS		
<i>P</i> value ² , OR (95% CI)	NS		
<i>P</i> value ³ , OR (95% CI)		0.0040, 1.72 (1.19-2.48)	
G908R			
Genotype			
GG	91 (82.73)	295 (81.00)	468 (86.83)
GC	17 (15.45)	65 (18.00)	69 (12.80)
CC	2 (1.81)	4 (1.00)	2 (0.37)
<i>P</i> value ¹	NS		
<i>P</i> value ²	NS		
<i>P</i> value ³		0.0420	
C allele	21 (9.54)	73 (10.00)	73 (6.77)
<i>P</i> value ¹ , OR (95% CI)	NS		
<i>P</i> value ² , OR (95% CI)	NS		
<i>P</i> value ³ , OR (95% CI)		0.0140, 1.53 (1.09-2.15)	
3020insC			
Genotype			
-	78 (70.90)	301 (82.69)	503 (93.32)
insC/-	28 (25.45)	57 (15.66)	35 (6.49)
insC/insC	4 (3.64)	6 (1.65)	1 (0.18)
<i>P</i> value ¹	< 0.0001		
<i>P</i> value ²	0.0200		
<i>P</i> value ³		< 0.0001	
insC allele	36 (16.36)	69 (9.47)	37 (3.43)
<i>P</i> value ¹ , OR (95% CI)	< 0.0001, 5.5 (3.39-8.94)		
<i>P</i> value ² , OR (95% CI)	0.0067, 1.87 (1.21-2.88)		
<i>P</i> value ³ , OR (95% CI)		< 0.0001, 2.95 (2.04-4.44)	

¹Childhood-onset *vs* controls; ²Childhood-onset *vs* adult onset; ³Adult onset *vs* controls. NS: Not significant.

frequency of 3020insC polymorphism was significantly higher in the paediatric cohort than in the adult-onset cohort ($P = 0.0067$).

Concerning the genotype-phenotype correlation ileal involvement was more frequent in individuals with at least one *NOD2/CARD15* polymorphism (78.25%) than in wild-type carriers (59%), in both cases of child- and adult-onset CD (OR = 2.46, 95% CI: 1.33-4.57, $P = 0.006$). The examined variants did not influence CD behavior in the present study.

Concerning the rs2241880 A/G polymorphism of the *ATG16L1* gene, the frequency of the G allele was increased in both pediatric and adult CD patients compared to controls ($P = 0.017$ and $P = 0.001$, respectively) as shown in Table 3. No association of the *ATG16L1*

Table 3 Genotype and allele frequencies of *ATG16L1* polymorphism rs2241880 and *IL23R* polymorphism rs11209026 in childhood-onset CD patients, adult-onset CD, and controls *n* (%)

	Childhood-onset CD (<i>n</i> = 110)	Adult-onset CD (<i>n</i> = 364)	Controls (<i>n</i> = 539)
rs2241880			
Genotype			
AA	17 (15.45)	46 (12.64)	104 (19.30)
AG	45 (40.91)	177 (48.63)	274 (50.83)
GG	48 (43.64)	141 (38.74)	161 (29.90)
<i>P</i> value ¹	0.0190		
<i>P</i> value ²	NS		
<i>P</i> value ³		0.0040	
G allele	141 (64.09)	459 (63.05)	596 (55.29)
<i>P</i> value ¹ , OR (95% CI)	0.0170, 1.44 (1.07-1.95)		
<i>P</i> value ² , OR (95% CI)	NS		
<i>P</i> value ³ , OR (95% CI)		0.0010, 1.38 (1.14-1.67)	
rs11209026			
Genotype			
RR	105 (95.45)	329 (90.38)	458 (84.97)
RQ	5 (4.54)	32 (8.79)	79 (14.66)
QQ	0	3 (0.82)	2 (0.37)
<i>P</i> value ¹	0.0120		
<i>P</i> value ²	NS		
<i>P</i> value ³		0.0220	
Q allele	5 (2.27)	38 (5.22)	83 (7.69)
<i>P</i> value ¹ , OR (95% CI)	0.0018, 0.28 (0.11-0.69)		
<i>P</i> value ² , OR (95% CI)	NS		
<i>P</i> value ³ , OR (95% CI)		0.0400, 0.66 (0.44-0.98)	

¹Childhood-onset *vs* controls; ²Childhood-onset *vs* adult onset; ³Adult onset *vs* controls.

polymorphism with early-onset CD was seen in our childhood-onset CD case-control analysis *vs* adult-onset CD analysis (Table 3). Furthermore, *ATG16L1* polymorphism did not influence the disease location and behaviour in the population studied.

The minor allele (Q) of the rs11209026 (R381Q) polymorphism of the *IL23R* gene was underrepresented in both childhood-onset and adult-onset CD, compared to controls (*P* = 0.0018 and *P* = 0.04, respectively) as shown in Table 3. No genotype-phenotype correlations were found among the CD patients studied with *IL23R* rs11209026 (R381Q) polymorphism.

DISCUSSION

Our present survey represents the first Greek study to document the frequency of the *NOD2/CARD15*, *ATG16L1*, and *IL23R* gene polymorphisms in childhood-onset CD, and compare them to those in an adult-onset CD cohort.

Our results confirm the previously reported association of *NOD2/CARD15* 3020insC mutation with early-onset CD^[9,28,29]. In our study, only the *NOD2/CARD15* 3020insC mutation was strongly associated with childhood-CD susceptibility, and its frequency was significantly

higher in the childhood cohort than in the adult-onset cohort, whereas in previous studies of early-onset CD patients, significantly higher carrier rates were found either for all the 3 *NOD2/CARD15* mutations^[10] or for G908R and/or 3020insC only^[12,30]. Others did not find any differences in the frequency of the 3 major *NOD2/CARD15* mutations between a childhood-onset and an adult-onset CD cohort^[31]. Both ileitis and ileocolitis were more frequent in carriers of *NOD2/CARD15* polymorphisms, indicating an association of *NOD2/CARD15* polymorphisms with ileal involvement. This confirms previous findings in both pediatric and adult patients^[8,10,12,30,32]. In contrast to other studies indicating an association between *NOD2/CARD15* polymorphisms and stricturing behaviour^[12] we did not find any significant association between *NOD2/CARD15* polymorphisms and CD phenotype. These conflicting results can be explained by the regional and ethnic differences in genotypes, and the relatively small numbers of patients included in these studies.

Recent studies have reported *ATG16L1* rs2144880 variant genotype association with adult-pediatric onset CD. So far, reports in the literature have been conflicting. Specifically, Prescott *et al*^[18] and Baldassano *et al*^[19] demonstrated an association of this variant with diagnosis at an earlier age. Van Limbergen *et al*^[20] and Latiano *et al*^[31] have suggested that the *ATG16L1* rs2144880 variant is associated with susceptibility to adult CD in Scotland, but not to early-onset disease. In our study in the Greek population, we were able to demonstrate an effect of this *ATG16L1* polymorphism on both paediatric and adult CD susceptibility. However, the allele and genotype frequencies in childhood-onset CD were comparable to that seen in adults and therefore, we can not support an association of *ATG16L1* with early-onset CD in Greece. In agreement with previous studies, in the genotype-phenotype analysis, no association was detected in the cases tested^[25,33].

Regarding the rs11209026 (R381Q) polymorphism of the *IL23R* gene, our study confirms the recently described associations between variants in the *IL23R* gene in both pediatric and adult-onset CD^[19-21,31]. Recently Yamazaki *et al*^[34] did not find any positive association of the *IL23R* gene polymorphism with CD in the Japanese population. Furthermore, in agreement with previous studies we did not observe any association of the rs11209026 (R381Q) polymorphism of the *IL23R* gene with the disease location and phenotype^[33,35]. This finding can be attributed to the distinct ethnic difference of genetic backgrounds of CD that has been reported previously for other genes between Japanese and Caucasian populations. It should be noted that the different results in allele frequencies between the studies can be explained by large regional and ethnic differences in genotypes, by the broad spectrum of clinical phenotypes of patients with CD, and by the relatively small numbers of cases included in most studies.

In conclusion, this study demonstrates that the 3020insC mutation in *NOD2/CARD15* gene is associated with CD in a Greek childhood-onset CD cohort. Moreover, the 3020insC mutation occurred significantly more often in childhood onset patients with CD than in

adult-onset CD patients. Our results provide an independent confirmation of the association of the *ATG16L1* rs2144880 and the *IL23R* rs11209026 (R381Q) polymorphisms with susceptibility to CD without supporting their implication in early-onset disease. Therefore, further studies are needed to specifically identify gene variants that predispose children to early paediatric onset disease.

COMMENTS

Background

As a multifactorial disorder, inflammatory bowel disease (IBD) is caused by a complex interaction of genetic, microbial, and immunological factors. Approximately 10%-20% of all IBD will present either in childhood or adolescence. Recent data have shown that a subgroup of patients with early-onset IBD may have specific phenotypes that differ from adult onset IBD, suggesting that the pathogenesis of paediatric IBD and adult IBD may differ. The study assesses whether the polymorphisms of *NOD2/CARD15*, autophagy-related 16-like 1 (*ATG16L1*), and interleukin-23 receptor (*IL23R*) genes play a more critical role in the susceptibility of childhood-onset than adult-onset Crohn's disease (CD).

Research frontiers

Although several gene loci have been associated with susceptibility to CD in adults, the aetiology of childhood CD is still unknown. The current study is one of the first studies assessing the impact of candidate gene's polymorphisms and disease susceptibility in childhood CD in a Greek cohort.

Innovations and breakthroughs

It is important to investigate the genetic variation in susceptibility to CD and identify markers that will facilitate identification of individuals at risk of developing this disease. The results suggest that the polymorphism 3020insC in *NOD2/CARD15* occurs statistically significantly more often in patients with childhood-onset CD than in patients with adult-onset CD. The *ATG16L1* and *IL23R* variants are associated with susceptibility to CD, but not early-onset disease.

Applications

The results of this study will help us to further understand the genetic determinants of childhood CD.

Peer review

The present study demonstrates that the 3020insC mutation in *NOD2/CARD15* gene is associated with CD in a Greek childhood-onset CD cohort. Moreover, the 3020insC mutation occurred significantly more often in childhood onset patients with CD than in adult-onset CD patients.

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Endoscopic submucosal dissection in dogs in a World Gastroenterology Organisation training center

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Abstract

AIM: To evaluate if canine models are appropriate for teaching endoscopy fellows the techniques of endoscopic submucosal dissection (ESD).

METHODS: ESD was performed in 10 canine models under general anesthesia, on artificial lesions of the esophagus or stomach marked with coagulation points. After ESD, each canine model was euthanized and surgical resection of the esophagus or stomach was carried out according to "The Principles of Humane Experimental Technique, Russel and Burch". The ESD specimens were fixed with needles on cork submerged in a formal solution with the esophagus or stomach, and delivered to the pathology department to be analyzed.

RESULTS: ESD was completed without complications using the Hook-knife in five esophageal areas, with a procedural duration of 124 ± 19 min, a length of 27.4 ± 2.6 mm and a width of 21 ± 2.4 mm. ESD was also completed without complications using the IT-knife2 in five gastric areas, with a procedural duration of 92.6 ± 19 min, a length of 32 ± 2.5 mm and a width of 18 ± 3.7 mm.

CONCLUSION: ESD is feasible in the normal esophagus and stomach of canine models, which are appropriate for teaching this technique.

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Key words: Endoscopic mucosal resection; Endoscopic submucosal dissection; Stomach neoplasms; Training

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INTRODUCTION

The incidence of esophageal adenocarcinoma is currently rising in Western countries and Latin America. Also, gastric cancer is the second leading cause of cancer-related death worldwide. Endoscopic submucosal dissection (ESD) has provided a new alternative for minimally invasive treatment of gastrointestinal (GI) early-stage cancer^[1-10].

With the purpose of preserving gastrointestinal function and obtaining specimens for precise histological evaluation, ESD^[11,12] has been developed for lesions ≥ 2 cm that are not amenable for endoscopic mucosal resection (EMR) because of their size^[1,13,14]. Adequate training is essential, because complications^[11,12] such as perforation are more likely with ESD than with EMR^[2,4,15-18]. In selected cases, ESD may replace surgery and provide clean margins for accurate histological diagnosis of the lesion borders and a complete curative treatment unlike other techniques such as piecemeal EMR^[2,4,15,16], cryotherapy, laser, argon plasma and photodynamic therapy because of their local recurrence rates.

There are few training centers around the world in which an endoscopy fellow can be trained in the ESD technique. There is probably only a formal ESD training program in Asian countries^[4-7,12,16], Germany^[8,9], recently in Portugal^[10] and at one site in Colombia. Animal models have been used to test ESD devices and new technology in this field, but a formal training program is still necessary to teach this technique in western countries and Latin America. For this reason, animal models are an invaluable learning resource.

Our aim was to evaluate if canine models are appropriate to teach endoscopy fellows the ESD technique.

MATERIALS AND METHODS

Ethics

This study was carried out in accordance with "The Principles of Humane Experimental Technique, Russel and Burch"^[19]. After receiving approval for the protocol from the ethics and animal research committees at our institution (GAS-14-09/09-1), the procedures were conducted on 10 mongrel dogs that weighed 18-20 kg that

had previously passed the quarantine period. All the procedures were done by a staff member of the Gastroenterology Department, who was trained at the Fujigaoka Hospital of the Showa University, Yokohama, Japan.

Cutting devices

Hook knife: The hook-type knife KD-620LR (Olympus Optical, Tokyo, Japan) was used to perform ESD in the esophagus^[6] of the canine models. It was right-angled 1 mm at the tip. Safety is improved compared with the use of a needle knife, because the submucosal tissue is hooked and pulled before incision. Safety is further improved when used in conjunction with a transparent soft cap D-201-11804 (Olympus Optical) because the tissue can then be pulled inside the hood (Figure 1E). The knife had a rotating function so that the operator could select the optimal direction of the hook.

Modified insulated-tipped knife 2: The modified insulated-tipped diathermic knife2 (IT-knife2) (KD-611L; Olympus Optical) was used to perform ESD in the stomach^[14] of the canine models. This knife consisted of a small ceramic ball attached to the tip of the blade, which functioned as an insulator so that the incision and dissection of the mucosa and submucosa could be performed safely.

A specialized feature of the modified IT-knife2^[6], which differs from the original IT-knife and other incision instruments, is that the portion between the insulator tip and the sheath, on the base of the ceramic ball, has a combination of the original IT-knife and the triangular knife that is used for removing the tissue with the electrodes on the distal side of the blade. This feature allows a less horizontal cutting position, which was necessary with the previous knife model.

Injection solution for mucosal and submucosal elevation

For all the canine models, we used normal saline solution with diluted epinephrine (1:10 000) and indigo carmine^[3,4,15], which were injected to elevate the lesion and separate the submucosal layer from the muscular layer, as required during all the steps of the ESD procedure, at a volume of 10-30 mL, depending on the size of the lesion. Also, this solution was used for small bleeding sites during ESD^[11,12], or when the cushion of the submucosal layer was insufficient for good dissection. Too much infiltration can obstruct good vision of the circumferential cutting, which interferes with the final cut of the lesion.

Electrosurgical HF-generator

The new electrosurgical HF-generator (ESG-100; Olympus Optical) allowed us to set the power for preprogrammed coagulation and cutting modes.

Endoscopic unit and video-endoscope

All the procedures were done with a CV-145 endoscopy unit (Olympus Optical) and a GIF-Q1145 video-endoscope (Olympus Optical).

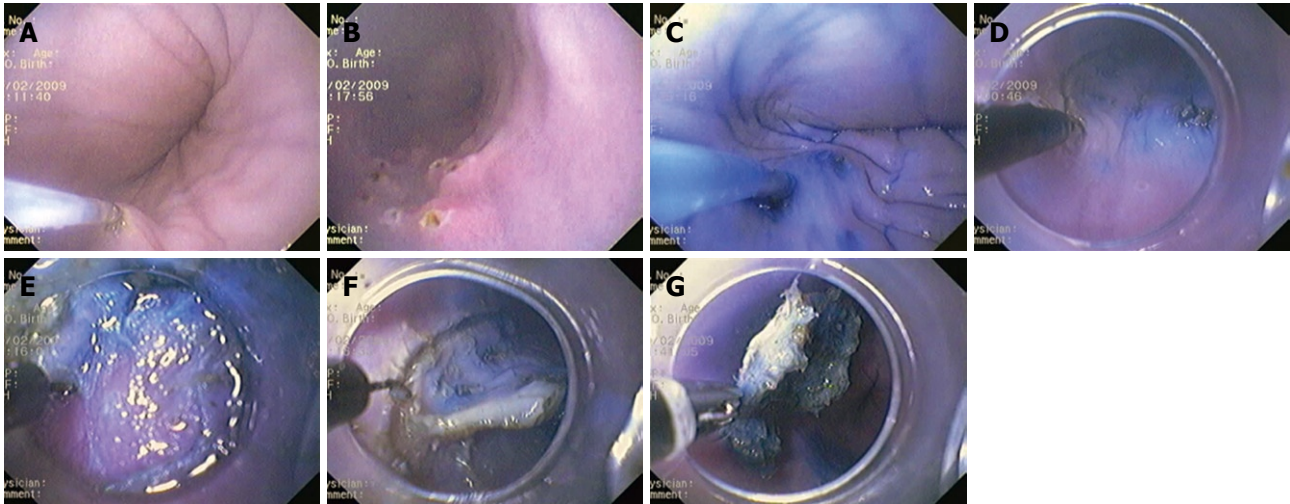


Figure 1 Procedure. A, B: Artificial lesion marked with coagulation points; C: Injection of normal saline with epinephrine and indigo carmine; D: Knife cutting of a circumference around the lesion; E: Transparent softcap provides lesion counter-traction; F: Soft cap attached to the tip of the endoscope during endoscopic submucosal dissection (ESD); G: Grasping forceps during retrieval of ESD specimens.

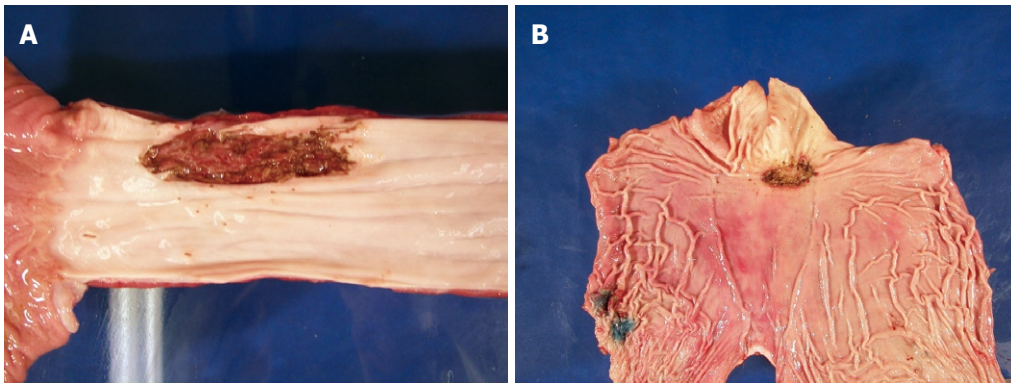


Figure 2 Photographs of the retrieved surgical specimens. Esophagus (A) and stomach (B) after ESD.

ESD procedure

The initial step was to produce an artificial lesion marked with coagulation points (Figure 1A and B) in the esophagus or stomach of 10 canine models (one at a time), under general anesthesia. The settings for marking the points with the precutting knife (KD-10Q-1; Olympus Optical) for the artificial lesions were: power, 20, and force coag2 mode. After marking, the above-mentioned solution was injected so that the peak of the lifting was outside of the markings (Figure 1C). ESD was performed with the Hook-knife in the esophagus or the IT-knife2 in the stomach. After that, we cut a circumference around the lesion with the knife (Figure 1D). We then used another injection to elevate the submucosal layer of the center of the lesion. The settings for the mucosal cutting of the circumference were power at 80 in pulse cut slow mode. Afterwards, we performed submucosal dissection using a soft cap D-201-11804 attached to the tip of the endoscope (Figure 1E) using the same settings as in the circumferential cut. This transparent soft cap provided lesion counter-traction (Figure 1F), which is similar to a surgeon's left hand, which made it easy to dissect the submucosal layer. The grasping forceps (FG-49L-1; Olympus Optical) were used to retrieve the ESD specimens (Figure 1G).

After ESD, each dog was euthanized and surgical resection of the esophagus or stomach (Figure 2A and B) was carried out according to “The Principles of Humane Experimental Technique, Russel and Burch”^[19]. The ESD specimens were fixed with needles on a piece of cork (Figure 3A and B) submerged in formol solution, together with the resected esophagus or stomach, and delivered to the pathology department for investigation.

RESULTS

The ESD procedure was completed without complications using the Hook-knife in five esophageal areas, with a procedural duration of 124 ± 19 min, a length of 27.4 ± 2.6 mm, and a width of 21 ± 2.4 mm. The ESD procedure was also completed without complications using the IT-knife2 in five gastric areas, with a procedural duration of 92.6 ± 19 min, a length of 32 ± 2.5 mm, and a width of 18 ± 3.7 mm.

There was no perforation in either group. This fact was corroborated by an expert pathologist^[8] after histological and macroscopic study of the specimens.

Descriptive statistics of the pilot study are shown in Table 1. The size of each specimen, the procedural duration, the site of ESD, and its complications are shown

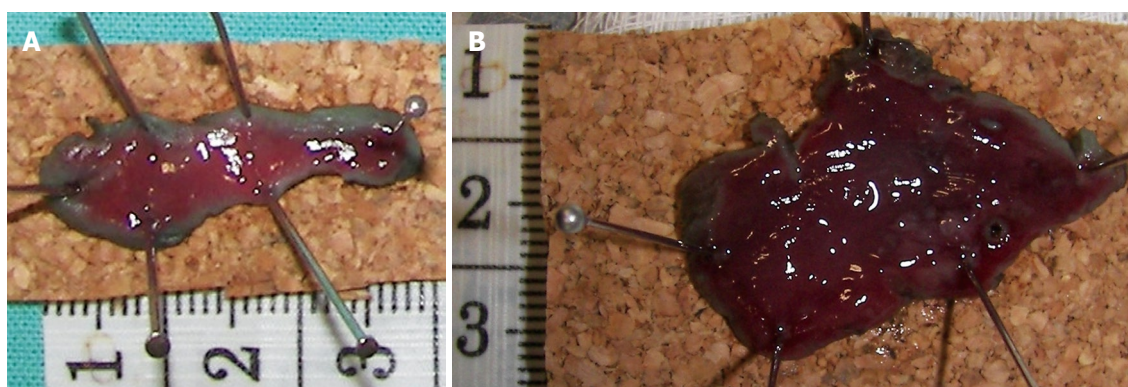


Figure 3 ESD specimens (A and B) fixed with needles in cork.

Table 1 Descriptive statistics of the endoscopic submucosal dissection (ESD) pilot study

Group	Values	Procedural duration (min)	Specimen length (mm)	Specimen width (mm)
Hook-knife (Esophagus)	n = 5			
	mean	124'	27.400	21.000
	SD	42' 778"	5.814	5.447
	SE	19' 131"	2.600	2.449
IT-knife2 (Stomach)	n = 5			
	mean	92' 60"	32.000	18.000
	SD	43' 518"	5.701	8.367
	SE	19' 462"	2.550	3.742
Total (Esophagus and stomach)	n = 10			
	mean	108' 30"	29.700	19.500
	SD	43' 919"	5.945	6.852
	SE	13' 888"	1.880	2.167
	Range	137'	23.000	20.000

Table 2 ESD in the esophagus using the Hook-knife (KD-620LR) and in the stomach using the IT-knife2 (KD-611L)

Size of specimen	Procedural duration (min)	Site of ESD	Complications
Hook-knife (Esophagus)			
30 mm × 20 mm	180	Lower	None
30 mm × 20 mm	160	Lower	None
30 mm × 20 mm	100	Lower	None
17 mm × 15 mm	90	Upper	None
30 mm × 30 mm	90	Mid	None
IT-knife2 (Stomach)			
25 mm × 10 mm	150	Lesser curvature of antrum	None
35 mm × 30 mm	120	Antrum posterior wall	None
30 mm × 20 mm	90	Lesser curvature of antrum	None
40 mm × 10 mm	60	Lesser curvature of antrum	None
30 mm × 20 mm	43	Upper greater curvature	None

in Table 2 for the esophagus using a Hook-knife and in Table 2 for the stomach using an IT-knife2.

DISCUSSION

ESD has provided a new alternative for minimally invasive treatment of gastrointestinal early-stage^[13] (T1mN0) cancer lesions > 2 cm, with minimal risk of deeper wall-layer involvement or lymph node metastases, as confirmed by endoscopic ultrasonography (EUS)^[1-7]. This technique provides a curative alternative that preserves gastrointestinal function and offers an accurate histological T stage that replaces, in selected cases, surgery or other therapeutic attempts like piecemeal EMR^[2,4,15,16], cryotherapy, laser, argon plasma and photodynamic therapy because of their local recurrence rates.

Multiple factors make esophageal, small intestine and colon ESD more difficult compared with gastric ESD, including difficulties in maintaining the position of the endoscope, the thinness of their walls, the luminal angulations, and peristalsis. To avoid complications such as perforation during the ESD procedure requires adequate training^[4-10,12,16], and for this reason, animal models are invaluable as a learning resource.

Injection of indigo carmine^[3,4,15] and the consequent blue staining of the submucosal tissue (Figure 1B) was used to identify the submucosal layer and the deep margin during the resection process.

Although there are several injection solutions commercially available in Japan for the submucosal cushion (Artz, 3.8% NaCl^[15], Suveniel, 20% glucose, and glyceol), we decided to standardize the use of normal saline with epinephrine and indigo carmine for all the ESD procedures, and reserve for difficult cases the use of Suprahyl (Asofarma de Mexico S.A. de C.V., Mexico) and sodium hyaluronate^[17] (Meiji Seika Kaisha LTD, Tokyo, Japan).

Also, the solution of normal saline, epinephrine and indigo carmine was used in cases with small bleeding sites during ESD. Fortunately, we did not have any bleeding complications^[11,12] during ESD. It is also very important to use hemostatic forceps as the submucosal dissection progresses for all the visible vessels below the lesion, to prevent bleeding obstructing vision of the cutting direction and post-ESD bleeding.

The electrosurgical HF-generator allowed us to set the power for preprogrammed coagulation and cutting

modes, which proved to be very important in performing the ESD without bleeding or perforation.

The right amount of infiltration always allows visualization of the circumference of the lesion for the final cut. Also, it is important to keep in mind how the muscular layer runs and to observe accurately the accumulation of liquids, to plan the cutting direction so that gravity can assist with the total dissection of the lesion.

Histopathological examination of the ESD specimens by an expert pathologist is essential to confirm clean margins, and complete removal of the mucosa and submucosa in all cases.

The limitations of this research were that it involved artificial non-neoplastic lesions and a small number of specimens in a pilot study.

ESD is a feasible technique in the normal esophagus and stomach of dogs and the use of these models is appropriate for teaching this technique.

ESD is a procedure that requires not only endoscopic skills, but also a good understanding of the endoscopy devices, techniques and technology to identify and treat EGC lesions. The minimum proficiency required by an endoscopy fellow to start ESD training is achieved after the appropriate knowledge is acquired. To begin with, the trainee assists an expert in the field, and eventually, he/she is assisted by an expert. Team work and coordination between the endoscopist and their assistants is also essential to perform ESD successfully.

Further studies and practice are needed to improve performance in canine models and to evaluate when the learning curve has been completed and a trainee is ready to perform ESD in patients. Also, in western countries and Latin America, multicenter studies are necessary before this technique can become a routine procedure.

The implementation of an ESD course at a WGO training center^[1] will not only help to open numerous research areas that could contribute to the treatment of early GI cancer lesions, but will also help the worldwide dissemination of these techniques.

COMMENTS

Background

The incidence of esophageal adenocarcinoma is currently rising in Western countries and Latin America. Also, gastric cancer is the second leading cause of cancer-related death worldwide. Endoscopic submucosal dissection (ESD) has provided a new alternative for minimally invasive treatment of gastrointestinal early-stage cancer, with the preservation of gastrointestinal (GI) function. Because of possible complications, such as perforation, adequate training is necessary to perform ESD successfully.

Research frontiers

Complete curative treatment of early GI cancer with ESD has been demonstrated in the Asian countries. However, after many years of investigation in this field, there are still a lack of multicenter studies that have incorporated this technique into the everyday practice in Western countries and Latin America. In the present study, the authors demonstrated the feasibility of using animal models as a learning resource to teach the ESD technique.

Innovations and breakthroughs

There are few training centers around the world at which an endoscopy fellow can be trained in the ESD technique. There is probably only a formal ESD training program in Asian countries, Germany, recently in Portugal, and at one site in

Colombia. Completion of the learning curve and implementation of training in the ESD technique at a health institute and World Gastroenterology Organisation training center that annually receives trainees from all over Mexico and Central and South America will not only help to open numerous research areas that could contribute to the treatment of early GI cancer, but will also help the worldwide dissemination of these techniques.

Applications

Until now, ESD has been considered technically difficult, hazardous and time consuming. However, new technology is allowing us to overcome these drawbacks. Animal models have been used to test ESD devices and new technology but a formal training program is still necessary to teach this technique in western countries and Latin America. For this reason, animal models are an invaluable learning resource.

Terminology

ESD is a technique that allows *en-bloc* resection of early GI cancers > 2 cm, with minimal risk of deeper wall-layer involvement or lymph node metastases, as confirmed by endoscopic ultrasonography. In selected cases, ESD may replace surgery and provide clean margins for accurate histological diagnosis of the lesion borders and complete curative treatment.

Peer review

This is an interesting investigation that presents the feasibility of using canine models for ESD training.

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Hepatitis B-related events in autologous hematopoietic stem cell transplantation recipients

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Abstract

AIM: To investigate the frequency of occult hepatitis B, the clinical course of hepatitis B virus (HBV) reactivation and reverse seroconversion and associated risk factors in autologous hematopoietic stem cell transplantation (HSCT) recipients.

METHODS: This study was conducted in 90 patients undergoing autologous HSCT. Occult HBV infection was investigated by HBV-DNA analysis prior to transplantation, while HBV serology and liver function tests were screened prior to and serially after transplantation. HBV-related events including reverse seroconversion and reactivation were recorded in all patients.

RESULTS: None of the patients had occult HBV prior to transplantation. Six (6.7%) patients were positive

for HBV surface antigen (HBsAg) prior to transplantation and received lamivudine prophylaxis; they did not develop HBV reactivation after transplantation. Clinical HBV infection emerged in three patients after transplantation who had negative HBV-DNA prior to HSCT. Two of these three patients had HBV reactivation while one patient developed acute hepatitis B. Three patients had anti-HBc as the sole hepatitis B-related antibody prior to transplantation, two of whom developed hepatitis B reactivation while none of the patients with antibody to HBV surface antigen (anti-HBs) did so. The 14 anti-HBs- and/or anti-HBc-positive patients among the 90 HSCT recipients experienced either persistent (8 patients) or transient (6 patients) disappearance of anti-HBs and/or anti-HBc. HBsAg seroconversion and clinical hepatitis did not develop in these patients. Female gender and multiple myeloma emerged as risk factors for loss of antibody in regression analysis ($P < 0.05$).

CONCLUSION: Anti-HBc as the sole HBV marker seems to be a risk factor for reactivation after autologous HSCT. Lamivudine prophylaxis in HbsAg-positive patients continues to be effective.

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Key words: Autologous stem cell transplantation; Hepatitis B reactivation; Occult hepatitis; Multiple myeloma; Lymphoma

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INTRODUCTION

Hepatitis B virus (HBV) infection is one of the major human health problems in the world. It is estimated that 350-400 million (approximately 5%) people worldwide are affected by HBV infection^[1,2]. Turkey is one of the endemic areas for HBV infection. The spectrum of HBV-related disease ranges from asymptomatic HBV carrier state, chronic hepatitis B, acute hepatitis B, to rarely fulminant hepatitis. Chronic HBV infection is also associated with cirrhosis and hepatocellular carcinoma^[1]. A new clinical status of persistence of HBV genomes in the liver tissue and/or serum in HBV surface antigen (HBsAg)-negative individuals is designated as “occult HBV infection”^[3,4]. Though suspected to exist since the early 1980s, this peculiar form of chronic viral infection has been better identified during the past 10 years. The availability of highly sensitive molecular biology techniques made it possible to disclose several of its virological aspects and to show its worldwide distribution, as well as revealing its possible implications in various clinical contexts^[5,6].

Intensive chemotherapy, radiotherapy, monoclonal antibody treatment and autologous and allogeneic hematopoietic stem cell transplantation (HSCT) give rise to immune dysfunction which consequently exposes the patients to the risk of many infections, including viral hepatitis^[7,8]. Exacerbation of hepatitis B is a serious cause of morbidity and mortality in patients undergoing cytotoxic or immunosuppressive therapy, particularly in areas where chronic HBV infection is endemic^[9]. Iwai *et al*^[10] reported lethal hepatic failure in an immunosuppressed patient after allogeneic bone marrow transplantation due to reactivation of latent HBV. The patient had antibody to HBV surface antigen (anti-HBs) and no viral DNA detected in the serum prior to transplantation^[10]. Carpenter *et al*^[11] described a patient with chronic myelogenous leukemia whose pretransplantation evaluation revealed normal serum aspartate and alanine aminotransferase levels, a negative serum for HBsAg, anti-HBs, antibody to HBV core antigen (anti-HBc), and HBV-DNA assessed by a sensitive real-time polymerase chain reaction (PCR). The donor was seropositive for anti-HBc, but serum HBV-DNA was negative by PCR. This case went on to develop acute HBV infection 7 mo after transplantation^[11].

To date, there have been several reports of reverse seroconversion/HBV reactivation in patients previously positive for anti-HBs after allogeneic or autologous HSCT^[12-19]. There is also growing evidence regarding increased frequency of occult HBV infection in areas where hepatitis B is endemic^[5,20-22]. However, information pertaining to prevalence of occult hepatitis B prior to HSCT and frequency of reverse seroconversion/HBV reactivation after HSCT has been scant.

This study aimed to determine: (1) prevalence of occult HBV infection in patients with various hematologic malignancies who are candidates for autologous HSCT; (2) frequency, course and results of HBV reactivation

and reverse seroconversion after autologous HSCT; and (3) risk factors for HBV reactivation and reverse seroconversion in patients undergoing autologous HSCT for various malignancies.

MATERIALS AND METHODS

Study population

Ninety consecutive patients who underwent autologous HSCT at the Stem Cell Transplantation Unit of Gazi University with the diagnosis of various hematological malignancies from September 2003 through July 2008 were included in the study.

Detection of hepatitis markers and HBV-DNA

HBV serology and liver function tests were screened before transplantation, at day +30 and every 3 mo thereafter. HBV-DNA was tested in all patients prior to transplantation, and after transplantation in the patients who had reverse seroconversion/reactivation of HBV or acute HBV infection. HBV serology (HBsAg, anti-HBs, anti-HBc, HBeAg, anti-HBe) was tested by ELISA. HBV-DNA was extracted using the MagAttract Virus Mini M48 kit (Qiagen, Hamburg, Germany) on the Bio-Robot M48 workstation (Qiagen, Hamburg, Germany) following the manufacturer's instructions. Besides HBV markers, all patients were screened for antibody to hepatitis C virus (anti-HCV) and anti-human immunodeficiency virus antibodies prior to transplantation.

Definitions of HBV-related events

Hepatitis was defined as a serum alanine aminotransferase level greater than 100 IU/mL on two consecutive determinations more than 5 d apart^[23].

Occult hepatitis B was defined as the presence of HBV-DNA and the absence of HBsAg in plasma^[3].

HBV reactivation was defined as seroconversion from HBsAg-negative to positive for HBsAg with an increase in HBV-DNA levels compared with baseline HBV-DNA levels, in the absence of clinical and laboratory features of acute infection with hepatitis A, hepatitis C, or other systemic infection^[24].

Reverse seroconversion was defined as appearance of HBsAg and disappearance of anti-HBs after HSCT in patients who had no HBsAg but did have anti-HBs or anti-HBc before transplantation^[14,19].

Loss of antibody was defined as disappearance of anti-HBs and/or anti-HBc after transplantation.

Lamivudine prophylaxis

All patients with a positive HBsAg received 100 mg/d lamivudine prophylaxis before the transplantation conditioning regimen. Lamivudine prophylaxis was maintained for at least 1 year after transplantation. Liver function tests were within normal limits in all patients before starting the conditioning regimen.

This study was approved by the Institutional Board of Gazi University Medical School.

Statistical analysis

Statistical analysis was performed using the program of SPSS for Windows, version 11.5. Relative risks for reactivation of HBV and loss of antibodies were calculated by logistic regression analysis. $P < 0.05$ was considered to be statistically significant.

RESULTS

Patient characteristics

Among the ninety (59 male and 31 female) patients included in the study, forty-six had multiple myeloma (MM), 23 Hodgkin's lymphoma (HL), 15 non-HL (NHL), 4 acute myeloblastic leukemia, 1 acute lymphoblastic leukemia, 1 primitive neuroectodermal tumor (PNET). The median age at transplantation was 48 years (range: 16-71 years). The median follow-up after autologous HSCT was 15 mo (range: 6-36 mo). Patients with MM comprised the most common subgroup (46 patients, 51.1%) as shown in Table 1.

Changes in HBV serologic markers

Pre-transplantation surveillance of HBV infection showed that 6 patients (6.7%) were HbsAg-positive; three of these patients were HBV-DNA-positive. None of the patients in our cohort had occult hepatitis B. Total numbers of patients with anti-HBs and anti-HBc were 30 (33.3%) and 23 (25.6%), respectively. Forty-nine patients (54.4%) had neither anti-HBs nor anti-HBc; 12 patients (13.3%) had both (Table 2).

Clinical hepatitis B infection was detected in three patients. Two of these infections were HBV reactivation while one patient developed acute hepatitis B. While none of the patients with positive HBsAg reactivated after autologous HSCT, 2 of the 3 patients with negative HBsAg and positive anti-HBc had hepatitis B reactivation. On the other hand, none of the patients with negative HBsAg and positive anti-HBs reactivated.

Six patients with pretransplantation HBsAg received prophylactic lamivudine. Autologous HSCT was performed under lamivudine prophylaxis in those 6 patients; none of whom had HBV reactivation in the post-transplantation period.

Reactivation case 1: A 55-year-old male patient with MM had anti-HBc antibody as the sole HBV-related marker at pretransplantation screening. HBsAg, anti-HBs, HBeAg, anti-Hbe and HBV-DNA were all negative. The patient had received four cycles of VAD (vincristine, adriamycin, dexamethasone) as first line treatment, and four cycles of thal-dex (thalidomide-dexamethasone) as second line treatment. Cyclophosphamide-etoposide and melphalan were administered as mobilization and conditioning regimens, respectively. At day 110 after autologous HSCT, HBsAg and HBV-DNA became positive. He was in partial remission at the time of hepatitis B reactivation. Lamivudine treatment was started on the same day. Although ALT was within normal limits ini-

Table 1 Clinical characteristics of patients

Characteristic	n (%)
Total	90 (100.0)
Gender (M/F)	59 (65.6)/31 (34.4)
Median age (yr)	48 (range: 16-71)
Underlying disease	
Multiple myeloma	46 (51.1)
Hodgkin's lymphoma	23 (25.6)
Non-Hodgkin's lymphoma	15 (4.4)
Acute myeloblastic leukemia	4 (4.4)
Acute lymphoblastic leukemia	1 (1.1)
Primitive neuroectodermal tumor	1 (1.1)
Conditioning regimen	
Melphalan	47 (52.2)
BEAM	35 (38.9)
Busulfex-Cy	4 (4.4)
Busulfex-Melphalan	1 (1.1)
TBI-Cy-Thiotepa	1 (1.1)
Thiotepa-Cy-Bu	1 (1.1)
Z-BEAM	1 (1.1)

BEAM: The combination of Carmustine (BCNU), Etoposide, Cytarabine (Ara-C) and Melphalan; Z-BEAM: The combination of Ybritumomab Tiuxetan (Zevalin®), Carmustine (BCNU), Etoposide, Cytarabine (Ara-C) and Melphalan; Cy: Cyclophosphamide; TBI: Total body irradiation.

tially, it increased to 590 U/L (0-49 U/L, reference value) at day 225 post-transplantation. Subsequently ALT levels decreased gradually and normalized within 2 wk. Anti-HBc was still positive at the time of HBV reactivation and it remained positive during follow-up period. Anti-HBe and anti-HBs have not become positive during the follow-up. HBsAg and HBV-DNA disappeared in the first year after autologous HSCT. HBV-DNA titer was 1.1×10^6 copies/mL at day +110, 1.9×10^6 copies/mL at day +183, 0.9×10^6 copies/mL at day +225, 1100 copies/mL at day +256 and negative at day 365 after autologous HSCT.

Reactivation case 2: A 55-year-old male patient with MM had anti-HBc antibody as the sole HBV-related marker at pretransplantation screening. HBsAg, anti-HBs, HBeAg, anti-Hbe and HBV-DNA were all negative in this patient as well. At day 148 after autologous HSCT, HBsAg and HBV-DNA became positive. Lamivudine treatment was started the same day. ALT level was normal initially, but it was measured as 1157 U/L at day +198. Subsequently, ALT level decreased gradually and returned to normal limits within 2 wk. HBV-DNA titer was 1000 copies/mL at the time of reactivation, then decreased gradually and it became negative at day +225. HBsAg also disappeared in patient's serum on day 365 after autologous HSCT. Anti-HBc was still positive at the time of HBV reactivation and it remained positive during follow-up. Anti-HBe and anti-HBs became positive at day +198 and day +457 after autologous HSCT, respectively. He was in complete remission at the time of hepatitis B reactivation.

Acute HBV infection case 1: A 48-year-old female

Table 2 Pretransplant HBV serologic results and HBV-related events after transplantation *n* (%)

Pretransplantation HBV serologic result					HBV-related events after transplantation	
HBsAg	Anti-HBs	Anti-HBc	Anti-HBe	Patients	HBV reactivation/total patients	Loss of antibody/total patients
Negative	Negative	Negative	Negative	45 (50.0)	1/45	1/45
Negative	Positive	Negative	Negative	15 (16.7)	-	8/15
Negative	Negative	Positive	Negative	3 (3.3)	2/3	1/3
Negative	Negative	Negative	Positive	1 (1.1)	-	-
Positive	Negative	Negative	Negative	3 (3.3)	-	-
Positive	Negative	Positive	Positive	3 (3.3)	-	-
Negative	Negative	Positive	Positive	5 (5.6)	-	-
Negative	Positive	Negative	Positive	3 (3.3)	-	-
Negative	Positive	Positive	Negative	6 (6.7)	-	4/6
Negative	Positive	Positive	Positive	6 (6.7)	-	-

HBV: Hepatitis B virus (HBV); HBsAg: HBV surface antigen; anti-HBs: Antibody to HBV surface antigen; anti-HBc: Antibody to HBV core antigen.

Table 3 Characteristics and serologic marker details of the patients with loss of antibody

Patients No.	Age (yr)	Gender	Antibody disappearance time (post-transplantation months)		Time of antibody reappearance (mo)	Disease
			Anti-HBs	Anti-HBc		
#1	71	F	+6	-	+18	MM
#6	59	F	+6	-	-	MM
#8	50	M	+12	-	+18	MM
#10	48	F	+9	-	-	MM
#12	52	F	+24	+6	-	MM
#27	46	F	+1	-	+12	MM
#28	59	M	+1	-	+8	MM
#39	59	M	+1	-	-	MM
#40	30	M	-	+1	+6	NHL
#41	68	M	-	+3	-	MM
#43	16	F	+1	-	-	PNET
#48	56	F	+1	+1	+6 (anti-HBc)	MM
#55	64	M	+6	-	-	MM
#89	23	F	+3	-	-	HD

MM: Multiple myeloma; NHL: Non-Hodgkin's lymphoma; PNET: Primitive neuroectodermal tumor.

patient with MM received methylprednisolone for autologous graft-versus-host disease (GVHD) treatment at day 18 post-transplantation. HBsAg, anti-HBs, anti-HBc, HBeAg, anti-HBe and HBV-DNA were all negative prior to transplantation in this patient. On day +210 post-transplantation, HBsAg, HBeAg and HBV-DNA became positive. Lamivudine treatment was started. ALT level, which initially was normal, gradually increased to 534 U/L at day 287 post-transplantation. She remained on steroid treatment for the treatment of grade IV chronic dermal GVHD. HBV-DNA titer was 5.3×10^7 copies/mL on the day of reactivation, 1×10^6 copies/mL at day +287, 3.3×10^4 copies/mL at day +365, and 2000 copies/mL at day 580 after autologous HSCT. On day 580 post-transplantation when the data of this study were analyzed, she was positive for HBsAg, HBeAg and HBV-DNA (gradually decreasing titer, YMDD mutation negative).

Loss of antibody

The 14 anti-HBs- and/or anti-HBc-positive patients who underwent autologous HSCT experienced persistent (8 patients) or transient (6 patients) disappearance of anti-

HBs and/or anti-HBc. Interestingly, neither seroconversion for HBsAg nor clinical hepatitis developed in these patients. Eleven of these 14 patients had MM, 1 had NHL, 1 had HL, and 1 had PNET. Anti-HBs or anti-HBc reappeared in 5 of these patients within 6 to 8 mo. Characteristics of these patients and details of the changes in serologic markers are shown in Table 3.

Possible risk factors for reactivation and loss of antibodies, including age, gender, underlying disease, the number of pre-transplant chemotherapy cycles and the mobilization and conditioning regimens, were tested both in univariate and multivariate logistic regression analysis. No specific risk factors were found for reactivation of HBV. Female gender ($P = 0.04$, OR = 3.4, CI: 1.17-9.81) and MM ($P = 0.035$, OR = 4.29, CI: 1.29-16.2) emerged as risk factors for loss of antibody in univariate analysis; whereas only MM was an independent risk factor in multivariate analysis ($P = 0.043$, OR = 3.6, CI: 1.1-14.4).

DISCUSSION

HBV carriers have increased liver-related morbidity and

mortality during chemotherapy of hematological disorders^[25]. Reactivation in patients supposedly immune to hepatitis B has also become an emerging problem during the treatment of various hematologic disorders with chemotherapeutic agents, monoclonal antibodies, immunosuppressive agents and HSCT^[26]. HBV is a latent virus which may persist for a long time despite the presence of anti-HBs and/or anti-HBc antibodies. Reverse seroconversion/reactivation may occur which results in increased liver-related morbidity and mortality^[25], particularly in areas with high hepatitis B prevalence. Turkey is among the middle-endemic regions for hepatitis B with a mean seroprevalence for hepatitis B among healthy blood donors reported as 4.19% (3.9%-12.9%)^[2,27].

Occult HBV infections are defined as the presence of HBV-DNA and the absence of HBsAg in liver tissue, plasma or serum of HBV-infected patients^[3-5]. The risk of reactivation and fulminant course is particularly high in this group of patients. We investigated the prevalence of occult HBV infection in patients with various hematologic malignancies who were candidates for autologous HSCT. We also investigated the frequency and the risk factors associated with HBV reactivation/reverse seroconversion after autologous HSCT.

Pre-transplantation surveillance of HBV infection showed that six patients (6.7%) were HbsAg-positive, three of whom were also HBV-DNA-positive. The 6.7% HBsAg seropositivity among patients with hematologic malignancies in the presented study seems to be similar to the frequency in the normal population.

There are several reports describing effective lamivudine prophylaxis in HbsAg-positive patients receiving chemotherapy or HSCT^[9,28-30]. None of our patients with HBsAg, with or without positive HBV-DNA, had hepatitis reactivation under lamivudine prophylaxis. Our study results might suggest an efficacy of lamivudine prophylaxis in HbsAg-positive patients undergoing autologous HSCT, although lamivudine resistance has also been reported in other series^[23,31,32].

Reactivation of HBV in patients previously positive for anti-HBs and/or anti-HBc, so called “reverse seroconversion”, has been reported in immunosuppressed patients including patients with acquired immunodeficiency and recipients of organ transplantation or HSCT^[14,33,34]. In particular, patients with lymphoma receiving rituximab with or without chemotherapy have an increased risk of reactivation^[26]. The precise frequency of reactivation in the setting of HSCT in anti-HBs- and/or anti-HBc-positive and HbsAg-negative patients is not known, though previous reports mention a frequency of 7% to 12%^[18,35,36]. Two among the 3 patients (66.7%), who had anti-HBc as the “only” hepatitis B-related marker prior to transplantation developed HBV reactivation after autologous HSCT. The cause of the hepatitis in the two patients presented above seems to be reactivation of a previous infection. Similarly, Matsue *et al*^[37] found an increased risk of reactivation in their patients who had anti-HBc antibodies, while none of the patients in their

series with anti-HBs developed a reverse seroconversion. In the absence of HBV-DNA prior to transplantation, patients with anti-HBc as the sole HBV-related marker might be a variant or subgroup of patients with occult hepatitis where the infection is limited to the liver. Our results suggest that patients with anti-HBc in the absence of anti-HBs seem to be a high risk group requiring monitoring and even prophylaxis, though these data warrant verification with further prospective randomized studies. In contrast to the patients presented by Matsue *et al*^[37], whose HBV reactivation was after corticosteroid therapy for chronic GVHD, our patients who had HBV reactivation were not receiving corticosteroids and were in remission from their underlying diseases. The immunosuppressive effect of autologous HSCT *per se* might be responsible from the reverse seroconversion in our cases.

At present, HBV serological markers including HBsAg, anti-HBs and anti-HBc may not be adequate to perceive the existence of HBV. Recent studies have demonstrated that improvement of PCR methods have favored the recognition of occult HBV infections in an increasing number of clinical settings and geographical areas. To date, documentation of occult HBV prevalence rates has been limited to blood or organ donors and selected patient populations such as hemodialysis patients with HCV^[38-40], AIDS patients and hemophiliacs. The prevalence of HBV viremia in adult hemodialysis patients is 3.8%-15%, or 4-20 times higher than what standard monoclonal antibody-based HBsAg testing would have suggested. It is possible that host immune mechanisms and viral interactions can maintain HBV infection in a latent state until more profound immunosuppression ensues^[38-40]. Recently, occult HBV prevalence has also been investigated in community-based populations^[21]. The prevalence of occult hepatitis was found to be 15.3% in a cohort of 124 consecutive HbsAg-negative stem cell donors in Hong Kong, which suggests occult hepatitis is a matter of concern in endemic areas^[13].

None of the patients presented in this study with various hematological malignancies had occult hepatitis B prior to transplantation. Uhm *et al*^[7] similarly have not detected occult HBV infection in their patients with hematologic malignancies in Korea, which is also an endemic area for HBV infection. Absence of occult hepatitis in a relatively high risk group of patients requires further elucidation. On the other hand, patients with anti-HBc as the only HBV marker might be a variant subgroup with occult hepatitis. More sensitive methods such as detection of covalently closed circular DNA - the key intermediate of replication of the virus - in liver biopsy specimens may be required in patients with immunosuppression, in order to exclude occult infection.

The rates of disappearance of anti-HBs, anti-HBc, and both (loss of antibody) were 40% (12/30 patients), 6.7% (2/30 patients), and 6.7% (2/30 patients), respectively, in our series. Total rate of disappearance of anti-HBs and/or anti-HBc was 46.7% (14/30 patients). Loss

of antibody was transient in 6 of these patients. Anti-HBs reappeared after 8-18 mo (median 15 mo) in 4 patients and anti-HBc reappeared after 6 mo in 2 patients (Table 3). The most common underlying disease among the patients who had loss of antibody (11/14 patients) was MM. MM was discovered to be an independent risk factor for loss of antibody in multivariate analysis ($P = 0.043$, OR = 0.27). The reason why the loss of antibody emerges more in MM patients who have undergone autologous HSCT remains to be elucidated.

None of the patients who lost their hepatitis B antibodies developed HBsAg seroconversion or clinical hepatitis during the median 15 mo follow up period. The actual risk of disappearance of anti-HBs and reverse seroconversion was estimated to be 75% and 39.8% at 2 years, and 100% and 70% at 5 years, respectively, in allogeneic HSCT recipients in the study of Onozawa *et al.*^[19]. Lower rates of disappearance of antibody and reverse seroconversion in our study could be explained by the fact that our study population consisted of patients who had undergone autologous HSCT which is less immunosuppressive than allogeneic HSCT.

In conclusion, HBV-related events such as reactivation, acute hepatitis and loss of antibody can develop in patients undergoing autologous HSCT. Patients with anti-HBc antibody as the only HBV-related serologic marker might be a special risk group where antiviral prophylaxis should be considered. Loss of anti-HBs and/or anti-HBc after transplantation is not a rare complication of autologous HSCT, especially in MM patients, and does not necessarily progress to reverse seroconversion and clinical hepatitis. Further prospective and randomized studies are required to validate the prognostic significance and treatment of anti-HBc-positive patients.

COMMENTS

Background

Hepatitis B virus (HBV) infection is one of the major human health problems in the world. Turkey is one of the endemic areas for HBV infection. Intensive chemotherapy, radiotherapy, monoclonal antibody treatment, and autologous and allogeneic hematopoietic stem cell transplantation (HSCT) give rise to immune dysfunction which consequently expose the patients to the risk of many infections, including viral hepatitis. Exacerbation of hepatitis B is a serious cause of morbidity and mortality in patients undergoing cytotoxic or immunosuppressive therapy, particularly in areas where chronic HBV infection is endemic.

Research frontiers

This study aimed to determine the prevalence of occult HBV infection in patients with various hematologic malignancies who are candidates for autologous HSCT. Frequency, course of disease, results of HBV reactivation and reverse seroconversion after autologous HSCT, and the risk factors for HBV reactivation and reverse seroconversion in patients undergoing autologous HSCT for various malignancies were also determined.

Innovations and breakthroughs

HBV-related events such as reactivation, acute hepatitis and loss of antibody can develop in patients undergoing autologous HSCT. Patients with anti-HBc antibody as the only HBV-related serologic marker might be a special risk group where antiviral prophylaxis should be considered. Loss of antibody to HBV surface antigen (anti-HBs) and/or anti-HBc after transplantation is not a rare complication of autologous HSCT, especially in multiple myeloma patients, and does not necessarily mean progression to reverse seroconversion and clinical

hepatitis. Further prospective and randomized studies are required to validate the prognostic significance and treatment of anti-HBc-positive patients.

Applications

Presence of anti-HBc as the sole HBV-related marker seems to be a risk factor for reactivation. Loss of anti-HBs and anti-HBc are frequently seen after autologous HSCT, not necessarily having a poor prognostic significance. Lamivudine prophylaxis in HBV surface antigen (HBsAg)-positive patients, and treatment in patients with HBV reactivation, continues to be effective.

Terminology

HBV reactivation was defined as seroconversion from HBsAg-negative to positive for HBsAg with an increase in HBV-DNA levels compared with baseline HBV-DNA levels, in the absence of clinical and laboratory features of acute infection with hepatitis A, hepatitis C, or other systemic infection. Reverse seroconversion was defined as appearance of HBsAg and disappearance of anti-HBs after HSCT in patients who had no HBsAg but did have anti-HBs or anti-HBc before transplantation. Loss of antibody was defined as disappearance of anti-HBs and/or anti-HBc after transplantation.

Peer review

In this paper, the authors provide consistent data which may help better understanding of the yet unsolved topic of HBV reactivation after immunosuppression, even if they do not change significantly current practices in this field.

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Identification of cerebral response to balloon distention of the bile duct

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Abstract

AIM: To identify the brain loci that process human biliary sensation.

METHODS: In 6 patients (age range: 42-74 years; 4 men), who underwent percutaneous transhepatic biliary drainage (PTBD), the distal biliary tract was stimulated by repeatedly inflating the balloon of the PTBD catheter so that it reached volumes that produced a definite painless sensation. The functional magnetic resonance imaging (fMRI) of the cortical response to biliary sensation was examined.

RESULTS: Biliary balloon stimulation elicited activation of the insular cortex, prefrontal cortex, and somato-sensory cortex ($P < 0.001$).

CONCLUSION: Biliary balloon stimulation evoked a cerebral cortical response detectable by fMRI.

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Key words: Biliary; Sensation; Functional magnetic

INTRODUCTION

Elevated pressure of the extrahepatic bile duct may elicit a variety of symptoms ranging from various degrees of abdominal distention, discomfort, dyspepsia, and pain. However, there is no information concerning the cerebral cortical response to sensory signals that originate in the biliary tract in humans. On the other hand, the cortical response to esophageal, stomach or rectal stimulation has been studied using several different modalities including evoked potentials^[1], functional magnetic resonance imaging (fMRI)^[2], and positron emission tomography^[3].

In the present study, we used fMRI to investigate the cerebral cortical response to biliary stimulation in humans.

MATERIALS AND METHODS

Patients

Six patients who underwent percutaneous transhepatic biliary drainage (PTBD) participated in this study after the study protocol was approved by the Institutional

Review Board of Juntendo University, and the patients provided their informed consent. The study population consisted of 4 men and 2 women with an average age of 58 years (range: 42–74 years). The indication for PTBD in these patients was the presence of intrahepatic stones. A balloon-affixed 18 French PTBD catheter was used in every patient to perform lithotripsy of intrahepatic stones under percutaneous transhepatic cholangioscopy.

Extrahepatic bile duct distention

Bile duct distention was achieved using the balloon-affixed 18 French PTBD catheter. Before each study, the balloons were inflated in 1 mL increments to determine the volume of air necessary to induce a definite but painless sensation, and the condition was confirmed by magnetic resonance cholangiopancreatography beforehand. The balloon catheter was located at 4 cm proximal to the papilla of Vater and inflated to the desired diameter by manually injecting the predetermined volume of air. Balloon distention was done by the same investigator in all studies. The subjects were asked to confirm the sensation of distention before and after the fMRI study.

MRI scanning

Functional MRI has been shown to accurately detect cortical activity with the use of the blood oxygenation level-dependent (BOLD) technique. The BOLD technique is based on the fact that deoxygenated and oxygenated blood have subtly different magnetic susceptibilities. This change in magnetic susceptibility alters the microscopic distribution of the magnetic field within a single MRI pixel and results in a change in signal intensity, greater oxygenation being associated with a brighter pixel contrast. Thus, the time course of gradient echo MRI images shows subtle changes in brightness in regions of altered oxygenation rate. Neuronal activity is associated with increased venous oxyhemoglobin levels; consequently, the anatomic location of the cortical regions that increase in brightness during application of a stimulus is associated with the sensation of that stimulus^[2,4].

Magnetic resonance images were acquired using a 1.5-Tesla Toshiba VISART EX system with a standard quadrature head coil. BOLD imaging was performed using a field-echo echo planar imaging (EPI) sequence [repetition time (TR) = 2000 ms, echo time (TE) = 45 ms, matrix = 96 × 96, flip angle 70° and field of view (FOV) = 26 cm]. EPI-MRI images were acquired in the axial plane for 13 contiguous slices, 8 mm in thickness, covering the whole brain.

Study protocol

After positioning the subject, echo planar MRI images were captured as follows: for a total of 180 s were captured over a period of 180 s with three 60 s cycles (30 s rests alternated with 30 s periods of sustained balloon distention). In the same subjects, sham biliary balloon distention was performed for control.

Statistical analysis

The functional data processing was performed on personal

Table 1 Cerebral cortical regions associated with biliary balloon distention

Subjects	Regions of activation				
	OFC	Ins	PFC	S	POC
1			B		L
2		B	R		
3				B	
4		B			
5	L	B		B	
6			B	R	

B: Bilateral; Ins: Insular cortex; L: Left; OFC: Orbitofrontal cortex; PFC: Prefrontal cortex; POC: Parieto-occipital cortex; R: Right; S: Somatosensory cortex.

computer (PC) workstations (Dynabook, Toshiba, Tokyo) using Statistical Parametric Mapping (SPM99) (Wellcome Department of Imaging Neuroscience) implemented in Matlab (Mathworks). We used SPM99 software to perform realignment for motion correction, normalization or deformation using the standard brain template from the Montreal Neurological Institute and conversion to the standard stereotaxic atlas of Talairach space and performing data analysis (using a threshold of $P < 0.01$) for individual subjects. A typical boxcar model convolved with a hemodynamic response of SPM was used. Data analysis was performed using the amplitude estimates (percent signal change) from the 6 patients.

RESULTS

The volume threshold of bile duct balloon distention at which a sensation of abdominal discomfort was induced was 4.5 ± 0.8 mL; the mean diameter of the balloon was 15.0 ± 1.7 mm, which was $159.0\% \pm 11.6\%$ greater than the bile duct diameter before balloon dilation. All subjects reported they felt a definite painless sensation when the balloon was inflated, before and after fMRI scanning. Bile duct balloon distention produced a cortical fMRI response in all subjects. At the corrected P level of less than 0.01, the cortical response was mostly observed in the insular cortex, prefrontal cortex, and somatosensory cortex (Table 1 and Figure 1). The fMRI signal increased to an average maximum value of $4.1\% \pm 0.6\%$ above the average baseline signal intensity.

With sham biliary balloon distention, all subjects felt no sensation during, before and after fMRI scanning, and the fMRI signal was not observed in all subjects.

DISCUSSION

In this study we characterized the cerebral cortical response to bile duct balloon distention. The technique used in this study is based on measurable changes in magnetic signal intensity due to changes in cerebral blood oxygenation, and the percent average signal change in our study was $4.1\% \pm 0.6\%$; the percent change in signal intensity accepted as significant has been reported for

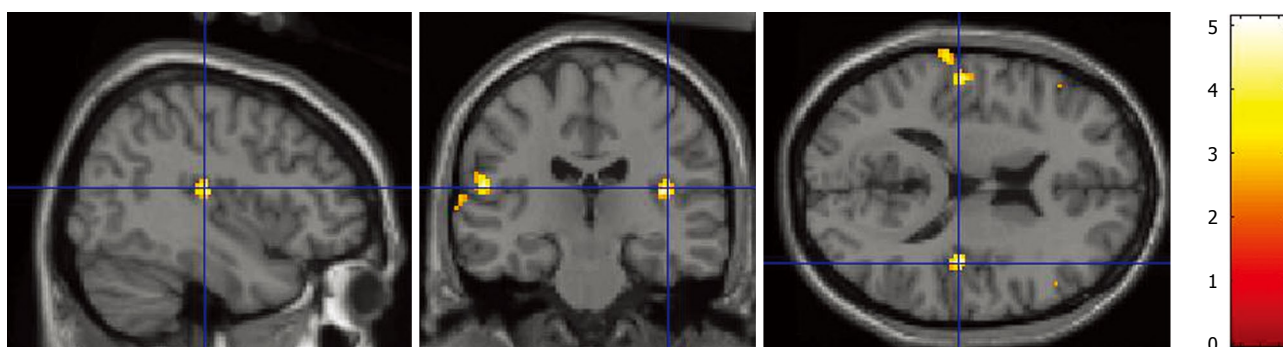


Figure 1 Sagittal, coronal and axial section showing increased activation in the bilateral insular cortex ($P < 0.01$).

simple motor tasks ($4.3\% \pm 0.32\%$)^[5], visual stimulation ($3\%-5\%$)^[6], and psychotropic drug effects ($2\%-3\%$)^[4].

The findings of the present study indicated that balloon stimulation of the bile duct at the definite sensation was relayed to the cerebral cortex and induced neuronal activity detectable by fMRI. Functional MRI technique has been previously used to study the cortical response to balloon dilation of the esophagus, stomach, and rectum^[2,7-9]. The present findings are the first step in the evaluation of cerebral response to bile duct balloon distention, and show the possibility of the presence of pressure sensitive vagal afferents in the biliary tract and the cortical relay of these afferents beyond the brainstem.

The cerebral cortical response to biliary balloon distention was mostly observed in the insular cortex, pre-frontal cortex, and somatosensory cortex. The spread of activation around these regions was comparable to that observed in a meta-analysis of studies on noxious somatic stimulation^[10]. Furthermore, insular activation was found to be the most consistent finding in visceral stimulation research^[9,11]. As described by Neafsey *et al.*^[12], electrical stimulation of the insula in rats, cats, dogs, monkeys, and human elicits changes in blood pressure, heart rate, respiration, piloerection, papillary dilation, gastric motility, peristaltic activity, salivation, and adrenalin secretion, and the insula is regarded as a key integrative visceral sensory area, mediating affective response to visceral stimulation^[11].

Visceral hypersensitivity, a condition characterized by lower thresholds for discomfort, pain, or other sensations during intraluminal balloon distention, has been demonstrated by fMRI in patients with different functional gastrointestinal disorders including functional dyspepsia, irritable bowel syndrome, and noncardiac chest pain^[8,9,13]. The present study suggests the possibility to detect hypersensitive conditions in functional biliary disorder using fMRI.

Potential weaknesses of this study include, first, the small number of patients. The current data need to be confirmed in larger groups of patients. Secondly, manual control of the volume used to induce biliary distension rather than barostat pressure controlled inflations was used due to difficulties with the use of a barostat in the MRI scanner room. A third limitation of this study was

the introduction of the PTBD catheter that may cause not only emotional distress, but also vagal activation. The possible impact of this on registered brain activation patterns was minimized by allowing at least 2 wk between introduction of the PTBD catheter and the MRI scan. However, a certain amount of stimulation at baseline by the presence of the catheter is unavoidable^[9,14].

In summary, biliary balloon stimulation, inducing painless distention, evokes a cerebral cortical response detectable by fMRI. Further functional imaging studies of biliary sensation are required to improve the confidence of interpretation of results.

COMMENTS

Background

Elevated pressure of the extrahepatic bile duct may elicit a variety of symptoms ranging from various degrees of abdominal distention, discomfort, dyspepsia, and pain. However, there is no information concerning the cerebral cortical response to sensory signals that originate in the biliary tract in humans.

Research frontiers

In the present study, the authors used functional magnetic resonance imaging (fMRI) to investigate the cerebral cortical response to biliary stimulation in humans.

Innovations and breakthroughs

The present findings are the first step in the evaluation of cerebral response to bile duct balloon distention.

Applications

The present study suggests the possibility to detect hypersensitive conditions in functional biliary disorder using fMRI.

Peer review

It is an interesting study. The authors are congratulated on making the first observation on the relay of mechanosensitive information from the biliary tract presumably via vagal afferents, the brain stem and on to specific regions of the cerebral cortex.

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Efficacy of early treatment with infliximab in pediatric Crohn's disease

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(23.1%, 3 of 13 patients) in group C was lower than that (61.5%, 8 of 13 patients) in group B ($P = 0.047$). At the 2 years follow-up, the relapse rate (38.5%, 5 of 13 patients) in group C was lower than that (76.9%, 10 of 13 patients) in group B ($P = 0.047$). Adverse events in group C were fewer than in groups A and B.

CONCLUSION: Early induction with infliximab at diagnosis, known as "top-down" therapy, was effective for reducing the relapse rate compared to conventional therapies for at least 2 years.

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Key words: Efficacy; Infliximab; Pediatric Crohn's disease; Relapse rate; Top-down treatment

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Abstract

AIM: To investigate the effectiveness of early infliximab use for induction and maintenance therapy in pediatric Crohn's disease.

METHODS: We performed a retrospective chart review of 36 patients with Crohn's disease. Ten patients (group A) were treated with mesalamine after induction therapy with oral prednisolone, and 13 patients (group B) were treated with azathioprine after induction therapy with oral prednisolone. Thirteen patients (group C) received infliximab and azathioprine for induction and maintenance therapy for the first year, and were treated with azathioprine after 1 year. All patients were followed for at least 24 mo. Efficacy was determined by the relapse rate using the pediatric Crohn's disease activity index score in each group at 12 and 24 mo.

RESULTS: At the 1 year follow-up, the relapse rate

INTRODUCTION

Although the etiology of Crohn's disease remains incompletely understood, environmental factors, infectious microbes, ethnic origin, genetic susceptibility, and immune system dysfunction have been implicated in the associated chronic mucosal inflammation^[1]. There is no known cure for Crohn's disease. Therefore, the goal of treatment is to mitigate inflammation and the associated clinical symptoms. The current treatment

guidelines are designed to maintain remission after induction therapy.

Conventional therapy for active disease includes treatment with corticosteroids. Initially, corticosteroids are very effective and fast acting, but long term exposure to corticosteroids becomes problematic with issues of dependency and/or resistance^[2]. Infliximab is an anti tumor necrosis factor (TNF)- α monoclonal antibody that was introduced for the treatment of moderate to severe Crohn’s disease^[3,4]. The efficacy of infliximab suggests a new concept; that early induction with intensive therapy may reduce complications associated with conventional treatment and may improve the quality of life. Therefore, intensive therapy with early use of this biological agent has been proposed, and is known as ‘top-down’ therapy. The results of some studies suggest that infliximab is more effective in children than in adults^[5,6].

Ethnic differences in the clinical characteristics of Crohn’s disease have been noted. Crohn’s disease is relatively common in developed countries, while it is not as common in Asian countries^[7]. In addition, recent studies from Far East Asian countries reveal that the prevalence of *NOD2* mutations, associated with increased susceptibility to Crohn’s disease in Western populations, is lower than that found in other populations^[8-10]. Therefore, the clinical response to treatment for Crohn’s disease in Asian countries might differ from patients in Western countries.

The purpose of this study was to evaluate the efficacy of the early use of infliximab, compared to conventional treatments, at 1 and 2 years of follow-up, in Korean pediatric patients with Crohn’s disease.

MATERIALS AND METHODS

Patients

We included newly diagnosed pediatric patients with Crohn’s disease. All included patients had not been treated with agents such as corticosteroids, mesalamine, or infliximab, and were followed for at least 24 mo at our clinic. Forty-three patients confirmed to have Crohn’s disease at Samsung Medical Center, Korea, between March 2001 and February 2007 were enrolled. Seven patients were excluded: four patients were lost to follow-up and three patients did not respond to the induction regimen with prednisolone or infliximab. Among three patients who did not achieve induction, 2 patients did not stop corticosteroids and one patient did not respond to three infusions of infliximab for induction. Therefore, 36 patients were eligible for inclusion in the study. A retrospective chart analysis was conducted of physician notes, laboratory studies, radiology reports, endoscopy records, and histology reports. Biopsies were obtained by endoscopy in all eligible patients. The diagnosis of Crohn’s disease was made in accordance with the ESPGHAN - Porto criteria^[11]. Infectious diseases such as tuberculosis were ruled out by taking a detailed family history, imaging

studies and confirmation of a negative purified protein derivative tuberculin (PPD) test result and a negative PCR-hybridization of *Mycobacterium tuberculosis* on biopsy tissue. Our study was approved by the institutional review board of our institution.

Grouping by treatment regimen

The patients ($n = 36$) were divided into three subgroups according to the treatment regimen. Ten patients (group A) were treated with mesalamine after induction therapy with oral prednisolone and 13 patients (group B) were treated with azathioprine after induction therapy with oral prednisolone. In the third subgroup, 13 patients (group C) received infliximab and azathioprine for induction and maintenance therapy for the first year, and were treated with azathioprine after 1 year. The patients in group A were treated mainly from 2001 to 2003. Treatment in groups B and C occurred mainly from 2003 to 2005 and from 2005 to 2007, respectively. The oral corticosteroid, prednisolone (1-2 mg/kg per day), was used for induction therapy. Mesalamine (Pentasa, 50-80 mg/kg per day) or azathioprine (Imuran, 2-3 mg/kg per day) was provided for maintenance therapy as the conventional treatment. Infliximab (Remicade, 5 mg/kg) was administered by intravenous infusion at weeks 0, 2 and 6, in combination with daily azathioprine and subsequently every 8 wk for 10 mo. After treatment with infliximab and azathioprine for the first 12 mo, only azathioprine therapy was continued.

The group treated with early infliximab was not previously treated with other medications such as corticosteroids or immunomodulators. All patients were followed for at least 24 mo.

Adverse events

Adverse events and laboratory results for side effects were investigated. Patients treated with mesalamine underwent evaluations for hypersensitivity, rash, alopecia, anorexia, headache, elevated liver enzyme, pancreatitis, serum creatinine and urine analysis. For azathioprine, patients were evaluated for pancytopenia, pancreatitis, hepatotoxicity, rash, alopecia, anorexia and arthralgia. Patients receiving infliximab were assessed for anaphylaxis, dyspnea, rash, headache, nausea, elevated liver enzyme, pancreatitis, pancytopenia and serious infections. Evaluation of the well-known adverse effects of prednisolone was not included in the analysis.

Relapse rate

We defined remission of disease as a pediatric Crohn’s disease activity index (PCDAI) score of less than 10 points and a relapse was defined as greater than 10 points^[12,13]. The relapse rate was defined as the rate of the presence of relapse, more than once, after a remission was achieved with treatment. Moderate to severe disease was defined by a score greater than 30 points by the PCDAI. The relapse rate was obtained using the

Table 1 Baseline characteristics

Gender (male/female)	27/9
Median age (range, yr)	13 (1-16)
Duration of follow up [mean (range), mo]	51.9 (24-101)
Disease location	
Small intestine	3
Colon	10
Small intestine & colon	23
PCDAI at diagnosis (mean \pm SD)	32.9 \pm 8.6

PCDAI: Pediatric Crohn’s disease activity index.

Table 2 Differences in baseline characteristics of each treatment group

	Group A	Group B	Group C	P
Median age (range, yr)	13 (10-14)	12.5 (4-14)	14 (1-16)	0.520
Duration of follow up (mo) (mean \pm SD)	82.7 \pm 29.1	50.3 \pm 15.5	29.6 \pm 7.4	0.234
PCDAI (mean \pm SD)	30.3 \pm 6.5	33.1 \pm 9.9	34.8 \pm 8.8	0.464

Group A: Treated with mesalamine after induction therapy with oral prednisolone; Group B: Treated with azathioprine after induction therapy with oral prednisolone; Group C: Treated with infliximab and azathioprine for induction and maintenance therapy.

PCDAI score. The efficacy of the early use of infliximab was evaluated by the relapse rate in each group at 12 and 24 mo.

Statistical analysis

A comparison of the study groups was performed using the χ^2 test for variables, and Fisher’s exact test was used to compare each of the measurements. All statistical analyses utilized a 0.05 level of significance.

RESULTS

Baseline characteristics

The study population at diagnosis included 27 males (75.0%) and nine females (25.0%), with a mean age of 11.9 ± 3.5 years (Table 1). The mean age of the patients in group A was 12.9 ± 1.4 years, for group B it was 11.5 ± 2.7 years and for group C, 12.9 ± 4.0 years; there were no statistical differences between these groups ($P = 0.520$). The mean overall follow-up period was 51.9 mo (range: 24-101 mo) and the minimal duration of follow-up was 24 mo. The location of disease was the terminal ileum in three patients (8.3%), the colon in 10 patients (27.8%) and the ileocolon in 23 patients (63.9%). The mean PCDAI score at baseline was 32.9 ± 8.6 points. There was no significant difference in the PCDAI scores between the groups. The mean PCDAI score was 30.3 ± 6.5 in group A, 33.1 ± 9.9 in group B and 34.8 ± 8.8 in group C ($P = 0.464$). Baseline characteristics were not significantly different between the treatment groups (Table 2).

Relapse rate

The cumulative relapse rate, including infliximab use, was

Table 3 Relapse rate by characteristics

	n (%)	P
Age at diagnosis		
< 13 yr (n = 13)	9 (69.2)	0.553
≥ 13 yr (n = 23)	15 (65.2)	
Involvement		
Colon (n = 10)	5 (50.0)	0.416
Terminal ileum (n = 3)	2 (66.7)	
Small and large bowel (n = 23)	17 (73.1)	
PCDAI at diagnosis		
< 30 (n = 11)	7 (63.6)	0.544
≥ 30 (n = 25)	17 (68.0)	

52.8% (19 patients) at 12 mo and 66.7% (24 patients) at 24 mo; there was no significant difference in the relapse rate between the different age groups ($P = 0.553$); 69.2% (9 out of 13 patients) for patients less than 13 years of age and 65.2% (15 out of 23 patients) for those more than 13 years of age (Table 3). The relapse rate in patients who had colon only involvement was 50.0% (5 out of 10 patients). The relapse rate in patients with terminal ileum only involvement was 66.7% (2 out of 3 patients), and for patients with ileocolonic involvement the relapse rate was 73.9% (17 out of 23 patients). There was no difference in the relapse rate between patients with mild disease, with a PCDAI score less than 30 points at diagnosis (7 out of 11 patients, 63.6%), and moderate to severe disease, with a PCDAI score more than 30 points (17 out of 25 patients, 68.0%).

The relapse rate at 1 year was 23.1% [95% confidence interval (CI): 0.2%-46.0%] in group C (3 out of 13 patients), 61.5% (95% CI: 35.1%-88.0%) in group B (8 out of 13 patients) and 80.0% (95% CI: 55.2%-100.0%) in group A (8 out of 10 patients). At 1 year, the absolute difference between groups C and B was 38.5% (95% CI: 3.5%-73.4%) and was 56.9% (95% CI: 23.2%-90.7%) between groups C and A. There was a significant difference between each of the groups ($P = 0.047$, $P = 0.012$) (Figure 1A). At the 2 years follow up, the relapse rate in group C [38.5%, 5 out of 13 patients (95% CI: 12.0%-64.9%)] was lower than the relapse rate in groups B [76.9%, 10 out of 13 patients (95% CI: 54.0%-99.8%)] and A [90.0%, 9 out of 10 patients (95% CI: 71.4%-100.0%)]. At 2 years, the absolute difference between groups C and B was 38.5% (95% CI: 3.5%-73.4%) and was 51.5% (95% CI: 19.2%-83.9%) between groups C and A. There was a statistically significant difference between each group ($P = 0.047$, $P = 0.029$) (Figure 1B).

Adverse events

Patients treated with mesalamine had three adverse events. Five patients treated with azathioprine were found to have adverse events. Only one patient had dyspnea and tachycardia after the third infusion of infliximab (Table 4).

DISCUSSION

To our knowledge, this is the first study reported in Asia

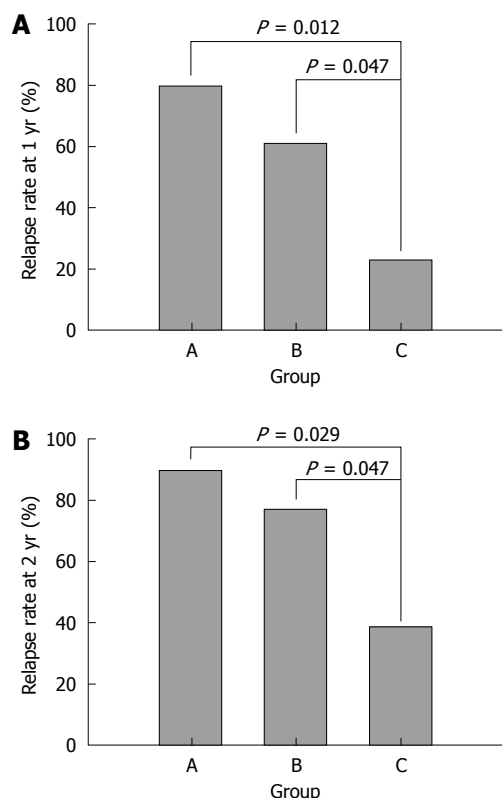


Figure 1 Relapse rate at 1 year (A) and 2 years (B). Group A: Treated with mesalamine after induction therapy with oral prednisolone; Group B: Treated with azathioprine after induction therapy with oral prednisolone; Group C: Treated with infliximab and azathioprine for induction and maintenance therapy.

on the long-term efficacy of ‘top-down’ infliximab treatment for newly diagnosed pediatric Crohn’s disease. We have used infliximab as induction therapy since 2005. Our patients had a better response to infliximab treatment compared to conventional therapy. The improved response, when compared to Western data, suggests that there might be ethnic differences in the response to treatment. A review of the medical literature showed that the incidence and prevalence of Crohn’s disease in Korea is lower than in developed countries; however, it is rapidly increasing^[7]. In addition, the pathogenesis associated with the common *NOD2* variant in Asian populations differs from Western countries.

The results of our study showed that induction and maintenance therapy with infliximab and azathioprine was more effective than conventional treatments in children. After discontinuation of infliximab maintenance therapy for 1 year, we showed that the relapse rate in group C was lower than that in the other groups. The remission rate achieved with infliximab was higher in our study than in the study reported by Hyams *et al.*^[14]; at 1 year, 55.8% (29 out of 52) of patients in the study by Hyams receiving infliximab every 8 wk were in clinical remission; on the other hand, 76.9% (10 out of 13) of the patients in our study group C achieved remission. There was no difference in the PCDAI score between the study by Hyams and our study, 42.1 ± 9.2 and 34.8

Table 4 Adverse events

	<i>n</i> (%)	Details
Group A (mesalamine)	3 (30.0)	Anorexia (<i>n</i> = 2), Pancreatitis (<i>n</i> = 1)
Group B (azathioprine)	5 (38.5)	Anorexia (<i>n</i> = 1), Pancytopenia (<i>n</i> = 3), Pancreatitis (<i>n</i> = 1)
Group C (infliximab)	1 (8.3)	Dyspnea and tachycardia (<i>n</i> = 1)

± 8.8 , respectively. The reason why remission was maintained longer in our study compared to the findings from other developed countries remains unclear. Three hypotheses can be posited: first, it is likely that there are ethnic differences in the clinical response to infliximab, as seen with *NOD2* variations; second, we used infliximab as ‘top-down’ therapy, while in the study by Hyams the patients who had received other drugs such as oral corticosteroids and aminosalicylates were also included in the infliximab treatment group; finally, the number of patients in our study was relatively small, and a selection bias cannot be ruled out. Further study is needed to confirm our findings. However, even with the differences in the number of patients and inclusion criteria, the clinical response to early use of infliximab in our report was much better than the responses reported by Hyams.

Pediatric Crohn’s disease is characterized by frequent relapses, a widespread disease extent, a high prevalence of extraintestinal manifestations, and in general a severe clinical course^[15,16]. In pediatric patients, both the disease itself and the treatment used to control it, most commonly corticosteroids, can seriously impair quality of life as well as cause significant side effects such as growth failure and an increased risk of infections. For these reasons clinicians tend to use corticosteroids for short durations. Although corticosteroids are effective in inducing clinical remission in patients with active Crohn’s disease, these agents have limited efficacy for maintaining remission and the healing of mucosal lesions^[17].

Infliximab, is an anti TNF- α monoclonal antibody that was initially used in corticosteroid-dependent and corticosteroid-refractory Crohn’s disease^[18]. The use of infliximab has been well documented as an effective treatment for moderate to severe Crohn’s disease in children, in several studies^[14,19]. Infliximab, as a scheduled treatment, is effective for the induction and maintenance of remission in patients with Crohn’s disease^[20,21]. Early use of infliximab with immunosuppressants, known as ‘top-down’ therapy, in patients with newly diagnosed Crohn’s disease has resulted in better outcomes in adult patients^[22]. An 8-wk maintenance treatment schedule with infliximab has been shown to be a cost-effective approach in adult patients with active luminal or fistulizing Crohn’s disease^[23]. Such therapy offers the potential for altering the natural history of Crohn’s disease, and is changing treatment paradigms. One possible explanation for the prolonged remissions with infliximab treatment

is that this medication might help heal the mucosal lesions in patients with severe as well as those with mild disease^[24]. However, the new concept of an early aggressive or ‘top-down’ treatment approach is not widely accepted yet, especially in children, even though a few studies have reported that infliximab was more effective in children than in adults^[5,6].

The limitations of this study include the following: This was a single center study with a small number of patients who were reviewed retrospectively. As an open trial, the physicians and patients knew the treatment regimen, which could have biased the assessment of treatment efficacy. However, selection bias was unlikely because the distribution of treatment regimens among the three groups was made based on chronological grouping.

In conclusion, the results of this study showed that early and intensive treatment of pediatric Crohn’s disease patients with infliximab, at initial diagnosis, was more effective for maintaining remission and reducing flares. Treatment with azathioprine monotherapy, after discontinuation of infliximab, for 1 year, provided prolonged remission.

COMMENTS

Background

The etiology of Crohn’s disease remains incompletely understood. There is no known cure for Crohn’s disease. Therefore, the goal of treatment is to mitigate inflammation and the associated clinical symptoms. Conventional therapy for active disease includes treatment with corticosteroids, which is very effective and fast acting. However, long-term exposure to corticosteroids becomes problematic with issues of dependency and/or resistance.

Research frontiers

Infliximab is an anti tumor necrosis factor α monoclonal antibody that was introduced for the treatment of moderate to severe Crohn’s disease. The efficacy of infliximab suggests a new concept; that early induction with intensive treatment, known as ‘top-down’ therapy, may reduce complications associated with conventional treatment and may improve quality of life.

Innovations and breakthroughs

This is the first study reported in Asia on the long-term efficacy of ‘top-down’ infliximab treatment for pediatric Crohn’s disease. The results of this study showed that early and intensive treatment using infliximab, at initial diagnosis, was more effective for maintaining remission and reducing flares for 2 years.

Applications

Early induction treatment with infliximab at diagnosis, known as “top-down” therapy, was effective in reducing relapse rate and adverse events compared to conventional therapies for at least 2 years.

Peer review

This is a useful cohort that will serve as a useful reference for pediatricians considering infliximab induction therapy for Crohn’s disease.

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Diabetic factors associated with gastrointestinal symptoms in patients with type 2 diabetes

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categories (upper and lower GI symptoms), and consisting of 11 individual symptoms. In the diabetic patient group, diabetic complications including peripheral neuropathy, nephropathy and retinopathy, glycosylated hemoglobin (HbA1c) level and diabetes duration were evaluated.

RESULTS: Among the total 190 diabetic patients and 190 controls enrolled, 137 (72%) of the diabetic patients and 116 (62%) of the controls had GI symptoms. In the diabetic patient group, 83 (43%) had upper GI symptoms and 110 (58%) lower GI symptoms; in the control group, 59 (31%) had upper GI symptoms and 104 (55%) lower GI symptoms. This difference between the two groups was significant for only the upper GI symptoms ($P = 0.02$). Among the diabetic factors, the HbA1c level was the only independent risk factor for upper GI symptoms in the multiple logistic regression analysis (odds ratio = 2.01, 95% confidence interval: 1.02-3.95).

CONCLUSION: Type 2 diabetes was associated with an increased prevalence of upper GI symptoms and these symptoms appeared to be independently linked to poor glycemic control, as measured by the HbA1c levels.

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Key words: Diabetes; HbA1c; Upper gastrointestinal symptoms

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Kim JH, Park HS, Ko SY, Hong SN, Sung IK, Shim CS, Song KH, Kim DL, Kim SK, Oh J. Diabetic factors associated with

Abstract

AIM: To determine whether gastrointestinal (GI) symptoms are more frequent in type 2 diabetic patients and to examine which diabetic factors are associated with the symptoms.

METHODS: Consecutive subjects with diabetes and age-/gender-matched normal controls were recruited for this study. GI symptoms were assessed using a structured questionnaire divided into two GI symptom

gastrointestinal symptoms in patients with type 2 diabetes. *World J Gastroenterol* 2010; 16(14): 1782-1787 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v16/i14/1782.htm> DOI: <http://dx.doi.org/10.3748/wjg.v16.i14.1782>

INTRODUCTION

Diabetes mellitus (DM) is becoming increasingly common because of the epidemic of obesity and sedentary lifestyles in South Korea and worldwide^[1-3]. The prevalence of gastrointestinal (GI) symptoms in diabetic patients has been investigated previously in several studies; however, the results are inconsistent due to the different ethnic groups and populations studied^[4-9]. Diabetes-related GI motor dysfunction is common and affects the esophagus, stomach and the lower GI tract^[10]. Many patients with diabetes have upper and lower GI symptoms. The complications involving the GI tract are now recognized to be an important cause of morbidity in patients with diabetes^[11]. Although several pathogenic mechanisms may be involved in these GI symptoms, such as autonomic neuropathy, diabetic peripheral neuropathy, glucose imbalance, diabetic duration, and psychiatric disorders, there is substantial controversy about their etiology.

Therefore, the aim of this study was to determine the frequency of GI symptoms in type 2 diabetic patients and whether GI symptoms are more common in diabetic patients than normal controls. In addition, the diabetic factors associated with the GI symptoms were studied.

MATERIALS AND METHODS

Subjects

We performed a prospective study of a consecutive series of outpatients with type 2 diabetes who visited Konkuk University Medical Center from October 2005 to September 2007 for the first time. All patients were referred by an endocrinologist after a comprehensive evaluation at the endocrine center. These patients underwent screening with esophago-gastro-duodenoscopy (EGD) and colonoscopy (or sigmoidoscopy with fecal occult blood test) to rule out upper and lower GI organic disorders, such as a malignancy, peptic ulcer, or erosive esophagitis. The control subjects were carefully matched for age and gender, and were randomly selected from subjects who underwent a screening EGD and colonoscopy at the Health Promotion Center of Konkuk University Medical Center. The exclusion criteria were the presence of upper and lower GI organic disorders on the EGD; a history of upper and lower GI malignancy, peptic ulcer, major abdominal surgery, or underlying psychiatric illness; a medical history of taking a proton pump inhibitor within the last month; severe liver, lung, renal, or hematological disorders. Patients were also excluded if they were unwilling or unable to provide informed consent, or if they could not complete all phases of the study. Subjects pro-

vided written informed consent before enrollment, and the study protocol was carried out in accordance with the Declaration of Helsinki, Good Clinical Practice, and was approved by the human ethics review board of Konkuk University Medical Center. After enrollment, each subject completed a structured questionnaire to precisely assess the GI symptoms, in the absence of organic, systemic or metabolic diseases; data on smoking and alcohol consumption (> 40 g/d) was included. In addition, for the diabetic patients, diabetic complications including peripheral neuropathy, nephropathy and retinopathy, glycosylated hemoglobin (HbA1c) level, the treatment of diabetes and the duration of diabetes were recorded.

Symptom assessment

The questionnaire contained questions regarding GI symptoms and consisted of two subgroups: an upper GI symptom group and a lower GI symptom group. The upper GI symptom group included six items (globus, heartburn, acid regurgitation, non-cardiac chest pain (NCCP), ulcer-like dyspepsia and dysmotility-like dyspepsia) and the lower GI symptom group included five items (irritable bowel syndrome, abdominal bloating, constipation, diarrhea and anal discomfort). A 'predominant upper GI symptoms' classification was defined as: more frequent and/or more severe upper GI symptoms than lower GI symptoms reported on the questionnaire, and assessed separately. A 'predominant lower GI symptoms' classification was defined in the same way. The questions were analyzed inclusive of all symptoms regardless of the severity or frequency of each item. An interview, using the structured questionnaire, was conducted by two investigators, who provided the patients with standard explanations of the questions and definitions of the symptoms. All symptoms that were not completely self-explanatory were explained by a standard description. The 11 items used for the GI symptoms were constructed to comply as closely as possible with the Rome II criteria for functional GI disease^[12].

Diabetic factors

In the diabetic patient group, diabetic complications were evaluated including peripheral neuropathy, nephropathy and retinopathy. In addition, the HbA1c level and duration of diabetes in each patient were evaluated. The diabetic complications were classified according to the following definitions: (1) Nephropathy was defined as prominent proteinuria on the urine analysis or a serum creatinine that exceeded 133 $\mu\text{mol/L}$; (2) Peripheral neuropathy was assessed by the recommended protocol of nerve conduction study (NCS), including six sensory nerves and six motor nerves^[13]; and (3) Retinopathy was diagnosed based on fundoscopic examination by a skilled ophthalmologist. In addition, HbA1c level was measured using the high performance liquid chromatography method within 1 mo of the questionnaire study. Furthermore, treatment of diabetes included oral hypoglycemic agents and insulin.

Table 1 Baseline characteristics *n* (%)

	DM group (<i>n</i> = 190)	Control group (<i>n</i> = 190)	<i>P</i> -value
Age (mean ± SD, yr)	57.1 ± 12.5	57.0 ± 10.6	NS
M/F	86 (45)/104 (55)	86 (45)/104 (55)	NS
Smoking	52 (27)	61 (32)	NS
Alcohol use	51 (27)	78 (41)	NS

DM: Diabetes mellitus; NS: Not significant.

Statistical analysis

Statistical analysis was performed using the χ^2 test for comparison of discrete variables and the *t*-test was used for comparison of continuous variables. The continuous variables measured in this study are expressed as the mean ± SD. Multivariate analysis was performed using logistic regression. To examine the association between GI symptoms and type 2 diabetes, multivariate models included adjustment for smoking and alcohol as categorical factors. In the models used to examine the diabetic factors associated with upper GI symptoms, adjustments for smoking, alcohol, the treatment of diabetes, and other diabetic factors were included. For each variable, the odds ratio (OR) and 95% confidence interval (CI) were determined. A two tailed *P* value of < 0.05 was considered statistically significant.

RESULTS

Ten out of a total of 200 subjects with type 2 diabetes who were recruited for the study were excluded because they were unwilling or unable to provide informed consent, or could not complete all phases of the study. Finally, 190 subjects with type 2 diabetes and 190 controls were included in this study. The diabetic and normal subjects were well matched in terms of age and gender (86 men and 104 women with a mean age of 57 years). The clinical factors, including a history of current smoking and alcohol use are shown in Table 1.

The frequency of GI symptoms (any or several) was 72% in the diabetic subjects and 62% in the controls (*P* = NS). Among the upper GI symptoms and the lower GI symptoms, the multiple logistic regression analyses showed that the diabetic patients presented with a significantly higher frequency of upper GI symptoms than the controls (43% *vs* 31%, *P* < 0.05, OR = 1.68, 95% CI: 1.07-2.63); however, no differences were observed for the lower GI symptoms (Table 2). When the individual items of the upper GI symptoms were analyzed separately, globus, heartburn and dysmotility-like dyspepsia were more common in the diabetic patients than in the controls (Figure 1).

The demographic and diabetic characteristics according to the presence or absence of upper GI symptoms in the diabetic patient group are shown in Table 3. Subjects with upper GI symptoms tended to have more complications (66% *vs* 46%), a higher HbA1c level (8.06% *vs* 7.39%) and a longer duration of symptoms (10.4 years

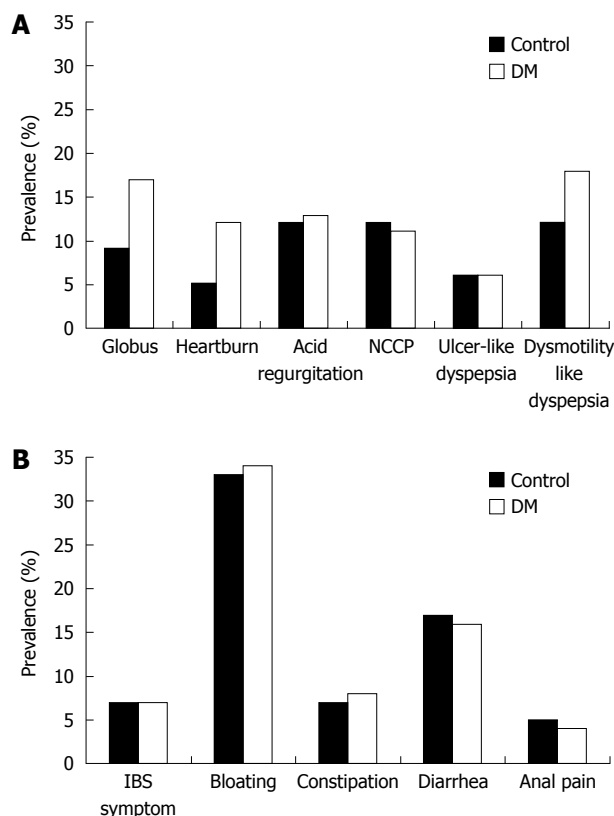


Figure 1 Differences in individual upper (A) and lower (B) gastrointestinal (GI) symptoms between diabetic patients and control groups. A: The diabetic patients had a higher frequency of globus, heartburn and dysmotility-like dyspepsia than the controls; B: There was no difference for any item of the lower GI symptoms between the two groups. DM: Diabetes mellitus; NCCP: Non-cardiac chest pain.

vs 6.5 years) than the upper GI symptom-negative group. On multiple logistic regression analyses, only the higher HbA1c level was significantly associated with smoking, alcohol, the treatment of diabetes, and other covariate factors by the adjusted OR for upper GI symptoms (OR = 2.01, 95% CI: 1.02-3.95) (Table 4).

The relationship of the HbA1c level with upper GI symptoms was studied using the normal HbA1c group (HbA1c < 6%) as the reference standard. There was a significant increase in the prevalence of upper GI symptoms in subjects with an 8% ≤ HbA1c < 9% (OR = 3.38%, 95% CI: 1.06%-10.71%), in subjects with a HbA1c ≥ 9% (OR = 3.23%, 95% CI: 1.13%-9.24%) (Figure 2), and in subjects with HbA1c ≥ 8%. All individual upper GI symptoms including globus, heartburn, acid regurgitation, NCCP, ulcer-like dyspepsia and dysmotility-like dyspepsia were more common than in subjects with a HbA1c < 8% (Figure 3).

DISCUSSION

The prevalence of DM worldwide is estimated to be around 200 million people, more than 5% of the adult population, globally. The current high prevalence of type 2 diabetes is likely to eventually result in a heavy burden of diabetes complications; this will pose a significant

Table 2 Symptomatic characteristics *n* (%)

	DM group (<i>n</i> = 190)	Control group (<i>n</i> = 190)	Unadjusted		Adjusted ¹	
			OR (95% CI)	<i>P</i> -value	OR (95% CI)	<i>P</i> -value
GI symptom	136 (72)	118 (62)	1.54 (1.03-2.31)	0.020	1.45 (0.92-2.29)	0.110
UGI symptom	83 (43)	59 (31)	1.7 (1.15-2.50)	0.005	1.68 (1.07-2.63)	0.020
LGI symptom	110 (58)	105 (55)	1.11 (0.76-1.61)	0.340	1.1 (0.71-1.70)	0.680

¹Adjusted for smoking and alcohol use. OR: Odds ratio; CI: Confidence interval; GI: Gastrointestinal; UGI: Upper GI; LGI: Lower GI.

Table 3 Characteristics according to the presence or absence of upper GI symptoms in the diabetic patient group *n* (%)

	UGI symptoms (+) (<i>n</i> = 83)	UGI symptoms (-) (<i>n</i> = 107)	Total (<i>n</i> = 190)
Age (mean ± SD, yr)	57.3 ± 13.1	57.1 ± 12.1	57.2 ± 12.5
M/F	34 (40)/52 (60)	52 (50)/52 (50)	86 (45)/104 (55)
Smoking	24 (29)	28 (26)	52 (27)
Alcohol use	19 (23)	32 (30)	51 (27)
Diabetic treatment	76 (92)	97 (91)	175 (91)
Complication	55 (66)	49 (46)	104 (55)
Peripheral neuropathy	49 (59)	44 (41)	93 (49)
Nephropathy	33 (40)	23 (22)	56 (30)
Retinopathy	32 (39)	16 (15)	48 (25)
HbA1c			
mean ± SD	8.06 ± 1.90	7.39 ± 1.94	7.68 ± 1.95
≥ 8	38 (46)	26 (24)	64 (34)
Duration (yr)			
mean ± SD	10.4 ± 7.3	6.5 ± 5.9	8.2 ± 6.8
≥ 10	44 (53)	35 (33)	79 (42)

Table 4 Diabetic factors associated with upper GI symptoms

	Unadjusted		Adjusted ¹	
	OR (95% CI)	<i>P</i> -value	OR (95% CI)	<i>P</i> -value
Complication	2.35 (1.27-4.33)	0.005	1.64 (0.81-3.32)	0.170
HbA1c level	2.63 (1.39-4.96)	0.003	2.01 (1.02-3.95)	0.040
Diabetes duration	1.67 (0.84-3.29)	0.006	1.67 (0.84-3.29)	0.140

¹Adjusted for age, gender, smoking, alcohol use and other covariate factors.

challenge to individuals, communities and healthcare systems during the coming decades, worldwide^[14].

Chronic GI symptoms may represent a clinically important problem in a substantial number of patients with diabetes^[15]. There are several papers which report the association of GI symptoms with diabetes. Epidemiological data regarding the association of GI symptoms with diabetes are, however, inconsistent^[6], and the reported frequency of upper and lower GI symptoms varies among different ethnic groups/populations, although population-based studies, in general, have demonstrated an increase in upper and lower GI symptoms^[16]. Our present study is the first study in Korea to examine the GI symptoms in type 2 diabetic patients and to analyze the diabetic factors associated with these symptoms. We found that the frequency of overall GI symptoms, upper GI symptoms and lower GI symptoms in the 190 patients with diabetes studied was 72%, 43% and 58%,

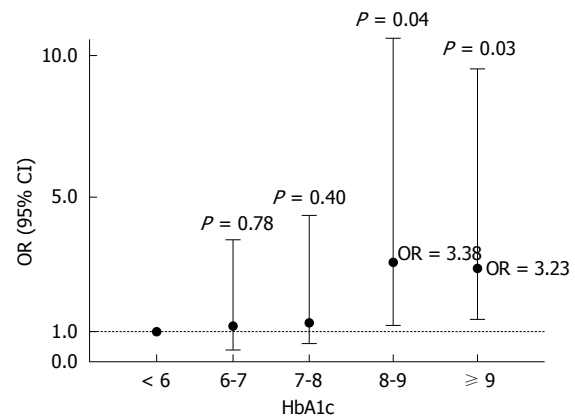


Figure 2 Differences in upper GI symptoms according to the HbA1c level. There was a significant increase in the prevalence of upper GI symptoms in subjects with 8% ≤ HbA1c < 9% and in subjects with HbA1c ≥ 9%; subjects with normal HbA1c (HbA1c < 6%) were used as the reference group. OR: Odds ratio.

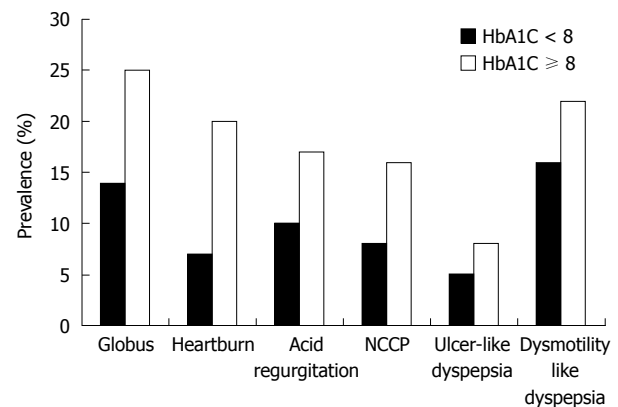


Figure 3 Differences in individual upper GI symptoms according to the HbA1c Level. In subjects with an HbA1c ≥ 8%, all upper GI symptoms were more common than in subjects with an HbA1c < 8%.

respectively. Comparison of the frequency of the overall GI symptoms, upper GI symptoms and lower GI symptoms between the diabetic patient group and the age- and gender-matched control group showed 1.45 times as many overall GI symptoms, 1.68 times as many upper GI symptoms and 1.10 times as many lower GI symptoms in individuals with diabetes. The risk of only upper GI symptoms in this group showed a statistically significant increase, with adjustments for age, gender, smoking and alcohol consumption.

The natural history and pathogenesis of GI symp-

toms in patients with diabetes remains poorly defined. Several pathogenic mechanisms such as autonomic neuropathy, diabetic peripheral neuropathy, glucose imbalance, diabetic treatment, diabetes duration, and psychiatric disorders may be involved in GI symptoms. Traditionally, GI symptoms in diabetic patients have been attributed to disordered motor function as a result of the irreversible autonomic neuropathy that frequently accompanies diabetes^[17]. Also, the hypothesis that poor glycemic control by itself is a major cause of chronic GI symptoms has been raised recently, based primarily on data from large population studies^[16,18,19] as well as small physiological studies^[17,20-22]. Other factors that may be important in the etiology of GI symptoms in patients with diabetes include the duration of diabetes^[23] and psychiatric comorbidity^[24,25].

Systematically, we attempted to evaluate the relationship between upper GI symptoms and the various features of diabetes such as diabetic complications (including peripheral neuropathy, nephropathy and retinopathy), HbA1c level, the treatment of diabetes and the duration of diabetes. Among several diabetic factors, only the HbA1c level reflecting glycemic control was found to be significantly associated with the upper GI symptoms in the diabetic patients when the individual factors of diabetes were analyzed separately by multiple logistic regression analyses. Hyperglycemia has been shown to affect the perception of GI sensations^[17,20-22,26] such as nausea and fullness, produced by distension of the proximal stomach or duodenum; such sensations are more intense during hyperglycemia than during euglycemia. Acute changes in the blood glucose concentration have also been shown to impair autonomic nerve function^[27] and lower pain thresholds in patients with diabetes^[28]; although there appear to be regional variations in the effects of the blood glucose concentration on both GI motility and the perception of sensations from the GI tract^[21,29]. In addition, these effects have been reported to have less of an impact on lower GI symptoms, similar to the results of this study.

In our study, we focused on the association between upper GI symptoms and HbA1c levels. As a result, we found 3.38 times as many upper GI symptoms in the cases with HbA1c $\geq 8\%$ compared to those with HbA1c $< 6\%$; all individual upper GI symptoms were common in the cases with HbA1c $\geq 8\%$. This result is in agreement with the recommendation for a maintenance of HbA1c $< 8\%$ to prevent serious diabetic complications^[30].

The limitations of this study include the following: only the presence or absence of individual GI symptoms, not the severity and/or frequency, was examined in the symptom assessment, regardless of the major symptoms. There were no data collected on coexisting psychiatric disorders associated with GI symptoms in patients with diabetes; this might be an important factor according to the recent reports by Quan *et al.*^[24,25]. Nevertheless, our present study is a case-control study with age- and sex-matched controls and is the first of its kind

performed in Korea. In addition, this study has a large methodological advantage with regard to the well established subjective analysis for diabetic complications such as peripheral neuropathy, and not objective answers such as the 'yes' or 'no' of self-reports.

In conclusion, upper GI symptoms were more common in patients with type 2 diabetes than in well-matched control subjects. The results of this study provide evidence that upper GI symptoms appear to be independently linked to poor glycemic control as measured by HbA1c level. Therefore, we cautiously suggest that chronic upper GI symptoms may be reversible with tight control of blood glucose level.

COMMENTS

Background

Diabetes is known to be associated with gastrointestinal (GI) symptoms and several mechanisms have been implicated in the pathogenesis of GI symptoms. However, there is little information from Korea as well as few reports from Asia.

Research frontiers

Researchers have previously assessed the increase of the prevalence of GI symptoms in diabetic patients and the diabetic factors associated with the GI symptoms. However, results of the prevalence of GI symptoms in diabetic patients and the diabetic factors associated with these GI symptoms are inconsistent due to the different ethnic groups and populations assessed.

Innovations and breakthroughs

This study is a case-control study with age- and sex-matched controls and is the first study of its kind performed in Korea. In addition, this study has a methodological advantage with regard to the well established subjective analysis for diabetic complications such as peripheral neuropathy.

Applications

Upper GI symptoms were more common in patients with type 2 diabetes than in well-matched control subjects and appeared to be independently linked to poor glycemic control as measured by the HbA1c levels in Korean individuals. Therefore, chronic upper GI symptoms may be reversible with tight control of blood glucose level.

Peer review

This is a purely observational, prospectively planned study. Prevalence of GI symptoms in diabetic patients and non-diabetic healthy subjects has been widely studied and well documented. The fact that poor glycemic control is associated with increased frequency of upper and lower GI tract symptoms also has been well established. On the other hand, this topic still may be interesting to readers since the study focused on different ethnic groups.

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Inflammatory cytokine gene polymorphisms increase the risk of atrophic gastritis and intestinal metaplasia

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Abstract

AIM: To investigate the effects of interleukin-8 (*IL-8*), macrophage migration inhibitory factor (*MIF*) gene polymorphisms, *Helicobacter pylori* (*H. pylori*) infection, on the risk of developing severe chronic atrophic gastritis (SCAG) and intestinal metaplasia (IM).

METHODS: A total of 372 cases were selected from a cohort study in Linqu County, a high risk area for gastric cancer (GC) in northern China. To obtain a sufficient group size, patients with normal or superficial gastritis were included. Based on an average follow-up period of 56 mo, the 372 cases were divided into no progres-

sion group (no histological progression from normal or superficial gastritis, $n = 137$), group I (progressed from normal or superficial gastritis to SCAG, $n = 134$) and group II (progressed from normal or superficial gastritis to IM, $n = 101$). *IL-8*, *MIF* gene polymorphisms were detected by polymerase chain reaction-based denaturing high-performance liquid chromatography analysis and DNA sequencing.

RESULTS: An increased risk of SCAG was found in subjects with *IL-8*-251 AA genotype [odds ratio (OR) = 2.62, 95% CI: 1.23-5.72] or *IL-8*-251 A allele carriers (AA + AT) (OR = 1.81, 95% CI: 1.06-3.09). An elevated risk of IM was found in subjects with *IL-8*-251 AT genotype (OR = 2.27, 95% CI: 1.25-4.14) or *IL-8*-251 A allele carriers (OR = 2.07, 95% CI: 1.16-3.69). An increased risk of SCAG was found in subjects with *MIF*-173 GC genotype (OR = 2.36, 95% CI: 1.38-4.02) or *MIF*-173 C allele carriers (GC + CC) (OR = 2.07, 95% CI: 1.21-3.55). An elevated risk of IM was found in subjects with *MIF*-173 CC genotype (OR = 2.27, 95% CI: 1.16-4.46) or *MIF*-173 C allele carriers (OR = 3.84, 95% CI: 1.58-9.34). The risk of SCAG and IM was more evident in subjects carrying *IL-8*-251 A allele (OR = 6.70, 95% CI: 1.29-9.78) or *MIF*-173 C allele (OR = 6.54, 95% CI: 2.97-14.20) and positive for *H. pylori* infection.

CONCLUSION: *IL-8*-251 and *MIF*-173 gene polymorphisms are significantly associated with the risk of SCAG and IM in a population with a high risk of GC in Linqu County, Shandong Province, China.

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Key words: Chronic atrophic gastritis; Gene polymorphisms; *Helicobacter pylori*; Interleukin-8; Intestinal metaplasia; Macrophage migration inhibitory factor

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INTRODUCTION

Atrophic gastritis (AG) and intestinal metaplasia (IM) are two important precursor lesions of intestinal type gastric cancer (GC)^[1]. These precursor lesions may significantly elevate the risk of intestinal type GC^[2,3].

Some bacterial factors, such as the pathogenic island of *Helicobacter pylori* (*H. pylori*) including *cagA*, *slm1 vacA*, *babA2*, *sabA*, and *oipA*, are correlated with the severity of atrophic gastritis and occurrence of IM^[4-9]. However, bacterial factors alone are not sufficient to explain the diverse results of *H. pylori*-related diseases. Our previous study has shown that the proportion of *cagA* + *H. pylori* strains in children living in Linq County, an area with a high risk of GC in China, is very high (88.5%)^[10]. It has also been demonstrated that almost 100% of *H. pylori* strains isolated from Chinese population are *cagA* positive^[11].

There is increasing evidence that host inflammation-related cytokines and their gene polymorphisms are related with atrophic gastritis and IM^[12,13]. Interleukin-8 (IL-8), a member of Cys-X-Cys (CXC) chemokine family, is an activator and chemoattractant of neutrophils and lymphocytes^[14]. Gastric mucosal levels of IL-8 increase significantly after *H. pylori* infection and parallel to the severity of gastritis^[15]. Macrophage migration inhibitory factor (MIF), an important activator of T lymphocytes and macrophages, plays a pivotal role in inflammatory and immune diseases^[16,17]. *H. pylori* infection is associated with an increased expression of MIF mRNA and protein in gastric epithelial and inflammatory cells. Increased expression of the MIF protein correlates with histological severity of GC and its precursor^[18].

As these inflammatory cytokines and their gene polymorphisms may potentially influence the outcome of *H. pylori* infection, a few studies have investigated the association of gene polymorphisms in these inflammatory cytokines with the risk of atrophy and IM^[15,19,20].

However, these studies were limited by their single time-point assessment for pathological diagnosis. Therefore, we conducted a prospective study to investigate the association of IL-8 gene polymorphisms with the risk of atrophic gastritis, and MIF gene polymorphisms with IM, showing that the high expressing genotypes of IL-8 are significantly associated with the increased risks of severe chronic atrophic gastritis, and MIF is significantly associated with IM.

MATERIALS AND METHODS

Study protocol

The initial study included over 3399 people from Linq

County, a rural area of Shandong Province, China, which has one of the highest GC mortality rates in the world (70/10000 males and 25/10000 females per year)^[21]. In brief, we launched an endoscopic-pathological screening program for GC and precancerous lesions of GC in 3399 residents from 14 randomly selected villages of Linq County in autumn of 1989 or in spring of 1990. In 1994, a follow-up endoscopic screening was performed in 83% of eligible members. People, diagnosed as normal or superficial gastritis in 1989 or in 1990, were subsequently genotyped for IL-8 and MIF. We analyzed the relation between genotypes and progression of gastric disease. The study design received approval from the Institutional Review Board of Peking University School of Oncology, and was conducted in accordance with the Helsinki Declaration. Informed consent was obtained from all participants.

Endoscopy and pathological diagnosis

Seven biopsies were taken from standard locations in each subject. The procedures and histopathologic criteria have been described elsewhere^[22,23]. We reviewed all slides of 372 patients in our study according to the updated Sydney system^[24]. Each subject was assigned a global diagnosis based on the most severe diagnosis among the seven biopsies. Independent examinations were performed by three experienced gastroenterologists. If there was any disagreement, the final decision was made based on the majority or subsequent discussion of the case.

Grouping

After the first examination in 1989, 372 of the 3399 subjects including 172 males and 200 females with a mean age of 42.2 years (range: 24-65 years) were enrolled in this study. All these 372 subjects were initially diagnosed as normal or as superficial gastritis (baseline). After a 56-mo follow-up, these 372 subjects were subdivided into group I (*n* = 134), group II (*n* = 101) and no progression group (*n* = 137). Lesions in patients of group I were progressed from baseline to severe chronic atrophic gastritis (SCAG). According to the updated Sydney system, recognition of minor degrees of atrophy without intestinal metaplasia in the antrum was difficult, marked degrees of atrophy without intestinal metaplasia in the antrum were selected as the presence of atrophic gastritis. Lesions in patients of group II were progressed from baseline to IM, and lesions in patients of no progression group did not progress from baseline lesion.

Blood sample collection

In 1994, blood samples were collected and allowed to clot for 30-40 min at room temperature in the dark. Serum was harvested, the clot was frozen immediately at -20°C, and stored at -70°C for 2 or 3 d. During the transfer, dry ice was used.

Diagnosis of *H. pylori* infection

The detailed serologic assay has been described elsewhere^[22]. Serum level of *H. pylori*-specific IgG and IgA in all samples was measured by enzyme-linked

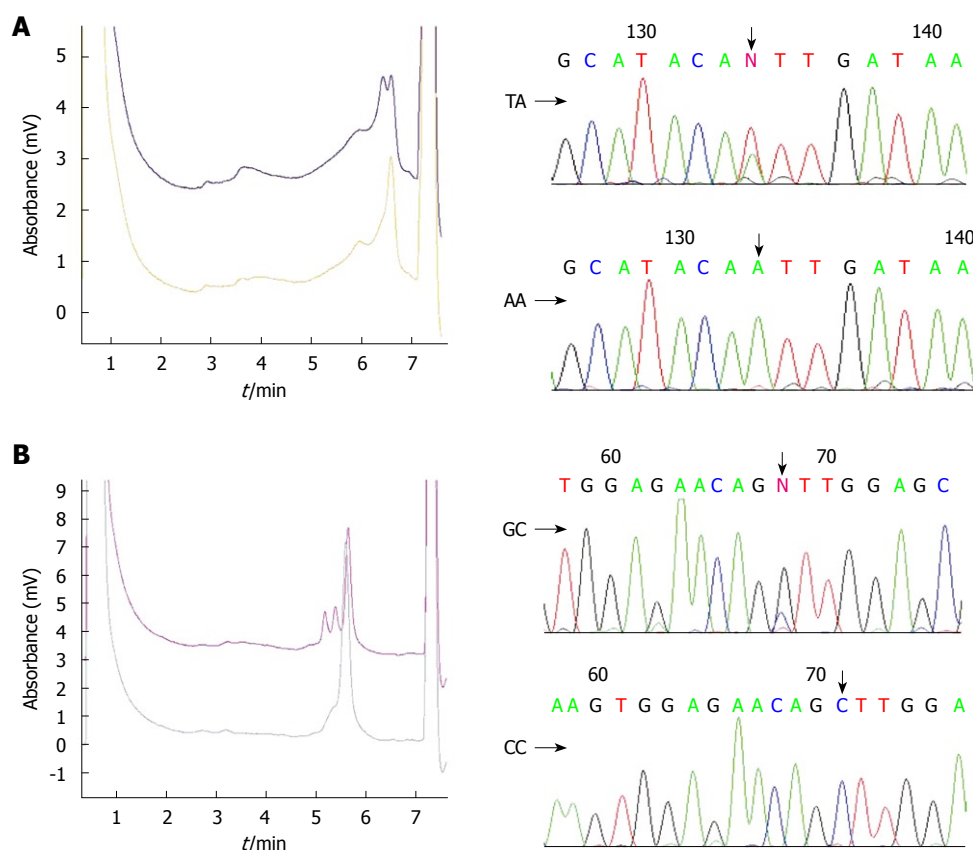


Figure 1 Denaturing high-performance liquid chromatography (DHPLC) and sequence analysis. A: Interleukin-8 (IL-8)-251 A/T polymorphism; B: Macrophage migration inhibitory factor (MIF)-173 C/G polymorphism.

immunosorbent assay (ELISA). Quality-control samples were assayed at Vanderbilt University (Nashville, TN). An optical density ratio (ODR) value > 1.0 and < 1.0 was considered seropositive and negative, respectively.

The presence of *H. pylori* was further confirmed by immunohistochemistry (IHC). Briefly, paraffin-embedded tissue sections were stained using IHC with an avidin-biotin complex immunoperoxidase kit. Polyclonal rabbit anti-*H. pylori* (ab7788, Abcam, Cambridge, UK) was used as a primary antibody as previously described^[25].

DNA preparation

Blood clots were thoroughly washed with Tris-EDTA (TE) buffer containing 50 mmol/L Tris-HCl (pH 8.0) and 1 mmol/L EDTA. After centrifugation, pellets were incubated with rotation in a lysis buffer (TE buffer containing 2 g/L SDS and 200 μ g/mL proteinase K) overnight at 37°C. Lysate was then extracted with phenol and precipitated with isopropanol. The precipitate was washed with 70% ethanol, dried, and dissolved in TE buffer. Concentration and purity of DNA were determined by spectrophotometry at A_{260} nm and A_{280} nm. Then, DNA was aliquoted and stored at -80°C until use.

Cytokine genotyping

Polymerase chain reaction (PCR) was performed in a 25 μ L reaction mixture containing 100ng of DNA, 0.1 μ mol/L of each primer, 0.2 mmol/L of deoxynucleosidetriphosphate, 1.0 U of *Taq* DNA polymerase (Promega, Madison, WI), and 1 \times reaction buffer. The primer sequences of MIF-173, IL-8-251 have been described elsewhere^[26]. PCR-based denaturing high-performance

liquid chromatography (DHPLC) and single nucleotide polymorphisms (SNP) were further confirmed by direct sequencing.

DHPLC analysis was performed on a transgenomic WAVE system (Transgenomic Inc., Omaha, NE). The detailed genotyping process has been described elsewhere^[27]. In brief, PCR products were denatured for 1 min at 94°C and then gradually re-annealed by decreasing the sample temperature from 94°C to 45°C for 30 min to form homo- and/or hetero-duplexes. The PCR products were then applied to the DHPLC column at an optimal oven temperature and eluted with a linear acetonitrile gradient at a flow rate of 0.9 mL/min. The results of DHPLC were further confirmed by DNA sequencing with an ABI Prism 377 DNA sequencer (Applied Biosystems, Foster City, CA) (Figure 1).

Statistical analysis

Data were analyzed using the SPSS 13.0 (SPSS Inc., Chicago, IL, USA). Multiple linear regression analyses were performed with gender, age, smoking, and drinking as explanatory variables to determine which factors influence the progression of gastric lesions. Genotypes of IL-8-251 and MIF-173 loci were also included as explanatory variables when difference in groups I and II and no progression group was detected. Odds ratios (OR) with 95% confidence interval (CI) were computed. $P < 0.05$ was considered statistically significant.

RESULTS

Demographic characteristics and *H. pylori* infection with

Table 1 Parameters of patients with or without progression *n* (%)

	No progression group (<i>n</i> = 137)	Group I (<i>n</i> = 134)	<i>P</i> value	Group II (<i>n</i> = 101)	<i>P</i> value
Age (yr)					
≤ 40	32 (23.4)	45 (33.6) ^a	0.045	35 (34.7) ^b	0.027
40-50	76 (55.5)	60 (44.8)		42 (41.6)	
≥ 50	29 (21.1)	29 (21.6)	0.330	24 (23.7)	0.200
mean ± SD	44.2 ± 0.7	43.8 ± 0.6		46.7 ± 0.7	
Sex			0.600		0.260
Male	59 (43.1)	62 (45.9)		51 (50.5)	
Female	78 (56.9)	72 (54.1)		50 (49.5)	
<i>H. pylori</i> infection			< 0.001		0.100
Negative	59 (43.1)	30 (22.4)		33 (32.7)	
Positive	78 (56.9)	104 (77.6) ^c		68 (67.3)	

^a*P* = 0.045 *vs* patients at the age of 40-50 years ($\chi^2 = 4.04$); ^b*P* = 0.027 *vs* patients at the age of 40-50 years ($\chi^2 = 4.87$); ^c*P* < 0.001 *vs* patients with no progression ($\chi^2 = 13.13$). *H. pylori*: *Helicobacter pylori*.

Table 2 Cytokine genotype frequencies in patients with or without progression *n* (%)

	No progression group (<i>n</i> = 137)	Group I (<i>n</i> = 134)	Group II (<i>n</i> = 101)
IL-8-251			
TT	59 (43.1)	39 (29.1)	25 (24.8)
TA	64 (46.7)	70 (52.2)	65 (64.4)
AA	14 (10.2)	25 (18.7)	11 (10.9)
MIF-173			
GG	100 (73.0)	71 (53.0)	62 (60.4)
GC	34 (24.8)	54 (40.3)	29 (28.7)
CC	3 (2.2)	9 (6.7)	10 (10.9)

IL-8: Interleukin-8; MIF: Migration inhibitory factor. GC: Gastric cancer.

progression and no progression are listed in Table 1. The number of patients under the age of 40 years was greater in groups I and II than in no progression group. The percentage of *H. pylori* infection was significantly higher in group I than in no progression group.

The patients expressed Alleles at the individual loci were expressed in patients showing no progression of the lesions, with no significant χ^2 values.

Effect of IL-8-251 polymorphism on development of SCAG and IM

Multivariate analysis showed that the frequencies of IL-8-251 in groups I and II were significantly different from those in no progression group (Table 2). Compared with IL-8-251 TT genotype, IL-8-251 AA genotype and IL-8-251 A allele carriers exhibited a significantly increased risk for progression from baseline lesions to SCAG (Table 3). The patients with IL-8-251 TA genotype or IL-8-251 A allele carriers had an increased risk for progression from baseline lesions to IM.

Effect of MIF-173 polymorphism on development of SCAG and IM

Multivariate analysis showed that the MIF-173 GC geno-

Table 3 Relation between cytokine gene polymorphisms and development of precursory lesions of GC *n* (%)

Genotype	Group I (<i>n</i> = 134) OR (95% CI)	Group II (<i>n</i> = 101) OR (95% CI)
IL-8-251		
TT	1.00	1.00
TA	1.64 (0.96-2.79)	2.27 (1.25-4.14)
AA	2.62 (1.23-5.72)	1.20 (0.76-1.90)
TA + AA	1.81 (1.06-3.09)	2.07 (1.16-3.69)
MIF-173		
GG	1.00	1.00
GC	2.36 (1.38-4.02)	1.50 (0.57-3.94)
CC	1.92 (0.95-3.87)	2.27 (1.16-4.46)
GC + CC	2.07 (1.21-3.55)	3.84 (1.58-9.34)

OR and 95% CI were calculated by logistic regression, with no progression group as a reference group and adjusted for age, sex. OR: Odds ratio.

type or MIF-173 C allele carriers were significantly associated with an increased risk for progression from baseline lesions to SCAG and IM (Table 3).

The risk for SCAG in association with IL-8-251 and MIF-173 genotypes was further examined with stratification by *H. pylori* infection. The OR for development of SCAG in subjects carrying IL-8-251 A allele or with *H. pylori* infection alone was 2.34 (95% CI: 0.95-2.83) or 3.28 (95% CI: 1.09-9.78), respectively. However, the OR was significantly elevated in subjects carrying the AA genotype and with *H. pylori* infection (OR = 6.70, 95% CI: 1.29-9.78) (Table 4). There was an interaction between IL-8-251 A allele carriers and *H. pylori* infection, with a relative risk for development of SCAG due to the interaction of 6.70, and a synergy index of 1.57.

A similar trend to develop SCAG was observed between the MIF-173 C allele carriers and *H. pylori* infection. The OR of developing SCAG significantly increased in subjects carrying at least one MIF-173 C allele and with *H. pylori* infection (OR = 6.54, 95% CI: 2.97-14.20) (Table 4). An interaction between the MIF-173 C allele carriers and *H. pylori* infection was observed (OR = 2.26, synergy index = 3.15).

The association of IM and IL-8-251 with MIF-173 genotypes was further examined with stratification by *H. pylori* infection. However, the OR for IM in subjects carrying MIF-173 C allele and with *H. pylori* infection was elevated significantly (OR = 2.93, 95% CI: 1.28-6.60) (Table 5). There was also an interaction between the MIF-173 C allele carriers and *H. pylori* infection (OR = 2.20, synergy index = 1.25).

DISCUSSION

In this study, all tested genetic polymorphisms in IL-8 and MIF increased the risk of SCAG and IM. IL-8 and MIF are inflammatory cytokines expressed in injured mucosa after *H. pylori* infection. IL-8 is an important mediator of the inflammatory response and increases mucosal injury in *H. pylori* infected patients because IL-8 is a major activator and chemokine for neutrophils which

Table 4 Risk of SCAG in patients with IL-8-251 and MIF-173 genotypes and *H. pylori* infection

<i>H. pylori</i> infection	IL-8-251 genotype				MIF-173 genotype			
	TT	OR (95% CI) ¹	TA + AA	OR (95% CI) ¹	GG	OR (95% CI) ¹	C carriers	OR (95% CI) ¹
Negative	5/22	1.00	25/37	2.34 (0.95-2.83)	17/40	1.00	13/19	1.60 (0.64-3.97)
Positive	34/34	3.28 (1.09-9.78)	70/44	6.70 (1.29-9.78)	54/60	2.11 (1.07-4.13)	50/18	6.54 (2.97-14.20)

¹OR and 95% CI were calculated by logistic regression, with no progression group as a reference group and adjusted for age, sex. SCAG: Severe chronic atrophic gastritis.

Table 5 Relation between risk of IM and MIF-173 genotypes infection

<i>H. pylori</i> infection	MIF-173 genotype			
	GG ¹	OR (95% CI) ²	C carriers ¹	OR (95% CI) ²
Negative	19/40	1.00	14/19	1.55 (0.63-3.72)
Positive	43/60	1.51 (0.76-2.94)	25/18	2.93 (1.28-6.60)

¹Group II/No progression, "Group II" means lesions were progressed from normal or superficial gastritis to IM after follow up; ²OR and 95% CI were calculated by logistic regression, with no progression group as a reference group and adjusted for age, sex.

contribute to mucosal damage by secreting NO and H₂O₂^[28] and significantly augments T helper 1 (Th1) immune response by inducing proinflammatory cytokines such as TNF- α , interferon- γ , and IL-1 β secretion. It has been shown that Th1 predominant immune responses inhibit acid secretion from gastric glands, and cause gastric atrophy and metaplasia in a *H. pylori* infected mouse model^[29,30].

The transcript activity is significantly higher in the IL-8-251 A promoter than in the IL-8-251 T promoter^[31]. Furthermore, the DNA sequence around the IL-8-251 A allele region may produce a potential binding site for C/EBP, and induce IL-8 expression through the nickel subsulphide dependent pathway^[32].

In this study, the risk of progression from baseline lesions to SCAG and IM was significantly increased in patients carrying the IL-8-251 AA and IL-8-251 AT genotype or the IL-8-251 A allele, which is consistent with the reported findings^[19,31]. Furthermore, SCAG occurred due to the interaction between IL-8-251 A allele carriers and *H. pylori* infection. A previous study on the same population also demonstrated that the IL-8-251 AA genotype significantly increases the risk of GC^[33]. In the present study, IL-8-251 A allele carriers were positively correlated with the development of SCAG and IM, implying that *IL-8-251* gene polymorphism plays an important role in the development of GC.

MIF promotes the recognition of Gram-negative bacteria by the innate immune system^[34]. The *MIF* gene appears to be a strong candidate susceptibility gene for *H. pylori*-related diseases. Xia *et al*^[17] reported that both mRNA and protein levels of MIF are up-regulated in *H. pylori*-infected patients and parallel to the severity of gastritis. Moreover, the expression level of MIF protein is markedly different in patients with gastritis, IM, DYS, GC^[17,18].

Functional studies, both *in vivo* and *in vitro*, demonstrated that the mutant allele MIF-173 C is associated with an

increased MIF protein production^[31,35]. The presence of MIF-173 C allele stimulates protein 4 (AP-4) transcription factor binding site that may up-regulate MIF expression^[36].

In our study, the MIF-173 C allele was found to be associated with the high risk of SCAG and IM. Moreover, an interaction occurred between MIF-173 C allele carriers and *H. pylori* infection, thus promoting progression from baseline lesions to SCAG and IM. Other studies found that MIF not only modulates the expression of proinflammatory mediators such as TNF- α , IL-1 β , IL-8, IFN- γ , but also regulates the activation of T cells^[35,37].

Therefore, we hypothesize that variants of *MIF* gene polymorphism may contribute to the different outcomes of *H. pylori*-related gastritis. Moreover, *MIF* gene polymorphisms may be another important candidate gene marker for the outcomes of patients infected with *H. pylori*.

The fact that the population in our study lived in a relatively closed society and had similar living conditions or habits, may minimize the effects of other mixed factors such as intake of fresh vegetables, salt consumption, water intake. Furthermore, the subjects in our study were followed up for an average period of 56 mo, and the final pathological diagnosis was made in 1994. Although more recent pathological assessments may provide additional insights, an average follow-up period of 4-5 years provides a more dynamic process for the assessment of risks than a single time point analysis^[15,19,20].

In summary, *H. pylori* infection and variants in IL-8-251 or MIF-173 polymorphisms influence the occurrence of SCAG and IM. Because of the high prevalence of *H. pylori* infection, antibiotic resistance, and some potential drawbacks associated with *H. pylori* eradication therapy (e.g. reflux esophagitis), our study may provide a reasonable basis for therapeutic decisions even at the early stage of GC.

COMMENTS

Background

Atrophic gastritis and intestinal metaplasia are two important precursory lesions of intestinal type gastric cancer (GC), except for factors of *Helicobacter pylori* (*H. pylori*). Host gene polymorphisms also play a very important role in the development of GC.

Research frontiers

To date, a few studies are available on the relation between gene polymorphisms of inflammatory cytokines and risk of atrophy and intestinal metaplasia (IM).

Innovations and breakthroughs

In this study, the authors conducted a prospective study using the data obtained during the 56-mo follow-up (including both gastroscopic and histopathological information). The relation between interleukin-8 (IL-8), migration inhibitory factor (*MIF*) gene polymorphisms and risk of atrophic gastritis and IM was also studied.

Applications

Because of the high prevalence of *H. pylori* infection, antibiotic resistance, and some potential drawbacks in *H. pylori* eradication therapy (e.g. reflux esophagitis), the result of this study may provide some new evidence for the selection of patients infected with *H. pylori* and for the prevention of progression of *H. pylori*-related gastritis.

Terminology

IL-8, a member of Cys-X-Cys (CXC) chemokine family, is an activator and chemoattractant of neutrophils and lymphocytes, thus playing an important role in the development of gastritis and GC. Macrophage MIF, an important activator of T lymphocytes and macrophages, plays a pivotal role in inflammatory and immune diseases. The expression of MIF is increased after *H. pylori* infection and related with histological severity of GC and its precursor.

Peer review

The authors studied the role of different IL-8 and MIF genotypes in the development of precancerous gastric lesions, severe atrophic gastritis or intestinal metaplasia, in a cohort of 372 patients. The study defined *H. pylori*-infected patients at the risk for GC.

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Does (supra)gastric belching trigger recurrent hiccups?

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Abstract

Twenty-four hours multichannel intraesophageal impedance and pH monitoring in a patient who suffered from recurrent hiccups for more than a year revealed frequent supragastric belching and pathological oesophageal acid exposure. Furthermore, a temporal relationship between the start of a hiccup episode and gastric belching was observed. The data support the hypothesis that there is an association between supragastric belching, persistent recurrent hiccups and gastro-oesophageal reflux disease, and that gastric belching may evoke hiccup attacks.

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Key words: Hiccup; Impedance and pH monitoring; Gastro-oesophageal reflux; Supragastric belching

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INTRODUCTION

Hiccups are mostly a transient phenomenon that do not require medical attention. Prolonged hiccups are rare and can be indicative of a serious underlying disease process. Gastro-oesophageal reflux disease (GORD) has been associated with protracted hiccups^[1-3]. Previous reports on functional disturbances of the oesophagus in patients with hiccups are sporadic and confined to conventional manometry and pH monitoring. Multichannel oesophageal impedance measurement is a recently developed technique that detects both liquid and gas transport in the oesophagus. In this report we present detailed results of 24 h intraesophageal impedance measurement and pH monitoring in a patient who suffered from persistent recurrent hiccups for more than a year. In addition, we had the unique opportunity to monitor intraluminal pressure and impedance signals of the oesophagus during the spontaneous onset of a hiccup attack.

CASE REPORT

A 74-year-old man suffered from chronic recurrent hiccups for more than 18 mo. Upper gastrointestinal endoscopy showed reflux oesophagitis grade A (Los Angeles classification). Proton pump inhibitor (PPI) therapy was successful for heartburn, but hiccups and belching persisted. Extensive clinical evaluation did not reveal other abnormalities that could explain the hiccups. Treatment with metoclopramide, baclofen and gabapentin did not relieve symptoms. During hiccup episodes, hiccups

Table 1 24 h ambulatory impedance-pH measurement

	Off PPI	On PPI	Normal
% time pH < 4.0			
Total	50	27	< 4.0%
Upright	45	10	< 4.7%
Supine	57	55	< 2.0%
Supragastric belches			
Total	188	17	Absent
Symptom episodes			
Severe belching	4	-	-
Heartburn	5	-	-
% time with hiccup	> 60%	> 60%	Absent

PPI: Proton pump inhibitor.

were continuous, lasted all day and were even present at night. Severe belching sometimes preceded the start of a hiccup attack. Hiccups were absent approximately 1 d per week.

24-h ambulatory impedance-pH monitoring

Electrical impedance was recorded at 3, 5, 7, 9, 15 and 17 cm and pH at 5 cm above the upper border of the lower oesophageal sphincter (LOS) with a transnasally positioned disposable catheter (reference code K6011-EI-0632 Unisensor AG, Attikon, Switzerland). A commercially available ambulatory system (Ohmega, MMS, Enschede, The Netherlands) was used for storage and subsequent analysis of data according to previously described criteria^[4-7]. The study was performed once on and once off PPI therapy.

pH-metry off PPI revealed severely pathologic oesophageal acid exposure (Table 1). Basal impedance was below 1000 Ohm for more than 90% of the time at all measurement sites in the oesophagus, prohibiting reliable detection of swallow- or reflux-induced impedance drops. Impedance patterns consistent with “supragastric belches (SGBs)” were observed frequently^[6]. These consisted of impedance increments exceeding 3000 Ohm with a rapid aboral propagation (velocity > 10 cm/s). These impedance peaks started in the proximal oesophagus and were followed by a retrograde return of impedance to baseline (Figure 1). A total of 188 SGBs were observed in our patient (Table 1). Based on these findings the patient was treated with PPI (20 mg rabeprazole twice daily) and referred for behavioral therapy in an attempt to reduce supragastric belching.

Twenty-four hours ambulatory impedance-pH monitoring on PPI 1 mo after behavioral therapy revealed a considerable reduction of SGBs by 90% (Table 1). The treatment by a speech therapist focused on teaching abdominal breathing and controlled swallowing to avoid influx of air. Oesophageal acid exposure was reduced (Table 1) and heartburn completely disappeared after PPI therapy. However, after 1 year follow-up, the patient reported only a minor and transient reduction of hiccups.

Oesophageal impedance measurement and perfusion manometry

Stationary manometry and impedance measurements were performed with a perfused multi-lumen manometric and impedance assembly in combination with a sleeve sensor (reference code CE5-0010, Dentsleeve international, Mississauga, Ontario, Canada) connected to the Solar GI system (MMS) as described previously^[8]. Intraesophageal pressures and impedance signals at 2, 7, 12 and 17 cm above the upper border of the LOS revealed ineffective oesophageal motility characterized by non-transmitted contractions with absence of pressure responses at 2 cm and in 3 out of 14 liquid swallows also at 17 cm. In 2 out of 14 swallows a simultaneous contraction was observed with mean amplitude > 30 mmHg. In addition, impedance tracings showed abnormal bolus transport as only 41% (normal ≥ 80%) of saline swallows were followed by normal transit. During these routine measurements hiccups were absent. Recording was continued for 3 h with the patient comfortably seated in a chair. After 20 min an episode of hiccups began. Approximately 20 s before the onset of the hiccups a sequence of gastric belching followed by simultaneous oesophageal contractions was observed (Figure 2).

DISCUSSION

In our patient presenting with recurrent hiccups, excessive supragastric belching was found by 24-h impedance measurement. In addition, gastric belching and oesophageal spasms were observed by combined oesophageal impedance monitoring and manometry immediately preceding the onset of a hiccup attack.

The typical impedance pattern of SGBs was previously observed in patients with severe belching by Bredenoord *et al*^[6]. It was attributed to rapid entrance of air into the oesophagus from proximal that was almost immediately expelled. The pattern was clearly distinct from normal air swallowing in which a fall in impedance was associated with a more slowly propagated impedance peak and also from gas reflux or gastric belches in which the impedance peak started in the distal oesophagus and moved in a retrograde direction^[4-6]. SGBs were not reported in healthy controls and in patients with functional dyspepsia^[4-6]. In patients with GORD SGBs were found to occur more frequently, either as a putative trigger of reflux episodes or as a response to a perceived reflux episode^[9].

The pathophysiology of hiccups is not clear. It has been suggested that sudden rapid distension of the proximal oesophagus by excessive food ingestion, carbonated beverages or aerophagia can trigger the hiccup reflex^[10,11]. The present report provides evidence for an association between belching and hiccups. The observation that two gastric belches preceded the onset of a hiccup episode in our patient supports the hypothesis that

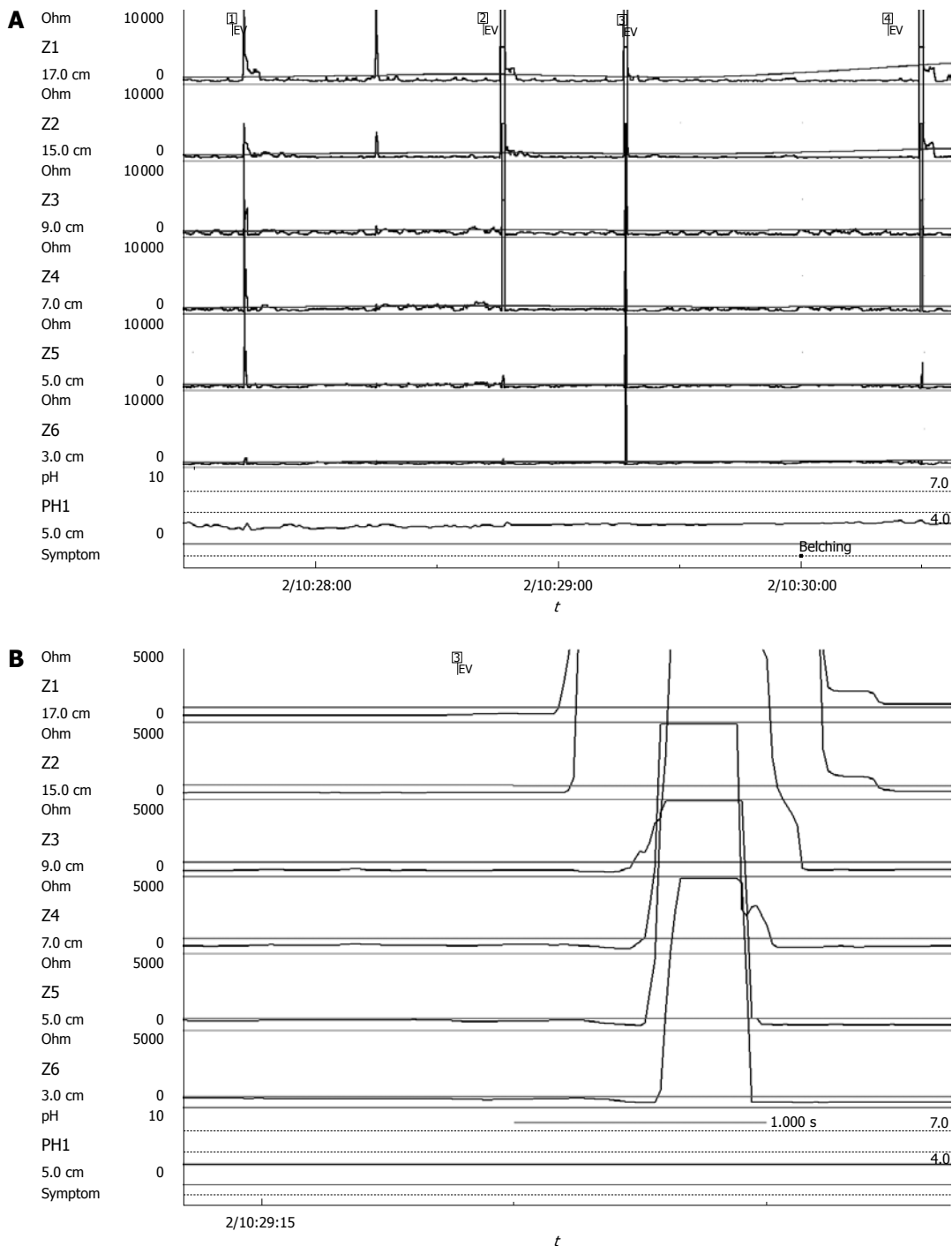


Figure 1 Oesophageal intraluminal impedance monitoring combined with oesophageal pH measurements. A: Three minutes stretch of the tracings showing an abnormally low basal impedance with repetitive impedance peaks in all channels marked EV 1-4; B: Zoomed-in segment of the recording shows that an impedance peak (EV3) starts in the proximal channel, moves in the antegrade direction and is cleared in the retrograde direction. The horizontal bar represents 1.000 s. The patient reported severe belching at 10:30:00 during this period of repetitive supragastric belches (SGBs). Oesophageal pH is continuously below 4.

rapid distension by oesophageal gas transport can trigger hiccups. Our patient also suffered from severe pathological acid reflux, ineffective oesophageal motility and impaired transit for liquid boluses. GORD was associated with protracted hiccups in previous case reports^[1-3]. Disappearance of protracted hiccups after treatment with PPI in some patients suggests that reflux may precipitate hiccups^[2]. However, persistence of hiccups after

antireflux surgery in some reports^[1,3] or treatment with PPI, as in our patient, suggests that other factors may be involved in patients with GORD.

In conclusion, in a patient with recurrent hiccups and GORD multichannel intraluminal impedance, pH and pressure measurements show (1) excessive supragastric belching and (2) a temporal relationship between the start of a hiccup episode and gastric belching.

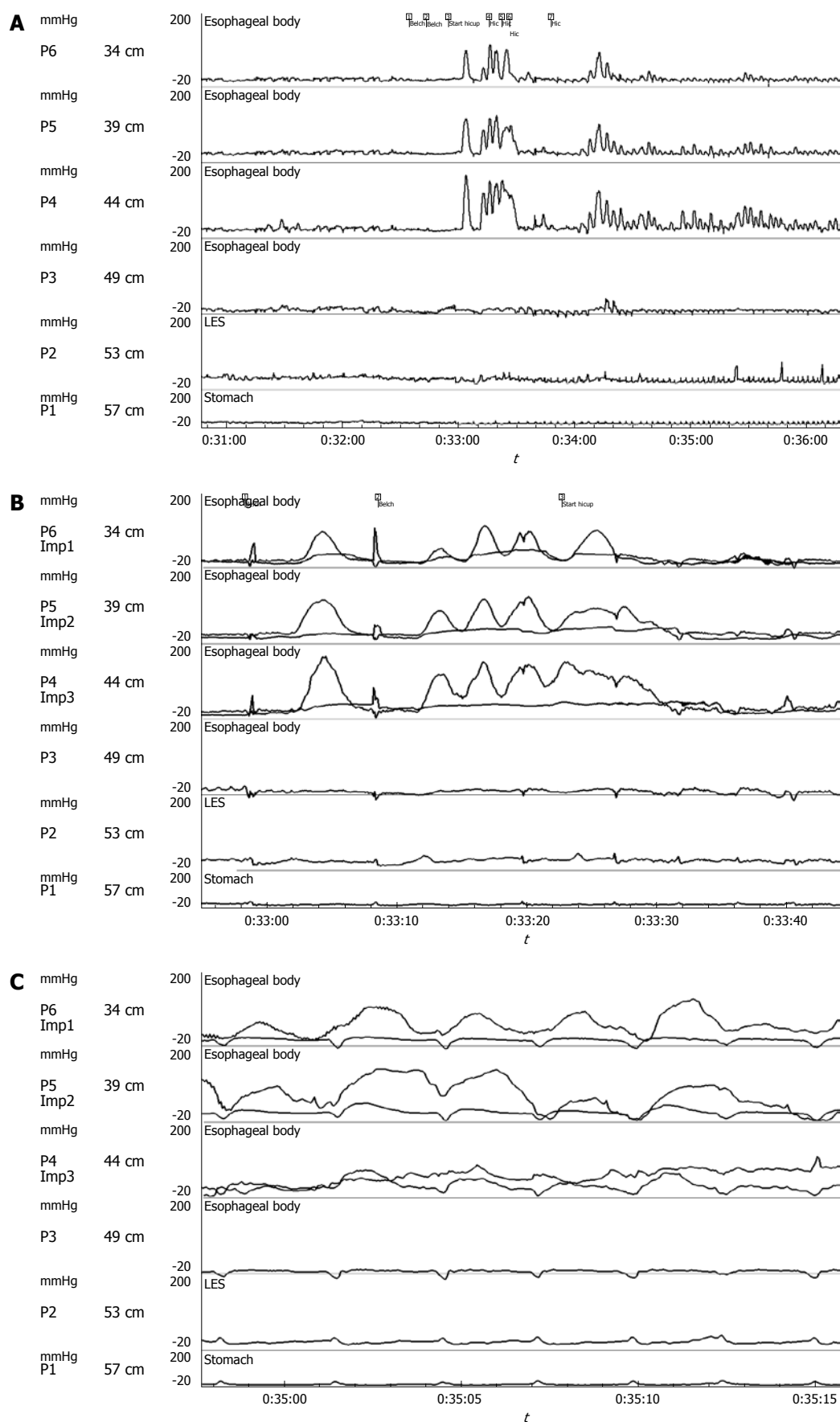


Figure 2 Combined impedance and perfusion manometry. A: Pressure tracings from 31-36 min during spontaneous onset of a hiccup attack at time 33:20 min; B: Combined impedance and manometry of a zoomed in segment of Figure 2A shows that the hiccup episodes are preceded by 2 gastric belches and high pressure simultaneous, repetitive contractions; C: Another zoomed in segment of Figure 2A shows that hiccups are accompanied by sharp positive pressure peaks in the lower oesophageal sphincter and stomach, and by negative peaks in the oesophagus. The upper border of the lower oesophageal sphincter (LOS) was located at 51 cm from the nostrils.

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Surgical spacer placement and proton radiotherapy for unresectable hepatocellular carcinoma

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Abstract

Few potentially curative treatment options exist apart from hepatic resection for patients with huge hepatocellular carcinoma (HCC). Proton radiotherapy is a promising new modality which has an inherent anti-tumor effect against HCC. However, the application of proton radiotherapy for tumors adjacent to the gastrointestinal tract is restricted because the tolerance dose of the intestine is extremely low. A novel two-step treatment was developed with surgical spacer placement and subsequent proton radiotherapy to administer proton radiotherapy with curative intent. This report presents a case of a patient with a huge unresectable HCC treated by this method who achieved disease-free survival of more than 2 years. This new strategy may potentially be an innovative and standard therapy for unresectable HCC in the near future.

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Key words: Hepatocellular carcinoma; Proton radio-

INTRODUCTION

Hepatocellular carcinoma (HCC) accounts for between 85% and 90% of primary liver cancer and is one of the most frequent malignancies^[1]. Currently, several treatment modalities are available for the treatment of patients with HCC, including hepatic resection, liver transplantation, transarterial chemoembolization (TACE), percutaneous ethanol injection, percutaneous microwave coagulation therapy, radiofrequency ablation, and radiotherapy^[2,3]. Although TACE is the only nonsurgical treatment option for HCC measuring above 10 cm × 10 cm, the 5-year survival rate is less than 10%, and its efficacy remains uncertain^[4]. At present, a hepatic resection is regarded as the only curative treatment for a huge HCC, if the patient's hepatic functional reserve is acceptable for resection^[5,6].

Proton radiotherapy is a new mode of radiotherapy that has an inherent advantage over photon radiotherapy. Owing to the defined range of protons exhibited by the Bragg peak, they allow reduction of the irradiated volume and the dose administered to the normal tissues^[7,8]. Recently, many reports have demonstrated the effectiveness and safety of proton radiotherapy and it has

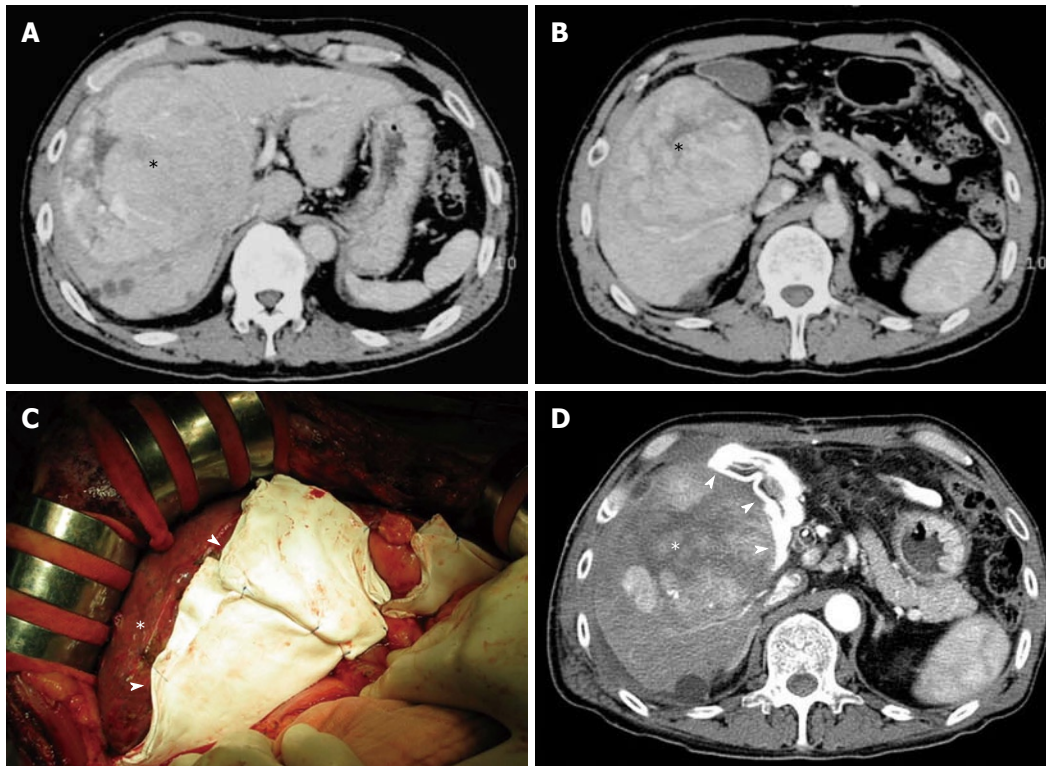


Figure 1 A huge tumor occupying a wide area of the right lobe. A: Abdominal computed tomography (CT) showing a pre-treatment dominant lesion located widely in the right lobe (asterisk); B: The tumor (asterisk) has extended contact with the gastrointestinal tract; C: Intraoperatively, Gore-Tex sheets (arrowheads) maintained a space between the tumor (asterisk) and the gastrointestinal tract (under the fingertips); D: Post-operative abdominal CT showing the spacer (arrowheads) around the tumor (asterisk); the spacer maintained a sufficient open space between the tumor and the gastrointestinal tract.

become an acceptable modality for various malignancies including HCC^[7-9]. However, the application of proton radiotherapy for huge HCC is restricted because most of these lesions are in contact with the gastrointestinal tract that cannot tolerate a radical dose of proton beams^[10,11]. Recently, we developed a novel 2-step treatment with surgical spacer placement and subsequent proton radiotherapy^[12]. In this case report, we employed this strategy for a huge unresectable HCC and achieved a long-term survival benefit without recurrence.

CASE REPORT

A 59-year-old male was referred for the treatment of a huge HCC in the right lobe of the liver. He had developed hepatitis B 50 years earlier and received no medical treatment. A physical examination revealed general fatigue and abdominal distension as a result of the huge tumor. Abdominal computed tomography (CT) showed a massive tumor, measuring 12 cm × 12 cm, occupying a wide area of the right lobe (Figure 1A). There was no sign of tumor vascular invasion in the hepatic or the portal vein and the tumor broadly abutted the gastrointestinal tract on CT images (Figure 1B). Liver function tests showed a total bilirubin of 1.0 mg/dL, aspartate aminotransferase of 33 IU/L, alanine aminotransferase of 24 IU/L, prothrombin time-international normalized ratio of 1.16, and an indocyanine green retention rate at 15 min of 19.6%. Serological analysis showed that the

serum level of α -fetoprotein and PIVKA II (protein induced by vitamin K absence II) were 3.7 ng/mL and 3910 mAU/mL, respectively. There was no regional lymph node metastasis. A right trisegmentectomy was not acceptable judging from the criteria for a safe hepatic resection^[13]. Proton radiotherapy with curative intent also was not acceptable since the tumor was located broadly adjacent to the gastrointestinal tract. Therefore, a novel 2-step treatment was employed with surgical spacer placement and subsequent proton radiotherapy^[12]. Eight Gore-Tex sheets (20 cm × 15 cm × 2 mm) were superimposed and applied as a spacer and sutured tightly to the inferior surface of the liver to keep the gastrointestinal tract away from the tumor (Figure 1C). No part of the tumor was resected during the spacer placement surgery, because it was just a first step to allow proton radiotherapy. Abdominal CT after the operation showed the spacer maintained a sufficient open space between the tumor and the gastrointestinal tract (Figure 1D). The postoperative course was uneventful and 1 mo later, proton beams of 84GyE were administered in 20 fractions over 30 d (Figure 2). No acute or late treatment-related toxicities (grade 2 or more) were observed and liver function was unchanged for up to 2 years after the proton radiotherapy. In addition, abdominal CT showed regression of the tumor. Areas of high signal intensity on the CT images correlated with liquefaction necrosis, granulation tissue, and the tumor (Figure 3A). Notably, the serum PIVKA II level remained within normal limits for up to 2 years,

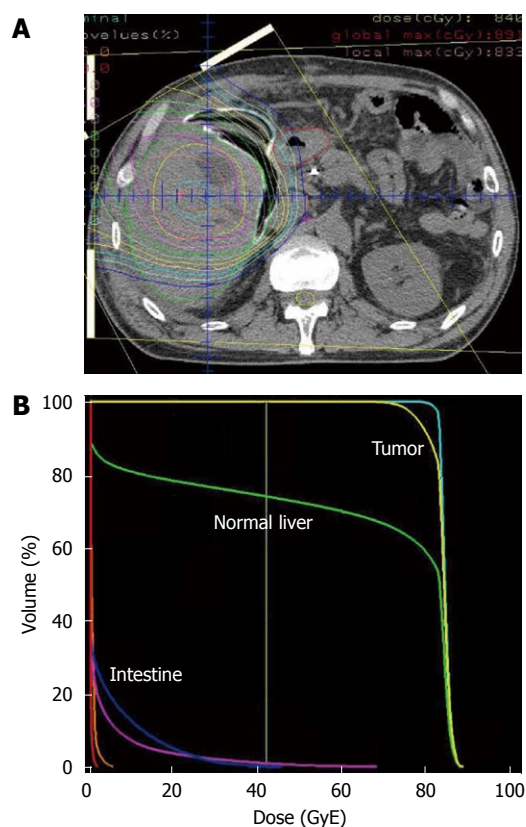


Figure 2 Treatment plan for proton radiotherapy with a total dose of 84 GyE in 20 fractions. A: Isodose curves demonstrate 100% of the prescribed dose at the center and decreasing by 10% of the dose from the inside out; B: Dose-volume histogram of the target volume and the intestine shows that the tumor is entirely irradiated by almost 100% of the prescribed dose, and the intestine is hardly irradiated.

indicating no sign of recurrence, consistent with other modalities (Figure 3B). As there was no convincing evidence for the safety of hepatic resection after proton radiotherapy, we decided not to perform any surgery even after shrinkage of the tumor.

DISCUSSION

Among various malignancies, HCC is one of the most suitable malignancies for the treatment of particle radiotherapy with several excellent treatment results^[7,14,15]. However, the application of particle radiotherapy for tumors adjacent to the gastrointestinal tract is restricted because the tolerance dose of the intestine is extremely low^[14,16]. An adequate margin of more than 10 mm is absolutely necessary for the administration of full-dose irradiation^[17].

There have been many reports describing surgical methods for removal of the gastrointestinal tract from the irradiation field to prevent radiation-induced gastrointestinal damage during conventional photon radiotherapy^[10,11]. However, the dose distribution of photon beams is generally inadequate for local tumor control, and the impact of combination therapy with surgery and subsequent photon radiotherapy has remained uncertain.

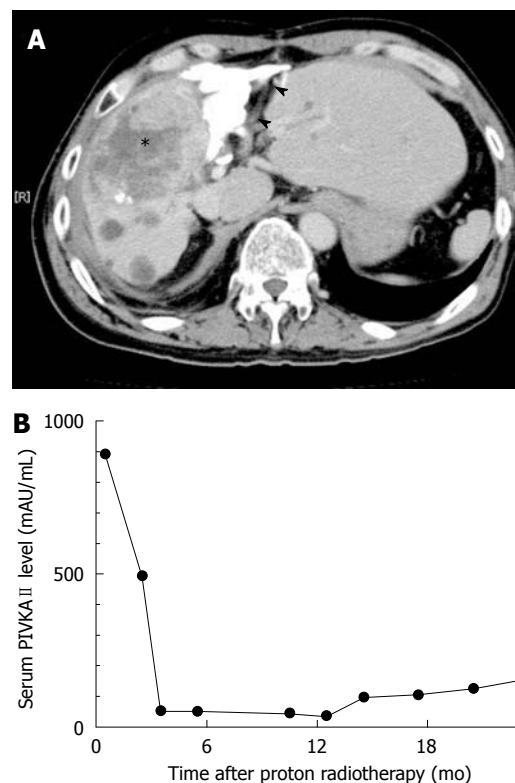


Figure 3 Two years after the proton radiotherapy abdominal CT reveals distinct shrinkage of the tumor (asterisk). Arrowheads indicate the gastrointestinal tract (A) along with reduced serum levels of the tumor marker, PIVKA II (protein induced by vitamin K absence II) (B).

Unlike conventional photon radiotherapy, proton radiotherapy is considered a curative treatment. Tumors can be controlled at a high rate as long as a sufficient dose can be administered. The 2-step treatment is an evolving strategy that enables the delivery of a sufficient dose to the tumor.

Curative treatments for HCC are limited to hepatic resection, liver transplantation and local ablative therapies^[3]. In addition, in the absence of other treatment options, hepatic resection is the only treatment modality for a huge HCC that can produce notable survival, but the 5-year survival rate is only 16.7%-33%^[6]. There was no possibility of treating this case in a curative manner or achieving disease-free survival of more than 2 years using a conventional strategy. The 2-step treatment achieved complete local control of the huge primary HCC and excellent disease-free survival. Surgical spacer placement can maintain a safety margin from the gastrointestinal tract and full-dose particle radiotherapy can be achieved without serious toxicities under that condition. It is highly important in terms of a new option for curative treatment of a huge unresectable HCC. This new strategy might potentially be an innovative and standard therapy for unresectable HCC in the near future.

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Intestinal anisakiasis can cause intussusception in adults: An extremely rare condition

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INTRODUCTION

New food culture may bring a new cause of disease. The culture of eating raw fish, such as sushi and sashimi, has been spreading worldwide and now exists in the United States, European and Asian countries. This may provide the possibility of accidentally contracting anisakiasis, a parasitic disease. We report an extremely rare case of adult intussusception caused by anisakiasis.

CASE REPORT

A 41-year-old man was admitted into our hospital for right lower abdominal colicky pain that had abruptly occurred at midnight of the day before, and which had gradually worsened.

Physical examination revealed a 5 cm-sized, hard and non-mobile mass with extreme tenderness in his right middle abdomen. Ultrasonography (US) showed multiple

Abstract

We report an extremely rare case of adult intussusception caused by anisakiasis. A 41-year-old man was admitted into our hospital for right lower abdominal colicky pain. Ultrasonography and computed tomography revealed the presence of intussusception. As pneumodynamic resolution by colonoscopy failed, surgery was performed. The anisakis body was found in the submucosal layer of the resection specimen. The patient was discharged 9 d after the operation. Anisakiasis may cause intussusception in any country where sushi or sashimi now exists as a popular food. If suspicious, detailed clinical interview as to food intake prior to symptom development is crucial.



Figure 1 Abdominal contrast-enhanced computed tomography (CT) imaging. The coronal multiplanar reconstruction imaging in abdominal contrast-enhanced CT clearly reveals the invaginations of the ascending colon itself (arrows).



Figure 2 A gross view of the resection specimen. The extirpated mass is 4 cm in diameter and its surface is smooth, yellowish, edematous and slightly soft (arrow).

concentric ring signs and hay-fork signs. Thus, he was diagnosed as having intussusceptions. Abdominal X-ray revealed null colon gas but feces in the right lower abdomen. Contrast-enhanced abdominal computed tomography (CT) showed invaginations of the ascending colon (Figure 1), the wall of which looked highly edematous. As the ascending colon was obstructed by these invaginations, the colonic lumen was filled with stool at the oral side of this obstruction. No solid tumor was detected at the top of these invaginations and the colonic wall was well enhanced, indicating no ischemic change existed.

First of all, we performed colonoscopy an hour after admission, hoping for both differential diagnosis of intussusceptions and pneumo-dynamic resolution. The leading top of the invaginations was yellowish, edematous and tensioned. No tumor was discernible. As pneumo-dynamic resolution of the intussusceptions by colonoscopy was not achieved, open surgery was performed 3 h after admission.

Normograde invaginations of the ascending colon were demonstrated during the operation. The ascending colonic wall was edematously swollen and showed no apparent ischemic change. Since the manipulative resolution of the invaginations was impossible, ileocecal resection was performed.

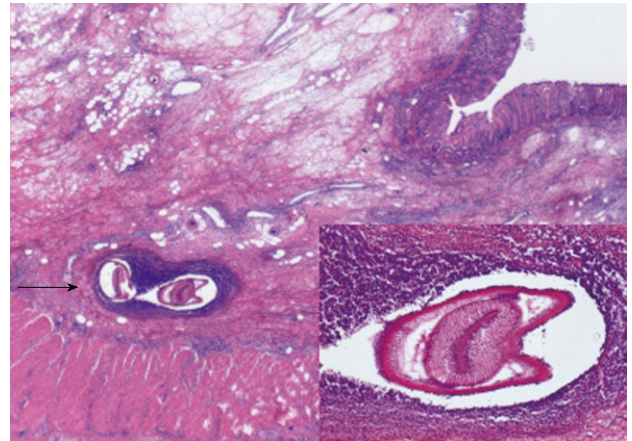


Figure 3 Histopathological findings of the resection specimen. An anisakis body (arrow) is demonstrated in the edematously inflamed submucosa of the extirpated ascending colon (hematoxylin and eosin stain; $\times 20$). Note that many eosinophils and lymphocytes infiltrate around the anisakis body, forming an eosinophilic granuloma. A magnified picture of the anisakis body is shown in the inset below right (hematoxylin and eosin stain; $\times 40$).

The resection specimen contained a 4 cm-sized yellowish inflammatory mass (Figure 2) at the leading top of these invaginations. The mass was edematous, well tensioned and the mucosal surface that surrounded the mass was normal.

We pathologically demonstrated the body of anisakis in the midst of eosinophilic granuloma in the submucosal layer (Figure 3). Marked inflammation and edema in the submucosal layer formed an inflammatory mass.

A retrospective clinical interview was performed where the patient told us that he had eaten marinated mackerel 2 d before admission. A retrospective analysis of his serum revealed that an anti-anisakis antibody test was positive. Based on these findings, we finally diagnosed this case as intussusceptions of the ascending colon caused by anisakiasis.

The patient's abdominal pain immediately disappeared after surgery. He clinically recovered well and was discharged 9 d after the operation.

DISCUSSION

Adult intussusception (AI) occurs much less often than in children and accounts for only 5% to 10% of all intussusceptions^[1]. More than 90% of AI derive from tumorous lesions^[1], 60% of which are malignant and the remaining 40% are benign tumors^[2]. Thus, almost all surgeons agree that operation is strongly recommended as a treatment for AI because of the high incidence of malignancy^[1].

US^[3-5] and CT are very useful for diagnosis of AI. There are specific US findings named as "multi concentric ring sign"^[4], "target ring sign"^[3], or "hay-fork sign"^[5]. However, these signs are often hard to determine, being masked by a fully gas-filled bowel^[6].

CT is the best modality to diagnose AI, visualizing "target signs", or "bowel-within-bowel" appearances^[7].

Table 1 Characteristics of affected patients in English and Japanese literature

Author	Age (yr) /sex	Symptom	Food	Intake to onset	Site of intussusception	Operation	Tenderness/palpable mass/ascites/US/CT/eosinophilic granuloma/anti-anisakis antibody	Reduction of intussusception	Anisakis body
Hayashi <i>et al</i> ^[19]	61/M	Left lower abdominal pain	Sliced raw mackerel	Half a day	Jejunum	Partial jejunectomy	+/-/ND/-/-/+ / +	ND	In the resected specimen
Kim <i>et al</i> ^[20]	38/M	General abdominal pain	Marinated mackerel	Half a day	Ileocecum	Ileocecal resection	+ / + / - / + / + / + / +	Failure, barium enema	In the resected specimen
Mitani <i>et al</i> ^[21]	66/M	Lower abdominal pain	Salted squid cuttlefish	Several hours	Sigmoid colon	Sigmoidectomy	+ / + / - / ND / + / + / ND	Success, barium enema	On the resected specimen
Iwakami <i>et al</i> ^[22]	46/F	General abdominal pain, nausea	Marinated mackerel	Half a day	Jejunum	Partial jejunectomy	+ / - / - / + / + / + / ND	ND	In the resected specimen
Hirano <i>et al</i> ^[23]	35/F	Epigastralgia	Sliced raw mackerel	1 d	Jejunum	Partial jejunectomy	+ / - / + / + / + / + / ND	Failure, barium enema	On the resected specimen
Kanisawa <i>et al</i> ^[18]	67/M	Severe upper abdominal pain and vomiting	Raw squid	2 d	Ascending colon	None	+ / + / ND / + / ND / - / ND	Success	Removed under colonoscopy
Sugiyama <i>et al</i> ^[24]	71/M	General abdominal pain	Horse mackerel	1 d	Transverse colon	Transverse colectomy	+ / - / - / + / + / + / ND	Failure, barium enema	On the resected specimen
Hamanaka <i>et al</i> ^[25]	47/M	General abdominal pain	Salmon and salmon caviar	7 d	Ileocecum	Ileocecal resection	+ / - / + / + / + / + / +	Failure, barium enema	In the resected specimen
Tsuji <i>et al</i> ^[26]	56/F	Epigastralgia, nausea	Vinegared sardine	Half a day	Jejunum	Partial jejunectomy	+ / - / + / + / + / + / ND	ND	In the resected specimen
Chikamori <i>et al</i> ^[17]	54/F	Severe upper abdominal pain and vomit	Raw bonito	2 d	Jejunum	Laparoscopic jejunectomy	+ / - / + / + / + / ND / ND	ND	On the resected specimen
Hibi <i>et al</i> ^[27]	35/M	Epigastralgia, nausea	Sliced raw cuttlefish	Several hours	Jejunum	Partial jejunectomy	+ / - / - / ND / + / + / ND	ND	In the resected specimen
Present case	41/M	Severe right lower abdominal pain	Marinated mackerel	1 d	Ascending colon	Ileocecal resection	+ / + / + / + / + / + / +	Failure	In the resected specimen

M: Male; F: Female; ND: Not documented.

The location or nature of tumors if present, soft tissues involved in the invaginations, obstruction and threatening signs of bowel viability, can all be evaluated by CT^[7]. Recently, multiplanar reconstruction (MRP) imaging by the multi-detector row CT has been developed and is more useful for diagnosing intussusceptions than other imaging examinations^[8].

Most surgeons agree that surgery is preferable as a treatment for AI because of the high frequency of malignancy^[1,2,9]. The resolution of intussusceptions has some risks, such as (1) intraluminal seeding and dissemination, (2) perforation and peritoneal dissemination and (3) anastomotic complication with edematous and fragile bowel wall^[1,2,9,10].

Anisakiasis is one of the parasitic diseases and is regarded as larva migrans of *Anisakis*^[11]. A person is accidentally infected by eating raw or uncooked fish containing the parasites. The larva of *Anisakis* penetrates into the mucosa of the digestive tract and causes anisakiasis^[12]. The preferential location of anisakiasis in Japan is the stomach (96%) rather than the intestine (4%)^[13]. On the other

hand, Van Thiel *et al*^[14] and Smith *et al*^[15] reported that intestine was more frequent than stomach in European countries. The symptoms of intestinal anisakiasis are non-specific, sometimes colicky and diffuse abdominal pain, nausea and vomiting^[12]. Patients are often misdiagnosed as having acute appendicitis or terminal ileitis^[12]. Clinical symptoms appear within 48 h after food intake, continue for 1 to 5 d and spontaneously recover^[16]. Our case had an operation since the conservative therapy was not effective in the resolution of intussusception.

AI caused by anisakiasis is extremely rare. There have previously been two English and nine Japanese reports about AI caused by anisakiasis (Table 1)^[17-27]. All cases ate raw or marinated fish from half a day to 7 d before the symptom onset. Colon and jejunum were usually involved. The common symptom was abdominal tenderness, although with no palpable mass in jejunum cases. Since the ascending colon is fixed in the retroperitoneum and exists near the abdominal wall, we can physically detect a mass at ease. On the other hand, jejunum, transverse colon and sigmoid colon are not fixed and not

necessarily near to the abdominal wall, so it is usually hard to detect a mass. Pneumo-dynamic resolution of AI by colonoscopy was successful in two of the twelve cases in whom emergency operation could be avoided. Correct diagnosis could be preoperatively made in only one of these two by a detailed clinical interview. Ten of the twelve cases had eosinophilic granuloma. Thus, it has been shown from the literature that if raw fish intake is not discovered at interview, or incidental detection of anisakis body fails, it is hard to determine what caused intussusception prior to surgery. That is the reason why surgery was indicated in almost all cases. Therefore, AI is, in general, a good indication for operation although we do not know what would happen if surgery was not performed.

We would like to draw attention to the risk of anisakiasis since it may occur in Japanese, Chinese and European countries and also the United States of America where sushi or sashimi is now prevailing as a popular cuisine. Thus, we believe that if suspicious, detailed clinical interview as to food intake prior to symptom development is crucial.

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Gastrointestinal stromal tumor of stomach with inguinal lymph nodes metastasis: A case report

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Abstract

Gastrointestinal stromal tumor (GIST) is the most common mesenchymal tumor in the alimentary tract. To the best of our knowledge, few cases have been reported in the literature about the peripheral lymph node metastasis of GIST. Here we report an unusual case of gastric GIST with inguinal lymph nodes metastasis. After the metastatic lymph nodes were resected, the patient started to take imatinib 400 mg/d for 12 mo. There were no signs of tumor recurrence at follow-up after 29 mo. This case suggests that the inguinal lymph nodes can be a potential metastatic site of GIST.

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Key words: Gastrointestinal stromal tumor; Inguinal; Lymph node; Metastasis

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INTRODUCTION

Gastrointestinal stromal tumor (GIST) is the most common gastrointestinal (GI) mesenchymal tumor, which accounts for 0.2% of all GI tumors and 80% of sarcomas^[1]. GIST most commonly arises from the stomach and accounts for about 1% of gastric malignancies^[2]. Liver and peritoneal metastatic lesions are often observed but lymph nodes metastasis is rarely found in GIST^[3]. Moreover, there are few reports about the peripheral lymph nodes metastasis of GIST in literature. Here we report one interesting GIST case with inguinal lymph nodes metastases 3 years after distal gastrectomy and hepatectomy due to gastric GIST with liver metastasis.

CASE REPORT

A 60-year-old Chinese man came to the clinic on August 8th, 2007, complaining of his feeling of an inguinal mass gradually growing for 5 mo. He received distal gastrectomy, perigastric lymphadenectomy and hepatectomy in July 2004 for gastric GIST with liver metastasis. Physical examination revealed a 4 cm × 3 cm round and nontender mass which was located in the right inguinal region. Inguinal ultrasonography showed a low echo-level mass (Figure 1). Computed tomography (CT) revealed a 4 cm × 5 cm low density mass (CT value = 68.8 HU) which was unenhanced during contrast-enhanced scan (Figure 2). A complete gross excision of the mass was carried out for this patient on August 13, 2007. Grossly, the surgical specimen consisted of a 4.0 cm × 3.5 cm × 3.0 cm large, nodular, whitish-gray and firm lymph node. Microscopically, it was confluent and was infiltrated with spindle cells. These spindle cells were predominantly arranged in inter-

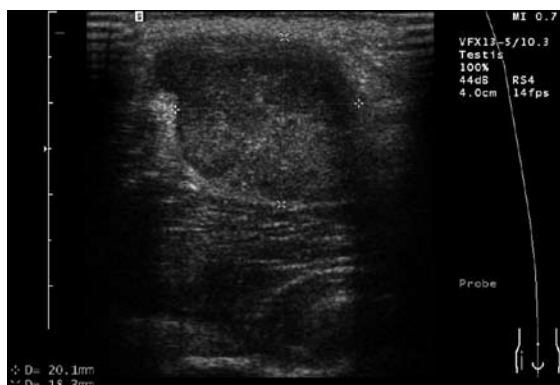


Figure 1 Ultrasonography. Inguinal ultrasonography shows a low echo-level lesion in the right inguinal region.



Figure 2 Computed tomography (CT). A low density lesion with weak, uneven enhancement during contrast-enhanced scan (arrow).

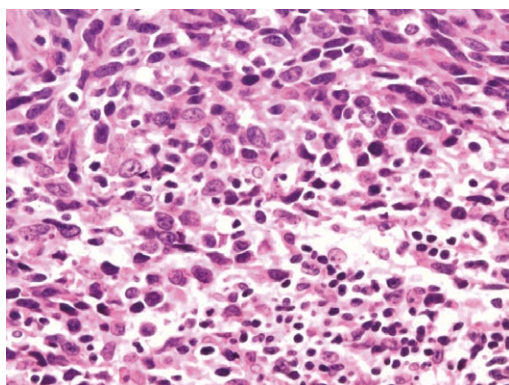


Figure 3 Hematoxylin & eosin (HE) staining. Spindle cells were predominantly arranged in interweaving fascicles (HE stain, $\times 100$).

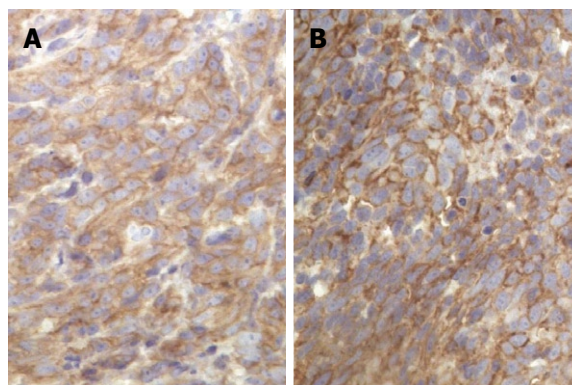


Figure 4 Immunohistochemical staining. A strong positivity for both CD117 (A) and CD34 (B) (CD117 stain, $\times 400$, CD34 stain, $\times 400$).

weaving fascicles (Figure 3). Immunohistochemical stains demonstrated a strong positivity for both c-kit (CD117) and CD34 (Figure 4), which was consistent with the primary tumor. Gene sequence analysis showed that all the primary tumors, the metastatic tumor in liver and the resected inguinal lymph nodes harbored deletion mutations affecting codons 557/558 of exon 11 in the KIT. After the operation, the patient took imatinib 400 mg/d for 1 year. And there was no sign of tumor recurrence during 29 mo of follow-up.

DISCUSSION

The case we reported herein is a high grade GIST according to the Bucher grading system^[4]. Unlike gastric adenocarcinoma, regional lymph nodes including perigastric and mesenteric lymph nodes metastasis of GIST is unusual. The prevalence is reported to range from 1.1% to 3.4%^[5-7]. In this case, perigastric lymphadenectomy was performed and no metastasis was observed in all of 19 lymph nodes at the first operation, although liver metastasis was detected.

To the best of our knowledge, this is the first reported case of GIST with inguinal lymph nodes metastasis. However, the distinct pattern of inguinal lymph node metastasis of gastric neoplasm is unclear. In the case of

gastric adenocarcinoma, tumor cells can metastasize to para-aortic lymph nodes which have a chance of retrograde dissemination to the pelvic lymph nodes and also inguinal lymph nodes. It is unclear whether this metastatic mechanism is applicable for GIST with inguinal lymph nodes metastasis. In this case, since liver is the first organ of metastasis occurrence, we can hypothesize that when tumor cells invade the liver, they have a higher probability to spread into peripheral blood, and tumor cells can migrate into peripheral lymph node *via* high endothelial venule, which finally results in lymph nodes metastasis.

At present, surgery remains the mainstay of treatment in GIST patients with isolated resectable GIST. Imatinib is currently indicated for the first-line treatment of metastatic or unresectable KIT-positive GIST. The persistent use of imatinib was necessary for this patient after complete resection of primary gastric and hepatic metastatic GIST. Unfortunately, this patient did not take imatinib after his first operation which led to further metastasis. After his second operation, the patient started imatinib treatment and survived without recurrence till now.

In conclusion, GIST can involve the inguinal lymph nodes as a metastatic site and further studies are necessary to clarify the mechanism of this metastasis.

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- 11 **Lam SK**. Academic investigator's perspectives of medical treatment for peptic ulcer. In: Swabb EA, Azabo S. Ulcer disease: investigation and basis for therapy. New York: Marcel Dekker, 1991: 431-450

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

Statistical data

Write as mean \pm SD or mean \pm SE.

Statistical expression

Express *t* test as *t* (in italics), *F* test as *F* (in italics), chi square test as χ^2 (in Greek), related coefficient as *r* (in italics), degree of freedom as *v* (in Greek), sample number as *n* (in italics), and probability as *P* (in italics).

Units

Use SI units. For example: body mass, *m* (B) = 78 kg; blood pressure, *p* (B) = 16.2/12.3 kPa; incubation time, *t* (incubation) = 96 h, blood glucose concentration, *c* (glucose) 6.4 ± 2.1 mmol/L; blood CEA mass concentration, *p* (CEA) = 8.6 $24.5 \mu\text{g/L}$; CO₂ volume fraction, 50 mL/L CO₂, not 5% CO₂; likewise for 40 g/L formaldehyde, not 10% formalin; and mass fraction, 8 ng/g, etc. Arabic numerals such as 23, 243, 641 should be read 23 243 641.

The format for how to accurately write common units and quantum numbers can be found at: http://www.wjgnet.com/1007-9327/g_info_20100315223018.htm.

Abbreviations

Standard abbreviations should be defined in the abstract and on first mention in the text. In general, terms should not be abbreviated unless they are used repeatedly and the abbreviation is helpful to the reader. Permissible abbreviations are listed in Units, Symbols and Abbreviations: A Guide for Biological and Medical Editors and Authors (Ed. Baron DN, 1988) published by The Royal Society of Medicine, London. Certain commonly used abbreviations, such as DNA, RNA, HIV, LD50, PCR, HBV, ECG,

WBC, RBC, CT, ESR, CSF, IgG, ELISA, PBS, ATP, EDTA, mAb, can be used directly without further explanation.

Italics

Quantities: *t* time or temperature, *c* concentration, *A* area, *l* length, *m* mass, *V* volume.

Genotypes: *gyrA*, *arg 1*, *c myc*, *c fos*, etc.

Restriction enzymes: *EcoRI*, *HindI*, *BamHI*, *Kho I*, *Kpn I*, etc.

Biology: *H. pylori*, *E. coli*, etc.

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Role of monocytes and macrophages in experimental and human acute liver failure

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Abstract

Acute liver failure (ALF) is a devastating clinical syndrome characterised by progressive encephalopathy, coagulopathy, and circulatory dysfunction, which commonly leads to multiorgan failure and death. Central to the pathogenesis of ALF is activation of the immune system with mobilisation of cellular effectors and massive production of cytokines. As key components of the innate immune system, monocytes and macrophages are postulated to play a central role in the initiation, progression and resolution of ALF. ALF in humans follows a rapidly progressive clinical course that poses inherent difficulties in delineating the role of these pivotal immune cells. Therefore, a number of experimental models have been used to study the pathogenesis of ALF. Here we consider the evidence from experimental and human studies of ALF on the role of monocytes and macrophages in acute hepatic injury and the ensuing extrahepatic manifestations, including functional monocyte deactivation and multiple organ failure.

INTRODUCTION

Acute liver failure (ALF) is characterised by overwhelming hepatic injury with failure of hepatocyte function, resulting in a devastating clinical syndrome of coagulopathy, encephalopathy, and circulatory dysfunction^[1,2]. Phenotypically, there are striking similarities between septic shock and ALF. Both conditions are characterised by activation of a systemic inflammatory response syndrome (SIRS), resulting in circulatory dysfunction with systemic vasodilatation and refractory multiorgan failure^[3]. ALF, like septic shock, is associated with an overwhelming activation of the immune response, including the production of inflammatory cytokines and mobilisation of cellular components of the immune system. Monocytes and macrophages are key orchestrators of the innate immune system and are postulated to play a pivotal role in the initiation, propagation and resolution of ALF.

The paucity of effective clinical treatments for ALF, except for supportive care and liver transplantation, re-

flects our poor understanding of the pathogenesis of ALF. Clinical trials and studies on patients with ALF are limited by small numbers of subjects with heterogeneous causes of ALF and are confounded by inherent difficulties in studying patients with rapidly progressive pathology. There are a number of animal models of acute liver injury commonly used to study ALF: murine models of acetaminophen (APAP) toxicity, concanavalin-A (Con-A) T-cell mediated hepatitis, carbon tetrachloride (CCl₄)-induced injury, and other models including galactosamine/lipopolysaccharide (Gal/LPS) (for a review^[4]). Unfortunately, these are models of acute liver injury and cannot truly reflect the human syndrome of ALF.

In this article we consider the role of macrophages, monocytes, and the inflammatory cytokines they produce, in the progression of acute liver failure in human and experimental models. We consider monocyte/macrophage function in the hepatic and systemic compartments, including their role in initial injury, the process of recruitment of monocytes to the injured liver and the possible influence of hepatic inflammatory events on the functional monocyte deactivation encountered in ALF.

RECRUITMENT OF MONOCYTES TO THE INJURED LIVER

Expansion of hepatic macrophage populations

Following an acute hepatic insult, there is a rapid and marked increase in the number of inflammatory cells within the liver. Recent studies have shed light on the process by which circulating monocytes are recruited to the injured liver, where, due to their plasticity, macrophages are implicated in both tissue destructive and reparatory processes of the inflammatory response.

Karlmark *et al.*^[5] demonstrated, in a murine model of acute CCl₄ hepatotoxicity, that a sub-population of infiltrating macrophages account for 10%-12% of total hepatic cells 24-48 h after the onset of liver injury. This massive recruitment of macrophages and their contribution to evolving acute liver failure have attracted much interest recently. Two distinct populations of macrophage have been described in both APAP and acute CCl₄ models of hepatotoxicity^[5,6]. The first is a CD11b⁺ F4/80⁻ population that resembles the sub-group of circulating monocytes that are the origin of stable populations of tissue macrophages. This population shows only a modest increase in number following acute liver injury by CCl₄. The second population is CD11b⁺ F4/80⁺ and this resembles the subset of circulating monocytes from which activated macrophages in inflammatory conditions are thought to be derived. It is this population that shows marked expansion in number following acute hepatic injury^[5].

Holt *et al.*^[6] 2008, in an APAP model of hepatotoxicity, showed that these "inflammatory macrophages" were derived from circulating monocytes rather than from proliferation of the resident Kupffer cell (KC) population. They noted preservation of the macrophage response to acute APAP toxicity when the KC population was depleted prior to APAP challenge. Conversely, when

circulating monocytes were depleted by bone marrow irradiation prior to APAP, the number of intrahepatic macrophages seen after tissue injury was diminished.

The role of chemokine: Monocyte chemoattractant protein-1

Monocyte chemoattractant protein-1 (MCP-1) is a member of the C-C chemokine family, which acts on the chemokine (C-C motif) receptor 2 (CCR2) that is expressed on macrophages, monocytes, and to a lesser extent, T and NK cells^[7]. Though initially described as a monocyte chemokine it also plays a role in the recruitment of NK cells and T cells in a wide range of inflammatory conditions^[8]. An early increase in hepatic expression of MCP-1 and other chemokines is a consistent feature of murine models of ALF. An increase in MCP-1 mRNA or total hepatic levels of MCP-1 have been reported within 12 h of liver insult in APAP, Con-A, Gal/LPS, and CCl₄ models of acute liver failure^[5,9-12]. The origin of hepatic MCP-1 in ALF is thought to be both resident KCs and injured hepatocytes^[13].

MCP-1/CCR2 interaction in animal models

Experiments using CCR2 -/- knockout mice suggest that functional CCR2 is necessary for egress of F4/80⁺ monocytes from the bone marrow. In the acute liver injury model induced by CCl₄, CCR2 -/- mice demonstrated a decreased number of hepatic and serum CD11b⁺ F4/80⁺ cells following liver injury, but an increase in this cellular fraction in bone marrow when compared with wild-type mice^[10]. When this population of monocytes was adoptively transferred into the circulation of CCR2 -/- mice they were able to traffic unhindered into the injured liver^[5]. Similarly to the liver injury model, CCR2 has been shown to be essential for monocyte mobilisation from the bone marrow, but not for efflux into inflamed tissues, such as in murine CMV, *L. monocytogenes*, and urinary tract infection models^[8,14,15].

This evidence suggests that hepatic-derived MCP-1 is able to stimulate the expansion of a bone marrow population of CD11b⁺ F4/80⁺ monocytes, and CCR2 is necessary for these cells to exit the bone marrow, but not essential for influx into the injured liver^[5].

MCP-1 in human ALF

In human ALF, there is also evidence of early upregulation of hepatic chemokines. Leifeld *et al.*^[9] demonstrated serum MCP-1 levels that were, on average, five times higher in patients with ALF when compared to patients with self-limiting acute hepatitis. MCP-1 levels were also significantly elevated above healthy control populations and those with chronic liver disease. There was a trend suggesting that higher serum MCP-1 levels were associated with poor outcome (death or transplantation) from ALF. Upregulation of chemokines, including MCP-1, was demonstrated by reverse transcriptase-polymerase chain reaction (RT-PCR) and immunohistochemistry. The latter technique showed that MCP-1 expression was not limited to inflammatory cells only, but was also de-

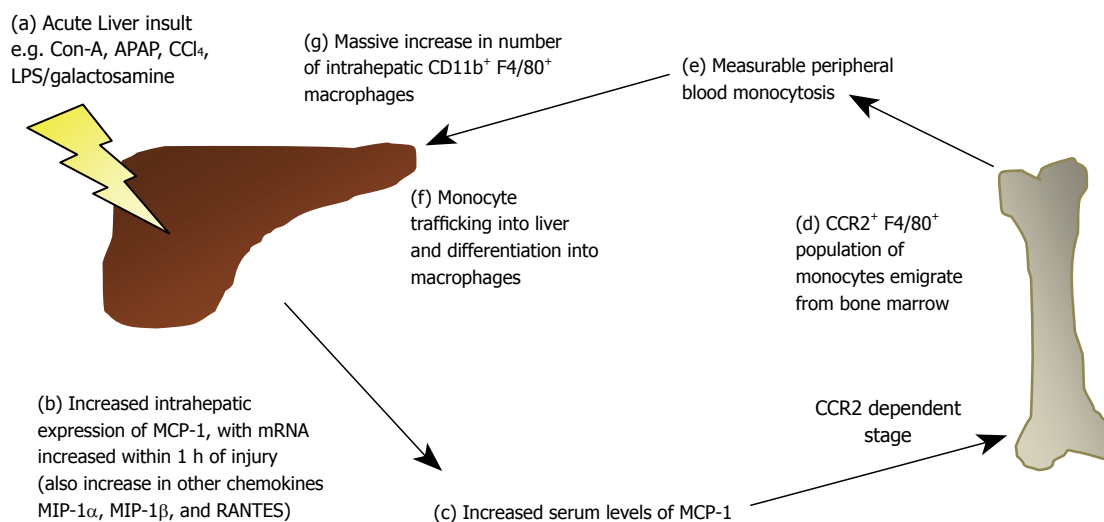


Figure 1 Summary of monocyte/macrophage recruitment to the liver following an acute hepatic insult. Con-A: Concanavalin-A; APAP: Acetaminophen induced hepatotoxicity; CCl₄: Carbon tetrachloride; LPS: Lipopolysaccharide; CCR2: Chemokine (C-C motif) receptor 2; MCP-1: Monocyte chemoattractant protein-1; MIP: Macrophage inflammatory protein; RANTES: Regulated upon Activation, Normal T-cell Expressed, and Secreted, abbreviated (CCL5).

tected in hepatocytes, biliary epithelial cells, and sinusoidal endothelial cells (SEC) in human ALF.

These findings have been replicated in patients with acute severe alcoholic hepatitis with clinical liver failure. MCP-1 is seen to be upregulated throughout the liver, with expression observed in hepatocytes and sinusoidal endothelial cells^[16]. Conversely, in control patients with stable alcoholic liver disease, MCP-1 expression is limited to non-parenchymal cells.

In paediatric patients with acute acetaminophen hepatotoxicity, serum MCP-1 levels showed a strong correlation with clinical parameters of severe liver injury, including elevated levels in patients in whom treatment with N-acetyl cysteine was delayed^[17].

Thus evidence from both murine and human studies suggests that early release of chemokines from injured hepatocytes, SECs and KCs in ALF is responsible for the mobilisation and subsequent trafficking of a population of activated monocytes/macrophages to the liver from the bone marrow (summarised in Figure 1).

THE ROLE OF HEPATIC MACROPHAGES IN THE PROGRESSION OF ACUTE LIVER FAILURE

Having established that macrophages are recruited to the liver in acute injury, the challenge that remains is determining what role these cells play in liver damage or repair.

Two early studies in animal models of APAP-induced hepatotoxicity suggested that depletion of macrophage numbers with gadolinium chloride or dextran sulphate prior to APAP dosing offered protection from liver damage^[18,19]. However, neither of these studies confirmed successful depletion of intrahepatic macrophages, and in one study, the number of intrahepatic F4/80⁺ cells post-APAP treatment was actually increased, suggesting their prior treatment with KC depleting agents had not

prevented an expansion in the macrophage population following the toxic insult^[19]. Subsequent studies have used the more effective technique of macrophage depletion with liposome/clodronate. An APAP model of acute liver failure has shown macrophage depletion increases sensitivity to toxic injury, as measured at 8 and 24 h post-APAP alanine transaminase (ALT) values^[20]. Conversely, in an acute CCl₄ model, macrophage depletion was reported to have no effect on ALT levels four and 24 h after toxin administration^[5].

Prevention of liver injury-induced macrophage population expansion by inhibition of the MCP-1/CCR2 interaction has been demonstrated in a number of studies using CCR2 knockout mice. As described above, these animals show a marked reduction in intrahepatic macrophages following liver injury compared with wild-type (WT) mice, due to failure of monocyte recruitment from bone marrow. Two studies in CCR2^{-/-} mice treated with toxic doses of APAP have shown ALT levels in the first 24 h comparable to WT controls^[6,13]. Histological evidence of liver damage was also comparable between CCR2^{-/-} and WT mice at 24 h in both studies. At later time points, the two studies showed divergent results, with Dambach *et al*^[13] showing less inflammation in CCR2^{-/-} mice. However, Holt *et al*^[6] described CCR2^{-/-} mice showing persisting necrosis and ongoing inflammation at 72 h, whereas the wild-type animals showed almost complete histological resolution. Hogaboam *et al*^[12] used a similar model of acute APAP toxicity in CCR2^{-/-} mice and results seemed to concur with Holt's study, showing a worsening of liver injury by ALT values and histology in the knockout animals at all time points when compared to WT controls.

Overall current evidence in murine models is conflicting (Table 1). It appears that depletion of resident KCs prior to, and at the onset of, liver injury reduces the extent of liver damage. Kupffer cells are partly responsible for the initial pro-inflammatory response to injury with cytokine production and the recruitment of multiple in-

Table 1 Literature summary on the effect of hepatic macrophage depletion on the extent of liver injury in experimental models of ALF

Study	Model	Method of macrophage depletion/inhibition	Affect on severity of acute liver injury	Conclusion
Laskin <i>et al</i> ^[18] , 1995	APAP (rat)	Gadolinium chloride/dextran sulphate	Decreased ALT at 24 h in treated groups; Decreased necrosis	Macrophage depletion was protective
Michael <i>et al</i> ^[19] , 1999	APAP (mouse)	Gadolinium chloride/dextran sulphate	Decreased ALT at 8 h in treated groups	Macrophage depletion was protective
Hogaboam <i>et al</i> ^[12] , 2000	APAP (mouse)	CCR2 -/-	ALT at 24 and 48 h, hepatic necrosis and TUNEL staining all increased in KO; Increase in IFN- γ and TNF- α	CCR2 KO - macrophage depletion worsened liver injury
Dambach <i>et al</i> ^[13] , 2002	APAP (mouse)	CCR2 -/-	ALT levels similar in WT and KO mice; Histologically KO mice showed less inflammation at 72 h	CCR2 KO - macrophage depletion, caused less inflammation at 72 h but no overall difference in outcome
Ju <i>et al</i> ^[20] , 2002	APAP (mouse)	Liposome/clodronate	Increased ALT at 8 and 24 h in treated group	Macrophage depletion increased liver damage
Holt <i>et al</i> ^[6] , 2008	APAP (mouse)	CCR2 -/-	ALT same at 10 and 24 h; Comparable histological necrosis at 24 h but delayed recovery at 48 and 72 h in CCR2 -/-	Reduction in infiltrating macrophage population causes delayed recovery
Karlmark <i>et al</i> ^[5] , 2009	CCl ₄ (mouse)	Liposome/clodronate	Unaltered ALT level at 4 and 24 h post-CCl ₄	Reduction in infiltrating macrophages had no effect on severity of liver damage

ALF: Acute liver failure; ALT: Alanine transaminase; WT: Wild-type; KO: Knockout; APAP: Acetaminophen induced hepatotoxicity; CCl₄: Carbon tetrachloride; CCR2: Chemokine (C-C motif) receptor 2.

flammatory effector cells including neutrophils, NK cells, NKT cells, and T cells that mediate tissue injury. However, there is some evidence to suggest that the population of hepatic macrophages derived from newly recruited infiltrating monocytes possess anti-inflammatory activity and may play a role in recovery from acute liver damage. Though it is beyond the scope of this review to discuss in detail, evidence also suggests that it is this same population of immigrant macrophages that is responsible for driving the fibrosis that follows acute liver injury^[5,10].

THE BALANCE BETWEEN PRO- AND ANTI-INFLAMMATORY CYTOKINES

Macrophage-derived inflammatory cytokines in experimental models of ALF (Table 2)

Tumour necrosis factor- α : Tumour necrosis factor- α (TNF- α), a macrophage derived pro-inflammatory cytokine, has a central role in the pathogenesis of many inflammatory clinical syndromes. TNF- α can enhance the production of other pro-inflammatory cytokines and provoke cellular recruitment to sites of injury. It also has an important direct effector function by inducing hepatocellular apoptosis through its interaction with TNF-receptor 1. This cell surface receptor is connected to an intracellular death domain whose activation ultimately leads to the induction of effector caspases and the execution of cellular apoptosis.

In experimental models of acute liver failure, elevated serum levels of TNF- α and increased mRNA have been observed shortly after the initiating insult^[21,25,48]. Studies in the murine Gal/LPS and Con-A models of hepatic failure have shown that treatment with neutralising anti-TNF antibodies provides protection against liver damage^[24,25]. A recent study using targeted antisense oli-

gonucleotides to inhibit expression of the TNF- α gene, limited the hepatic damage and subsequent mortality from Gal/LPS in a rat model of acute hepatic failure^[26]. TNF- α -driven hepatocyte necrosis through the TNF-R1 pathway has been well described in a number of experimental models of acute liver injury^[21,23,49].

Interferon- γ : Interferon- γ (IFN- γ) is a pro-inflammatory cytokine released by T-cells, NK cells, and macrophages. It has a variety of anti-viral, anti-bacterial and pro-inflammatory effects. In models of acute liver injury, increases in serum IFN- γ and intrahepatic IFN- γ mRNA are seen as part of the initial response to injury^[28,29]. IFN- γ -/- knockout mice show resistance to toxic and T-cell mediated acute liver failure, with improved survival and reduced hepatic necrosis and apoptosis^[30]. Neutralisation of IFN- γ with antibodies in WT mice confers a dose-dependent protective effect following APAP or Con-A administration^[28,29].

IFN- γ deficient mice show a decrease in Fas induction after liver injury; hence it has been postulated that IFN- γ contributes to liver injury by the upregulation of Fas on hepatocytes, rendering them susceptible to apoptotic cell death^[30].

Macrophage migration inhibitory factor: Macrophage migration inhibitory factor (MIF) is a multi-functional inflammatory cytokine that is constitutively expressed in many cell types, including centrilobular hepatocytes and Kupffer cells^[50]. Pre-formed MIF is released early in response to stress (glucocorticoid) and infectious stimuli (LPS). It has an important role in stimulating the inflammatory response through cellular recruitment, stimulation of cytokine production, and counteracting the inhibitory effect of glucocorticoid on the production of pro-inflammatory cytokines. Though initially described as a T-cell cytokine, it has since been

Table 2 Pro- and anti-inflammatory cytokines in experimental models of ALF

Cytokine	Model	Source	Mechanism of action
TNF- α	Murine hepatic ischaemia ^[21,22] Murine α -amanitin or actinomycin D ^[23] Murine Gal/LPS ^[24] Murine Con-A ^[25] Rat Gal/LPS ^[26]	Macrophage	Hepatocellular apoptosis Cellular recruitment
IFN- γ	Murine Gal/LPS ^[27] Murine APAP ^[28] Murine Con-A ^[29,30]	Macrophages NK and T cells	Induce iNOS Upregulates Fas, sensitising hepatocytes to apoptosis Upregulates adhesion molecules and chemokines resulting in leucocyte accumulation
MIF	Guinea pig halothane ^[31] Murine APAP ^[31] Murine LPS in BCG-primed mice ^[32] Rat ethanol ^[33]	Preformed stores released from hepatocytes and KCs early in injury	Stimulate release of proinflammatory cytokines Counter-act glucocorticoid anti-inflammatory effects Cellular recruitment
IL-6	Murine APAP ^[22,34,35] Murine alcohol and TNF- α ^[36] Murine Con-A ^[37,38] Murine Acute CCl ₄ ^[39] Murine Fas-mediated apoptosis ^[40] Murine ischemia/reperfusion ^[41]	Released from macrophages and T cells	Reduced TNF- α secretion Activation of STAT3 signalling pathway Induction of anti-apoptotic proteins e.g. Bcl-2, Bcl-x _L , FLIP
IL-10	Murine Gal/LPS ^[27,42,43] Murine CCl ₄ ^[44] Rat CCl ₄ ^[45] Murine APAP ^[46] Murine Con-A ^[47]	Macrophages/monocytes and injured hepatocytes	Inhibition of TNF- α , IFN- γ secretion

Con-A: Concanavalin-A; Gal/LPS: Galactosamine lipopolysaccharide; TNF- α : Tumour necrosis factor- α ; IFN- γ : Interferon- γ ; MIF: Macrophage migration inhibitory factor; IL-6: Interleukin-6.

established that macrophages are an important source of MIF^[51]. An increase in MIF mRNA is seen in KCs following acute injury^[50].

MIF has a role in a number of experimental models of acute liver injury^[31-33]. Significant increases in serum MIF are detectable in the early stages following acute liver injury by toxins or LPS^[31,32]. MIF -/- knockout mice demonstrate resistance to APAP-induced hepatotoxicity, displaying lower ALT levels, less histological necrosis, and improved survival compared to WT counterparts^[31]. In a BCG-LPS model of murine ALF pre-treatment with anti-MIF antibodies prevented the development of liver failure and death in response to LPS challenge^[32].

Interleukin-6: Interleukin-6 (IL-6) is a macrophage and T cell-derived multifunctional cytokine with both pro- and anti-inflammatory actions. In a number of acute liver injury models, IL-6 has been shown to play an important protective role. IL-6 -/- knockout mice display increased sensitivity to liver injury, impaired regeneration, and poor outcomes in APAP^[22,34], alcohol and TNF- α -mediated liver cell apoptosis^[36], acute CCl₄ toxicity^[39], Fas-mediated apoptosis^[40], and ischaemia-reperfusion injury^[41]. In a number of these experiments, the treatment of knockout and wild-type mice with recombinant IL-6 prior to the liver insult conferred protection against hepatic damage^[37,39-41]. Experimental evidence suggests the hepatoprotective effects of IL-6 in acute liver damage are mediated through the activation of the STAT3 signalling pathway, with induction of anti-apoptotic proteins such as Bcl-2, Bcl-x_L, and FLIP and inhibition of NKT cells *via* targeting of CD4⁺ T cells^[38,39,52].

Interleukin-10: IL-10 is a pleiotropic anti-inflammatory cytokine that is capable of downregulating several aspects of the pro-inflammatory cascade, including activated macrophage function. IL-10 is secreted from various cell types, including monocytes and macrophages, in response to injury. The liver is a major source of IL-10 in systemic inflammatory conditions.

In experimental models of acute liver injury, IL-10 has consistently been shown to be protective against liver damage^[27,42-47]. IL-10 serum levels and mRNA expression in liver tissue are seen to rise early after hepatic insult in APAP, Con-A, and Gal/LPS models^[42,46,47]. The kinetics of IL-10 production parallel TNF- α secretion in a murine Gal/LPS model, suggesting both pro- and inflammatory cytokines are released together and act concurrently during the early acute inflammatory response^[42].

In a Con-A model of ALF, neutralisation of IL-10 with monoclonal antibodies exacerbated liver damage, as measured by serum transaminases and liver histology. Serum levels of TNF- α , IFN- γ , and IL-12 were augmented by neutralisation of IL-10^[47]. Similar findings were demonstrated in an APAP model utilising IL-10 knockout mice, where the absence of IL-10 was associated with increased ALT, worse histological necrosis, and reduced survival. Again this increased susceptibility seemed to be attributable to upregulation of pro-inflammatory cytokines and the effectors TNF- α , IL-1, and iNOS^[46]. Treatment with recombinant IL-10 in Gal/LPS models caused a dose-dependent reduction in liver damage and hepatic and serum TNF- α expression^[27,42,43]. *In vitro* work on isolated rat hepatocytes demonstrated that IL-10 was expressed early following LPS challenge. Treatment of

KCs with exogenous IL-10 downregulated production of superoxide dismutase and TNF- α responses following LPS challenge; a phenomenon analogous to the functional monocyte deactivation described later^[45].

Thus, in animal models of acute liver injury there is consistent evidence that IL-10 is expressed in the early phases of liver damage, simultaneous to pro-inflammatory effectors. It acts to abrogate liver injury by limiting the release of pro-inflammatory cytokines and effectors. *In vivo* it is likely to be the balance of these inter-related and mutually regulating factors that determine the extent and outcome of the hepatic insult.

Macrophage-derived inflammatory cytokines in human studies of ALF

As in experimental models of acute liver failure, ALF in humans is known to be associated with massive activation of pro- and anti-inflammatory mediators. A number of studies have looked at patient populations and correlated serum levels of inflammatory cytokines with clinical parameters of ALF severity. TNF- α has been shown in a number of clinical cohorts to be elevated in patients with ALF compared with controls with acute hepatitis or chronic liver disease^[53-57]. In two of these studies, elevated TNF- α levels correlated with poor clinical outcomes^[53,58]. IL-6 and IL-10 have been shown to possess hepatoprotective properties in experimental models of ALF models^[53-56,59]. However, human studies of ALF have detected higher levels of IL-6 and -10 in non-surviving ALF patients compared to spontaneous survivors^[53,56,59].

Interestingly, a number of recent studies have measured proxy markers of macrophage and monocyte activation and shown that increases in these markers correlate with poor outcome in patients with ALF. CD163 is a macrophage lineage-specific scavenger receptor that is expressed by activated macrophages (and to a lesser extent by circulating monocytes) and can be shed in the circulation as a soluble receptor^[60]. Hiraoka *et al.*^[60] showed that, in a small population of patients with ALF, mean levels of serum CD163 were elevated when compared with either healthy controls or patients with acute hepatitis. There was a trend towards increasing CD163 in non-survivors, however this did not reach significance. Møller *et al.*^[61] used a larger cohort from the US ALF study group and replicated the finding of elevated CD163 in ALF patients compared with controls. They also showed a significant difference between CD163 levels on day three between survivors and non-survivors.

FUNCTIONAL MONOCYTE DEACTIVATION IN HUMAN ALF

Infectious complications are a common feature of established acute liver failure and account for a significant proportion of overall mortality^[1,62]. A number of recent studies have investigated the phenomenon of functional monocyte deactivation that occurs in patients with acute and acute-on-chronic liver failure, and that might be partially responsible for the observed susceptibility of these

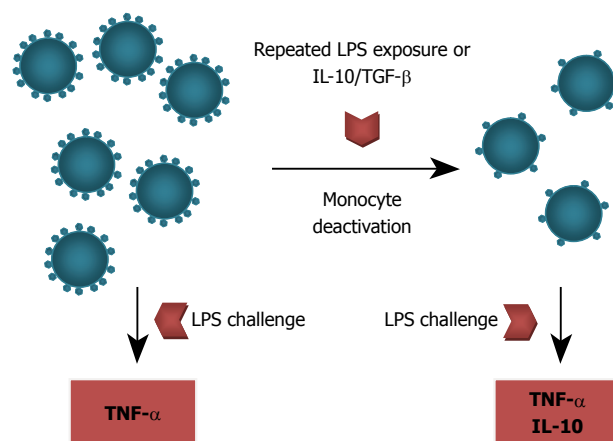


Figure 2 Circulating monocytes express abundant cell surface HLA-DR. Initial exposure to LPS provokes a massive tumour necrosis factor- α (TNF- α) response. Repeated exposure to LPS or the presence of interleukin-10 (IL-10)/transforming growth factor- β (TGF- β) causes monocyte deactivation with downregulation of cell surface HLA-DR expression, impaired antigen presenting capability, and an attenuated TNF- α response to future LPS challenge.

patients to overwhelming sepsis^[53,63-65].

Monocyte deactivation was first described in the early 1990s in clinical populations of post-trauma and sepsis patients^[58,66]. This phenomenon shares many features with the experimental model of “endotoxin tolerance”, which is characterised by a refractory monocyte phenotype with decreased expression of HLA-DR, impaired ability to present antigens, and a profoundly attenuated TNF- α response to LPS challenge (Figure 2). This monocyte phenotype can be induced experimentally by repeated LPS challenge (hence endotoxin tolerance) or exposure to IL-10 and TGF- β ^[66].

Antoniades *et al.*^[53] demonstrated monocyte deactivation in a group of patients with acetaminophen-induced acute liver failure (AALF). Patients with AALF who had adverse outcomes (death or transplantation) had significantly lower expression of HLA-DR than transplant-free survivors with AALF, control patients with chronic liver disease, and healthy controls. A reduction in the percentage and total number of HLA-DR positive monocytes was observed, as was a reduction in HLA-DR expression on individual monocytes. Longitudinal follow up of non-surviving and surviving patients showed the former group had persisting evidence of monocyte deactivation, with suppressed HLA-DR at days 3-6, whereas the later group showed recovery of monocyte function between day 1 and days 3-6.

Three studies have looked at this phenomenon in patients with acute-on-chronic liver failure and shown a similar reduction in monocyte HLA-DR in this group^[63-65]. Furthermore, they all demonstrated that monocytes derived from acute-on-chronic liver failure patients show impaired *ex vivo* TNF- α secretion in response to LPS exposure. Berres *et al.*^[64] and Xing *et al.*^[65] also replicated the finding that low monocyte HLA-DR has a strong association with poor outcome in liver failure patients.

The confirmation of immune dysfunction with functional monocyte deactivation in ALF begs the question

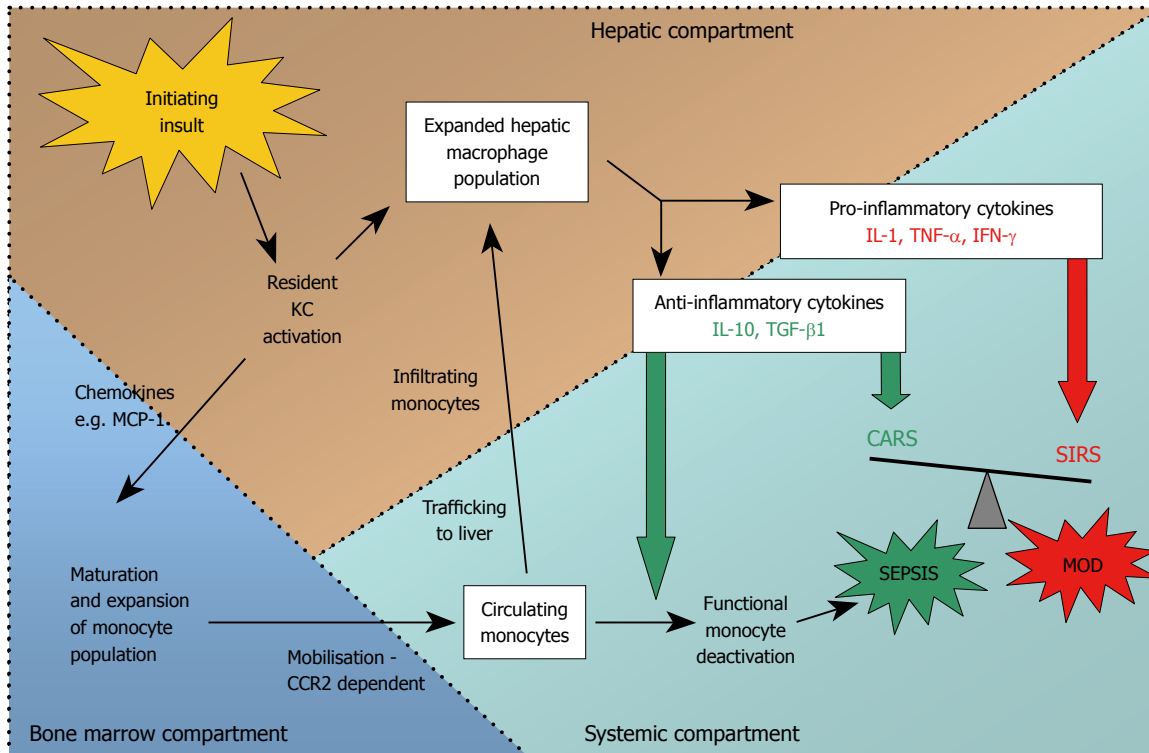


Figure 3 Summary of pathways. The initiating hepatic insult causes early release of chemokines, which promote the maturation of a population of bone marrow monocytes. These activated monocytes enter the circulation and some will traffic to the liver and differentiate into macrophages, accounting for the marked expansion in hepatic macrophage numbers seen after acute liver injury. Pro and anti-inflammatory cytokines are released simultaneously, early in the course of ALF. High levels of both pro and anti-inflammatory cytokines can be measured in the systemic circulation and drive the systemic inflammatory response (SIRS) and compensatory anti-inflammatory response (CARS) respectively. Imbalance in these two opposing forces in favour of an anti-inflammatory milieu can lead to functional monocyte deactivation, recurrent sepsis and multiple organ dysfunction (MOD). MCP-1: Monocyte chemoattractant protein-1; CCR2: Chemokine receptor 2.

of what is driving this phenomenon and how might it be modulated to improve clinical outcomes. The presence of high levels of circulating anti-inflammatory cytokines, IL-10, TGF- β as part of the compensatory anti-inflammatory response (CARS) in ALF are obvious culprits, in that *in vitro* studies have clearly demonstrated their ability to induce monocyte deactivation (Figure 2). Thus it appears that the anti-inflammatory cytokines that provided early hepatic protection in acute liver failure, can, if unchecked, cause subsequent systemic immune paralysis that leads to a worse outcome.

CONCLUSION

Monocytes and macrophages are central players in the complex process of initiation, propagation, and resolution of acute liver injury. Current evidence from experimental and human models suggests that macrophages are activated early in the evolution of acute liver injury and respond with vigorous release of chemokines and pro-inflammatory cytokines that drive the acute inflammatory response to injury. Simultaneously, macrophages initiate a counter-acting anti-inflammatory cascade that moderates the degree of acute hepatic inflammation. As ALF progresses, circulating monocytes are recruited to the liver and are implicated in the resolution of inflammation and tissue repair processes. Meanwhile, the intricate balance between pro- and anti-inflammatory

cytokines released from blood monocytes, hepatic macrophages, and other immune cells, is maintained through multiple interrelated regulatory mechanisms. However, in ALF, the immune response is skewed towards an anti-inflammatory-predominant environment, which could account for functional monocyte deactivation encountered and its attendant clinical sequelae of recurrent sepsis and multiple organ failure (Figure 3).

FUTURE DIRECTIONS

Further studies should aim to dissect the temporal relationship of macrophage recruitment and activation, in both circulating and hepatic inflammatory compartments, during the evolution of acute liver failure. Insight into the mechanisms that influence macrophage function during the process of acute hepatic injury will enable the development of therapeutic strategies that can be administered in a timely fashion to modulate the activity of this pivotal immune cell during acute liver failure.

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Modifier-concept of colorectal carcinogenesis: Lipidomics as a technical tool in pathway analysis

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Abstract

In the modifier concept of intestinal carcinogenesis, lipids have been established as important variables and one focus is given to long-chain fatty acids. Increased consumption of long-chain fatty acids is in discussion to modify the development of colorectal carcinoma in humans. Saturated long-chain fatty acids, in particular, are assumed to promote carcinogenesis, whereas polyunsaturated forms are likely to act in the opposite way. At present, the molecular mechanisms behind these effects are not well understood. Recently, it has been demonstrated by lipidomics and associated molecular techniques, that activation and metabolic channeling of long-chain fatty acids are important mechanisms to modify colorectal carcinogenesis. In this Editorial, an overview about the present concept of long-chain fatty acids and its derivatives in colorectal carcinogenesis as well as technical algorithms in lipid analysis is given.

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Key words: Colorectal neoplasms; Carcinogenesis; Long-chain fatty acids; Acyl-CoA; Lipidomics

INTRODUCTION

Colorectal adenomas and carcinomas are much more common than small bowel tumors, and colorectal cancer (CRC) is a leading cause of cancer deaths in Europe and the United States. The clinical symptoms of bleeding and/or obstruction found in the presence of large intestinal neoplasias are similar regardless of histologic type or etiology. It has been suggested that there is an association between colorectal carcinogenesis and pathways of fatty acid metabolism, with long-chain acyl-CoA thioesters as essential intermediates and cyclooxygenases (COXs) and acyl-CoA synthetases (ACSLs) as important enzymes. This observation is determined by molecular data indicating high functional diversity of activated lipid intermediates including formation of complex lipids (e.g. ceramide) and utilization in beta-oxidation as well as regulation of cellular signaling, transcriptional activity, cellular structure, and cellular behavior. Dietary lipids have been assumed as important additional variables in colorectal carcinogenesis.

In the following paragraphs the modifying role of lipids in colorectal carcinogenesis and the innovative technical approaches to elucidate the complex molecular network of lipids are addressed in detail.

EPIDEMIOLOGY AND DIVERSITY OF COLORECTAL CANCER

CRC continues to be one of the most common malignancies worldwide and the third leading cause of death among cancers^[1]. In industrialized Western countries, the incidence and prevalence are higher than in Asia or developing countries. Although the molecular mechanism of CRC has become better understood in the past 2 decades, the prognosis of CRC, especially advanced cancer, has not significantly improved. Five-year survival rates have risen from 56.5% for patients diagnosed in the early 1980s to only 63.2% for those diagnosed in the early 1990s, and most recently to 64.9%^[2]. The sparsely improving trend is most likely due to the fact that the prognosis for patients with CRC is highly dependent on disease stage at the point of diagnosis. Only 10% of CRCs are diagnosed early, and in most patients advanced disease is found. Therefore, early detection of CRC is essential in improvement of a patient's prognosis.

CRCs comprise a heterogeneous group of malignancies including hereditary forms and so-called sporadic tumors^[3,4]. At the current state of knowledge, the majority of CRCs are sporadic. However, experimental and epidemiological data give evidence that the class of sporadic CRCs comprises a heterogeneous group of malignancies with differences in pathogenesis and prognosis. It is suggested that the pathogenesis of some sporadic CRCs does not follow the traditional adenoma-carcinoma sequence. Recently, a new pathway in "sporadic" CRC development, the serrated pathway, has been discovered^[5]. The molecular alterations seen in serrated tumors are different from those traditionally found in the adenoma-carcinoma sequence. In serrated lesions, loss of heterozygosity and mutations in *APC* or *TP53* are rare, whereas hypermethylation of CpG islands and microsatellite instability are frequent. At present, 2 categories of serrated carcinogenesis are defined including 2 basic pathways, the sessile serrated pathway and the traditional serrated pathway. While in the sessile serrated pathway *BR4F* mutation and high-level microsatellite instability are molecular hallmarks in carcinogenesis, and tumor location is predominantly in the right colon, *KRAS* mutation and microsatellite stability have been characterized as important steps in the traditional serrated pathway, where cancers arise mainly in the left colon.

The diversity in colorectal carcinogenesis is accompanied by numerous genetic and epigenetic alterations. Epidemiological data indicate that the heterogeneity of such genomic lesions is determined by additive factors, called the modifiers of colorectal carcinogenesis^[6]. Evidence is given that diet and nutrition are key factors acting as carcinogenesis modifiers. This activity is probably relevant in both steps of colorectal tumorigenesis, initiation and progression. Therefore, identification and characterization of nutritional components as promoters or silencers of intestinal cancer are of high interest in tumor preventive strategies, tumor epidemiology, and the health economy.

THE MODIFIER CONCEPT OF COLORECTAL CARCINOGENESIS

At present, the model of tumor genotype to phenotype/histomorphology correlation, best-known as the adenoma-carcinoma sequence, is well accepted. The genetic changes that occur in colorectal carcinogenesis are paralleled by the progression from small premalignant lesions to advanced carcinomas indicating that each successive genetic injury/modification confers an advantageous characteristic upon the expanding tumor. The MAPK-ERK (mitogen-activated protein kinase-extracellular signal-regulated kinase) pathway, which mediates cellular responses to many extracellular inputs, is involved in regulation of apoptosis, cell growth, secretion, and cellular differentiation. K-ras and B-raf are key players in this signaling cascade, because mutations result in continuous gene activation leading to autonomous cell proliferation and tumor growth. The *KRAS* gene belongs to the Ras oncogene family and encodes a 21 kDa protein with intrinsic GTPase activity. This protein is regulated by a cycle of de- and re-palmitoylation, which promotes its rapid exchange between the plasma membrane and the Golgi apparatus. While numerous different genes can become altered in carcinogenesis, it has been recently proposed that the number of deregulated signaling pathways is relatively small^[7].

Over the last few decades it has been shown that aberrant activity of some cellular pathways is crucial in colorectal carcinogenesis. Alterations in the coding genes, e.g. *APC* or *TP53* mutations and loss of heterozygosity, were elucidated as key mechanisms for misdirected signaling^[8]. Important insights into such principles of cancer-relevant molecular signaling were given by the characterization of hereditary colorectal carcinoma syndromes, i.e. hereditary non-polyposis colon cancer, familial adenomatous polyposis, and MUTYH-associated polyposis. Genotype to phenotype correlations in affected patients revealed that the type of variation in gene sequence and epigenetics is essential for onset and progress in cancer development. However, some inter-individual discrepancies were found in this correlation analysis, indicating additional molecular mechanisms that are likely to modify pathway execution^[9-11]. These observations were substantiated by several experiments including studies in *APC* mice revealing examples of modifying genetic loci and non-genetic co-factors, e.g. nutritional components^[12]. In mice, modifying genetic loci were addressed with the acronym Mom (modifiers of min) and demonstrated the genetic-based principle of modulation in gene expression with relevance to disease severity. One interesting candidate gene, firstly identified in the *APC* mice, is *Pla2g2a* coding secretory phospholipase A2 (PLA2)^[13]. Activity of this enzyme (prototype group I) is associated with diverse functions including digestion, smooth muscle contraction, and cell proliferation. In addition, PLA2s of prototype group II are involved in inflammatory conditions and are upregulated

by pro-inflammatory cytokines such as tumor necrosis factor and interleukin-1B. Recently, the PLA2-receptor (PLA2R) has been identified as a potential new tumor suppressor gene crucial in the induction of cellular senescence through activation of the p53 pathway^[14].

In addition to genes acting as defined modifiers in colorectal carcinogenesis, matrix composition has been identified as an important factor to modify epithelial cell behavior and intestinal tumorigenesis^[15]. The heterogeneity in matrix composition is marked and is a result of the complex mixture of non-epithelial cells embedded in a well-adapted set of extracellular stored lamellar and non-lamellar molecules. Additionally, different types of secreted intestinal products, such as mucins and other glycoproteins which are non-cellular matrix-components have been identified as important modifiers in intestinal pathogenesis^[16]. Muc2, expressed by goblet cells, is the most abundant secreted intestinal mucin and important in intestinal homeostasis. The impaired expression and/or secretion of Muc2 have been correlated with the development of inflammatory intestinal conditions and colorectal carcinogenesis^[17-20]. Recently, the molecular basis for this phenomenon was identified, when an interaction of Muc2 and Apc resulting in modification of Wnt signalling was determined^[21,22].

In addition to Muc2, other molecules such as cytokines are suggested as important mediators in chronic intestinal inflammation and modifiers of intestinal tumor development. In the intestine, the main sources of cytokines and inflammation-triggered molecules are lymphocytes. The pathophysiological association of chronic inflammatory activity and intestinal tumorigenesis has been substantiated by epidemiological data demonstrating a high incidence of CRC in patients with chronic inflammatory bowel disease, a condition additionally associated with disturbances in composition of non-cellular matrix components.

Over the last decade, an additional modifier of colorectal carcinogenesis and pathogenesis of non-neoplastic intestinal diseases has been identified and characterized, namely the microbiome. The term intestinal microbiome describes the taxonomically complex and micro-ecological highly dynamic community of micro-organisms on the gut mucosal surface^[23]. The interaction of surface lining mucosal cells and the microbiome has an impact on the metabolome and lipidome, the maturation and proliferation of intestinal cells along the crypt-plateau axis, tissue homeostasis, and can be a factor in various diseases, such as inflammatory bowel disease and obesity^[24,25]. The successful use of designed bacterial strains in the treatment of inflammatory intestinal conditions, such as necrotizing enterocolitis in preterm infants, underline that the microbiome modificatory activity is fundamental in intestinal homeostasis^[26].

In summary, colorectal carcinogenesis is based on a sequence of molecular events in enterocytes including gene mutations, epigenetic modifications, and aberrant signaling in basic cellular pathways. However, proliferation, cellular activities, and cell death of enterocytes and the execution of so-called modifier genes of CRC-relevant pathways

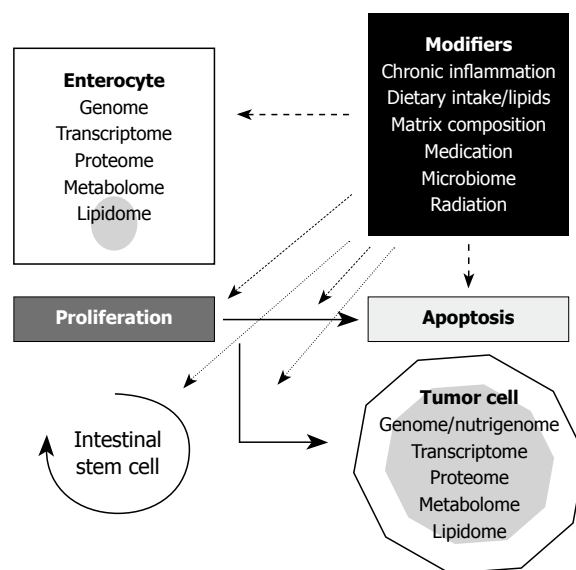


Figure 1 The modifier concept of colorectal carcinogenesis. The important key points in enterocytic life span, proliferation and apoptosis, are connected by basal molecular pathways. Such pathways, as well as cell behavior determining molecular entities such as the genome, transcriptome, proteome, metabolome, and lipidome, are exposed to cellular stress and act as modifiers. In addition to enterocytes, the intestinal stem cells are affected by this evolutionary principle. The aberrant modification of pathways is suggested as an important variable in colorectal carcinogenesis. The term nutrigenome of tumor cells reflects the genome modification of malignant cells by dietary components with expression of so-called modifier genes as demonstrated in APC mice.

strongly depend on additional variables including matrix composition, the microbiome, chronic inflammatory activity, environmental effects, and dietary components. In particular, nutrition and diet, as stated below, are suggested as fundamental in modifying the initiation and progression of CRCs^[6]. Key aspects in the current view of the CRC carcinogenesis modifier concept are summarized in Figure 1.

NUTRITION AND CRC - DIETARY FAT AND INTESTINAL LIPIDS

Among the many aspects of industrialization, such as obesity, exposure to electromagnetic fields, radiation, medications, and a reduced physical activity, diet seems to be a plausible variable in colorectal carcinogenesis because of its direct contact with the intestinal mucosa, i.e. the surface lining epithelial cells^[27]. The literature is marked by epidemiological studies aiming to elucidate the constituents of a CRC-preventive diet, the optimal amount of dietary fiber, and to define biologic markers for nutritional epidemiology of CRC^[28]. A reason for the controversial outcome of such studies could be that the entire diet, with its balance of animal and vegetable products, and grains that have been processed to varying degrees, is highly variable and not a single entity. However, some dietary components are described as putative candidates in modifying colorectal carcinogenesis. An increased risk for cancer development is found in individuals consuming diets high in red and processed meat^[29]. In

addition, alcohol in combination with a diet low in folate and methionine appears to increase cancer risk^[30-32]. Vitamin D and calcium are suggested to have a protective effect in CRC carcinogenesis, but the molecular mechanism behind this has not been elucidated. Recently, it has been shown that deep-fried/oxidized fats such as hydroxyl- and hydroperoxy fatty acids, are able to influence lipid metabolism by activation of the transcription factor, peroxisome proliferator-activated receptor α (PPAR α)^[33]. Oxidized fats are established in diets containing large quantities of polyunsaturated fatty acids (PUFAs) by heating. Expression of PPAR α -related genes is involved in degradation of fatty acids, affects the synthesis of cholesterol/triacylglyceride, and is associated with cellular behavior. These data indicate that lipid metabolism activity and cellular lipid-dependent signaling could be modified by a diet enriched in deep-fried fats.

In the last few decades, the highly diverse class of lipids has become the focus of intensive research activities, because epidemiological data have indicated a link between the intake of dietary lipids and development of (sporadic) CRCs^[34,35]. In this concept, the composition of the different types of fatty acids and the expression/activity of lipid-metabolizing enzymes such as COXs and ACSLs are of high importance^[36]. A growing number of reports support the findings that bioactive dietary components containing long-chain PUFAs modulate important determinants that link inflammation to cancer development and tumor progression^[37], whereas short-chain fatty acids, e.g. acetate, propionate, and especially butyrate, mainly produced by the microbiome using fermentable dietary polysaccharides, are suggested to be cancer-preventive^[38,39]. Propionate and acetate are utilized preferentially by extra-intestinal tissues, whereas butyrate is suggested to play an important role in intestinal mucosa homeostasis as an energy source for enterocytes. It has been shown that cancer-preventive intestinal butyrate levels correlate with increased epithelial cell differentiation, cell cycle arrest, and apoptosis of enterocytes. Additional aspects of butyrate activity, namely inhibition of the enzyme histone deacetylase, and a decrease in the transformation of primary to secondary bile acids as a result of colonic acidification, could be of relevance in colorectal carcinogenesis^[40]. Recently, multiple free fatty acid receptors (FFAR) have been identified in the intestine^[41]. The receptor activity highly depends on the length of the free fatty acids. Short-chain fatty acid signaling is mediated by FFAR2 and FFAR3, whereas FFAR1 and GPR120 can be activated by medium- and long-chain free fatty acids. It is suggested that defects in FFAR3 are associated with reduced extraction of energy from short-chain free fatty acids. The first experimental evidence was that at least FFAR2 could be associated with cellular transformation and colorectal carcinogenesis. In this experimental setting the putative mitogenic action of FFAR2 was aggravated by short-chain free fatty acids.

The important role of lipids, especially fatty acids, in the modifier concept of CRCs is underlined by the plethora of intestinal long-chain fatty acid (LCFA) activi-

ties. LCFAs were suggested as important cell cycle modifiers, particularly in enterocytes. In ACSL5-overexpressing enterocytes, an increase in apoptosis susceptibility was found, probably as a result of an aberrant and locally restricted increase in LCFA derivatives^[42]. These investigations underline the importance of ACSL-mediated metabolic channeling of fatty acids in the regulation of cell behavior^[43]. In addition, lipidation of proteins is suggested as a basic mechanism in the translation of LCFA modifier activities in several signaling cascades and receptor structures^[44]. This mechanism was recently demonstrated for the CD95 death-inducing signaling complex (DISC)^[45]. In the earliest events in DISC formation, palmitoylation of CD95 on cysteine 199 enhances receptor aggregation to a high molecular complex. Palmitoylation, as an important mechanism in fatty acid-based protein modification *via* thio-ester linkage to cysteine (S-palmitoylation), has been recently classified into 5 general classes^[46]. The first class comprises transmembrane proteins S-acylated on cysteines adjacent to or in the transmembrane domain. In the second class, proteins are typified by Ras family molecules, where S-palmitoylation is dependent on prior prenylation within the C-terminal "CAAX" box. In the third class, palmitoylated proteins include molecules with S-palmitoylation at one or more cysteine residues near the N- or C-terminus. Dual acylation is found in the fourth class proteins such as members of the Src and G protein families. N-myristoylation is suggested to improve accessibility of the protein to a membrane-bound palmitoyl acyl transferase and subsequent protein palmitoylation. The hallmark of class 5 proteins is covalent binding of palmitate *via* amide-linkage to an N-terminal cysteine residue.

In summary, dietary fatty acids are suggested as important substrates for protein modification *via* direct molecular interaction. A complex metabolic conversion of absorbed lipids into cell-synthesized LCFA is not mandatory. However, synthesis of acyl-CoA derivatives from dietary fatty acids seems an important step for the modifying activity and bioavailability of lipids. Palmitoylation has been shown as a basic molecular mechanism in lipid-dependent protein modification. As detailed below, lipidomics is a powerful technical algorithm to elucidate key aspects in the interaction of dietary lipids and cellular lipid metabolism.

LIPIDOMICS - A PROMISING TOOL IN CRC-RELATED LIPID ANALYSIS

In the last 2 decades, laboratory technology to investigate carcinogenesis has primarily focused on genomic and proteomic analyses^[47]. Many research-based studies on CRC combined genetic, translation-related and/or biochemical approaches to discover clinically useful DNA- or protein-based biomarkers for CRC screening, diagnosis and response to treatment. Since the crucial roles of lipids in numerous signaling pathways including carcinogenesis, apoptosis and proliferation, has been identified, the necessity to understand and analyze the impact of lipids in

colorectal carcinogenesis, diagnosis, prognosis and treatment becomes more and more important.

In recent years, lipidomics, a novelty in the “-omic” research, has emerged as a promising tool to investigate cellular lipid metabolism and pathogenic alterations in lipid pathways^[48]. Lipidomics, defined as the large-scale study of the pathways and networks of cellular lipids, is a chromatography- and mass-spectrometry-based research area that includes the study of cellular and functional lipidomics as well as lipids in health and disease. The cellular lipidome comprises over 1000 different, highly diverse and complex lipids of which many are metabolically interconvertible and structurally similar^[49]. Because of this complexity, comprehensive lipid analyses are required to elucidate the physiological properties and functionality of different lipid metabolites. The ability to analytically distinguish different lipid species is critical. Despite these experimental challenges and the fact that lipidomics has only emerged as a distinct field within the past few years, the number of compounds that can be analyzed is growing rapidly^[50]; including fatty acids, sphingolipids, glycerolipids, glycerophospholipids, sterol lipids and prenol lipids, each containing distinct classes and subclasses of molecules^[51-56]. The following section gives a short overview of classical and state-of-the-art analytical techniques that are commonly used by lipidologists to investigate lipids in health and disease.

Thin layer chromatography (TLC) has been used for the qualitative study of lipids since the early 1960s^[57,58]. The technique uses a thin layer of a stationary phase such as silica or cellulose on a flat support, usually a glass or aluminum plate. Although the combination of a wide variety of silica-based solid-phase and organic mobile-phase protocols enables the separation of almost all lipid classes, TLC does not offer sufficient structural specificity and sensitivity as other methods of lipid detection. This has led to its use primarily as a screening tool prior to use of more sensitive and elaborated methods.

High pressure liquid chromatography (HPLC), a more sophisticated chromatographic technique, is routinely employed in lipidomic analysis for separation and identification of lipid species. HPLC provides resolution of lipids based on headgroup/class (normal phase) or fatty acid residues (reverse phase). For example, fatty acids and acyl-CoA are almost always separated on a reverse-phase column where the separation occurs on the basis of polarity, effective chain length, and degree of unsaturation^[51], whereas separation of phospholipids can be achieved by either normal-phase HPLC^[59] or reverse-phase HPLC^[60]. However, HPLC alone can not guarantee the accurate quantification of lipids since this often depends on definite resolution and structural elucidation.

Mass spectrometry based techniques are currently the analytical tool of choice in lipidomic methodology. Two powerful approaches that provide sensitivity and structural specificity are electrospray ionization (ESI) and matrix-assisted laser desorption ionization (MALDI) mass spectrometry. ESI allows lipids in solution to be continuously infused directly into the ion source of a

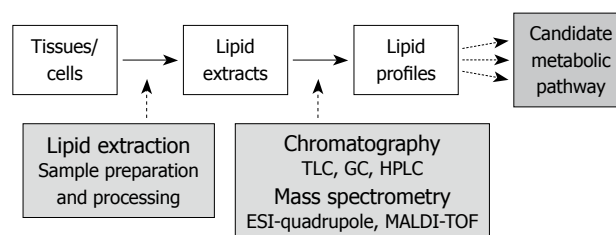


Figure 2 Summary of the lipidomics work flow - lipid extraction and analysis. TLC: Thin layer chromatography; GC: Gas chromatography; HPLC: High pressure liquid chromatography; ESI: Electrospray ionization; MALDI-TOF: Matrix-assisted laser desorption ionization-time-of flight.

mass spectrometer, typically a triple quadrupole mass analyzer. By applying a high voltage to the infusion capillary, an electric field is created along which the charged droplets travel. As they travel, the solvent rapidly evaporates and droplets divide into individual charged ions to enter the mass spectrometer. The combination of classical separation techniques such as HPLC with state of the art ESI-mass spectrometry provides the necessary quantitative accuracy, sensitivity and structural specificity to detect and quantify a wide range of lipid species. Thus, liquid chromatography-mass spectrometry (LC-MS) and tandem mass spectrometry (LC-MS/MS)-based methods are currently one of the most popular technologies in lipid research^[52,61-63].

In contrast to the LC-based lipid analysis, the recently developed direct infusion-based shotgun lipidomics approach, an analytical platform without direct coupling of any chromatography for lipid separation, allows the absolute quantification of hundreds of lipid species in small quantities with high throughput. This powerful strategy provides a promising future application in clinical studies and medical approaches to investigate pathogenic changes in lipid metabolism and lipid-mediated disorders^[64,65].

In addition to ESI mass spectrometry, MALDI mass spectrometry, typically used in conjunction with time-of flight mass analyzers, is a laser-based soft ionization method that has been successfully used for large lipid species. Here, the lipid sample is mixed with an energy absorbing matrix and irradiated with a laser to ionize the lipid molecules which then enter the mass spectrometer. Although MALDI is most often used for protein analyses and only used to a small extent for lipid analyses, the power of this technology lies in its application for tissue-imaging mass spectrometry. Direct tissue analysis of lipid species provides the opportunity to identify not only quantities but also the distribution and subcellular localization of lipids within the tissue^[66,67]. MALDI tissue imaging (MSI), thus, greatly enhances the application spectrum of lipidomic analysis in disease-related alterations in lipid metabolism. A typical lipidomic work flow, including sample preparation and analysis is summarized in Figure 2.

The potential and the feasibility of lipidomic technologies in studies of diabetes, obesity, atherosclerosis and Alzheimer's disease have been described recently^[68]. A lipidomic study of breast cancer pathogenesis demon-

strated that the complete profile of adipose tissue lipids in patients with benign and malignant breast tumors, rather than a single lipid has the capability to quantify the dietary part of breast cancer risk and to identify dietary modification in order to reduce breast cancer occurrence^[69]. As the relevance of lipids as potential biomarkers becomes apparent, an increasing number of studies have focused on the discovery of reliable lipid biomarkers for disease prognosis, prevention and therapeutic application. A recent study showed that the analysis of fatty acyl chains from ethanolamine-containing plasmalogens provided a suitable biomarker to identify malignancy and metastatic capacity of frequent human cancers, such as breast, lung, and prostate cancer^[70]. Although there are currently not many studies focusing on lipidomics-based approaches in colorectal carcinogenesis, the implications of this strategy for the future of CRC treatment and prevention are vast. As the field of lipidomics advances, the role of the lipidome in cellular functions and pathologic states will become clearer, and the identification and establishment of preventive and therapeutic approaches will become more focused. The integration of lipidomic data with genetic, proteomic and metabolomic data will provide a powerful analytical platform for elucidating the mechanism of lipid-based disease, for biomarker screening, and for monitoring pharmacological therapy^[71].

CONCLUSION

In current opinion, the basic molecular pathways determining colorectal carcinogenesis are few in number. However, the number of mechanisms that could act in modifying initiation and progression of intestinal neoplasia is constantly growing. Among dietary components, the modifying capacity of lipids, especially of LCFAs, has been demonstrated by experimental and epidemiological studies. Lipidomics, the large-scale study of the pathways and networks of lipids in cells or tissues, is established as a modern and powerful tool to further elucidate the colorectal carcinogenesis modifying activities of lipids.

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Risk factors in familial forms of celiac disease

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Abstract

Celiac disease has been reported in up to 2% of some European populations. A similar risk has been identified in the America and Australia where immigration of Europeans has occurred. Moreover, an increasing number of celiac disease patients are being identified in many Asian countries, including China and India. Finally, celiac disease has also been detected in Asian immigrants and their descendants to other countries, such as Canada. Within these so-called "general" celiac populations, however, there are specific high risk groups that have an even higher prevalence of celiac disease. Indeed, the single most important risk factor for celiac disease is having a first-degree relative with already-defined celiac disease, particularly a sibling. A rate up to 20% or more has been noted. Risk is even greater if a specific family has 2 siblings affected, particularly if a male carries the human leukocyte antigen-DQ2. Both structural changes in the small bowel architecture occur along with functional changes in permeability, even in asymptomatic first-degree relatives. Even if celiac disease is not evident, the risk of other autoimmune disorders seems significantly increased in first-degree relatives as well as intestinal lymphoma. Identification of celiac disease is important since recent long-term studies have shown that the mortality of celiac disease is increased, if it is unrecognized and untreated.

INTRODUCTION

Only a few decades ago, celiac disease was considered an uncommon disorder present mainly in Europe or in countries where Europeans had emigrated, including Canada and Australia^[1]. In Ireland, and in particular, Galway in the West of Ireland, appears to have an especially high prevalence of celiac disease with an estimate of up to 1 in 300 persons^[2]. Similar experiences have been noted in Scandinavian countries. In the United States, however, earlier reports suggest that the detection of celiac disease is surprisingly low^[1,3]. With the emergence of modern serological screening tests for celiac disease and increased efforts to use these screening measures, celiac disease has become more readily detectable.

EMERGING RISK ESTIMATES

In recent years, changes have occurred with an estimate of overall prevalence rates increased in most countries, often in the region of about 1%-2%^[4]. Interestingly, in a longitudinally-based study from Olmstead County in the United States over many decades, increased detection has been reported, in part, due to increased use of serological screening^[5]. Recently, a report from Hangzhou in China has also suggested that the prevalence of adult celiac disease may be more common in China than previously appreciated^[6]. Of note, celiac disease has also been

reported in immigrants to Canada from China, Japan and South Asia, particularly from the Punjab region of India^[7]. Even in the United States, celiac disease is now generally believed to affect 0.5%-1.0% of the general population^[8].

HIGH RISK GROUPS FOR CELIAC DISEASE

These reported numbers often reflect prevalence estimates, usually resulting from screening of selected groups, e.g. blood bank serum samples, thought to be representative of the entire population from a specific geographic area. Confirmatory small intestinal biopsies have often been done in sero-positive cases to provide an overall “minimal” estimate of population prevalence (since all sero-positive patients may not be available for biopsy). As shown in Table 1, there are also some high risk groups within the general celiac population, often without typical clinical symptoms, such as diarrhea or weight loss, that may have even higher prevalence rates. Among these factors that specifically denote a higher risk for celiac disease, the single most important is a familial history of biopsy-defined celiac disease with some estimates up to 20% or more of first-degree relatives. Diagnosis of celiac disease in all of these high risk groups, however, is especially important since failure to detect celiac disease coupled with failure to treat the disease may lead to an increased morbidity, and critically, a nearly 4-fold increased risk of mortality from celiac disease^[9].

FAMILIAL RISK FACTORS

Evidence for a familial risk in celiac disease has been accumulated from many sources (Table 2), including biopsy and serological studies in families with known celiac disease^[10-17], animal model studies in the Irish Setter dog^[18,19], functional studies based on evaluation of intestinal permeability or other absorptive indices^[20-23], human leukocyte antigen (HLA)-genotyping studies^[24-26], and more recently, genome-wide expression and linkage studies^[27].

Biopsy and serological studies

Early family studies using small intestinal biopsies have provided strong evidence for the familial nature of celiac disease^[10,11]. A systematic review of these earlier studies using MEDLINE and EMBASE databases concluded that up to 20% of first-degree relatives of European descent may be at risk for celiac disease^[12]. Similar results have been reported from non-European populations, including a recent report from the Punjab region of India^[13]. In addition, more extensive studies in biopsy-defined celiac disease have further confirmed these findings, particularly in “at-risk” first-degree (1:22) and second-degree relatives (1:39), compared to a control “not-at-risk” group (1:133)^[14]. This risk appears to be especially increased in families with at least 2 siblings diagnosed as celiac disease^[15], and in this setting, more males were detected compared to females^[16]. Particularly significant predictors

Table 1 High risk populations for celiac disease

Relatives, especially first-degree
Anemia, especially iron deficiency
Osteopenic bone disease
Insulin-dependent diabetes (type 1), especially children
Liver disorders, especially AIH and PBC
Genetic disorders, including Down and Turner's syndrome
Autoimmune endocrinopathy, especially thyroid disease
Skin disorders, particularly dermatitis herpetiformis
Neurological disorders, including ataxia, seizures, MG
Others, including IgA nephropathy

AIH: Autoimmune hepatitis; PBC: Primary biliary cirrhosis; MG: Myasthenia gravis.

Table 2 Evidence of familial risk

Biopsy and serological studies
Irish Setter dog model studies
Functional permeability studies
HLA marker studies
Genome wide expression/linkage studies

HLA: Human leukocyte antigen.

of familial risk include carrying HLA-DQ2 and being a sibling of the proband^[17].

Animal model studies

Animal studies have provided some indirect, but important evidence. Gluten-sensitive enteropathy has been reported in a family of Irish Setter dogs^[18] along with the association of a canine major histocompatibility complex (MHC) DQ haplotype^[19].

Functional studies

Earlier studies showed that an intentional increase in gluten intake may provoke jejunal mucosal architectural changes in some first-degree relatives along with reduced xylose absorption, even in those without clinically overt disease^[20], suggesting that, in the genetically-predisposed, both structural and functional changes may be induced in familial celiac disease. Abnormal permeability to lactulose and mannitol in first-degree relatives also suggests that functional changes may occur in biopsy-defined disease^[21] as well as in first-degree relatives with apparently normal small bowel biopsies^[22]. Importantly, this familial risk in first-degree relatives is genetically-based and not related to their daily calculated gluten intake^[23]. In other words, familial celiac disease is not due to a common environment with increased intake of gluten-containing foods, but has a definitive genetic basis.

Human leukocyte antigen marker studies

HLA markers have been popularly used for decades in clinical studies of celiac disease. These clinical studies have served to emphasize the strong genetic component involved in the development of celiac disease. In large part, possession of the HLA-DQ2 variant, necessary for presentation of dietary antigens to intestinal T-cells, is

critical. Non-HLA genetic factors are also increasingly recognized as a very important, and possibly even a more significant and critical risk factor in the development of familial celiac disease. Furthermore, a recent HLA-genotyping study (i.e. DQA1*0501, DQB1*0201, DRB1*04) with small bowel biopsies from Finland has suggested a practical means of exclusion of some first-degree relatives without celiac disease for screening purposes^[24]. However, it has been reported that some asymptomatic first-degree relatives may show only minimal changes in intra-epithelial lymphocyte numbers^[25]. In addition, with a prolonged follow-up, flat small bowel mucosal biopsies have been reported in familial disease without initially abnormal biopsies^[26]. These findings are important and need to be confirmed as further longitudinal studies may be required in first-degree relatives without initial biopsy evidence of celiac disease.

Genome-wide studies

Most exciting are the recent genome-wide expression and genome-wide linkage studies done to explore a number of clinical disorders, including celiac disease. A recent meta-analysis of 8 genome-wide linkage studies in celiac disease permitted definition of both HLA and non-HLA chromosome regions that appear to predispose to celiac disease and suggested the possibility of different types of disease-predisposing variants^[27].

RISK OF ASSOCIATED DISEASE IN RELATIVES

Of potentially even greater significance are reports on relatives without celiac disease at risk for other diseases, often associated with celiac disease. For example, a serological survey study of first-degree relatives of children with documented celiac disease suggested that autoimmune diseases may be increased in addition to biopsy-defined, but “sub-clinical” celiac disease^[28]. In addition, first-degree relatives may also develop the closely linked dermatological disorder, dermatitis herpetiformis^[29]. Moreover, patients with type-1 diabetes have an increased risk of celiac disease and biopsy-defined, but essentially asymptomatic and unrecognized celiac disease^[30]. Finally, although lymphoma risk seems to have fallen in the past 4 decades, individuals with a sibling affected with celiac disease have an increased lymphoma risk^[31]. These studies, then, have important implications for risk of familial forms of celiac disease in relatives, particularly first-degree, but also for a host of other genetically-related clinical disorders, even in the absence of celiac disease. In future, expression of different celiac disease phenotypes and their individual specific risks of different diseases may be more readily defined with precise genetic markers or more precise genetic signatures.

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Chemokines and hepatocellular carcinoma

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INTRODUCTION

Chemokines in humans are a family of small proteins of 45-50 kb which contain a structural homologous conservative family of cysteine residues. Chemokines are classified into four families, namely CXC, CC, CX3C and C according to the presence of four cysteine residues in conserved locations. CXC are subdivided into two categories, those with a specific amino acid sequence (or motif) of glutamic acid-leucine-arginine (or ELR for short) immediately before the first cysteine of the CXC motif (ELR-positive), and those without an ELR motif (ELR-negative). CXC and its cognate receptor are combined and activated. The receptors are mainly found in neutrophils, lymphocytes, endothelial cells and epithelial cells. Typical chemokine proteins begin with a signal peptide of approximately 20 amino acids that is cleaved from the mature portion of the molecule during its secretion from the cell. All chemokines share a typical 3-dimensional structure which is stabilized by two disulfide bonds joining the first to the third, and the second to the fourth cysteine residues. CC receptors are mainly distributed in dendritic cells, lymphocytes, macrophages, eosinophilic leukocytes, and natural killer cells, but they do not activate neutrophils. Currently, more than 50 kinds of chemokines have been found with only 20 kinds of chemokine receptors, therefore, in this system a considerable number of useless receptors (receptor, R)/ligand (ligand, L) interact with each other.

Chemokines play a central role in many biological events, such as embryonic development, wound healing, angiogenesis, Th1/Th2 development, leukocyte homeostasis, lymphatic organ development, inflammatory diseases, and tumors. Previous studies suggest that

Abstract

Chemokines play a paramount role in tumor progression. In hepatocellular carcinoma (HCC) progression, chemokines and their receptors play an intricate role. Currently, chemokines and their receptors such as the CXCL12-CXCR4 axis, CX3CL1-CX3CR1 axis and the CCL20-CCR6 axis have received much research attention. Although a large number of studies show that these axes are strongly associated with HCC, the exact mechanism by which these axes promote the growth and progression of HCC remains unknown. In this paper, several chemokines and their receptor interactions in HCC progression, growth and metastasis and immune response to HCC are reviewed.

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Key words: Chemokines; Hepatocellular carcinoma

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chemokines are soluble molecules, and are involved in inflammatory response by regulating the transfer of white blood cells. Chemokines are now considered to play a critical and complicated role in a variety of cells such as tumor cells. Chronic inflammation is a long-term and low-grade injury caused by chemical factors, bacteria, and viruses, and there is a strong association between chronic inflammatory conditions in a particular organ and cancer specific to that organ such as colon cancer and ulcerative colitis, colorectal cancer and inflammatory bowel disease, pancreatic cancer and chronic pancreatitis, esophageal adenocarcinoma and Barrett's esophagus, liver cancer and hepatitis^[1-3]. The most striking evidence of the direct regulation of tumors and tumor growth by chemokines is the over-expression of GRO (CXCL1, growth-related oncogene), α , β , γ in human melanoma cells, and its tumorigenicity in nude mice from *in vitro* research^[4,5]. Recent studies have been focused on the roles of chemokines and their corresponding receptors in the growth and progression of tumors. The chemokines and their receptors involved in these processes are known as axes. At present, a large number of studies have demonstrated that the CXCL12-CXCR4 axis may be related to distant organ metastasis of tumors while the CCL21-CCR7 axis may be associated with lymph node metastasis.

Many studies have been conducted on chemokines and their receptors in hepatocellular carcinoma (HCC). However, various axes may not play the same role in HCC as they do in other tumors. Currently, chemokines and their receptors such as the CXCL12-CXCR4 axis and the CCL20-CCR6 axis have received much research attention. This paper will analyze the relationship between chemokines and their receptors in HCC.

CXCL12-CXCR4 AXIS IN HCC

The CXCL12-CXCR4 axis is considered to be an important factor in the regulation of angiogenesis which is essential for growth and progression of HCC. Using immunohistochemistry, Li *et al.*^[6] found a much higher expression of the CXCL12-CXCR4 axis in HCC specimens than in adjacent, cirrhosis, liver adenocarcinoma, and normal liver tissues. The findings of the study by Sutton *et al.*^[7] indicated that the CXCL12-CXCR4 axis in HCC cell lines promoted the growth, invasion and metastasis of these cell lines. Liu *et al.*^[8] suggested that CXCR4 and CXCL12 may play an important role in the metastasis of HCC by promoting the migration of tumor cells. In addition, some researchers found that fucoidan exhibited antitumor activity toward Huh7 cells through the down-regulation of CXCL12 expression^[9]. Data from recent research strongly suggests that CXCL12-CXCR4 stimulates the proliferation of oval cells, and HCC occurrence correlates with the abnormal differentiation of these cells^[10]. All these studies reveal a strong association between CXCL12-CXCR4 and the incidence of HCC development, however, the exact mechanism remains unknown. Chu *et al.*^[11] conducted studies on HCC cell lines which suggested that the CXCL12-

CXCR4 system mediates active MMP-9 and MMP-2 secretion, which facilitates the consequent metastasis of those cells. Schimanski *et al.*^[12] found that in patients with HCC, the expression of CXCR4 significantly correlated with the progression of local tumor, distant dissemination of lymphatic metastasis, as well as a decreased 3-year-survival rate. Strong expression of CXCR4 was significantly associated with HCC progression. They also found that loss of p53 function did not impact on CXCR4 expression, which indicated that CXCR4 influences HCC progression through other channels. However, Schimanski's work did not suggest which channel may be involved.

Some researchers have shed doubt on whether CXCL12-CXCR4 promotes the growth and progression of HCC. Using immunohistochemistry and RT-PCR, Shibuta *et al.*^[13] detected the down-regulation of CXCL12/CXCR4 expression in HCC and also found that CXCR4 expression correlated with the cell cycle when studying HCC cell lines. Nahon^[14] conducted a retrospective analysis on 120 HCV-infected HCC patients, and suggested a lack of association between CXCL12/CXCR4, death and HCC recurrence. New research shows that SDF-1-3'A (CXCL12) gene polymorphisms are considered to be relevant factors in HCC occurrence and development which have little to do with CXCR4^[15]. Different findings from previous studies can be attributed to the various research methods and statistical methods adopted. These studies need to be improved. For example, in Nahon's study, results could be achieved if the sample size was increased. In addition, most of the HCC studies did not include different forms of hepatitis (HBV and HCV) as a variable.

Therefore, even though most of the research data show that the CXCL12-CXCR4 axis is important in HCC, its specific role is still unknown and some evidence to the contrary can not be explained. Further study and discussion are needed to clarify these issues.

CCL20-CCR6 AXIS IN HCC

There is currently an increasing interest in the CCL20-CCR6 axis in HCC. Fujii *et al.*^[16] found in *in vitro* experiments that the CCL20-CCR6 axis may promote the growth of the hepatoma cell line Huh7 through phosphorylation of mitogen-activated protein kinase (MAPK).

Rubie *et al.*^[17] also studied CCL19, CCL20, CCL21, CXCL12 and the expression of their receptors in HCC, and found that all chemokines were found to be expressed in normal liver and HCC tissues, yet CCL20 was the only chemokine which showed significant up-regulation in HCC tissues. The only chemokine receptor to show significant up-regulation in HCC tissues was CCR6. Clinicopathological analysis revealed a strong association between the levels of expression and the degrees of differentiation. That is, with high differentiation, the expression was low. Therefore, the authors proposed that the CCL20-CCR6 axis plays an important role in the growth and progression of HCC, namely a distinct increase in CCL20 expression rates in HCC tis-

sues of grade III tumors in comparison to grade II tumors. In addition, high expression of CCL20 is often accompanied by high expression of CCR6. Therefore, the authors suggested that CCL20-CCR6 may be involved in the formation and development of HCC.

Uchida *et al.*^[18] observed the formation of pseudo-podia in HCC cell lines with high expression of CCR6, which was not observed in cells low in CCR6. The incidence of intrahepatic metastasis was higher in patients with increased expression of CCR6 than in patients with low expression of CCR6. Disease-free survival was significantly poorer in the high CCR6 expression group than in the low CCR6 expression group. The author concluded that CCR6 might be associated with the intrahepatic metastasis of HCC and may be used as a prognostic factor after hepatic resection for HCC.

In addition, researchers have studied the differences in RNA and protein expression of the chemokines CXCL12, CCL19, CCL20, CCL21 and their receptors in primary liver cancer and colon cancer metastasis; even though they have a high expression of CCL20 and CXCR4, the expression in colon cancer liver metastasis was significantly higher than that in primary liver cancer, thus CXCR4 is important in differentiating primary and secondary liver tumors, while CCL20 and its receptor CCR6 may promote tumor growth in addition to their role in identifying primary and secondary liver tumors^[19].

Although a large number of studies show that the CCL20-CCR6 axis is strongly associated with HCC, the exact mechanism by which the CCL20-CCR6 axis promotes the growth and progression of HCC remains unknown.

FRACTALKINE-CX3CR1 AXIS IN HCC

Fractalkine (CX3CL1) and its receptor CX3CR1 are expressed in DC, NK cells, CD8+ T cells, and macrophages. Although studies have shown that fractalkine can regulate the host immune response, the relationships between the fractalkine-CX3CR1 axis, tumor biochemistry and HCC are unclear. According to recent studies, the fractalkine-CX3CR1 axis is critical in the diagnosis of HCC, because it can not only regulate the immune response, but can also regulate the cell cycle of HCC^[20]. Relevant experiments indicate that fractalkine can enhance the anti-tumor effect of the immune system against HCC in mice. Tang *et al.*^[21] observed that CX3CL1 could elicit tumor-specific cytotoxic T cells and an increased production of IL-2 and IFN- γ capable of inhibiting tumor growth. Matsubara *et al.*^[20] found that HCC patients with an high expression of fractalkine and its receptor CX3CR1 have a low rate of recurrence of intrahepatic metastasis or extrahepatic metastasis, which suggest that the expression of fractalkine and its receptor CX3CR1 may be related to the prognosis of HCC patients, and may be involved in tumor immunity by killing tumor cells.

However, some researchers have proposed that there is a lack of association between the CX3CL1-CX3CR1 axis and HCC, as studies have demonstrated that CX3CR1

is not a risk factor for HCC^[22]. These researchers believe that tumor-derived chemokines have dual roles. Leukocyte aggregation following a signal of increasing chemokine concentration may not only be beneficial to the host, but also contribute to tumor growth. The roles virus-related chemokines play in tumors may be multiple, therefore the specific role of CX3CR1 in the formation of chronic hepatitis and liver disease needs to be elaborated.

In addition, the up-regulated expression of CX3CL1-CX3CR1 in ischemia-reperfusion injury suggests that CX3CL1-CX3CR1 is crucial in reperfusion injury^[23].

OTHER RELATED CHEMOKINES IN HCC

Recent studies have found that the combination of CCL5 with the CCR1 receptor can promote metastasis and invasion of the HCC cell line Huh7^[24]. Furthermore, studies of the expression of CCL3 and its receptor CCR1 in liver cancer have shown a significantly higher level of expression in cancer tissues. Animal experiments also confirmed the essential contribution of CCL3 and CCR1 to the growth and progression of HCC^[25]. Recent studies indicated that CCL3 induced by IL-1 combined with its effective receptor CCR1 may be involved in the growth and progression of HCC. The authors studied six chemokines in six types of HCC cell lines and found that all the hepatoma cell lines constitutively and exclusively expressed CCR1 mRNA and its protein on their cell surface, however, the expression of CCR1 was not detected in normal liver cells^[26]. This research suggested a close relationship between the CCL3-CCR1 axis and HCC.

VCC-1 (VEGF correlated chemokine-1) is a newly discovered chemokine, and has a high expression in breast and colon cancer. Studies have demonstrated that it promotes tumor growth by increasing angiogenesis^[27]. Our recent studies have shown (findings are not yet published) that the expression of VCC-1 in cancer tissues of HCC patients is much higher than that in paracancerous tissues and normal liver tissues. This expression is related to tumor size and tumor differentiation. The findings suggest that VCC-1 may be involved in the growth and progression of HCC, but whether it plays a role in HCC recurrence and metastasis remains unknown. In addition, it is not clear with which receptor VCC-1 interacts.

CHEMOKINES AND THEIR RECEPTORS INVOLVED IN HCC TUMOR IMMUNITY

As mentioned above, not all chemokines promote tumor growth and metastasis. Fractalkine/CX3CL1 is believed to be able to inhibit tumor growth^[21]. Hirano *et al.*^[28] discovered that some chemokines induced by IFN- γ such as Mig (CXCL9) and IP-10 (CXCL10) can promote the gathering of lymphocytes in HCC, and are essential in tumor immunity.

Despite lymphocyte infiltration in liver tumors, the tumor grows and spreads rapidly. Liu *et al.*^[29] studied the expression of chemokines in HCC tissues and HCC cell lines, and found that IP-10 and Mig were expressed in

serum and tissues. The expression of IP-10 in cancer tissues was higher than that in paracancerous tissues. Auto-crane chemokines in HCC cell lines can lead to CXCR3-specific lymphocytes infiltration. However, if the liver cancer cell lines and normal lymphocytes are cultivated, the expression of CXCR3 in lymphocytes shows a distinct decrease. As a result, the combination of CXCR3 and IP-10 and metastasis of T cells to tumor cells are both reduced. *In vitro* experiments show weakening chemotactic effects of CD4+ and CD8+ T cells from liver cancer cells on IP-10, whereas the chemotactic effects of T-cells from normal tissues on IP-10 were enhanced. This study demonstrated the functional desensitization of the chemokine receptor CXCR3 in lymphocytes from HCC patients by CXCR3 ligands secreted by tumor cells. This may cause lymphocyte dysfunction and subsequently impaired immune defense against the tumor.

CONCLUSION

In summary, the roles of chemokines in HCC are multiple, and range from promoting to inhibiting. But which role is dominant? HCC may be regulated by complicated chemokines and their receptors. Current studies show that in tumors such as lung cancer, colon cancer and prostate cancer, the CXCL12-CXCR4 axis may be related to tumor metastasis of distant organs and the CCL21-CCR7 axis may be associated with lymph node metastasis. However, further study is needed to clarify these associations in HCC. In addition, there are few reports on the expression of the CCL21-CCR7 axis in HCC although much research has been carried out on the relationship of this axis with lymph node metastasis in other tumors.

Researchers are expected to explore the possibility of chemokines and their receptors being target genes and to utilize their anti-tumor function when treating HCC. Recent studies have shown that co-expression of suicide genes and MCP-1 (monocyte chemotactic protein-1) in rat liver and colon cancer has a significant anti-tumor effect^[30]. Gene therapy based on a variety of chemokine axes such as the CXCL12-CXCR4 axis is being studied. The desired effect can only be achieved by combining several chemokine axes rather than focusing on a single axis.

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Role of HSP-90 for increased nNOS-mediated vasodilation in mesenteric arteries in portal hypertension

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Abstract

AIM: To explore the role of heat shock protein-90 (HSP-90) for nitrgenic vasorelaxation in the splanchnic circulation in rats with and without portal hypertension.

METHODS: Neuronal nitric oxide synthase (nNOS) and HSP-90 were analyzed by immunofluorescence, western blotting and co-immunoprecipitation in the mesenteric vasculature and isolated nerves of portal-vein-ligated (PVL) rats and sham operated rats. *In vitro* perfused de-endothelialized mesenteric arterial vasculature was precontracted with norepinephrine (EC₈₀) and tested for nNOS-mediated vasorelaxation by periaarterial nerve stimulation (PNS, 2-12 Hz, 45V) before and after incubation with geldanamycin (specific inhibitor of HSP-90 signalling, 3 µg/mL) or L-NAME (non-specific NOS-blocker, 10⁻⁴ mol/L).

RESULTS: nNOS and HSP-90 expression was signifi-

cantly increased in mesenteric nerves from PVL as compared to sham rats. Moreover, nNOS and HSP-90 were visualized in mesenteric nerves by immunofluorescence and immunoprecipitation of nNOS co-immunoprecipitated HSP-90 in sham and PVL rats. PNS induced a frequency-dependent vasorelaxation which was more pronounced in PVL as compared to sham rats. L-NAME and geldanamycin markedly reduced nNOS-mediated vasorelaxation abrogating differences between the study groups. The effect of L-NAME and geldanamycin on nNOS-mediated vasorelaxation was significantly greater in PVL than in sham animals. However, no difference in magnitude of effect between L-NAME and geldanamycin was noted.

CONCLUSION: HSP-90 acts as a signalling mediator of nNOS-dependent nerve mediated vascular responses in mesenteric arteries, and the increased nitrgenic vasorelaxation observed in portal hypertension is mediated largely by HSP-90.

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Key words: Heat shock protein-90; Nitric oxide; Vasodilation; Portal hypertension; Mesenteric circulation

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INTRODUCTION

Arterial vasodilation and vascular dysregulation represent the initiating as well as perpetuating mechanism in the pathophysiology of the hyperdynamic circulatory syndrome in portal hypertension^[1]. This arterial vasodilation occurs early and is most pronounced in the splanchnic circulation which therefore has been suggested to be the main culprit for reduced peripheral vascular resistance occurring with progression of portal hypertension^[2,3]. A strong line of evidence suggests that vascular overproduction of nitric oxide (NO) plays a pivotal role for the development of splanchnic arterial vasodilation in this condition^[4-6]. In fact, non-specific inhibition of NO synthesis has been shown to restore mesenteric vascular responsiveness^[7] and to almost normalize splanchnic vascular resistance^[8] thereby markedly ameliorating portal-systemic shunting and the hyperdynamic circulation^[9,10].

Three isoforms of NO-synthases (NOS) have been cloned. Vascular overproduction in the splanchnic circulation in portal hypertension is derived from endothelial (eNOS) and neuronal (nNOS) but not inducible NOS (iNOS)^[4,11,12]. eNOS as well as nNOS-derived NO production are regulated through distinct posttranslational modifications, one being the interaction of NOS with regulatory proteins^[13]. The molecular chaperone heat shock protein-90 (HSP-90) facilitates folding and stabilization of cellular proteins thereby promoting specific signalling pathways^[14]. In support of this concept, interaction of eNOS and nNOS with HSP-90 has recently been shown to facilitate NO synthesis^[15,16]. In fact, in portal hypertension we have observed increased eNOS activity and an associated enhanced eNOS-dependent vasorelaxation in mesenteric arteries, which was markedly inhibited by geldanamycin, an ansamycin antibiotic and specific HSP-90 antagonist^[17]. Whether HSP-90 likewise plays a crucial role in nNOS-mediated vasodilation in the splanchnic circulation in healthy as well as portal hypertensive conditions is currently unknown.

Chronic specific nNOS-inhibition markedly attenuates the hyperdynamic circulatory syndrome in experimental cirrhosis^[18] underlining the relative importance of nNOS-derived NO in this scenario. Moreover, we recently reported an increased nNOS expression in the whole mesenteric vascular bed of portal vein-ligated (PVL) rats^[12], and nNOS has been demonstrated to be up-regulated and to be highly expressed in vascular smooth muscle cells of the mesenteric arteries during portal hypertension^[19]. nNOS expression and function in mesenteric nervous tissue independent of the arterial smooth muscle layer, however, has not been investigated so far.

Thus, the aim of our study was to analyse (1) nNOS expression in mesenteric nerves during portal hypertension; (2) whether HSP-90 co-localizes with and regulates nNOS-mediated vasorelaxation in the mesenteric arterial vasculature; and (3) if this is the case, whether alterations in HSP-90 function occur during portal hypertension.

MATERIALS AND METHODS

Animals

The investigation was performed in male Sprague-Dawley rats (Harlan Sprague Dawley Laboratories, Indianapolis, IN), weighing 300-399 g. Rats were caged at a constant room temperature of 21°C, exposed to a 12:12 hour light: dark cycle, and allowed free access to water and standard rat chow *ad libitum*. All experimental procedures in this study were conducted according to the German Physiological Society principles for the care and use of laboratory animals (Granted permission number 621-2531.1-23/00, Government of Oberpfalz, Bavaria).

Drugs

Acetylcholine, Sodium-Nitroprusside, Norepinephrine, Guanethidine, Atropine, Timolol, L-NAME and geldanamycin were purchased from Sigma (Deisenhofen, Germany).

Induction of portal hypertension

A prehepatic portal hypertensive animal model extensively studied in our laboratory was used^[20]. Portal hypertension was induced surgically in aseptic conditions. Briefly, the rats were anaesthetized with ketamine hydrochloride (Ketalar, 100 mg/kg bw; Parke, Davis, Avon, CT). After a midline abdominal incision, the portal vein was freed from surrounding tissue. A ligature (silk gut 3-0) was placed around a 20-gauge blunt-tipped needle lying alongside the portal vein. Subsequent removal of the needle yielded a calibrated stenosis of the portal vein. In sham-operated rats, the same operation was performed with the exception that after isolating the portal vein no ligature was placed. All studies were performed in 12-18 h fasted animals 10-14 d after surgery.

In vitro perfusion

The *in vitro* perfusion system used was a partial modification of that originally described by McGregor and used extensively in previous studies from our laboratory and others^[5,21,22]. Briefly, the superior mesenteric artery (SMA) was cannulated with a PE-60 catheter and gently perfused with 15 mL warm Krebs solution to eliminate blood. After isolating the SMA with its mesentery, the gut was cut off close to its mesenteric border. The arterial vasculature was then transferred to a 37°C water-jacketed container and perfused with oxygenated 37°C Krebs' solution (95% O₂, 5% CO₂) using a roller pump (Ismatec, IPC 8-channel, Zürich, Switzerland). The Krebs solution had the following composition (mmol/L): NaCl, 118; KCl, 4.7; KH₂PO₄, 1.2; MgSO₄, 1.2; CaCl₂, 2.5; NaHCO₃, 25; disodium EDTA, 0.026; and glucose, 11.0; pH 7.4. The effluent of the perfused tissue was continuously removed from the perfusing chamber. The perfusion pressure was measured with a P-23-Db strain gauge transducer (Statham, Oxnard, CA) on a side arm just before the perfusing cannula and continuously

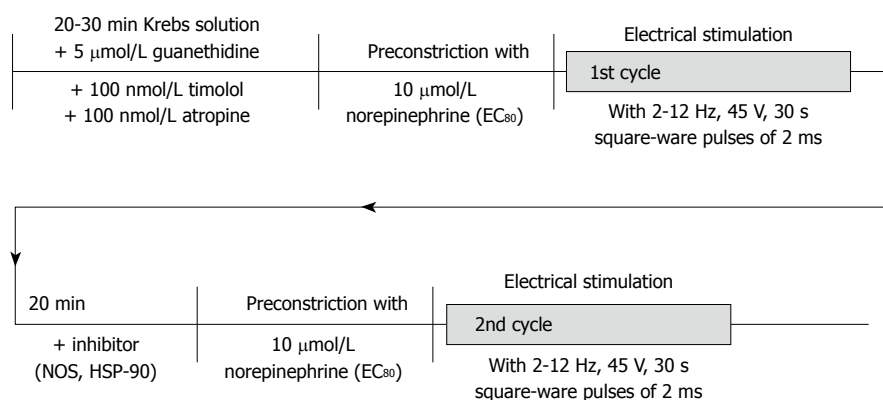


Figure 1 Experimental study protocol. In this illustration, the chronological order of applications of drugs and performance of peri-arterial nerve stimulation (PNS) is shown. EC₈₀: Concentration achieving 80% maximal vasoconstriction; NOS: Nitric oxide synthase; HSP-90: Heat shock protein-90.

recorded (Powerlab Quadbridge and Powerlab 4/20, from AD Instruments, Spechbach, Germany).

Where indicated endothelial denudation of the mesenteric vasculature was performed by a combined treatment of cholic acid (sodium salt) and distilled water as has been used before^[21]. In brief, after cannulation of the SMA and gentle flushing with 10 mL of warmed Krebs' solution to eliminate blood, perfusion with cholic acid (0.5%/1.5 mL for 10 s) followed by 15 mL of Krebs solution (to eliminate cholic acid) was performed. The preparation was then transferred to the 37°C waterjacketed container and perfused with oxygenated 37°C Krebs solution (4 mL/min) for 10 min. After the mesenteric vasculature was relaxed, 37°C warmed distilled water was perfused for 10 min before starting Krebs perfusion again. After an equilibration period of 45 min, experimental perfusion protocols were performed (Figure 1). At the end of each experiment we assessed whether the vessel was completely de-endothelialized and whether the smooth muscle function was maintained. The mesenteric preparation was kept precontracted with methoxamine (MT; α -1-agonist: 100 μ mol/L) and dose-dependent vasorelaxation to the endothelium-dependent vasodilator acetylcholine (ACh: 10^{-8} to 10^{-6} g, bolus of 0.1 mL) and the endothelium-independent vasodilator sodium nitroprusside (SNP: 10^{-6} to 10^{-5} g, bolus of 0.1 mL) was tested. Only experiments with ACh- and SNP-induced relaxation being less/more than 15%/60% were accounted as valid.

Periarterial nerve stimulation

Two platinum electrodes, one placed around the SMA and the other shaped as a wire grid the tissue was resting on, were used for transmural electrical field stimulation. The nerves of the preparation were stimulated by means of an electronic stimulator (I-ZQ4v, Hugo Sachs Electronics, Hugstetten, Germany), delivering single square-wave pulses (2 ms) at 45 V with a train duration of 30 s and frequencies of 2-12 Hz. In order to evaluate vasodilatory responsiveness vessels were precontracted submaximally (EC₈₀) using norepinephrine (NE 10^{-5} mol/L) before applying periarterial nerve stimulation (PNS) (Figure 1). nNOS-mediated vasorelaxation is known to be non-adrenergic and non-cholinergic in origin. Therefore, guanethidine (5×10^{-5} mol/L), atropine (10^{-9} mol/L) and timolol (10^{-9} mol/L) were added from the beginning in order to deplete endogenous noradrenaline stores and to prevent

its uptake as well as to avoid cholinergic stimulation. When a stable precontraction level was achieved, PNS was applied in a non-cumulative fashion. Sufficient time was allowed between each stimulation train for the perfusion pressure to return to a stable level, usually 5-10 min. PNS responses are expressed as percentage change of the pre-contraction level being present before PNS. Nitroergic vasodilation is known to be independent of prostaglandin synthesis^[23] and it is not subject to pre-junctional inhibition by α 2-adrenoreceptors^[23]. Moreover, in previous experiments tetrodotoxin has been used to evidence the neural origin of the hemodynamic response^[12].

Role of HSP-90 for nNOS-mediated vasorelaxation

Most importantly, all PNS experiments were performed in de-endothelialized mesenteric vasculature in order to exclude any hemodynamic effects of endothelium-derived vasodilators. In order to evaluate the magnitude of nNOS-mediated vasorelaxation, PNS experiments were performed in the absence (1st perfusion cycle) and presence of L-NAME (10 μ mol/L) (2nd perfusion cycle after incubation with L-NAME for 20 min). The differences in PNS-triggered vasorelaxation induced by L-NAME correspond to nNOS-mediated vasodilator effects. As for the role of HSP-90 in mediating this nNOS-vasodilation, vessel preparations were correspondingly pre-incubated with the HSP-90 inhibitor geldanamycin (GA 3 μ g/mL) for 20 min before performing the 2nd perfusion cycle (Figure 1). GA is a benzoquinone ansamycin antibiotic that binds to the nucleotide-binding site of HSP-90 and specifically blocks HSP-90 function^[24,25]. GA was first reported to inhibit iNOS activity in rat smooth muscle^[26], however, as reported earlier no iNOS protein was detected in the vasculature studied, ruling out a hemodynamic role of HSP-90 related to iNOS-derived NO. As shown previously, incubation with GA does not directly affect soluble guanylate cyclase or other smooth muscle cell machinery required for NO-dependent vasodilation since vasodilation is not altered in response to SNP^[17].

Immunofluorescence analysis

Mesenteric tissues were harvested by dissecting and removing the highly vascular tissue situated between the mesenteric lymph nodes and small intestine. The tissue was freed, washed in PBS, stored in 3.7% formaldehyde at 4°C over night, and then transferred into 20% sucrose solution

for more than 12 h at 4°C. After this procedure the SMA were embedded in tissue tek (Herstellter, Stadt, Land) and stored at -80°C. Frozen sections (Frigocut 2800 E, Leica, Wetzlar, Germany) were incubated overnight with monoclonal antibodies directed against nNOS or HSP-90, respectively (both BA, Transduction Laboratory, San Diego, CA, USA). Immunofluorescence staining was performed by utilizing either red fluorescent or green fluorescent anti-mouse IgG (Alexa 46, MoBiTec, Göttingen, Germany) as secondary antibodies. Sections treated with secondary antibody only or with control IgG (as first antibody) did not reveal an immunosignal (data not shown).

Immunoprecipitation and Western blotting

Mesenteric nerves located in near proximity to the SMA were dissected microscopically and washed in PBS and homogenized in a lysis buffer. Protein quantification of tissue samples was performed using the Lowry assay. In experiments examining the level of nNOS and HSP-90 protein in mesenteric tissue, 100 µg of protein were used for electrophoresis. For nNOS immunoprecipitation, about 1500 µg of detergent-soluble protein was incubated with excess nNOS mAb overnight after samples were pre-cleared with Pansorbin (Calbiochem, CA, USA). Immune complexes were precipitated by the addition of protein A-Sepharose. Protein samples were boiled in Laemmli loading buffer and separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) on a 7.5% acrylamide gel, and proteins were electroblotted onto nitrocellulose membranes. Subsequently, the membranes were washed with Tris-buffer saline with 1% Tween, blocked with 5% milk, and incubated with either HSP-90 mAb or nNOS mAb. Densitometry was performed with a scanner and analyzed with the Software Image Quant (Personal Densitometer Si, Amersham, Bioscience, UK).

Statistical analysis

Results were expressed as mean ± SE. Statistical analysis was performed using ANOVA (two-way, with repeated measurements) for comparison of study groups for PNS-induced vasorelaxation. Unpaired non-parametric tests were used for comparison of individual values between study groups. The level of statistical significance was set at $P < 0.05$.

RESULTS

Animals

There were no differences in body weight in the experimental groups (349 ± 36 g for PVL and 363 ± 42 g for sham rats, respectively). PVL rats showed elevated spleen weights, expressed in relation to body weight (PVL: 3.25 ± 0.30 g/kg bw *vs* sham: 2.48 ± 0.50 g/kg bw, $P < 0.0001$).

Western blotting and co-association of nNOS and HSP-90 in mesenteric nerves

To determine the importance of HSP-90 for neuronal vascular control in mesenteric arteries, we first examined expression, association and localization of nNOS and HSP-90 in mesenteric nerves. nNOS expression was

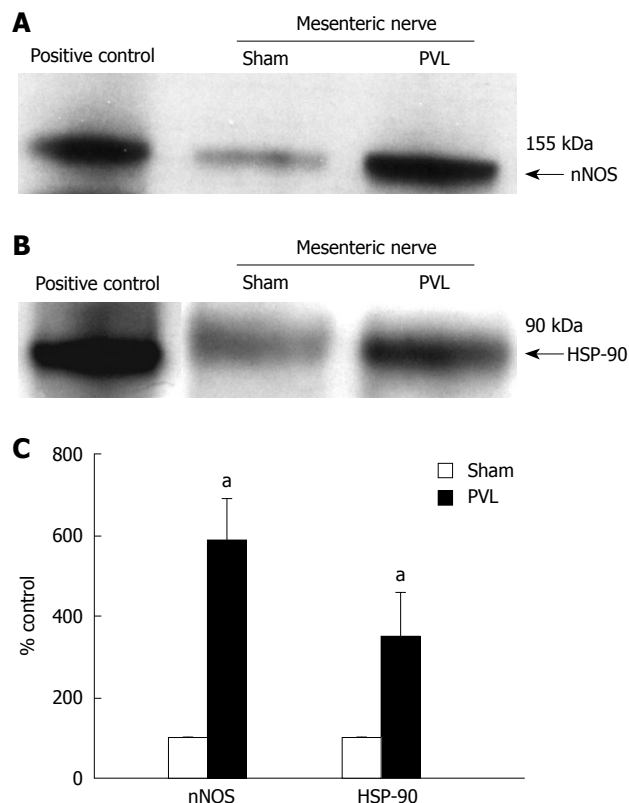


Figure 2 n-nitric oxide synthase (nNOS) and HSP-90 expression in mesenteric nerves. nNOS (A) and HSP-90 (B) in mesenteric nerves of portal-vein-ligated rats (PVL) and sham rats by Western blotting analysis. Protein lysate from rat brain tissue was used as positive control; C: Densitometric analysis of Western blottings revealed a more than 5-fold increase of nNOS and approximately 3-fold increase of HSP-90 protein expression in mesenteric nerves of PVL rats as compared to sham rats. ^a $P < 0.05$, $n = 3$ per group.

found to be increased in PVL rats as compared to sham rats (Figure 2A and C). In addition, HSP-90 expression was enhanced in mesenteric nerves in PVL rats as compared to sham rats (Figure 2B and C) and nNOS co-precipitated with HSP-90 from mesenteric nerves (Figure 3) evidencing direct protein-protein interaction.

Immunofluorescence of nNOS and HSP-90 in mesenteric nerves

We previously reported that both nNOS and HSP-90 are abundantly expressed in mesenteric vasculature in rats^[17]. Here we extend this information with a focus on the mesenteric neurons. In fact, immunofluorescence revealed co-localization, with nNOS and HSP-90 being distributed equally in perivascular neurons (Figure 4A and B). Indeed, identical localization and most obviously similar magnitude of protein expression for nNOS as well as HSP-90 were observed at the entry of the nerve supply into the mesenteric vasculature.

nNOS-dependent mesenteric vasorelaxation and HSP-90

Baseline perfusion pressures before removal of the endothelium were significantly lower in PVL as compared to sham rats (16.0 ± 6.1 mmHg *vs* 23.4 ± 8.0 mmHg, $P < 0.01$). After removal of the endothelium no difference in basal perfusion pressure between study groups was noted anymore (data not shown). The vasodilator response to

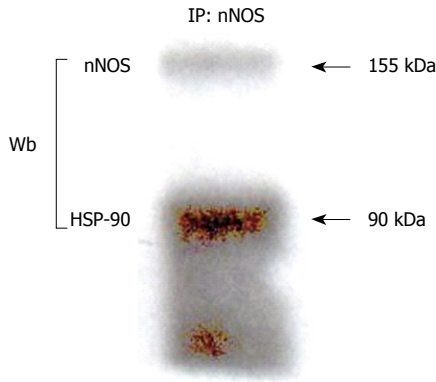


Figure 3 Co-immunoprecipitation. nNOS was immunoprecipitated from detergent-soluble protein lysates prepared from de-endothelialized mesenteric tissue harvested as described in materials and methods and prepared for gel electrophoresis or, alternatively, protein samples were directly prepared for gel electrophoresis. nNOS and HSP-90 are both abundantly expressed in mesenteric tissue and immunoprecipitation (IP) of nNOS coprecipitates HSP-90 under basal conditions. Wb: Western blotting.

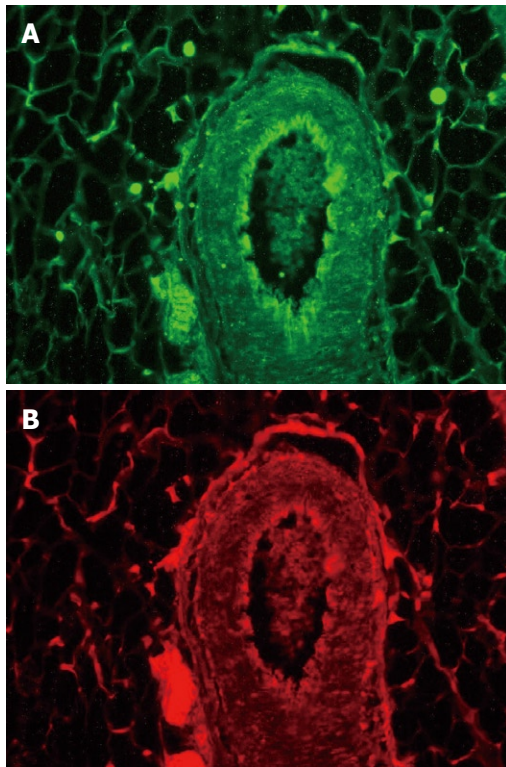


Figure 4 Immunofluorescence analysis of nNOS and HSP-90. As can be seen immunofluorescence shows strong signals for nNOS (A) and HSP-90 (B) mainly at the entry of the nerve bundle along the adventitia revealing identical localization of nNOS and HSP-90 protein in mesenteric nervous tissue.

acetylcholine at the highest dose used was $7.5\% \pm 5.0\%$ and $9.0\% \pm 6.0\%$ in PVL and sham rats, respectively (NS), demonstrating a sufficient de-endothelialization. The vasodilator response to sodium nitroprusside at the highest dose applied was $65\% \pm 7\%$ for PVL and $58\% \pm 9\%$ for sham rats (NS) demonstrating the functional integrity of the vascular smooth muscle cells in the vascular bed studied. NE-induced pre-constriction levels were similar in both study groups (95 ± 5 mmHg *vs* 88 ± 6 mmHg) indicating

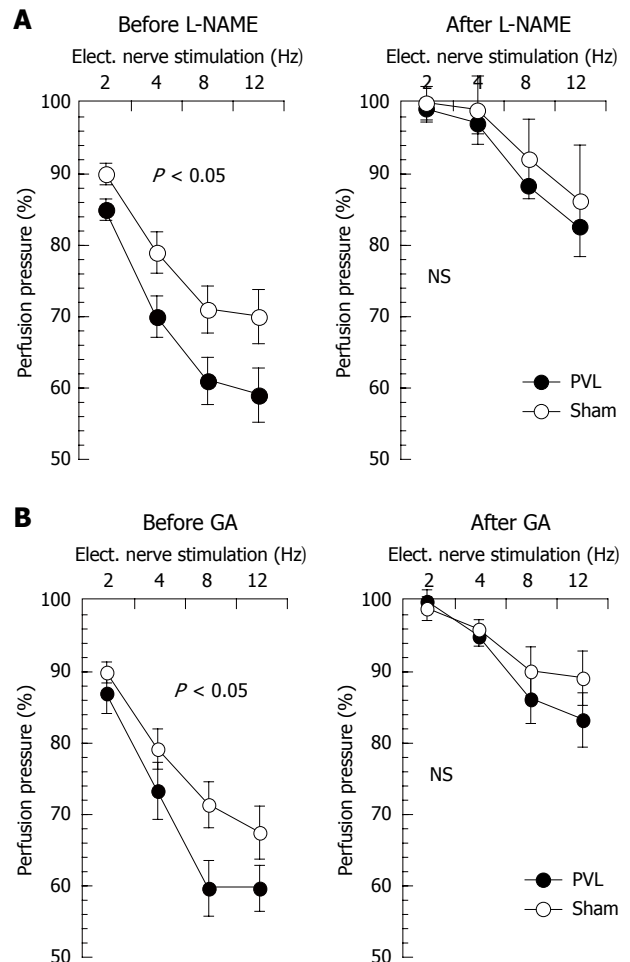


Figure 5 nNOS- and HSP-90-mediated neuronal vasorelaxation in PVL and sham rats. PNS-induced nitrgic vasorelaxation in de-endothelialized mesenteric vasculature is depicted before and after L-NAME (A) as well as before and after HSP-90-inhibition with geldanamycin (B). It can be appreciated that no more significant differences in frequency-dependent neuronal vasorelaxation were observed between the study groups after L-NAME as well as after geldanamycin. Values given as mean \pm SE. PVL-group: $n = 6$; Sham group: $n = 6$. $P < 0.05$ for ANOVA comparing study groups. Elect. nerve stimulation: Electric nerve stimulation; NS: Not significant.

similar vascular tone before neuronal stimulation. Incubation with either L-NAME or GA did not affect baseline or pre-constriction levels in either group. All vessels responded to PNS with a frequency-dependent decrease in perfusion pressure (Figure 5). This neurally mediated vasodilatory response was significantly more pronounced in PVL as compared to sham rats (Figure 5). Non-specific NO-inhibition by L-NAME (Figure 5A) abolished the difference in PNS-induced vasorelaxation between the study groups evidencing NO as being the vasodilator mediating this difference and thus reflecting nNOS-mediated vasorelaxation. Moreover, HSP-90 inhibition using GA likewise abolished the difference in PNS-induced vasorelaxation between PVL and sham rats (Figure 5B). This indicates that the observed increase in nNOS-mediated vasorelaxation in PVL rats is dependent on HSP-90 signalling.

When expressing PNS-induced vasorelaxation as percent change from the pre-constriction level a markedly increased response was observed in vessel preparations from

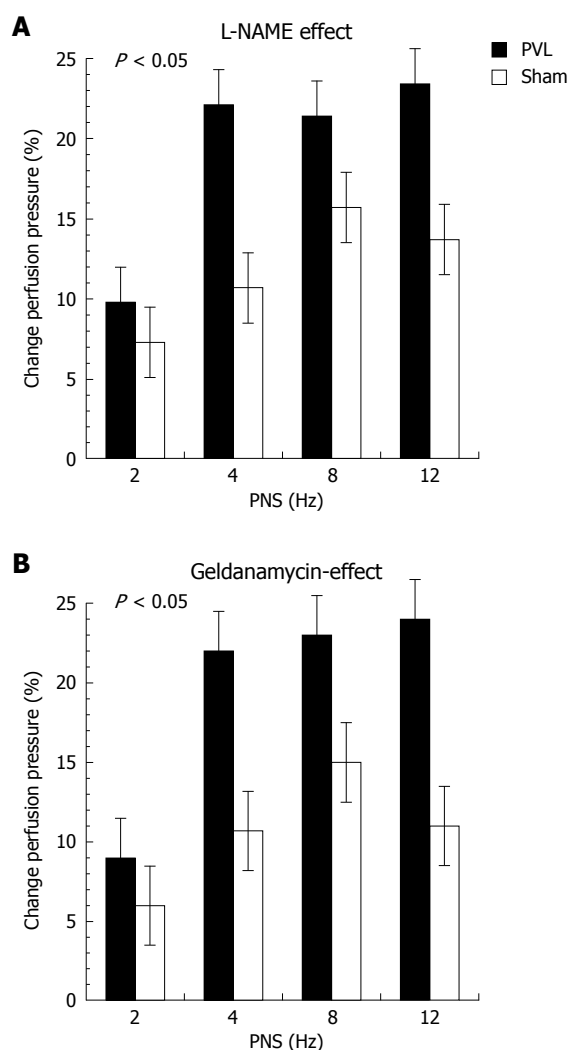


Figure 6 Changes in PNS-induced vasorelaxation induced by inhibition of NOS or HSP-90. Changes in pressure response to PNS induced by L-NAME (A) and geldanamycin (B) presented as percent change in perfusion pressure as compared to values obtained during the first perfusion cycle. $P < 0.05$ (ANOVA comparing all conditions between study groups) vs sham, respectively.

PVL rats as compared to sham rats (Figure 6). Maximal vasodilation was reached at 8-12 Hz, confirming previous investigations of SMA in rats^[27]. Finally, the effect of NO inhibition (Figure 6A) and HSP-90 inhibition (Figure 6B) expressed as percent change in perfusion pressure from PNS-induced vasorelaxation observed during the corresponding 1st perfusion cycle induced by L-NAME or GA was significantly more pronounced in PVL rats as compared to sham rats ($P < 0.05$). In addition, the magnitude of effect induced by L-NAME and GA was almost identical indicating that the majority of nNOS-mediated vasorelaxation is dependent on HSP-90 signalling.

DISCUSSION

HSP-90 acts as an intermediate in the signalling cascade leading to activation of nNOS^[28,29]. In this study, we examined the role of this protein interaction in mediating neuronal regulation of vascular tone in the mesenteric vascular bed, where vascular resistance contributes to the regulation

of portal pressure. The novel findings of this study include the interaction of HSP-90 and nNOS in mesenteric nerves and the participation of this interaction in regulating vasomotor function in resistance vessels based on inhibition of HSP-90 with GA. Moreover, we demonstrate that after PVL, enhanced HSP-90 signalling contributes to the well-known elevated nNOS-dependent vasorelaxation.

Numerous investigations have presented evidence that nitric nerves are involved in vascular homeostasis^[30,31]. However, mechanisms leading to nNOS-derived NO release from nitric nerve endings are missing. Under physiological conditions, NO production is strictly modulated to accommodate the need of homeostasis because abnormalities of NO formation cause disease^[32,33]. Since NO is highly diffusible and cannot be stored in intracellular compartments modulation of NO production in cells is primarily mediated through regulating NOS activity. Several mechanisms have been revealed in NOS regulation. These include protein-protein interactions, protein phosphorylation, and subcellular targeting^[34]. For example, regulation *via* protein-protein interaction such as HSP-90 and NOS appears particularly important. HSP-90 is one of the most abundant proteins in cells^[35] constituting 1%-2% of total intracellular proteins. As a molecular chaperone, HSP-90 has been shown to regulate more than 100 signal transduction pathways by controlling function, trafficking and turnover of a variety of signalling proteins^[36]. In respect to NOS regulation, HSP-90 was first reported to associate with eNOS acting as an allosteric enhancer^[15]. Subsequent studies with purified nNOS preparations indicated that HSP-90 likewise functions as an allosteric enhancer for nNOS and inhibition of HSP-90 results in the inability to form the dimeric active nNOS^[28]. Coupling of nNOS with HSP-90 is crucial for normal nNOS function^[37]. Here, we report co-localization of HSP-90 and nNOS in mesenteric nerves. In addition, nNOS of mesenteric nerves co-immunoprecipitates with HSP-90 indicating protein-protein interaction in this neuronal tissue. Finally, GA was used to probe the importance of HSP-90 in cellular pathways, since it directly binds to the amino-terminal ATP binding domain of HSP-90^[38] and specifically labels HSP-90 in cellular extracts^[25]. Since NANC-induced vasorelaxation in the mesenteric vasculature is reduced after inhibition of HSP-90 by GA, the hemodynamic functional relevance of the stated protein-protein interaction is demonstrated. To our knowledge, this is the first report to demonstrate a physiological role of HSP-90 for regulation of neuronal control of vascular tone via modulation of nNOS-derived NO release. This is in accordance with previous reports demonstrating that HSP-90 inhibition causes a decrease of NO production in nNOS-transfected cells^[16,39].

Portal hypertension is characterized by decreased systemic and particularly splanchnic vascular resistance which is the initiating mechanism for the development of the hyperdynamic circulatory syndrome in this entity^[40]. Therefore, any measure improving splanchnic vascular resistance is of high hemodynamic and clinical relevance. Vascular overproduction of NO has been shown to be the pathophysiological hallmark leading to this splanchnic vasodilation and to be due to a marked upregulation of

eNOS and nNOS-derived NO synthesis^[4-6,12]. Indeed, either non-specific or nNOS-specific NOS inhibition has been demonstrated to ameliorate the development of the hyperdynamic circulatory syndrome in experimental portal hypertension^[9,18]. In addition, enhanced eNOS and nNOS protein expression as well as an increased vasodilatory response to eNOS and nNOS stimulation has been reported by us and others in the mesenteric vasculature during experimental portal hypertension^[5,12,41]. Beside AKT phosphorylation^[42], a marked contributory role of HSP signalling for eNOS-derived NO overproduction has been reported by us previously^[17]. Here, we show that HSP-90 also largely mediates the enhancement in nNOS-dependent NO release in the mesenteric vasculature of pre-hepatic portal hypertensive rats. This is based on the observation of enhanced HSP-90 expression in mesenteric nerves of portal hypertensive rats and the finding that inhibition of HSP-90 signalling preferentially blocks NANC vasorelaxation in these animals.

Enhanced HSP-90 protein expression has been reported previously in whole mesenteric tissue of portal vein ligated rats^[43]. However, evaluation of HSP-90 expression in mesenteric nerves has not been reported before. Nonetheless, it has been evidenced in cell lines as well as in studies with purified nNOS that HSP-90 directly augments NO synthesis of nNOS in a dose-dependent manner^[16,37,44]. This effect has been attributed mainly to an increase in binding of calmodulin to nNOS mediated by HSP-90^[44]. By this mode of action, most importantly HSP-90 treatment has been shown to increase even the maximal nNOS activity and associated maximal nNOS-dependent NO synthesis achievable^[44]. Therefore, we propose that increased HSP-90 protein expression observed in mesenteric nerves of portal hypertensive rats results in enhanced nNOS-mediated vasorelaxation in the mesenteric vasculature of these animals due to its well-known augmenting effect on the nNOS-machinery. However, neither inhibition of NO nor HSP-90 did completely abrogate NANC vasorelaxation. Remaining vasodilation may well be mediated by other endogenous neurogenic vasodilators such as calcitonin-gene-related peptide or vasoactive-intestinal peptide known to be released from NANC nerves, particularly in rats. Nonetheless, these mediators are not mediating any differences in NANC-vasodilatory responses between the study groups since no significant difference in NANC vasorelaxation remained after NOS inhibition.

In summary, HSP-90 co-localizes with and binds to nNOS in mesenteric nerves playing a key role in nNOS-dependent vasoregulation in the mesenteric vasculature. This interaction serves to mediate the increased nitrgic vasorelaxation observed in the splanchnic circulation during experimental portal hypertension. In conjunction with our previous report on the excessive eNOS-derived NO overproduction depending mainly on HSP-90 signalling this study may provide the rational for pharmacological inhibition of HSP-90 as a novel target for the treatment of the hyperdynamic state in portal hypertension.

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COMMENTS

Background

Portal hypertension is the main cause of death in end-stage liver disease leading to multiple complications such as variceal hemorrhage, ascites and hepatorenal syndrome. Vasodilatation in the mesenteric circulation is of essential pathophysiological relevance in portal hypertension and n-nitric oxide synthase (nNOS) has gained much attraction as a vasodilation mediator in other clinical entities recently.

Research frontiers

Heat shock protein-90 (HSP-90) is a molecular chaperone required for the stability and activity of a diverse range of client proteins that have critical roles in signal transduction, cellular trafficking, cell growth, differentiation *etc.* but also vasoregulation. Among NOS it has been shown to be critical for full e- and nNOS activity. However, the role of HSP-90 in respect to neural nNOS-mediated vasoregulation has not been addressed in healthy or in portal hypertensive conditions. The strength of the current investigation relates to the utilization of the McGregor preparation enabling physiological and pharmacological testing of the whole mesenteric vasculature in its original anatomy and innervations. In contrast to arterial strips, this ensures testing of neural vasoregulation at close to *in vivo* conditions.

Innovations and breakthroughs

By this approach, pronounced nNOS-mediated vasodilation is confirmed in pre-hepatic portal hypertension and most strikingly is shown to be in large parts due to HSP-90 signalling. This is also the first time that HSP-90 has been established as a signaling mediator in neural vascular responses in healthy conditions.

Applications

Considering the well-known role of HSP-90 as an eNOS chaperone and enhanced eNOS-derived NO overproduction in portal hypertension, HSP-90 may well be an ideal target in treatment of portal hypertension.

Peer review

This is a good experimental investigation in which authors analyze the role of HSP-90 for neural vasoregulation in the mesenteric circulation in healthy and portal hypertensive conditions. The results are interesting and suggest that HSP-90 indeed is the main signaling intermediate translating into enhanced nNOS-dependent vasodilation during portal hypertension. Therefore, inhibition of HSP-90 could be used to treat portal hypertension.

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Different profiles of cytokine expression during mild and severe acute pancreatitis

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Abstract

AIM: To study secretion patterns of pro- and anti-inflammatory cytokines, and activation of various cellular subsets of leukocytes in peripheral blood.

METHODS: We have conducted a prospective observational study. One hundred and eight patients with a diagnosis of acute pancreatitis and onset of the disease within last 72 h were included in this study. The mRNA expression of 25 different types of cytokines in white blood cells was determined by quantitative real time polymerase chain reaction. Levels of 8 different cytokines in blood serum were measured by enzyme

linked immunosorbent assay. Clinical data and cytokine expression results were subjected to statistical analysis.

RESULTS: Severe and necrotizing acute pancreatitis (AP) is characterized by the significant depletion of circulating lymphocytes. Severe acute pancreatitis is associated with a typical systemic inflammatory response syndrome and over-expression of pro-inflammatory cytokines [interleukin (IL)-6, IL-8, macrophage migration inhibitory factor (MIF)]. Serum IL-6 and MIF concentrations are the best discriminators of severe and necrotizing AP as well as possible fatal outcome during the early course of the disease.

CONCLUSION: Deregulation of cellular immune system is a key event leading to severe and necrotizing AP. IL-6 and MIF could be used as early predictors of complications.

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Key words: Acute pancreatitis; Cytokines; Prognostic factors

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INTRODUCTION

Incidence of acute pancreatitis (AP) is about 30-40 cases per 100 000 individuals, and it carries an overall mortality

rate of 10%-15%^[1-3]. Mortality of patients with severe acute pancreatitis approaches 30%-40%^[4-6]. Pancreatic necrosis develops early in the course of the disease and usually is well established by 96 h after the onset of clinical symptoms^[7,8]. Both the risk of multiorgan failure (MOF) and infectious complications appear to be related to the degree of pancreatic necrosis^[9]. If patients survive the critical early stages of systemic inflammatory response, development of septic complications is a major determinant of survival. Infected pancreatic necrosis and sepsis-related multiple organ failure account for up to 40%-50% of all deaths among AP patients^[10-13].

At present it is widely accepted that the premature activation of digestive enzymes within pancreatic acinar cells is an initiating event that leads to the autodigestion of pancreas^[14-16]. Once the disease process is initiated, common inflammatory and repair pathways are invoked. There is a local inflammatory reaction at the site of injury; if marked, this leads to a systemic inflammatory response syndrome (SIRS). An imbalance between the early SIRS, and the later compensatory counter-inflammatory response (CARS), and development of MOF are considered to be the primary causes of morbidity and mortality in severe acute pancreatitis. Excessive leukocyte activation (including neutrophils and monocyte-macrophage lineage) with cytokinemia plays a critical role in the pathogenesis of pancreatitis and even more so, of the subsequent inflammatory response^[14-17]. It has been hypothesized that fatal pancreatitis is a consequence of abnormal phagocytic leukocyte hyperstimulation due to deregulation in T- and B-lymphocyte activation. However, the role of lymphocyte activation and its relation to the severity of disease in humans is still poorly understood^[18-21]. Another important drawback, in our opinion, is that the majority of information about the alterations of the immune system during the AP comes from *in vitro* and *in vivo* studies, and therefore it is not always directly applicable and relevant to the clinical situation in human acute pancreatitis.

Several methods for estimating severity of acute pancreatitis are widely used today and include APACHE II, Imrie and Ranson scores, the CT scoring system, and measurement of C-reactive protein and a number of laboratory markers^[22-28]. Most of these multifactorial scores and laboratory tests are very good at identifying the critical situation, when MOF or local complication supervene, however, none can accurately predict the disease severity, development of pancreatic necrosis and/or possible fatal outcome during the first hours of hospitalization.

Thus, the aims of this study have been to investigate the secretion patterns of pro- and anti-inflammatory cytokines, and to estimate the activation of various cellular subsets of leukocytes (including lymphocytes, neutrophils, and monocyte-macrophage lineage) in peripheral blood of patients with severe and mild AP. Another goal of this study was to identify the serum soluble molecules that are associated with the development of severe acute pancreatitis, and which could be used as early markers to assess the local (pancreatic necrosis) and systemic complications (SIRS, MOF) later in the course of the disease.

MATERIALS AND METHODS

Study design and patient population

We have conducted a prospective observational study in the period between June 2005 and December 2007. All patients admitted to the Department of Surgery at Kaunas University of Medicine Hospital (Lithuania) with a diagnosis of acute pancreatitis and onset of the disease within last 72 h were included in this study ($n = 108$). The Regional Ethics Committee approved the study (protocols No. BE-2-47 and P1-113/2005) and all the patients provided written informed consent.

The diagnosis was established on the basis of acute abdominal pain, at least 3-fold elevated levels of serum amylase and typical radiological findings. A contrast-enhanced CT scan was performed on days 4 to 7 after onset of the disease to demonstrate the presence of pancreatic necrosis. According to the clinical course and clinical severity scores (APACHE II > 7; Imrie-Glasgow > 2; MODS > 2), patients were stratified into mild and severe acute pancreatitis groups. Clinical data relating to the severity of disease, development of organ dysfunction and/or septic complications were prospectively collected in a standardized fashion. Patients with underlying chronic pancreatitis and patients with acute pancreatitis referred to our hospital from other institutions after management for more than 3 d were excluded from this study. Age- and sex-matched healthy subjects ($n = 18$) without previous medical history were enrolled as controls.

Peripheral blood samples from patients were drawn on admission to the hospital and, after centrifugation, samples were stored at -80°C until analysis. The blood samples of control group underwent a similar process. Blood sample analysis was uniformly performed at the Laboratory for Molecular Research of Pancreas, Department of Surgery, Heidelberg University (Germany). The mRNA expression of 25 different types of cytokines in white blood cells (WBCs) was determined by quantitative real time polymerase chain reaction (QRT-PCR). Levels of 8 different cytokines in blood serum were measured by enzyme linked immunosorbent assay (ELISA) according to the manufacturer's instructions (Sigma-Aldrich GmbH, Steinheim, Germany).

Statistical analysis

Clinical data and cytokine expression results were used for statistical analysis. Statistical analysis was performed using SPSS® for Windows release 14.0 (SPSS, Chicago, Illinois, USA). Discriminant function analysis was used to determine differences between the groups. The diagnostic performance of a test, with a particular cut-off value, was established using Receiver Operating Characteristic (ROC) curve analysis. The data are presented as mean \pm SE or median. For comparison between groups, the Mann-Whitney test or Student's *t* test were employed where appropriate. Results with $P < 0.05$ were considered statistically significant.

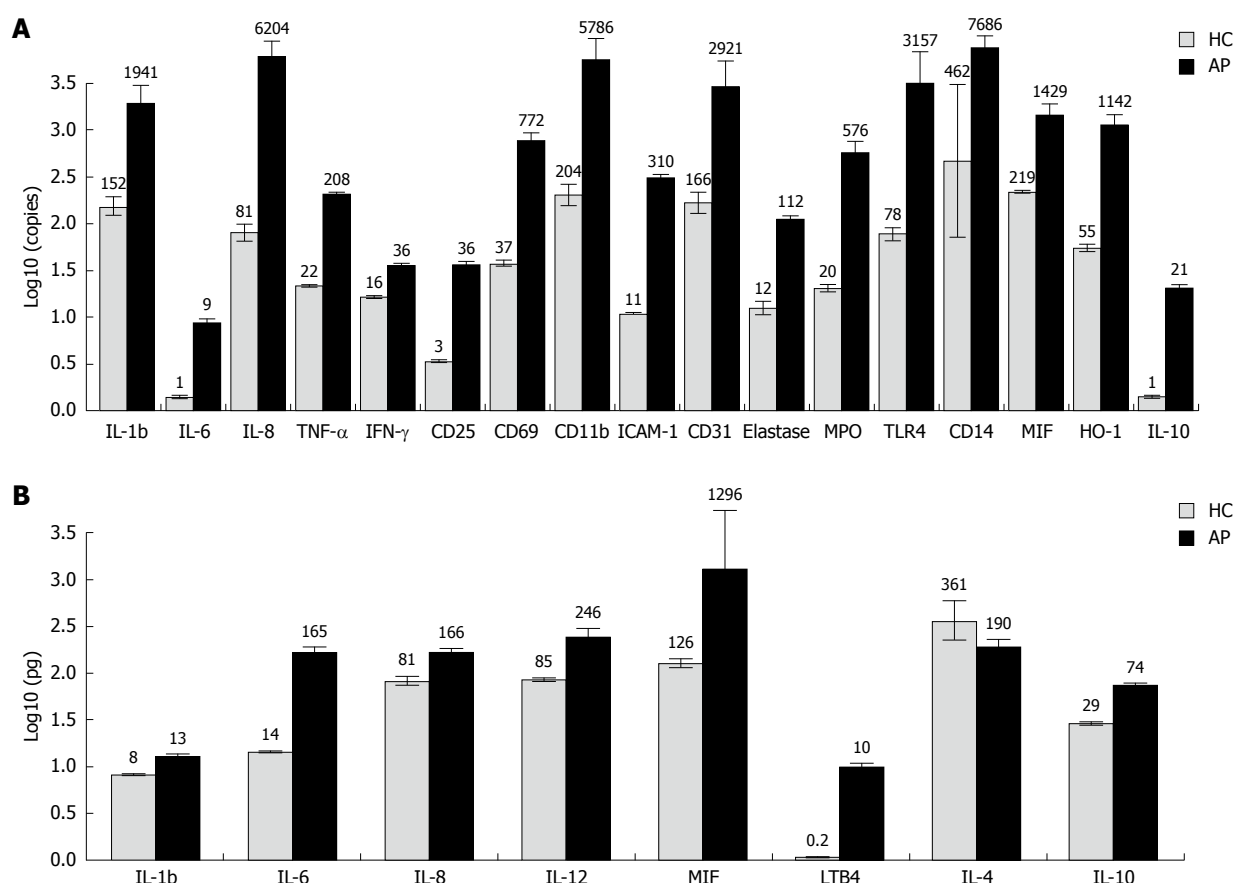


Figure 1 Cytokine expression at mRNA and protein level in peripheral blood of healthy subjects and acute pancreatitis (AP) patients. A: Gene expression in peripheral blood leukocytes of healthy subjects and AP patients. Gene expression at mRNA level of 25 different cytokines, adhesion molecules, lymphocyte activation markers, and other biologically active substances known to be linked to the inflammatory response were assessed by quantitative real time polymerase chain reaction (QRT-PCR) in peripheral blood leukocytes of AP patients and healthy controls. A median 18-fold (range: 2-77) increase in mRNA expression of pro-inflammatory molecules was observed in the AP group in comparison to healthy control subjects (HC). Activation of the anti-inflammatory system and some cytoprotective molecules was also notable in the AP patient group. Cytokine mRNA expression levels with statistically significant differences between AP and healthy control groups are shown; B: Cytokine concentration in blood serum of healthy controls and AP patients. Expression of eight different soluble molecules at protein level was assessed in serum of AP patients and healthy subjects using the enzyme linked immunosorbent assay (ELISA) method. A median 3-fold (range: 2-59) increase in protein expression of pro-inflammatory molecules was observed in the AP group when compared to HC. Cytokine expression levels with statistically significant differences between AP and healthy control groups are shown. IL: Interleukin; TNF- α : Tumor necrosis factor- α ; IFN- γ : Interferon- γ ; MPO: Myeloperoxidase; MIF: Macrophage migration inhibitory factor.

RESULTS

One hundred and eight patients (55 male and 53 female, mean age 54.1 ± 16.7 years) diagnosed with AP were included in the study. The main etiological factors of AP were alcohol (42.6%), high-fat diet (25.9%) and biliary stones (25.0%). Idiopathic pancreatitis was diagnosed in 7 cases (6.5%). Severe acute pancreatitis (APACHE II > 7) comprised 44.4% of all cases. Necrotizing AP was detected by contrast-enhanced CT in 52 patients (48.1%), and high volume ($> 30\%$) necrosis was present in 34 cases (31.3%). Multiple organ failure (> 2 systems) developed in 18 cases (16.7%). Overall mortality in our follow-up group was 12.9%; 9 patients (8.3%) died within first 2 wk of the disease and 5 patients (4.6%) later in the course of the disease. Eighteen healthy subjects (10 male and 8 female, mean age 40.1 ± 19.8 years) were studied as controls.

Gene expression at mRNA level of 25 different cytokines, adhesion molecules, lymphocyte activation markers, and other molecules known to be linked to the inflammatory response were assessed in peripheral blood

leukocytes of AP patients and healthy controls. Results showed a typical pro-inflammatory cytokine profile in AP patients with an activation of different subsets of leukocytes (lymphocytes, monocyte-macrophage lineage and neutrophils). Activation of anti-inflammatory system and some cytoprotective molecules was also notable in the AP patient group (Figure 1A). A median 18-fold (range: 2-77) increase in mRNA expression of pro-inflammatory molecules was observed in the AP group in comparison to healthy controls. Expression of eight different soluble molecules at protein level was also assessed in peripheral blood serum of AP patients and healthy subjects using the ELISA method. Analysis revealed a typical over-expression of pro-inflammatory cytokines and altered expression of anti-inflammatory cytokines in AP patients compared to the healthy volunteers (Figure 1B). A median 3-fold (range: 2-59) increase in protein expression of pro-inflammatory molecules was observed in the AP group in comparison to healthy controls. Since many of these changes have been shown in other animal and clinical studies, this served as a

Table 1 Differences in clinical course of mild and severe acute pancreatitis (mean \pm SD/count)

Clinical severity scores and patient characteristics	Mild AP	Severe AP	P
Apache II (score)	3.6 \pm 1.9	11.6 \pm 4.7	< 0.01
Imrie-Glasgow (score)	2.2 \pm 1.2	4.4 \pm 1.5	< 0.01
MODS (score)	1.1 \pm 1.3	4.3 \pm 3.4	< 0.01
Necrotizing AP (count)	16	36	< 0.01
C-reactive protein (mg/L)	91.8 \pm 18.7	152.9 \pm 21.5	< 0.01
Necrosis volume > 30% (count)	8	26	< 0.01
Infectious complications (count) ¹	2	12	< 0.01
Presence of MOF (count)	0	18	< 0.01
Need for surgery (count) ²	1	5	0.06
ICU stay (d)	1.0 \pm 1.0	9.0 \pm 2.0	< 0.05
Total hospital stay (d)	12.0 \pm 1.0	19.0 \pm 3.0	< 0.05
Number of early deaths (< 2 wk)	0	9	< 0.01
Number of late deaths (> 2 wk)	0	5	< 0.01

¹Infectious complications: proven sepsis, infected pancreatic necrosis, lung and urinary infection; ²Need for surgery: fasciotomy for intraabdominal compartment syndrome and/or necrosectomy. AP: Acute pancreatitis; MOF: Multiple organ failure; ICU: Intensive care unit.

control and proved that our methods function well.

For the next step of statistical analysis, AP patients were stratified into groups according to the severity of the disease based on presence of systemic (development of MOF) and local (pancreatic necrosis > 30%) complications, since these are the factors known to be associated with a poor outcome. Groups were formed on the basis of radiological findings on contrast-enhanced CT and APACHE II clinical score, where all patients with score > 7 were ascribed to the severe acute pancreatitis (SAP) group. Both SAP and necrotizing AP were associated with higher incidence of MOF, higher mortality and prolonged ICU and overall hospital stay. Whereas mild AP patients showed 0% mortality, overall mortality rate among SAP patients was 27% ($P < 0.01$) and early mortality (during first two weeks from the onset of the disease) was 64% ($P < 0.01$). In the necrotizing AP group overall mortality rate was 25% ($P < 0.01$), and early mortality was 62% ($P < 0.01$), compared to the 1.8% mortality rate seen in the edematous pancreatitis group. The main differences in clinical course and outcome of patients with mild and severe disease are revealed in Table 1. Interestingly, both severe and necrotizing AP were associated with a significant reduction in number of circulating blood lymphocytes, although there was no statistically significant difference in total WBC count (Figure 2). The mean WBC count in the SAP group was $14.76 \pm 0.95 \times 10^9/L$ compared to $13.92 \pm 0.62 \times 10^9/L$ in the mild AP group (n.s.). The mean WBC count in the necrotizing AP group was $14.38 \pm 0.91 \times 10^9/L$ compared to $14.27 \pm 0.75 \times 10^9/L$ in the edematous AP group (n.s.). However, the number of circulating lymphocytes in peripheral blood was reduced by 25% in the SAP compared to the mild AP group ($P < 0.05$), and by 17% in the necrotizing AP group compared to the edematous AP group ($P < 0.05$). Based on the significance and clinical relevance of these findings we investigated the presence of possible common molecular pathways leading to development of severe and necrotizing AP.

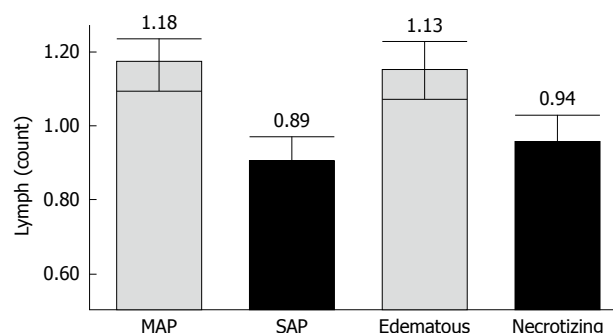


Figure 2 Reduction in number of lymphocytes during severe and necrotizing AP. Number of circulating lymphocytes in peripheral blood was significantly reduced in severe acute pancreatitis (SAP) compared to mild AP groups ($0.89 \pm 0.08 \times 10^9/L$ vs $1.18 \pm 0.07 \times 10^9/L$, $P < 0.05$), and in necrotizing compared to edematous pancreatitis groups ($0.94 \pm 0.08 \times 10^9/L$ vs $1.13 \pm 0.08 \times 10^9/L$, $P < 0.05$). These findings demonstrate the presence of possible common molecular pathways leading to the development of severe and necrotizing AP.

QRT-PCR analysis of cytokine mRNA expression levels in patients with severe and mild disease revealed some conflicting results (Figure 3A). Firstly, study showed that SAP is characterized by decreased intracellular mRNA levels of interleukin (IL)-1 and interferon- γ (IFN- γ). Secondly, SAP was also associated with a marked decrease in the levels of CD25 and CD69 mRNA in peripheral blood leukocytes. However, severe and necrotizing pancreatitis was associated with a very high activation of neutrophils and macrophages as demonstrated by nearly ten-fold higher mRNA expression of elastase and myeloperoxidase in peripheral WBCs. Another quite unexpected result showed a significantly lower mRNA expression of ICAM-1 and macrophage migration inhibitory factor (MIF) in peripheral WBCs (but not in the blood serum) from patients with SAP compared to that of mild AP patients. Analysis of cytokine protein expression levels showed that SAP was associated with a typical SIRS and 2-5 fold increase in expression of pro-inflammatory cytokines (IL-6, IL-8, MIF), as well as induction of compensatory and regulatory mechanism (IL-10) (Figure 3B). Enormously high levels of leukotriene B4 (LTB4) associated with neutrophil activation and injury to the vital organs were recorded in patients who died during the early stages of the disease.

Another important issue is whether the serum soluble molecules could be used as accurate early markers to predict the development of local (pancreatic necrosis) and systemic complications (SIRS, MOF) later in the course of the disease. Discrimination function analysis was used to determine differences between the groups of patients with mild and severe AP, edematous and necrotizing AP, and between survivors and those with fatal outcome. We included five variables (IL-6, IL-8, IL-10, MIF and LTB4) in a study in order to find out which serum markers are the best discriminators of the above mentioned groups. At each step, the variable that minimized the overall Wilks' Lambda was entered into the discriminant model thus revealing that IL-6 and MIF values were significant discriminators between groups of patients with mild and severe disease, as well as between survivors and non-survivors.

Table 2 Serum IL-6 and MIF are significant discriminators between groups of AP patients

Step	Entered	Wilks' Lambda							
		Statistic	df 1	df2	df3	Exact F			
						Statistic	df 1	df2	P
Severe <i>vs</i> mild									
1	IL-6	0.739	1	1	70.000	24.696	1	70.000	0.000
2	MIF	0.675	2	1	70.000	16.635	2	69.000	0.000
Edematous <i>vs</i> necrotizing									
1	IL-6	0.851	1	1	70.000	12.261	1	70.000	0.001
2	MIF ¹	0.943	1	1	70.000	4.247	1	70.000	0.043
Survivors <i>vs</i> non-survivors									
1	IL-6	0.761	1	1	70.000	22.005	1	70.000	0.000
2	MIF	0.709	2	1	70.000	14.153	2	69.000	0.000

¹Not included in the original model using the standard stepwise method due to its low discrimination value. At each step, the variable that minimizes the overall Wilks' Lambda is entered. IL-6: Interleukin-6; MIF: Macrophage migration inhibitory factor.

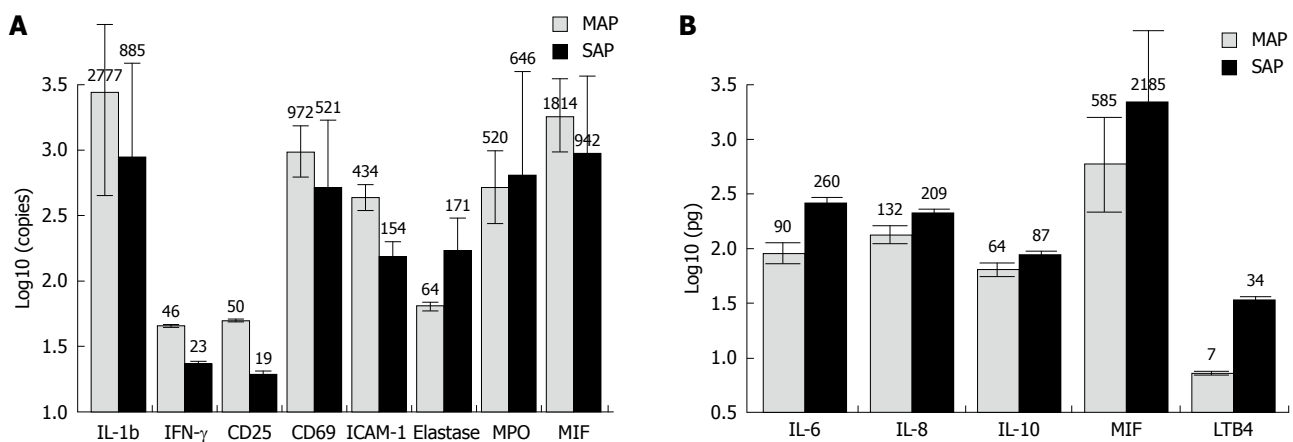


Figure 3 Cytokine expression at mRNA and protein level in peripheral blood of patients with mild and severe AP. A: Gene expression in peripheral blood leukocytes of MAP and SAP patients. QRT-PCR analysis revealed that SAP is characterized by significantly decreased intracellular mRNA levels of interleukin (IL)-1 and interferon (IFN)-γ. SAP is also associated with markedly lower mRNA expression levels of CD25 and CD69 in peripheral blood leukocytes. However, severe and necrotizing pancreatitis was associated with a very high activation of neutrophils and macrophages as demonstrated by nearly ten-fold higher mRNA expression of elastase and myeloperoxidase in peripheral white blood cells (WBCs). A significantly lower mRNA expression of ICAM-1 and macrophage migration inhibitory factor (MIF) was observed in peripheral WBCs (but not in the blood serum) from patients with SAP compared to that of MAP group. Cytokine mRNA expression levels with statistically significant differences between MAP and SAP groups are depicted; B: Cytokine concentration in blood serum of MAP and SAP patients. Analysis of cytokine expression at protein levels shows that SAP is associated with a typical systemic inflammatory response syndrome (SIRS) and 2-5 fold increase in expression of pro-inflammatory cytokines (IL-6, IL-8, MIF). Induction of a compensatory and regulatory mechanism (IL-10) is also observed in this group. Up to 4-5 fold higher levels of LTB4 were recorded in patients who died during the early stages of the disease. Cytokine expression levels with statistically significant differences between AP and healthy control groups are shown.

Statistical analysis revealed that IL-6 values differed significantly in groups of patients with and without pancreatic necrosis (Table 2). A particular cut-off value for IL-6 and MIF was established using Receiver Operating Characteristic (ROC) curve analysis to practically discriminate cases with severe, necrotizing and fatal acute pancreatitis (Figure 4A and B). Comparison of ROC curves for IL-6 and MIF showed no significant differences in diagnostic performance of these two routine tests, except for the discrimination of edematous and necrotizing pancreatitis where IL-6 proved to be of superior value.

DISCUSSION

Alteration of the immune system is one of the major mechanisms responsible for early and late mortality in severe AP. Excessive inflammatory reaction, known as

systemic inflammatory response syndrome (SIRS), is considered as the leading cause of death in early AP^[14,19,20,29]. The role of lymphocytes in this phenomenon has been partly studied and the knowledge of the mechanisms in humans is still incomplete^[20,30-35]. In agreement with previous studies, we found a significant depletion of circulating lymphocytes which was much more profound in the severe and necrotizing forms of pancreatitis^[20,30-35]. In our study, significant activation of lymphocytes was observed in the case of AP compared to healthy controls, as shown by strong expression of CD69 and CD25. The surface receptors CD69 and CD25 (IL-2 receptor) are early markers of lymphocyte activation. Increased expression of CD69 as well as of CD25 on CD3+, CD4+ and CD8+ cells have recently been reported in mild AP. It has also been suggested that the number of B-lymphocytes (CD19+) expressing CD69 was significantly lower in patients with

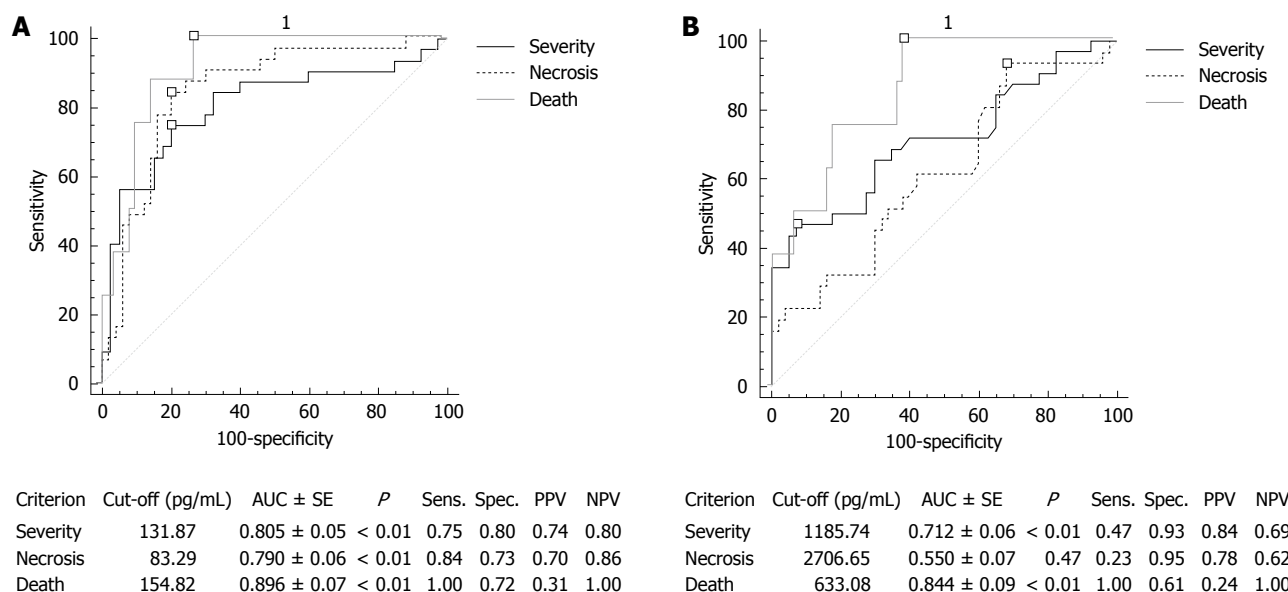


Figure 4 Prognostic utility of IL-6 and MIF in diagnosis of severe, necrotizing and fatal AP. A: Performance of serum IL-6 in diagnosis of severe, necrotizing and fatal AP. The diagnostic performance of a test for discriminating severe, necrotizing and fatal cases of AP was evaluated using Receiver Operating Characteristic (ROC) curve analysis. Study revealed that serum IL-6 concentration is a good predictor of the severe disease and systemic complications (SIRS, MOF); this marker could also be utilized for the stratification of patients with necrotizing AP and those with possible fatal outcome; B: Performance of serum MIF in diagnosis of severe, necrotizing and fatal AP. The diagnostic performance of a test for discriminating severe, necrotizing and fatal cases of AP was evaluated using ROC curve analysis. Study revealed that serum MIF concentration is a good predictor of the severe disease and systemic complications (SIRS, MOF); this marker could be used for early identification of patients with possible fatal outcome. Comparison of ROC curves for IL-6 and MIF showed no statistically significant differences in this respect ($P = 0.18$ for severity; $P = 0.58$ for mortality). However, serum MIF concentration has very poor prognostic value in predicting the development of pancreatic necrosis and is inferior to IL-6 serum concentration in this respect ($P < 0.01$). AUC: Area under curve; Sens.: Sensitivity; Spec.: Specificity; PPV: Positive predictive value; NPV: Negative predictive value.

severe pancreatitis than in patients with mild pancreatitis^[20,31,32]. We have also observed a significantly lower expression of both CD69 and CD25 surface receptors in peripheral blood leukocytes from SAP patients. Marked activation of different subsets of lymphocytes may explain why we have observed strong elevation of different cytokines in peripheral blood, despite a significant decrease in the number of circulating lymphocytes. One of the possible reasons for the decreased number of circulating lymphocytes is strong migration of activated cells to the sites of inflammation, including the pancreas and other tissues such as lungs or kidneys, as an element of SIRS^[16,20,36]. Another possible explanation is an excessive elimination of lymphocytes by apoptosis. Interestingly, the available data indicate that lymphopenia caused by the decrease of different lymphocyte subpopulations is also characteristic for other diseases with severe tissue damage, e.g. critical injury, sepsis and acute severe respiratory syndrome (ARDS)^[37-40].

Polymorphonuclear leukocytes, monocytes, tissue macrophages, lymphocytes, natural killer cells, and parenchymal cells are involved in a complex network of the host defense response in the case of tissue damage caused by severe AP, burns, accident trauma, major surgical interventions or sepsis^[20,39,41,42]. An overwhelming pro-inflammatory response (hyperinflammation) leads to the clinical manifestations of SIRS and finally to host defence failure expressed by multiple organ failure (MOF). The up-regulation of pro-inflammatory factors, such as TNF, IL-1 and IL-6, observed during the SIRS phase can be followed by

a second response that involves down-regulation of IFN- γ and increase in anti-inflammatory cytokines, such as IL-10 and transforming growth factor- β . This counter regulatory phenomenon is called the compensatory anti-inflammatory response syndrome (CARS)^[17,20,39]. However, this can result in the development of immunosuppression and is often associated with elevation of IL-10 and the shift of the Th1/Th2 balance towards a Th2 response^[17,20,39,42]. The network of various cytokines and other molecules participating in the regulation of the inflammatory processes is indeed very complex, and the precise timing of the release and activation of these mediators is not well known. Nevertheless, our study also shows that both SIRS and CARS occur in a real clinical situation and at a certain stage of the disease might even develop simultaneously. However, in the case of severe AP, the counter-regulatory mechanisms may lead to the dysfunction of immune cells mainly due to the significant decrease in total systemic lymphocyte count and the hyper-production of IL-10, which might suggest that T regulatory 1 cells (Tr1) are especially active in the course of severe disease^[43].

Involvement of other cellular subsets of leukocytes, mainly neutrophils and monocyte-macrophage lineage, in the development of SAP is also readily supported by our study. Neutrophils exert their toxicity by releasing myeloperoxidase (MPO), proteases such as elastase and reactive oxygen species (ROS) such as H₂O₂^[36]. In our study we have observed a very high mRNA expression of MPO and polymorphonuclear leukocyte elastase in cases of severe and necrotizing pancreatitis. Increased production

of platelet activating factor (PAF) and LTB4 is also closely associated with an activation of monocytes and neutrophils, and increased migration of neutrophils to pancreas and other target organs, including lungs and kidneys^[36,44,45]. We have also recorded noticeably higher levels of serum LTB4 in patients suffering from severe and fatal acute pancreatitis. Furthermore, patients with severe and necrotizing AP exerted very high serum levels of MIF. MIF is predominantly released by monocytes, macrophages and Th2 T-cell clones; it is associated with activation of immune cells and release of pro-inflammatory cytokines overriding glucocorticoid action at the site of inflammation. It is also known to be associated with a lung injury during SIRS through counter-regulation of the effect of glucocorticoids and through up-regulation of macrophage inflammatory protein-2, which itself serves as a potent chemo-attractant to the neutrophils^[46-49].

The infiltration of inflammatory cells into the pancreas is an early and central event in acute pancreatitis that promotes local injury and systemic complications of the disease. Recent research has yielded the important finding that resident cells of the pancreas (particularly acinar and pancreatic stellate cells) play an important role in leukocyte attraction *via* secretion of chemokines, cytokines and expression of adhesion molecules^[36,50-53]. We believe this could at least partially explain the occurrence of systemic lymphopenia, lower CD25, CD69 and ICAM-1 mRNA expression in cases of SAP. Activation of inflammatory cells and release of cytokines into the systemic bloodstream at the site of injury is possibly also an explanation for the discrepancy of low MIF mRNA expression in peripheral WBCs in association with high serum concentration of MIF in the case of severe and necrotizing pancreatitis.

Having identified the inflammatory mediators that are significantly up-regulated in serum of patients with severe and necrotizing AP (IL-6, IL-8, IL-10, MIF and LTB4) in comparison to those with mild disease, we have also evaluated the prognostic utility of these molecules in predicting the negative outcome of the disease. In accordance with the findings of other studies we have confirmed that both serum IL-6 and MIF concentrations are very good discriminators of severe AP accompanied by MOF, and could be used as early markers of possible fatal outcome. However, in contrast to the recent study of Rahman *et al.*^[54], we failed to demonstrate that MIF is an early specific prognostic marker for the development of pancreatic necrosis. In our experience, serum IL-6 concentration was superior in this respect^[28,55-57]. Nonetheless, serum MIF concentration may serve as an important prognostic marker that could identify patients likely to benefit from aggressive medical treatment and early transfer to specialist pancreatic units. At the molecular level, targeted disruption of these proteins with monoclonal antibodies may afford some therapeutic benefit in preventing the development of MOF, pancreatic necrosis, and septic complications.

Deregulation of the cellular immune system is a key event leading to the development of severe and necrotiz-

ing AP. Severe and necrotizing AP is characterized by the significant depletion of circulating lymphocytes in the bloodstream as a result of increased migration of these cells to the site of injury and other vital organs, including lungs and kidneys. Infiltration of pancreas and other peripheral organs by aberrantly activated inflammatory cells occurs within the first hours after onset of acute pancreatitis and leads to the development of SIRS and MOF. This whole process is regulated by a large number of cytokines and is an extremely complex network of intercellular interaction. It is clear that detailed elucidation of the numerous processes in the inflammatory cascade is an essential step towards the development of improved therapeutic strategies in acute pancreatitis. Published studies suggest that combination therapy targeting different steps of the inflammatory cascade may be the treatment of choice for this disease.

Data from other authors and from our current study demonstrate that serum IL-6 and MIF concentrations are very good discriminators of severe and necrotizing AP as well as of possible fatal outcome during the first hours and days after the onset of the disease. Therefore, these molecules could be used as markers for patient stratification and/or predicting the outcomes of AP in the early stages of the disease. However, their true prognostic utility, clinical and health care resource implications need to be further evaluated in a larger patient population and in the context of other currently existing diagnostic markers and indices.

COMMENTS

Background

Mortality of patients with severe acute pancreatitis (SAP) approaches 30%-40%. An imbalance between the early systemic inflammatory response syndrome (SIRS), and the later compensatory counter-inflammatory response, and development of multiple organ failure (MOF) are considered to be the primary causes of morbidity and mortality in SAP. Excessive leukocyte activation (including neutrophils and monocyte-macrophage lineage) with cytokinemia play a critical role in the pathogenesis of pancreatitis and even more so, of the subsequent inflammatory response. Thus, the aims of this study have been to investigate the secretion patterns of pro- and anti-inflammatory cytokines, and to estimate the activation of various cellular subsets of leukocytes (including lymphocytes, neutrophils, and monocyte-macrophage lineage) in peripheral blood of patients with severe and mild AP.

Research frontiers

It has been hypothesized that fatal pancreatitis is a consequence of abnormal phagocytic leukocyte hyperstimulation due to deregulation in T- and B-lymphocyte activation. However, the role of lymphocyte activation and its relation to the severity of disease in humans is still poorly understood. Another important drawback is that the majority of information about the alterations of the immune system during the AP comes from *in vitro* and *in vivo* studies, and therefore is not always directly applicable and relevant to the clinical situation in human AP.

Innovations and breakthroughs

This study by Dambrauskas *et al.* confirms that human severe and necrotizing AP is characterized by the significant depletion of circulating lymphocytes. SAP is associated with a severe systemic inflammatory response syndrome (SIRS) and over-expression of pro-inflammatory cytokines [interleukin (IL)-6, IL-8, macrophage migration inhibitory factor (MIF)]. The study demonstrates that serum IL-6 and MIF concentrations are the best discriminators of severe and necrotizing AP as well as possible fatal outcome during the early course of the disease. These cytokines could also be used as early predictors of local (pancreatic necrosis) and systemic complications (SIRS, MOF) later in the course of the disease. Deregulation of the cellular immune system is a key event leading to

the development of severe and necrotizing AP. Infiltration of peripheral organs by aberrantly activated inflammatory cells leads to the development of MOF.

Applications

Data from other authors and from the authors' current study demonstrate that serum IL-6 and MIF concentrations are very good discriminators of severe and necrotizing AP as well as possible fatal outcome during the first hours and days after the onset of the disease. Therefore, these molecules could be used as markers for patient stratification and/or predicting the outcome of AP in the early stages of the disease.

Terminology

Pancreatitis is a condition associated with development of acute and sudden inflammation of the pancreas. In about 85% of patients, acute pancreatitis is a mild disease and is usually associated with a rapid recovery within a few days of onset of the illness. However, in 15%-20% of patients with acute pancreatitis, severe damage to the pancreas may lead to a life threatening illness that is often associated with prolonged hospitalization, multiple surgical procedures, development of multiple organ failure, severe infective complications, and death in some patients.

Peer review

This prospective observational study seeks to characterise the cytokine expression profiles (at mRNA and protein levels) of patients with acute pancreatitis and to correlate the profiles with disease severity.

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Insulin-like growth factor-I receptor in proliferation and motility of pancreatic cancer

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Abstract

AIM: To develop a molecular therapy for pancreatic cancer, the insulin-like growth factor-I (IGF-I) signaling pathway was analyzed.

METHODS: Pancreatic cancer cell lines (MIA-Paca2, NOR-P1, PANC-1, PK-45H, PK-1, PK-59 and KP-4) were cultured in media with 10 mL/L fetal bovine serum. Western blotting analysis was performed to clarify the expression of IGF-I receptor (IGF-IR). Picropodophyllin (PPP), a specific inhibitor of IGF-IR, LY294002, a specific inhibitor of phosphatidylinositol

3 kinase (PI3K), and PD98059, a specific inhibitor of mitogen-activated protein kinase, were added to the media. After 72 h, a 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium inner salt (MTS) assay was performed to analyze cell proliferation. A wound assay was performed to analyze cell motility with hematoxylin and eosin (HE) staining 48 h after addition of each inhibitor.

RESULTS: All cell lines clearly expressed not only IGF-IR but also phosphorylated IGF-IR. PPP significantly suppressed proliferation of MIA-Paca2, NOR-P1, PANC-1, PK-45H, PK-1, PK-59 and KP-4 cells to $36.9\% \pm 2.4\%$ (mean \pm SD), $30.9\% \pm 5.5\%$, $23.8\% \pm 3.9\%$, $37.1\% \pm 5.3\%$, $10.4\% \pm 4.5\%$, $52.5\% \pm 4.5\%$ and $22.6\% \pm 0.4\%$, at $2 \mu\text{mol/L}$, respectively ($P < 0.05$). LY294002 significantly suppressed proliferation of MIA-Paca2, NOR-P1, PANC-1, PK-45H, PK-1, PK-59 and KP-4 cells to $44.4\% \pm 7.6\%$, $32.9\% \pm 8.2\%$, $53.9\% \pm 8.0\%$, $52.8\% \pm 4.0\%$, $32.3\% \pm 4.2\%$, $51.8\% \pm 4.5\%$, and $30.6\% \pm 9.4\%$, at $50 \mu\text{mol/L}$, respectively ($P < 0.05$). PD98059 did not significantly suppress cell proliferation. PPP at $2 \mu\text{mol/L}$ suppressed motility of MIA-Paca2, NOR-P1, PANC-1, PK-45H, PK-1, PK-59 and KP-4 cells to $3.0\% \pm 0.2\%$, 0% , 0% , $2.0\% \pm 0.1\%$, $5.0\% \pm 0.2\%$, $3.0\% \pm 0.1\%$, and $5.0\% \pm 0.2\%$, respectively ($P < 0.05$). LY294002 at $50 \mu\text{mol/L}$ suppressed motility of MIA-Paca2, NOR-P1, PANC-1, PK-45H, PK-1, PK-59 and KP-4 to $3.0\% \pm 0.2\%$, 0% , $3.0\% \pm 0.2\%$, 0% , 0% , 0% and $3\% \pm 0.1\%$, respectively ($P < 0.05$). PD98059 at $20 \mu\text{mol/L}$ did not suppress motility. Cells were observed by microscopy to analyze the morphological changes induced by the inhibitors. Cells in medium treated with $2 \mu\text{mol/L}$ PPP or $50 \mu\text{mol/L}$ LY294002 had pyknotic nuclei, whereas those in medium with $20 \mu\text{mol/L}$ PD98059 did not show apoptosis.

CONCLUSION: IGF-IR and PI3K are good candidates for molecular therapy of pancreatic cancer.

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Key words: Insulin-like growth factor-I receptor; Phosphatidylinositol 3 kinase; Pancreatic neoplasms

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INTRODUCTION

Molecular alterations of pancreatic cancer need to be clarified to develop molecular therapy. Growth factors transmit signals to stimulate tumor growth and enhance metastasis. Insulin-like growth factor (IGF)-I signaling plays an important role in the growth and development of many tissues^[1]. IGF-I signaling is also thought to be involved in tumorigenesis. Upon ligand binding, the tyrosine kinase of IGF-I receptor (IGF-IR) is activated, and results in autophosphorylation of tyrosine^[2]. This initiates a phosphorylation cascade to the phosphatidylinositol-3 kinase (PI3K) and mitogen-activated protein kinase (MAPK) pathways.

IGF-I is upregulated in human pancreatic cancer tissues but is not expressed in surrounding non-cancerous tissue^[3]. Serum level of IGF-I is elevated in pancreatic cancer patients^[4]. Histological analysis has shown that IGF-IR is positive in the membrane of cells of pancreatic cancer tissues but is not expressed in surrounding non-tumorous tissues^[3,5]. These facts imply that IGF-I acts as a growth factor for pancreatic cancer. IGF-IR is phosphorylated solely in pancreatic cancer tissues, and not in surrounding non-tumorous tissues^[6]. The present study suggests that IGF-IR transmits signals to downstream pathways. This hypothesis is supported by the facts that IGF-I stimulates DNA synthesis of PANC-1 and MIA Paca2 pancreatic cancer cell lines, and motility of HPAF-II, another pancreatic cancer cell line^[7,8].

IGF-IR is not involved in metabolism, therefore, it was expected that inhibition of IGF-IR and its downstream pathway would not cause any adverse effects^[1]. We therefore focused on the IGF-IR signaling pathway. Pancreatic cancer tissues express growth factors other than IGF-I^[9]. We used fetal bovine serum (FBS) as a growth model of pancreatic cancer cells instead of addition of IGF-I in serum-free medium, because pancreatic cancer tissues and serum from patients contain various growth factors.

MATERIALS AND METHODS

Culture and reagents

Pancreatic cancer cell lines, PANC-1, NOR-P1, PK-45H,

PK-1, PK-59, and MIA-Paca2 were purchased from RIKEN Cell Bank (Tsukuba, Japan)^[10]. NOR-P1 was cultured in Dulbecco's Minimum Essential Medium (DMEM): F12 medium and MIA-Paca2 was cultured in DMEM, and the others in Roswell Park Memorial Institute (RPMI)-1640 (Sigma, St. Louis, MO, USA). All the media were purchased from Sigma, and supplemented with 100 mL/L FBS (Trace Scientific, Melbourne, Australia). All the cell lines were cultured in 50 mL/L carbon dioxide at 37°C in a humidified chamber. LY294002, a specific inhibitor of PI3K, and PD98059, a specific inhibitor of MAPK, were purchased from Wako Pure Chemicals (Osaka, Japan), and picropodophyllin (PPP), a specific inhibitor of IGF-IR, was from Calbiochem (Darmstadt, Germany)^[11].

Western blotting analysis

Protein was isolated from cells after 72 h culture. Twenty micrograms of protein was subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and transferred to a nylon filter. Primary antibodies were polyclonal rabbit anti-IGF-IR antibody, anti-phosphorylated IGF-IR (Cell Signaling Technology, Danvers, MA, USA), and mouse monoclonal anti-tubulin- α antibody (Lab Vision, Fremont, CA, USA). Second antibodies were horseradish peroxidase (HRP)-linked anti-rabbit antibody (Amersham Bioscience, Tokyo, Japan) and HRP-linked anti-mouse antibody (Amersham Bioscience). Dilutions were 1:500 for primary antibodies, and 1:1000 for second antibodies. The filter was reprobed with anti-tubulin- α antibody. The specific antigen-antibody complexes were visualized by enhanced chemiluminescence (GE Healthcare Bio-Sciences Corp, Piscataway, NJ, USA).

Cell proliferation assay

Cells were trypsinized, harvested, and spread onto 96-well flat-bottom plates (Asahi Techno Glass, Tokyo, Japan) at a density of 1000 cells per well. Following 24 h of culture under RPMI-1640, DMEM, or DMEM: F12 with 100 mL/L FBS, medium was changed to RPMI-1640, DMEM, or DMEM: F12 without FBS, respectively, to quench the FBS effects. After 24 h of culture, the medium was changed to RPMI-1640, DMEM, or DMEM: F12 with 100 mL/L FBS, in addition to LY294002, PD98059, or PPP. After 72 h, a 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium inner salt (MTS) assay was performed according to the manufacturer's instructions (Promega Corporation, Tokyo, Japan). MTS was bio-reduced by cells into a colored formazan product that reduced absorbance at 490 nm. Absorbance was analyzed with a multiple plate reader at a wavelength of 490 nm with a BIO-RAD iMark microplate reader (Bio-RAD, Hercules, CA, USA).

Wound assay

Wound assays were performed according to a previously described procedure^[12]. Briefly, cells were spread onto four-well chambers (Becton Dickinson, Franklin Lakes, NJ, USA). Cells were then cut with a sterile razor, and stained with hematoxylin and eosin (HE). Five images were taken by a light microscope (Olympus, Tokyo, Japan). For each

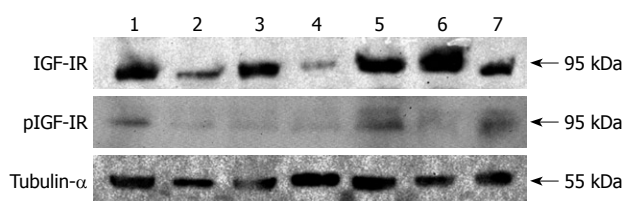


Figure 1 Western blotting analysis of pancreatic cell lines. Protein was isolated from cultured pancreatic cancer cell lines in 100 mL/L fetal bovine serum (FBS), and subjected to western blot analysis. It was shown clearly that all cell lines expressed IGF-IR as well as phosphorylated IGF-IR (pIGF-IR). Tubulin- α was used as an internal control. 1: MIA-Paca2; 2: NOR-P1; 3: PANC-1; 4: PK-45H; 5: PK-1; 6: PK-59; 7: KP-4.

experiment, the number of cells that migrated more than 150 μm per 100 μm cut surface was counted.

Statistical analysis

Cell proliferation and wound assay were analyzed statistically by one-factor analysis of variance. Statistical analysis was performed with JMP5.0J (SAS Institute Japan, Tokyo, Japan). $P < 0.05$ was accepted as statistically significant.

RESULTS

Expression and phosphorylation of IGF-IR in pancreatic cancer

To reveal involvement of IGF-IR in proliferation of pancreatic cancer cell lines stimulated with FBS, protein was isolated and subjected to Western blotting analysis. All cell lines clearly expressed not only IGF-IR but also phosphorylated IGF-IR, which suggested that IGF-IR played a role in proliferation with FBS (Figure 1). This result prompted us to analyze the effects of IGF-IR inhibitors.

Suppression of proliferation of pancreatic cancer with inhibitors

The MTS assay was performed to clarify whether PPP, LY294002, or PD98059 suppressed proliferation of pancreatic cancer cell lines. PPP suppressed proliferation of all the cell lines examined (Figure 2A). At 2 $\mu\text{mol/L}$, MIA-Paca2, NOR-P1, PANC-1, PK-45H, PK-1, PK-59 and KP-4 proliferation was reduced to $36.9\% \pm 2.4\%$ (mean \pm SD), $30.9\% \pm 5.5\%$, $23.8\% \pm 3.9\%$, $37.1\% \pm 5.3\%$, $10.4\% \pm 4.5\%$, $52.5\% \pm 4.5\%$ and $22.6\% \pm 0.4\%$, respectively ($P < 0.05$, $n = 3$). Next, we analyzed the downstream pathway of IGF-IR. LY294002 suppressed proliferation of all the cell lines examined (Figure 2B). At 50 $\mu\text{mol/L}$, proliferation of MIA-Paca2, NOR-P1, PANC-1, PK-45H, PK-1, PK-59 and KP-4 cells was reduced significantly to $44.4\% \pm 7.6\%$, $32.9\% \pm 8.2\%$, $53.9\% \pm 8.0\%$, $52.8\% \pm 4.0\%$, $32.3\% \pm 4.2\%$, $51.8\% \pm 4.5\%$, and $30.6\% \pm 9.4\%$, respectively ($P < 0.05$, $n = 3$). PD98059 did not suppress cell proliferation (Figure 2C). Although NOR-P1 and KP-4 cells showed a marginal decrease in proliferation in the presence of 20 $\mu\text{mol/L}$ PD98059, we could not analyze higher concentrations because PD98059 precipitated in the medium. Analysis with 50 $\mu\text{mol/L}$ PD98059 failed since PD98059 crystallized and precipitated. The other inhibitor of MAPK was not analyzed.

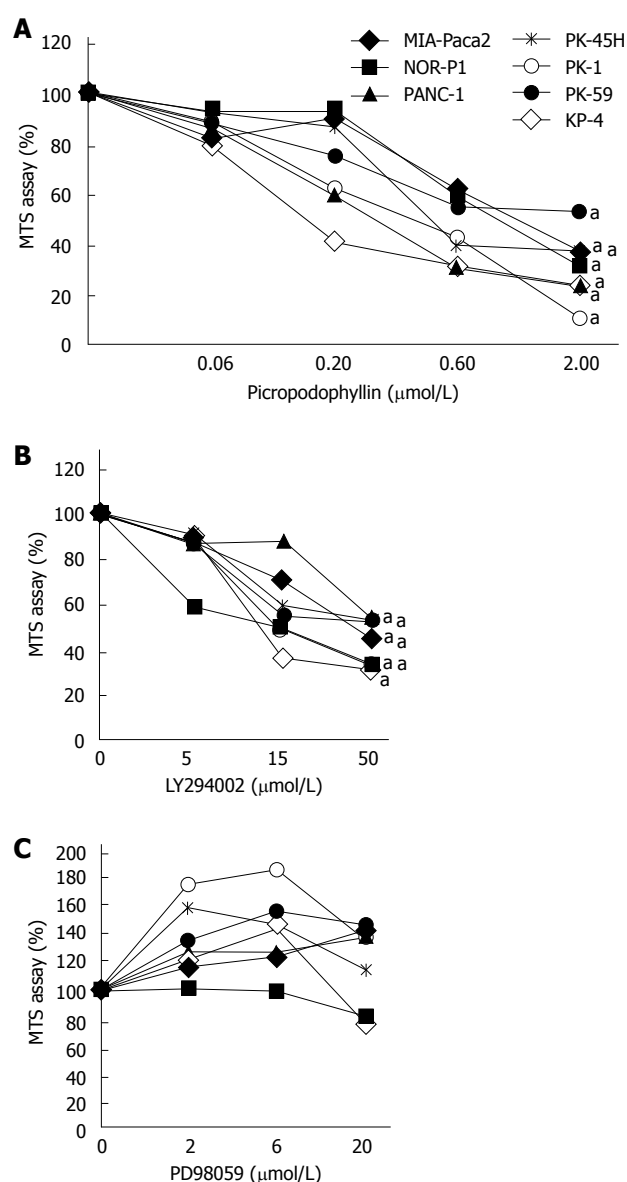


Figure 2 MTS assay of cells cultured with inhibitors. Pancreatic cell lines were cultured in 100 mL/L FBS with picropodophyllin (PPP) (A), LY294002 (B), or PD98059 (C). After 72 h, MTS assay was performed to analyze the change in cell numbers. PPP and LY294002 significantly suppressed cell numbers, whereas PD98059 did not ($^aP < 0.05$, $n = 3$).

Suppression of motility of pancreatic cancer with inhibitors

Suppression of motility is the initial step in the inhibition of metastasis. We analyzed changes in motility with inhibitors, by the wound assay (Figure 3). PPP at 2 $\mu\text{mol/L}$ suppressed motility of MIA-Paca2, NOR-P1, PANC-1, PK-45H, PK-1, PK-59 and KP-4 cells to $3.0\% \pm 0.2\%$, 0% , 0% , $2.0\% \pm 0.1\%$, $5.0\% \pm 0.2\%$, $3.0\% \pm 0.1\%$, and $5.0\% \pm 0.2\%$, respectively ($P < 0.05$, $n = 3$). LY294002 at 50 $\mu\text{mol/L}$ suppressed motility of MIA-Paca2, NOR-P1, PANC-1, PK-45H, PK-1, PK-59 and KP-4 cells to $3.0\% \pm 0.2\%$, 0% , $3.0\% \pm 0.2\%$, 0% , 0% , 0% and $3\% \pm 0.1\%$, respectively ($P < 0.05$, $n = 3$). PD98059 at 20 $\mu\text{mol/L}$ did not suppress motility, although NOR-P1 cells showed a marginal, non-significant decrease.

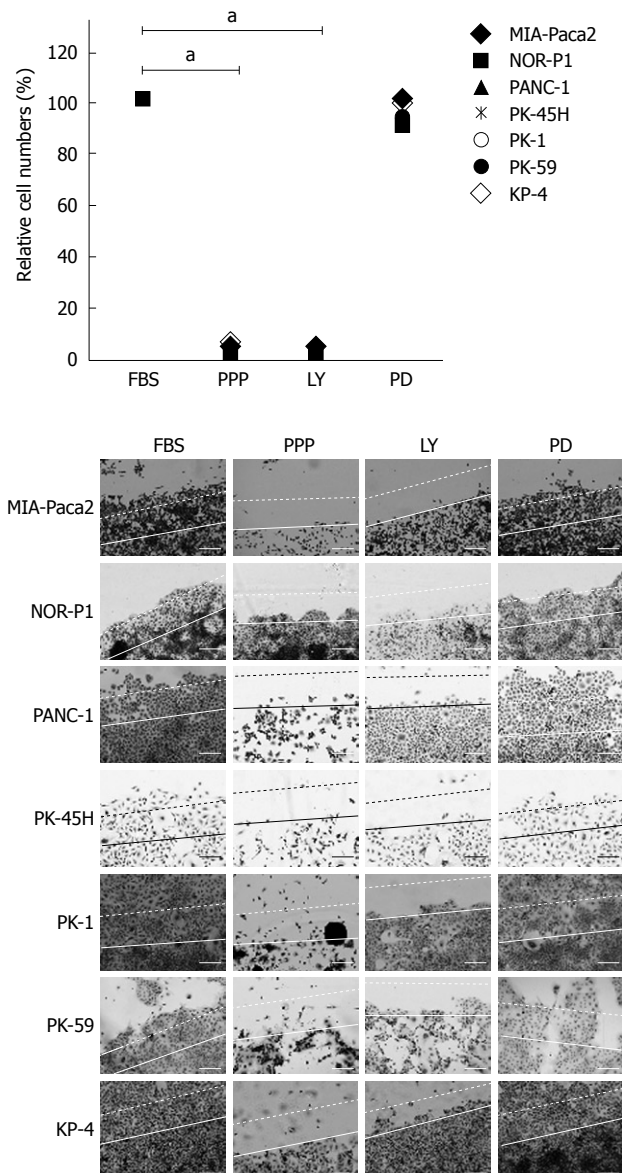


Figure 3 Wound assays with inhibitors. Wound assay was performed to analyze cell motility. Almost no cells cultured with PPP or LY294002 migrated $> 150 \mu\text{m}$, whereas those with PD98059 showed motility of $> 150 \mu\text{m}$ ($^*P < 0.05$, $n = 3$). Solid line: Edge of scratch; Dotted line: $150 \mu\text{m}$ from a solid line. Original magnification: $4\times$; Scale bar: $100 \mu\text{m}$. FBS: Medium with 100 mL/L FBS without any inhibitors; PPP: Medium with $2 \mu\text{mol/L}$ PPP; LY: Medium with $50 \mu\text{mol/L}$ LY294002; PD: Medium with $20 \mu\text{mol/L}$ PD98059.

Microscopic features of pancreatic cancer with inhibitors

Cells were observed under the microscope to analyze the morphological changes induced by inhibitors. Cells in medium with $2 \mu\text{mol/L}$ PPP or $50 \mu\text{mol/L}$ LY294002 had pyknotic nuclei, which suggested that they were apoptotic (Figure 4). Cells in medium with $20 \mu\text{mol/L}$ PD98059 did not show signs of apoptosis (Figure 4).

DISCUSSION

IGF-I stimulates thymidine incorporation of pancreatic cancer cell lines, such as MIA-Paca2, as strongly as FBS, which suggests that it is an autocrine growth factor^[13]. It

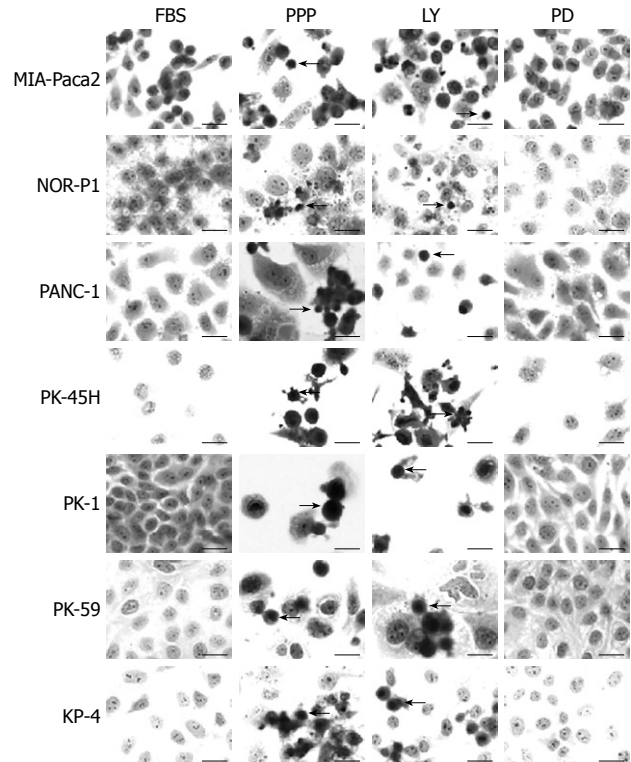


Figure 4 Apoptotic cells with PPP or LY294002. Under the microscope, cells with pyknotic nuclei (arrows) were observed in medium with PPP or LY294002, whereas there were no apoptotic cells in medium with PD98059. FBS: Medium with 100 mL/L FBS; PPP: Medium with $2 \mu\text{mol/L}$ PPP; LY: Medium with $50 \mu\text{mol/L}$ LY294002; and PD: Medium with $20 \mu\text{mol/L}$ PD98059. Original magnification: $20\times$; Scale bar: $25 \mu\text{m}$, the arrows indicate cells in apoptosis.

was expected that inhibition of IGF-IR activity would lead to pancreatic cancer regression. Antisense oligonucleotide to IGF-IR suppresses proliferation of ASPC-1, a pancreatic cancer cell line^[3]. Antisense oligonucleotide to K-ras synergistically enhances the suppression of pancreatic cancer cell lines by antisense oligonucleotide to IGF-IR^[14]. Antisense oligonucleotide is unstable in blood, therefore, small molecules that inhibit the tyrosine kinase of IGF-IR are desirable. NVP-AEW541, an IGF-IR inhibitor, suppresses growth of HPAF-II cells, a pancreatic cancer cell line^[15]. However, it is possible that NVP-AEW541 does not affect other cell lines. PPP is the first inhibitor to discriminate IGF-IR and insulin receptor^[16]. In our experiments, PPP suppressed proliferation of all pancreatic cancer cell lines. Previous studies and the present one indicate that IGF-IR is a good candidate for molecular therapy of pancreatic cancer.

The downstream pathway of the IGF-I receptor is intriguing since shutting down the pathway could reduce cancer. Antibody to IGF-IR suppresses phosphorylation of Akt, a downstream molecule of PI3K and MAPK^[13]. LY294002 significantly suppresses proliferation of PANC-1 and BxPC3 cells, and viability of KP-4 and PANC-1 cells^[7,17]. Our results showed that LY294002 suppressed proliferation of all the pancreatic cancer cell lines examined. In addition to proliferation, our data clearly demonstrated that LY294002 suppressed cell migration. We conclude that PI3K is a suitable target for molecular therapy of pancreatic cancer.

MAPK is another downstream pathway of IGF-IR. MIA-PaCa2 treated with 20 $\mu\text{mol/L}$ PD98059 in 100 mL/L FBS showed downregulation of phosphorylated extracellular signal-regulated kinase 1/2^[18]. Although it was expected that PD98059 would suppress proliferation of pancreatic cancer cells, 20 $\mu\text{mol/L}$ PD98059 did not suppress proliferation and motility of pancreatic cancer cells in our experiments. PD98059 at 20 $\mu\text{mol/L}$ does not suppress DNA synthesis stimulated by IGF-I in the culture medium, although phosphorylation of MAPK is suppressed^[19]. Curiously, PD98059 stimulates cell growth at concentrations of 0.1 $\mu\text{mol/L}$ to 0.1 pmol/L^[20]. SB203580, another inhibitor of MAPK, stimulates growth of PANC-1 cells in serum-free conditions^[21]. In our experiments, PD98059 marginally stimulated growth of PANC-1 cells with 10 mL/L FBS. These results suggest that inhibition of MAPK does not always suppress growth of pancreatic cancer cell lines with unknown mechanisms.

It may be concluded that IGF-IR and PI3K are good candidates for molecular therapy of pancreatic cancer.

COMMENTS

Background

Insulin-like growth factor-I receptor (IGF-IR) is expressed in pancreatic cancer tissues but not in surrounding non-cancerous tissues. Although it was speculated that IGF-I might play a role in proliferation of pancreatic cancer, the detailed mechanism is not known.

Research frontiers

IGF-IR is not involved in metabolism, therefore, it is expected that inhibition of IGF-IR and its downstream pathway should not cause adverse effects when used as a target for molecular therapy of pancreatic cancer.

Innovations and breakthroughs

IGF-IR was phosphorylated in cultured pancreatic cancer cell lines, which suggested that IGF-IR and its downstream pathway were activated. Each inhibitor of IGF-IR or phosphatidylinositol-3 kinase (PI3K) suppressed not only cell proliferation but also motility.

Applications

By revealing that IGF-IR was activated, this study indicated that IGF-IR might be a good candidate for molecular therapy of pancreatic cancer.

Terminology

IGF-I is a growth factor that is involved in cell proliferation and differentiation. Stimulation of IGF-I is transmitted via IGF-IR to downstream pathways, such as PI3K and mitogen-activated protein kinase.

Peer review

The authors have demonstrated that IGF-IR, phosphorylated and total, is involved in the proliferation of pancreatic cell lines. In Figure 1, we see that all cell lines expressed phosphorylated and total IGF-IR. Their experiments have shown that inhibition of IGF-IR activity results in a decrease in proliferation and motility of pancreatic cancer cell lines. These findings, along with the inhibition of PI3K, are interesting and show promise.

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Effect of S1P5 on proliferation and migration of human esophageal cancer cells

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and migration to S1P and S1P5 expression was evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide and migration assay, respectively.

RESULTS: Both normal human esophageal mucosal epithelium and Eca109 cells expressed S1P1, S1P2, S1P3 and S1P5, respectively. Esophageal mucosal epithelium expressed S1P5 at a higher level than Eca109 cell line. S1P5 over-expressing Eca109 cells displayed spindle cell morphology with elongated and extended filopodia-like projections. The proliferation response of S1P5-transfected Eca109 cells was lower than that of control vector-transfected cells with or without S1P stimulation ($P < 0.05$ or 0.01). S1P significantly inhibited the migration of S1P5-transfected Eca109 cells ($P < 0.001$). However, without S1P in transwell lower chamber, the number of migrated S1P5-transfected Eca109 cells was greater than that of control vector-transfected Eca109 cells ($P < 0.001$).

CONCLUSION: S1P binding to S1P5 inhibits the proliferation and migration of S1P5-transfected Eca109 cells. Esophageal cancer cells may down-regulate the expression of S1P5 to escape the inhibitory effect.

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Key words: Sphingosine 1-phosphate; Esophageal cancer; Sphingosine 1-phosphate 5; Proliferation; Migration

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Abstract

AIM: To investigate the sphingosine 1-phosphate (S1P) receptor expression profile in human esophageal cancer cells and the effects of S1P5 on proliferation and migration of human esophageal cancer cells.

METHODS: S1P receptor expression profile in human esophageal squamous cell carcinoma cell line Eca109 was detected by semi-quantitative reverse transcription polymerase chain reaction. Eca109 cells were stably transfected with S1P5-EGFP or control-EGFP constructs. The relation between the responses of cell proliferation

INTRODUCTION

Esophageal cancer is one of the most common malignancies worldwide, occurring mostly in developing countries with marked regional variations in incidence. Its mortality is very similar to its incidence due to relatively late diagnosis and inefficient treatment. About 300 000 people would die of esophageal cancer each year in the world^[1]. The incidence and mortality of esophageal cancer are unusually high in China, especially in Henan, Shanxi, Hebei and Sichuan Provinces^[2]. Even though a small number of esophageal cancer patients can survive more than 5 years after initial surgical treatment, over 60% of patients still die of metastasis and local recurrence^[3,4]. The ability to reverse the outcome of esophageal cancer is limited due to a poor understanding of its biology.

Sphingosine 1-phosphate (S1P) is a pleiotropic phospholipid mediator that mediates diverse cellular responses, such as cell proliferation, differentiation, migration, adhesion, and morphogenesis. S1P with a low nanomolar affinity binds to its 5-related G-protein coupled receptors (GPCR), and is designated as S1P1-5 (originally termed EDG-1, 3, 5, 6, and 8, respectively). S1P1, S1P2, and S1P3 receptors are ubiquitously expressed, whereas S1P4 is only expressed in lymphoid tissues and platelets, and S1P5 is predominantly expressed in the central nervous system^[5-11].

Previous reports have highlighted the importance of S1P and its receptors for the growth, metastasis and infiltration of tumors, such as gastric cancer^[5], thyroid cancer^[12], ovarian cancer^[13-16], and glioma^[17]. In general, S1P stimulates the migration of S1P3 dominantly expressing cell lines and inhibits the migration of S1P2 expressing cell lines. S1P1, S1P2 and S1P3 contribute positively to S1P-stimulated glioma cell proliferation, and S1P5 blocks glioma cell proliferation. S1P1 and S1P3 enhance glioma cell migration and invasion, while S1P2 inhibits cell migration but unexpectedly enhances glioma cell invasiveness by stimulating cell adhesion^[17].

The possible role and importance of S1P and its receptors in the growth and metastasis of human esophageal cancer have not been investigated. In this study, we focused on esophageal squamous cell carcinoma (ESCC), characterized S1P receptor expression patterns and investigated the role of S1P receptors in proliferation and migration of ESCC cells. ESCC cell line Eca109 expressed 4 S1P receptors (S1P1, S1P2, S1P3 and S1P5). Compared with normal esophageal mucosa epithelium, Eca109 cells expressed a very low level of S1P5. Furthermore, by over-expressing human S1P5 in Eca109 cells, the effects of S1P5 on esophageal cancer cells were partly clarified.

MATERIALS AND METHODS

Materials

S1P was obtained from Cayman Chemical (Ann Arbor, MI, USA). Dispase II, fibronectin and fatty acid-free bovine serum albumin (BSA) were purchased from Sigma

(St Louis, MO, USA). SV total RNA isolation system and pGEM-T easy vector system were bought from Promega (Madison, WI, USA). PrimeScript™ reverse transcription polymerase chain reaction (RT-PCR) kit and PrimerSTAR HS DNA polymerase with GC buffer were purchased from Takara Biotech (Dalian, China). Lipofectamine 2000 and G418 were obtained from Invitrogen (Carlsbad, CA, USA).

Preparation of normal human esophageal mucosal epithelium

Normal human esophageal mucosa samples were obtained from surgically resected esophagus of patients with esophageal carcinoma, and then confirmed by an experienced pathologist to contain neither macroscopic tumor nor histologically detectable metaplastic cells or cancer cells. Samples were collected with the signed informed consent from patients. Tunica mucosa was separated from the tissues with forceps, and cut into 1 cm × 1 cm sections which were then transferred to a 100 mm culture dish containing 10 mL PBS with 0.25% dispase II and incubated for 18-24 h at 4°C. Epithelial layers were separated from the full-thickness mucosa with forceps.

Cell culture

Human ESCC cell line Eca109 was obtained from Shanghai Institute of Cell Biology, Chinese Academy of Sciences. Eca109 cells were cultured in RPMI 1640 medium supplemented with 10% (v/v) fetal bovine serum (FBS) and 100 U/mL of penicillin and streptomycin at 37°C in a humidified incubator containing 5% CO₂.

Preparation of total RNA and semi-quantitative reverse transcription polymerase chain reaction

Total RNA was isolated with the SV total RNA isolation system following its manufacturer's instructions. RNA quality and integrity were checked by absorbance spectrometry and agarose gel electrophoresis. Semi-quantitative RT-PCR was performed to confirm differential expression of S1P receptors in Eca109 cell line. The sequences of primers used are as follows: 5'-TATCAGC GCGACAAGGAGAACAG-3' (sense) and 5'-ATAG GCAGGCCACCCAGGATGAG-3' (antisense) for S1P1 receptor (429-bp product), 5'-TCGGCCTTCATCGTC ATCCTCT-3' (sense) and 5'-CCTCCCGGGCAAACCA CTG-3' (antisense) for S1P2 receptor (220-bp product), 5'-CTGCCTGCACAATCTCCCTGACTG-3' (sense) and 5'-GGCCCGCCGCATCTCCT-3' (antisense) for S1P3 receptor (394-bp product), 5'-GAGAGCGGGGCCACC AAGAC-3' (sense) and 5'-GGTTGACCGCCGAGTTGA GGAC-3' (antisense) for S1P4 receptor (454-bp product), 5'-GGAGTAGTTCCCGAAGGACC-3' (sense) and 5'-T CTAGAATCCACGGGGTCTG-3' (antisense) for S1P5 receptor (236-bp product), 5'-GATGACATCAAGAAGG TGGTGAA-3' (sense) and 5'-GTCCTACTCCTTGGAG GCCATGT-3' (antisense) for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (246-bp product). Thirty-two cycles of amplification were performed using a thermal

cycler, Mastercycle[®] gradient (Eppendorf, Hamburg, Germany) at 94°C for 30 s, at 60°C for 30 s, and at 72°C for 1 min. The PCR products were electrophoresed on a 1.6% agarose gel and visualized with ethidium bromide staining.

Generation of stably-transfected Eca109 cell line

Human S1P5 open reading frame was cloned from normal esophageal mucosal epithelium cDNA *via* PCR using the PrimerSTAR HS DNA polymerase with GC buffer. The sequences of primers used are 5'-CATTG AAGCTTCCACCATGGAGTCGGGGCTGCTG-3' (sense) and 5'-CATTGTCTAGAGTCTGCAGCCG GTTCTGATA-3' (antisense). The sense primer was designed to add a *Hind*III site upstream of a consensus Kozak sequence and the S1P5 initiating Met (*italics*). The antisense primer was designed to remove the S1P5 stop codon and to add a *Xba*I site. PCR conditions, established to amplify the S1P5 receptor sequence, were 94°C for 1 min, followed by 35 cycles at 94°C for 30 s, at 60°C for 30 s, at 72°C for 2 min, and a final extension at 72°C for 8 min. The PCR products were electrophoresed on 0.8% agarose gel and the corresponding band was cut and purified. The purified PCR products were ligated into the pGEM-T easy vector and the sequence was confirmed by sequence analysis. The *S1P5* gene was spliced out with *Hind*III/*Xba*I and ligated into the Myc-His-EGFP-N1 frame, which was purified from similarly digested S1P1-Myc-His-EGFP-N1 as previously described^[18]. The initiating methionine of the GFP open reading frame in pEGFP-N1 was mutated to alanine. The resulting plasmid was designated as S1P5-EGFP. A *Hind*III/*Xba*I - digested DNA oligonucleotide containing both sense (5'-CATTGAAGCTTCCACCATGGAATTCTGCAGT CGACGGTACTCTAGACCG-3') and antisense (5'-CG GTCTAGAGTACCGTCGACTGCAGAATTCCATG GTGGAAGCTTCAATG-3') sequences was cloned into similarly digested Myc-His-EGFP-N1 frame as a control vector. The DNA oligonucleotide contained initiating Met and multiple cloning sites. The plasmid was designated as control-EGFP.

Cells were transfected using Lipofectamine 2000, according to its manufacturer's instructions. Briefly, 5×10^5 Eca109 cells in 2 mL of growth medium without antibiotics were seeded into each well of 6-well plates and grown overnight, then transfected using Lipofectamine 2000. The cells were split at a 1:20 dilution into a fresh growth medium 24 h after transfection. G418 was added at a concentration of 0.8 mg/mL the following day. Resistant colonies were picked to a 96-well plate 14 d after selection and several clones were analyzed. The cells were subsequently maintained in a medium containing G418 at 0.2 mg/mL.

Proliferation assay

Cells (5×10^3 /well in 100 μ L/well) were seeded into a 96-well plate and cultured in RPMI 1640 medium containing 10% FBS for 8 h at 37°C in an atmosphere

containing 5% CO₂ to allow attachment. The medium was then aspirated and a fresh medium containing 0.1% fatty acid-free BSA and varying concentrations of S1P was added into three replicate wells every 48 h. After incubation at 37°C in an atmosphere containing 5% CO₂ for 72 h, the number of living cells was calculated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. Briefly, a MTT solution was added into each well and the cells were incubated for an additional 4 h. The number of living cells was then counted by measuring the absorbance at 490 nm.

Migration assay

Migration assay was performed on a 6.5 mm-diameter transwell chamber with an 8 μ m pore size (Corning, NY, USA). The underside of transwell membranes was precoated with fibronectin (10 μ g/mL) and allowed to dry. Cells at 70%-80% confluence were serum starved overnight, detached from culture plates by 0.02% EDTA, washed and resuspended in RPMI 1640 medium with 0.1% fatty acid-free BSA at 1×10^6 cells/mL. For migration assay, cells (1×10^5 cells/well) were added into the upper chamber of transwell chambers separated by inserts with 8 μ m pores in the presence or absence of S1P. Following an 8-h incubation, nonmigrating cells on the upper surface of membranes were removed with cotton swabs, fixed and stained with 0.1% crystal violet in phosphate-buffered saline (PBS) at the ambient temperature, and then destained with PBS. Migrating cells were counted in 5 randomly selected high-power fields per membrane under an Olympus IX71 inverted microscope.

Statistical analysis

Data are expressed as mean \pm SD and shown as error bars in the figures. Statistical comparisons were made by Student's *t*-test. One-way ANOVA with a *post hoc* Bonferroni testing was employed in case of multiple comparisons. *P* < 0.05 was considered statistically significant.

RESULTS

Expression of S1P receptors in normal human esophageal mucosal epithelium and Eca109 cell line

Semi-quantitative RT-PCR of mRNA was performed to observe which S1P receptors are expressed in Eca109 cell line and normal human esophageal mucosal epithelium. For comparison, data were also normalized to the expression of the reference gene GAPDH, which gave similar results. Four S1P receptors were found in normal human esophageal mucosal epithelium with the following rank order of mRNA abundance: S1P1>S1P5>S1P3>S1P2. In contrast to Eca109 cell line, the mRNA abundance in S1P receptors was S1P3>S1P1>S1P5>S1P2. S1P4 expression was absent or minimal in Eca109 cell line and normal human esophageal mucosal epithelium (Figure 1). Eca109 cell line expressed S1P3 at a higher level than normal esophageal mucosal epithelium. In contrast, normal

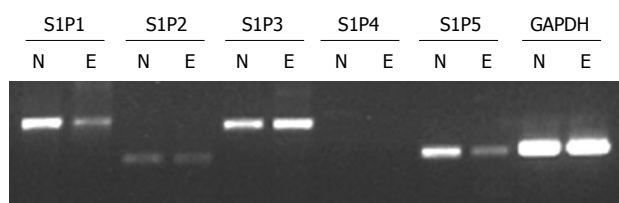


Figure 1 Expression of sphingosine 1-phosphate (S1P) receptors in normal human esophageal mucosal epithelium and Eca109 cell line. N: Normal human esophageal mucosal epithelium; E: Eca109 cell line.

esophageal mucosal epithelium expressed S1P1 and S1P5 at a higher level than Eca109 cell line. On the basis of these results, S1P5 was chosen for further study.

Generation of S1P5 overexpressing Eca109 cell line

Eca109 cells transfected with the S1P5-EGFP or Control-EGFP constructs were cultured in a medium containing 10% FBS. Their localization was visualized by fluorescence microscopy. As shown in Figure 2, control-EGFP was localized at the cytosol of Eca109 cells. In contrast, the S1P5-EGFP was localized at the plasma membrane. Interestingly, control-EGFP-transfected cells displayed the characteristic Eca109 cell morphology. In contrast, S1P5-EGFP-transfected cells displayed spindle cell morphology with elongated and extended filopodia-like projections in a medium containing 10% FBS or 0.1% fatty acid-free BSA.

Effect of S1P on proliferation of control-EGFP or S1P5-EGFP expressing Eca109 cells

Since S1P regulates the proliferation of many cell types, including cancer cells, it is of great interest to investigate whether S1P5 or S1P binding to S1P5 has an effect on the proliferation of control vector and S1P5 transfected Eca109 cells. To assess induction of proliferation, stably transfected cells in 96-well plates containing 10% FBS or 0.1% fatty acid-free BSA were counted after treatment with a vehicle (DMSO) and 10 or 100 nmol/L S1P for 72 h. The number of cells from both control vector and S1P5 transfectants did not increase in response to S1P ($P > 0.05$), suggesting that S1P does not behave as a mitogen at S1P5 (Figure 3).

Interestingly, the proliferation response of S1P5-transfected Eca109 cells was lower than that of control vector-transfected cells in a medium containing 10% FBS (8% lower, $P < 0.05$) or 0.1% fatty acid-free BSA with or without S1P (20%-29% lower, $P < 0.05$ or 0.01), suggesting that S1P5 has an intrinsic activity and inhibits cell proliferation.

Effect of S1P on migration of control-EGFP or S1P5-EGFP expressing Eca109 cells

Migration of Eca109 cells was quantified by transwell-based assay. Transwell membranes were precoated with fibronectin. At a concentration of 10 or 100 nmol/L S1P, the migration of S1P5-EGFP-transfected Eca109 cells was significantly lower than that of control-EGFP-transfected Eca109 cells (48% *vs* 84% at 10 nmol/L S1P,

39% *vs* 94% at 100 nmol/L S1P, $P < 0.001$) (Figure 4A and B). Interestingly, without S1P in the lower chamber, the migrated number of S1P5-EGFP transfected Eca109 cells (145 ± 10) was more than that of control-EGFP-transfected Eca109 cells (51 ± 4 , $P < 0.001$) (Figure 4A and C). Both cells failed to migrate on gelatin-coated or non-coated transwell membranes (data not shown). Control-EGFP and S1P5-EGFP transfected Eca109 cells exhibited spontaneous migration without S1P, and the addition of S1P to the lower chamber potentially inhibited cell migration.

DISCUSSION

In the present study, normal esophageal mucosal epithelium expressed S1P5 at a high level, and Eca109 cells expressed S1P5 at a low level. S1P5 was predominantly expressed in the white matter tracts and oligodendrocytes, and was particularly abundant in the anterior commissure, corpus callosum, and optic tract. S1P5 mRNA was detected in spleen, peripheral blood leukocytes, placenta, lung, aorta, and fetal spleen. A low level of signals has been detected in many tissues^[19,20]. In this study, normal human esophageal mucosal epithelium expressed S1P5 at a high level, suggesting that S1P5 is also a ubiquitous S1P receptor and plays an important role in different tissues and cells.

S1P is believed to be generated intracellularly by all cell types during sphingolipid degradation. Plasma S1P is mainly hematopoietic in origin with erythrocytes as a major contributor to the sources of extracellular S1P, whereas lymph S1P is from a distinct radiation-resistant source^[21,22]. The concentration of S1P in plasma and serum is 0.1-0.6 $\mu\text{mol/L}$ and 0.4-1.1 $\mu\text{mol/L}$, respectively, while the S1P level in tissues is 0.5-75 pmol/mg^[23]. Although S1P was originally thought to act as an intracellular second messenger, it has been widely accepted as an intercellular agonist. S1P binding to its receptors activates different intracellular signaling pathways depending on which intracellular $G\alpha$ protein they couple to. The S1P1 receptor primarily couples through $G\alpha_{i/o}$, whereas S1P2 and S1P3 can couple through $G\alpha_{i/o}$, $G\alpha_q$ and $G\alpha_{12/13}$, S1P4 and S1P5 can couple through $G\alpha_{i/o}$ and $G\alpha_{12/13}$ ^[6,7,24-26]. Signaling through $G\alpha_{i/o}$ has been associated with the activation of small guanosine triphosphatase (GTPase) Ras and extracellular signal-regulated kinase (ERK) to promote proliferation, and induction of Pi3K and small GTPase Rac to promote migration. Signaling through $G\alpha_{12/13}$ can promote the activation of small GTPase Rho and Rho-associated kinase (ROCK) to inhibit migration^[6]. Furthermore, S1P5 is also a constitutively active receptor with the ability to intrinsically stimulate various effector pathways *albeit* at a different extent in the absence of stimulating ligand^[27].

In the present study, normal esophageal mucosal epithelium expressed S1P5 at a higher level than Eca109 cell line. Therefore, Eca109 cells were transfected with the expression constructs of S1P5 or control vector in order to delineate the effects of S1P5 on the growth, migration

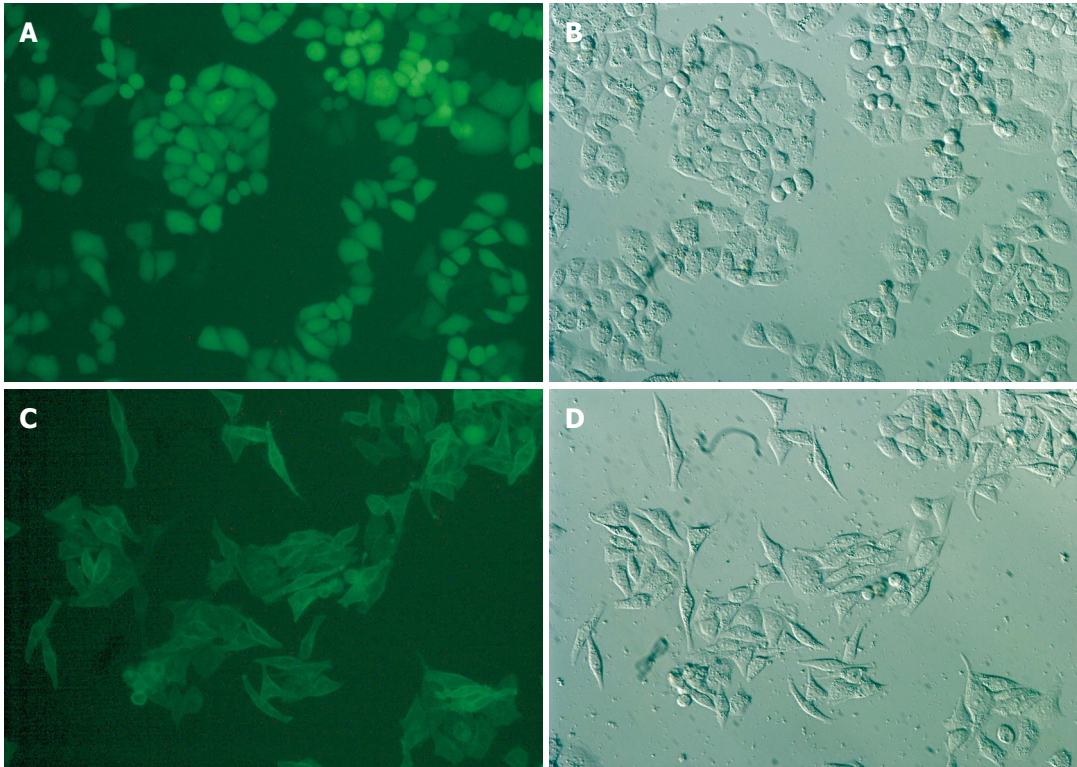


Figure 2 S1P5 receptor overexpression in Eca109 cells causes cell spindle change with elongated and extended filopodia-like projections in a medium containing 10% FBS or 0.1% fatty acid-free BSA ($\times 200$). A, B: Eca109/control-EGFP; C, D: Eca109/S1P5-EGFP.

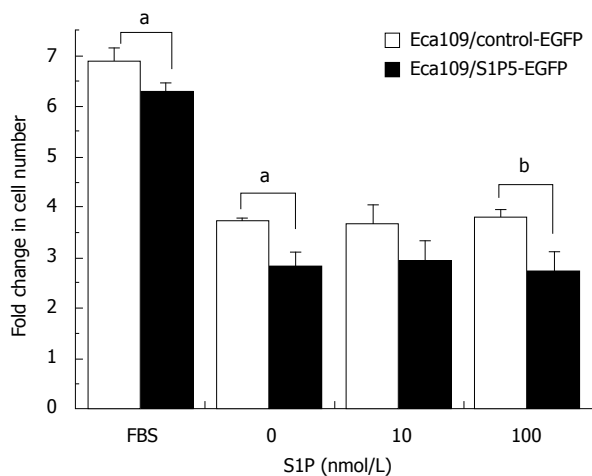


Figure 3 MTT assay showing proliferation of control-EGFP or S1P5-EGFP-transfected Eca109 cells. ^a $P < 0.05$, ^b $P < 0.01$ vs control group.

and morphogenesis of esophageal cancer cells. Eca109 cells that overexpress S1P5 were analyzed by phase contrast microscopy. Increased expression of S1P5 induced cell spindle change with elongated and extended filopodia-like projections in a medium containing 10% FBS or 0.1% fatty acid-free BSA. This morphological change is characterized by motile cells. However, transfection of several mammalian cell lines (CHO-K1, HEK293, NIH3T3, RH7777) with the S1P5 receptor induced cell rounding change, which is more pronounced in the presence of S1P-containing serum^[27]. Differences in morphological alteration may correlate well with cell type or malignant degree, because CHO-K1,

HEK293, NIH3T3 and RH7777 are the normally-derived mammalian cell lines.

In this study, the expression of S1P5 receptor in Eca109 cells reduced the proliferation of Eca109 cells whether or not the medium contained S1P, indicating that the S1P5 receptor is constitutively active in the absence of stimulating ligand. Antiproliferative effect is an intrinsic ability of the S1P5 receptor that is insensitive to ligand stimulation. Analogously, S1P5-transfected CHO cells exhibit an intrinsic activity and inhibit serum-stimulated cell proliferation^[28]. S1P5 can negatively and constitutively regulate cellular proliferation by reducing ERK1/2 activity *via* protein-tyrosine phosphatase. $G\alpha_{12/13}$ is a likely candidate G protein for the inhibitory effects of S1P5 on ERK activity. It has been shown that mutation-activated $G\alpha_{12/13}$, but not $G\alpha_q$, $G\alpha_s$, or $G\alpha_{i2}$, can inhibit EGF stimulation of ERK activity in COS-7 cells in a Ras- and Raf-independent manner^[29]. Down-regulation of S1P5 in esophageal cancer cells may weaken its antiproliferative effect.

In this study, overexpression of S1P5 receptor constitutively increased the migration of esophageal cancer cells to fibronectin in the absence of S1P, which was consistent with morphological alteration. However, addition of 10 and 100 nmol/L S1P significantly inhibited the constitutive migration of S1P5-transfected Eca109 cells. It has been shown that small GTPases of the Rho family, including Rac, Cdc42, and RhoA, are well-known regulators of cell migration^[5,30]. Cell migration induced by S1P receptors (S1P1 and S1P3) involves $G\alpha_{i/o}$ -mediated activation of phosphatidylinositol 3-kinase (Pi3K) and

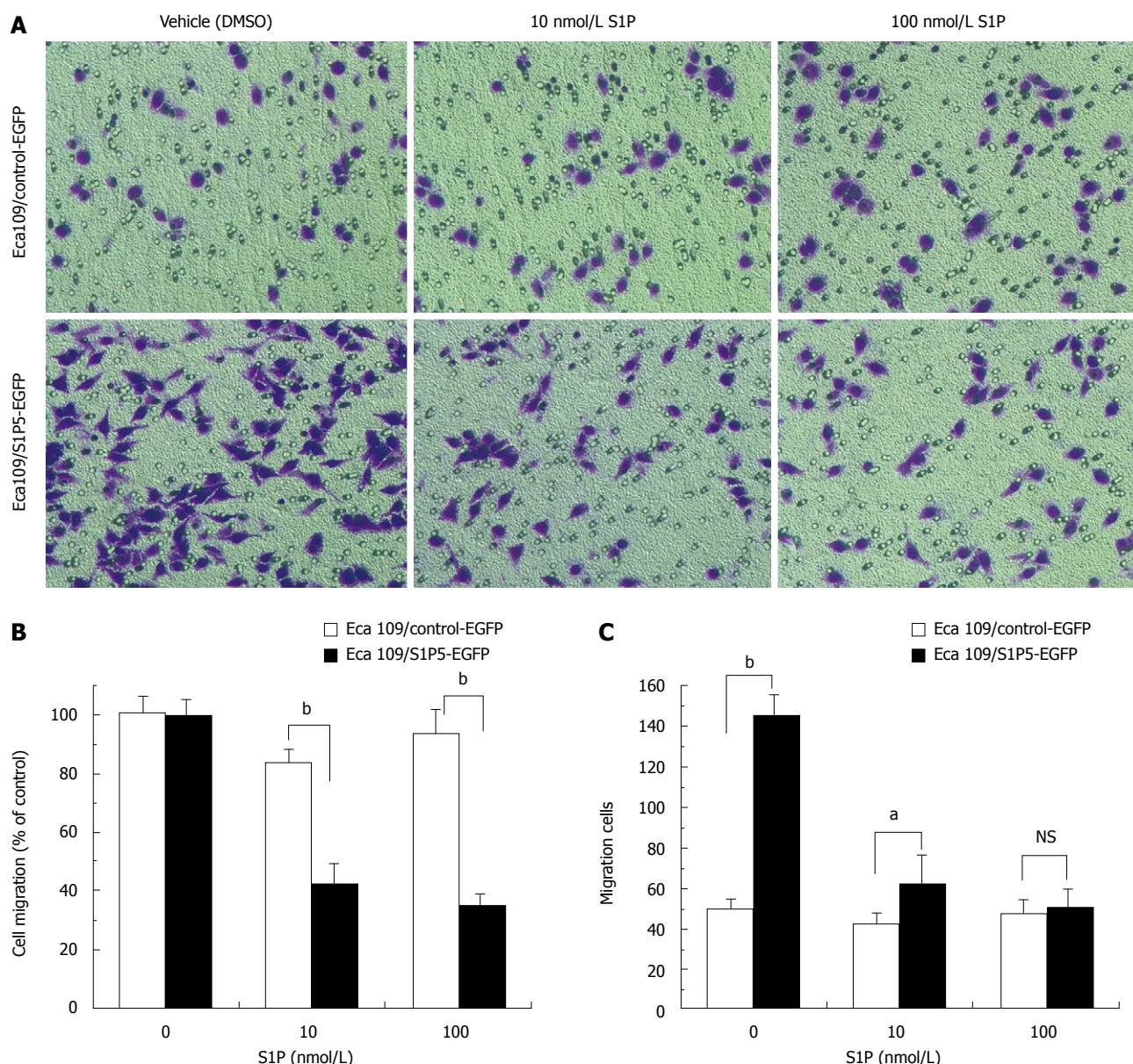


Figure 4 Effect of S1P on migration of S1P5-EGFP-transfected Eca109 cells and control-EGFP Eca109 cells. A: Eca109 cells are allowed to migrate for 8 h, and stained with crystal violet ($\times 200$); B: Results are expressed as percentage in cell migration of S1P-treated cells compared to vehicle (DMSO)-treated cells; C: Results are expressed as migration cell number. ^a $P < 0.05$, ^b $P < 0.001$ indicate a statistically significant effect between S1P5-EGFP and control-EGFP transfected Eca109 cells treated by indicated concentrations of S1P or vehicle. NS: Not significant.

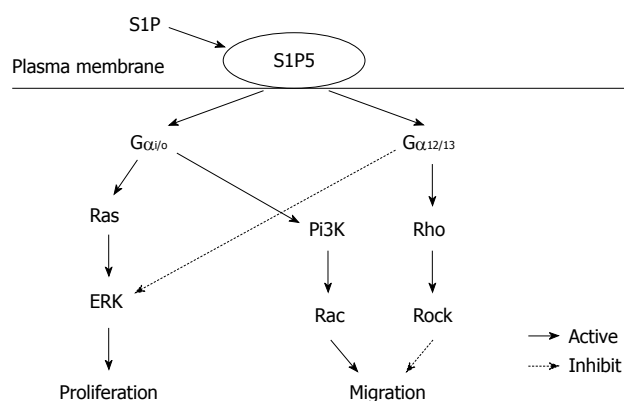


Figure 5 Putative signaling pathway of S1P5.

Rac. S1P receptors (S1P2 and S1P5) mediate inhibition of cell migration by $G\alpha_{12/13}$ -evoked stimulation of Rho^[12]. It was reported that fibronectin binding to $\alpha_5\beta_3$ integrin can decrease Rho activity and stimulate cell motility and spreading, suggesting that enhanced Rho activity may reduce integrin-driven cell migration^[7,31]. Our data support that S1P5 may constitutively induce cell migration due to $G\alpha_{i/o}$ -activated Rac and inhibit cell migration due to $G\alpha_{12/13}$ -stimulated Rho. In a microenvironment containing S1P interstitial fluid, esophageal cancer cells may escape the inhibitory effect of S1P-S1P5 on their migration by down-regulating the expression of S1P5. The putative signaling pathway of S1P5 is shown in Figure 5.

In summary, S1P5 is a constitutively active receptor

with the ability to intrinsically inhibit cell proliferation and induce cell migration. Cell migration can be inhibited by stimulating extracellular S1P. In normal esophageal mucosa cells, a high expression level of S1P5 may inhibit cell proliferation and migration while esophageal cancer cells may down-regulate the expression of S1P5 to promote cell proliferation and escape the inhibitory effect of S1P-S1P5 on cell migration. Our study is the first to show that the deficiency in inhibitory effect of S1P-S1P5 is of importance in the growth and metastasis of esophageal cancer cells. Further study is needed on the role of S1P and S1P5 receptor in esophageal cancer.

COMMENTS

Background

Esophageal cancer is one of the most common malignancies worldwide. Its mortality is very high due to relatively late diagnosis and inefficient treatment. The ability to reverse the outcome of esophageal cancer is limited due to a poor understanding of its biology. Progression of esophageal cancer may be associated with sphingosine 1-phosphate (S1P) and its receptors S1P1-5, which play an important role in other cancers.

Research frontiers

S1P is a pleiotropic phospholipid mediator. S1P binding to its 5 receptors S1P1-5 mediates diverse cellular responses such as cell proliferation, differentiation, migration, adhesion, and morphogenesis. Recent studies have shown that S1P and its receptors are related with cancer growth and metastasis. In this study, esophageal squamous cell carcinoma (ESCC), S1P receptor expression pattern, and the role of S1P receptors in proliferation and migration of ESCC cells were studied.

Innovations and breakthroughs

Recent reports have highlighted the importance of S1P and its receptors for the growth, metastasis and infiltration of tumors, such as gastric cancer, thyroid cancer, ovarian cancer, and glioma. This study showed that deficiency in inhibitory effect of S1P-S1P5 may be of importance in the growth and metastasis of esophageal cancer.

Applications

The results of this study indicate that deficiency in inhibitory effect of S1P-S1P5 may be of importance in the growth and metastasis of esophageal cancer. S1P5 or its associated signaling molecules may serve as a future strategy in biotherapy for esophageal cancer.

Terminology

S1P is a bioactive lipid that regulates central cellular processes such as cell proliferation, differentiation, migration, adhesion, and morphogenesis. S1P is present in plasma and serum at high nanomolar concentrations. S1P binding to related G-protein coupled receptors is designated as S1P1-5. S1P1, S1P2, and S1P3 receptors which are ubiquitously expressed, whereas S1P4 is restricted to lymphoid tissues and platelets and S1P5 is predominantly expressed in the central nervous system. Binding of S1P to its receptors activates different signaling pathways via the heterotrimeric G proteins.

Peer review

The paper is well written and presents an interesting aspect of the pathophysiology of esophageal mucosa which is related to the evolution of esophageal cancer.

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Incidence and risk factors for the development of anemia following gastric bypass surgery

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Abstract

AIM: To evaluate the incidence and risk factors for the development of anemia after Roux-en-Y gastric bypass (RYGB).

METHODS: A retrospective analysis of patients undergoing RYGB from January 2003 to November 2007 was performed. All patients had a preoperative body mass index $> 40 \text{ kg/m}^2$. A total of 206 patients were evaluated. All patients were given daily supplements of ferrous sulfate tablets for 2 wk following their operation. Hematological and metabolic indices were routinely evaluated following surgery. Patients were followed for a minimum of 86 wk.

RESULTS: There were 41 males and 165 females with an average age of 40.8 years. 21 patients (10.2%) developed post-operative anemia and 185 patients (89.8%) did not. Anemia was due to iron deficiency in all cases. The groups had similar demographics, surgi-

cal procedure and co-morbidities. Menstruation ($P = 0.02$) and peptic ulcer disease ($P = 0.01$) were risk factors for the development of post-operative anemia.

CONCLUSION: Iron deficiency anemia is frequent. RYGB surgery compounds occult blood loss. Increased ferrous sulfate supplementation may prevent iron depletion in populations at increased risk.

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Key words: Anemia; Complication; Gastric bypass surgery; Obesity

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Avgerinos DV, Llaguna OH, Seigerman M, Lefkowitz AJ, Leitman IM. Incidence and risk factors for the development of anemia following gastric bypass surgery. *World J Gastroenterol* 2010; 16(15): 1867-1870 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v16/i15/1867.htm> DOI: <http://dx.doi.org/10.3748/wjg.v16.i15.1867>

INTRODUCTION

Morbid obesity is one of the most common causes of morbidity and mortality in Western countries, and its incidence is rising with unpredictable consequences. It has been estimated that in the next 20 years, 40% of the population in the United States will be morbidly obese with a body mass index (BMI) greater than 30 kg/m^2 ^[1].

Roux-en-Y gastric bypass (RYGB) has become a common procedure for achieving short- and long-term weight loss. It has gained great popularity among surgeons and patients in recent years. Long-term complications are still being discovered, and a variety of nutritional problems have been reported^[2]. Because of the altered anatomy, absorption of iron from the proximal

gastrointestinal tract is impaired. Anemia develops in some patients with inadequate oral supplementation or chronic occult blood loss. The purpose of this study is to identify the incidence and risk factors of anemia in morbidly obese patients who underwent RYGB.

MATERIALS AND METHODS

Patients

After approval by the Institutional Review Board of Beth Israel Medical Center, a retrospective review of 206 medical records of patients who had undergone RYGB for the treatment of morbid obesity from January 2003 to November 2007 was conducted. Information gathered included demographic characteristics (age, gender, and ethnicity); BMI; past medical and surgical history; relevant laboratory values (pre- and post-operative levels of serum hemoglobin (Hg), hematocrit, mean corpuscular volume, iron, ferritin, and total iron binding capacity); length of bypass; and degree of weight loss after surgery.

One surgeon (IML) performed all gastric bypass operations using the same operative technique. All patients included in the study underwent standard RYGB surgery with the biliopancreatic limb measured to be 70 + BMI (in cm). The alimentary limb was 75 cm in length. Post-operatively, all patients were given a standard chewable multivitamin (Centrum Chewable, Wyeth Consumer Health Care) and ferrous sulfate tablets, 320 mg daily, starting on the 12th d after the operation. All patients were followed closely by a team of dietitians, bariatric surgeons, and psychologists, both before and after the operation. Laboratory data for hematologic and metabolic indices were obtained at frequent intervals post-operatively. Anemia was defined as serum Hg < 11 g/dL in men, and < 10 g/dL in women. Patients with epigastric pain and/or anemia underwent diagnostic upper gastrointestinal endoscopy. Marginal ulcer was treated with sucralfate slurry and oral proton pump inhibitors for a minimum of 2 wk and patients were reexamined endoscopically 6 wk later.

Statistical analysis

Statistical analysis using the Student *t*-test was used to compare pre- and post-operative serum levels of Hg, iron, and total iron binding capacity, and the χ^2 test was utilized to compare categorical data. The level of statistical significance was set at $P \leq 0.05$.

RESULTS

Of 206 patients analyzed, 41 (19.9%) were men and 165 (80.1%) women, with a mean age of 40.8 years (range: 18-60 years). A total of twenty-one (10.2%) patients developed anemia at some point during the post-operative period (Figure 1). Following statistical analysis, patients with the greatest risk for anemia were menstruating females ($P = 0.02$) and patients found to have marginal ulcer on endoscopy ($P = 0.01$). In all cases the anemia was due to iron deficiency (low serum ferritin, elevated total

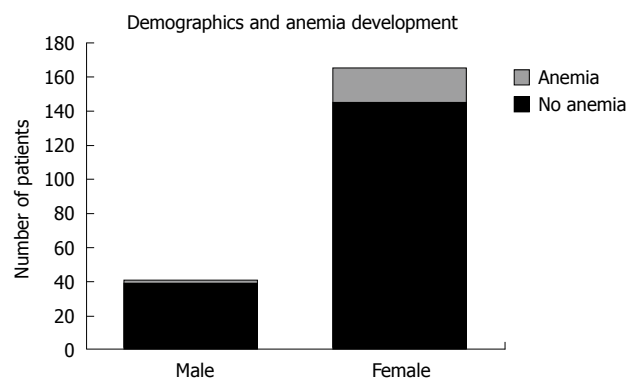


Figure 1 Number of patients who developed post-operative anemia.

Table 1 Associated co-morbidities in the patients who did not develop anemia compared with those who did develop anemia in the post-operative period

Co-morbidity	% of patients without anemia	% of patients with anemia	P
Hypertension	81.1	79.5	0.72
Diabetes mellitus	24.2	21.0	0.71
Asthma	29.5	19.8	0.89
Smoking	49.0	44.1	0.86
Menstruation	67.7	84.2	0.02
Peptic ulcer disease	6.0	22.0	0.01
Coronary artery disease	7.9	14.2	0.75
Thyroid disease	13.2	5.6	0.19

iron binding capacity, and low mean corpuscular volume). Table 1 shows the associated co-morbidities in the patients. The mean values of serum Hg and iron pre- and post-operatively (at 18, 44, 51, and 86 wk after the operation) for all patients are shown in Figure 2A and B. In the immediate post-operative period, serum Hg increased compared to the pre-operative state, and then decreased slowly over time. In contrast, serum iron dropped significantly in the immediate post-operative period, and then rose gradually with oral supplementation.

DISCUSSION

Nutritional deficiencies following RYGB have been previously reported. Vitamin deficiencies^[3], disorders of calcium^[4] and copper homeostasis^[5] have been reported elsewhere. Iron deficiency anemia presents a special clinical challenge. Disturbing behaviors such as pica (eating of non-food substances) and pagophagia (excessive ice-eating) have been observed in patients with anemia following gastric bypass surgery^[6,7]. Severe iron deficiency anemia may require parenteral injection of iron or even packed red blood cell transfusion^[8].

Anemia after RYGB may be multi-factorial; resulting from impaired absorption due to the surgically altered gastrointestinal tract, inadequate oral intake, or due to occult blood loss. Anemia may result from bleeding due to the operation itself, such as oozing from the staple or suture lines, marginal ulcers, gastritis, and anastomotic bleeding or due to malabsorption of compounds im-

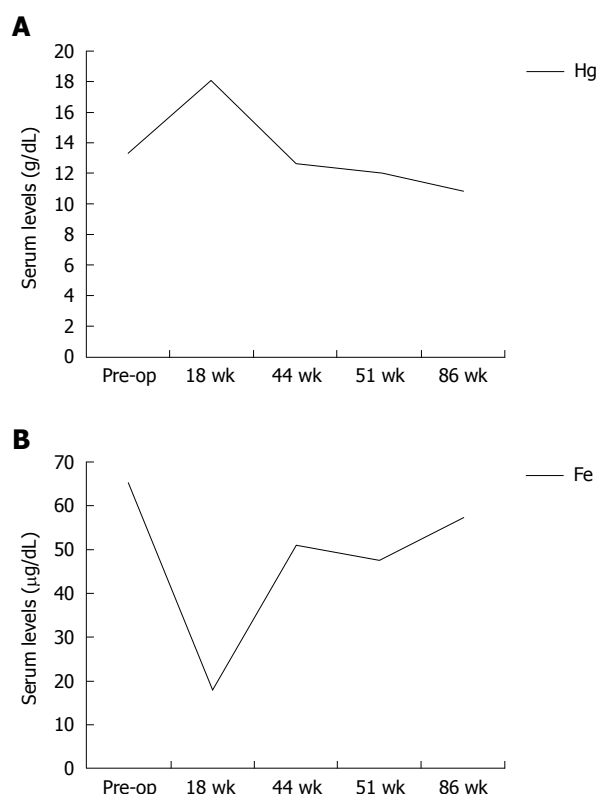


Figure 2 Serum hemoglobin (Hb) (A) levels (g/dL) and iron (Fe) (B) levels (µg/dL) pre- and post-operatively.

portant for the metabolism of Hg, such as iron, folate, thiamine, vitamin B₁₂, niacin, riboflavin, vitamin C, zinc, and copper^[1,9]. Diminished intake of red meats (a major natural source of iron) after gastric bypass surgery may further contribute to iron deficiency in these patients^[10]. The most prevalent type of anemia is iron deficiency, since iron is absorbed by the duodenum, and this type of anemia results from direct malabsorption due to exclusion of the duodenum from orally ingested nutrients. In addition, the food bolus does not encounter normal amounts of gastric acid secreted by the distal stomach which results in impaired conversion of ingested ferric iron to absorbable ferrous iron^[9]. Although prophylactic multivitamin supplements are routinely prescribed for RYGB patients, there are limited data in the medical and surgical literature demonstrating the efficacy of these supplements in the prevention of anemia after gastric bypass surgery^[11].

Symptoms of iron deficiency anemia may be nonspecific but include fatigue and muscle weakness, dyspnea, and chest discomfort^[12]. Typical laboratory findings include low serum ferritin, elevated total iron binding capacity, low mean corpuscular volume, and decreased intracellular Hg concentration. As such, iron deficiency anemia is typically hypochromic and microcytic^[13].

The results of the present study show that 10.2% of our patients developed iron deficiency anemia after RYGB, which is lower than the published data^[14]. It has been reported that anemia correlated best with serum transferrin saturation^[15]. Coupaye *et al*^[8] also reported

an incidence of anemia in 10% of patients. The present study demonstrates that risk factors for the development of iron deficiency anemia following RYGB are menstruation ($P = 0.02$) and peptic ulcer disease ($P = 0.01$). It is important to note that these patients developed anemia at some point during the post-operative period, despite being on standard iron supplementation. This may indicate that the current iron supplementation regimens used may not be adequate to prevent the development of iron deficiency anemia in certain patients after RYGB surgery. Love and Billett^[16] suggested that the risk of anemia following bariatric surgical procedures continues for life. The incidence of anemia does not appear to be greater following duodenal switch operations^[17].

Brolin *et al*^[11] found that multivitamin supplements lowered the incidence of folate deficiency, but did not prevent iron or vitamin B₁₂ deficiency. However, the authors did not suggest the prophylactic supplementation of vitamin B₁₂, simply because the patients did not develop clinically important deficiency of this vitamin post-operatively. On the other hand, they found that iron deficiency is very resistant to oral iron supplementation, especially in menstruating women, leading them to prescribe prophylactic high doses of oral iron (50 mg or more)^[11]. Varma *et al*^[18] found that menstruating females were more likely to require parenteral iron therapy after bariatric surgery. Other authors have suggested the addition of vitamin C to oral iron supplementation, but without great success^[19]. Previous reports suggest early parenteral iron replacement therapy in patients who do not respond to oral iron therapy in order to prevent anemia-related complications and to maintain patients' quality of life^[2,17]. In a prospective, double-blind, randomized study, Brolin *et al*^[20] found that prophylactic oral supplementation of 320 mg iron twice daily consistently prevented iron deficiency in menstruating women after RYGB.

In conclusion, it is characteristic that iron deficiency anemia secondary to malabsorption may not start until 2-3 mo after RYGB and can be present up to 2 years later^[1] as well as refractory to oral supplementation^[20]. As a result, it is of utmost importance that a protocol of long-term follow-up and close biologic and metabolic monitoring of patients after RYGB and the need for a more aggressive therapeutic approach when anemia has been diagnosed is established. Increased ferrous supplementation may be necessary to prevent iron depletion in populations at increased risk of developing iron deficiency anemia after RYGB surgery, such as menstruating women and patients with peptic ulcer disease.

COMMENTS

Background

With the wide application of bariatric surgery in young patients, long-term follow-up is necessary. Iron deficiency and other causes of anemia are prevalent in a large percentage of patients.

Research frontiers

Females of childbearing age and patients with peptic ulcer disease are at the greatest risk for anemia following gastric bypass surgery.

Innovations and breakthroughs

Routine blood work with regular complete blood counts and serum iron levels are required to prevent severe anemia, which may become refractory to oral supplementation.

Applications

Patients with anemia should have endoscopic evaluation to determine possible peptic ulcer disease or other etiologies. Women with heavy menstrual flow should be counseled about the need for routine supplementation.

Terminology

Bariatric surgery is performed for patients with morbid obesity (body mass index greater than 40 kg/m² or greater than 35 kg/m² for patients with weight-related morbidities) hypertension, non-insulin diabetes or obstructive sleep apnea.

Peer review

This paper looked for the anemia commonly encountered problem after bariatric surgery.

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Ischemic preconditioning-induced hyperperfusion correlates with hepatoprotection after liver resection

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tissue (2.7 segments) were similar in both groups. In controls (PM), on reperfusion of liver remnants for 15 min, portal perfusion markedly decreased by 29% while there was a slight increase of 8% in the arterial blood flow. In contrast, following IP + PM the portal vein flow remained unchanged during reperfusion and a significantly increased arterial blood flow (+56% vs baseline) was observed. In accordance with a better postischemic blood supply of the liver, hepatocellular injury, as measured by alanine aminotransferase (ALT) levels on day 1 was considerably lower in group B compared to group A (247 ± 210 U/I vs 550 ± 650 U/I, $P < 0.05$). Additionally, ALT levels were significantly correlated to the hepatic artery inflow.

CONCLUSION: IP prevents postischemic flow reduction of the portal vein and simultaneously increases arterial perfusion, suggesting that improved hepatic macrocirculation is a protective mechanism following hepatectomy.

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Key words: Ischemic preconditioning; Reperfusion injury; Liver; Surgery; Liver blood flow

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Abstract

AIM: To characterize the impact of the Pringle maneuver (PM) and ischemic preconditioning (IP) on total blood supply to the liver following hepatectomies.

METHODS: Sixty one consecutive patients who underwent hepatic resection under inflow occlusion were randomized either to receive PM alone ($n = 31$) or IP (10 min of ischemia followed by 10 min of reperfusion) prior to PM ($n = 30$). Quantification of liver perfusion was measured by Doppler probes at the hepatic artery and portal vein at various time points after reperfusion of remnant livers.

RESULTS: Occlusion times of 33 ± 12 min (mean \pm SD) and 34 ± 14 min and the extent of resected liver

INTRODUCTION

The common strategy to reduce intraoperative blood loss in human liver resection consists of temporary clamping

of the portal triad (Pringle maneuver, PM)^[1]. The extent of bleeding during surgery is associated with higher postoperative complication rates^[2], and the need for autologous blood transfusion may correlate with earlier recurrence of malignancies^[3]. The length of the ischemic time strongly determines the release of liver enzymes after hepatectomy^[4] indicating considerable hepatocellular injury caused by PM^[5]. After declamping of the portal triad, reperfusion of the remnant liver causes additional damage to its parenchymal and non-parenchymal cells^[6] which may cause the loss of functional integrity and consecutive hepatic failure^[7,8].

Several strategies against the deleterious ischemia-reperfusion (I/R)-induced complications have been suggested^[9-11], but these were not routinely introduced in the field of hepatic surgery in humans. This is mainly because of the complex mechanisms involved in I/R, including metabolic, immunological, and microvascular changes which exhibit numerous interactions, rendering the liver a difficult target for preventive strategies.

A new experimental approach to reduce I/R-related injury in the myocardium was firstly presented by Murry^[12] who referred to “ischemic preconditioning” (IP) as an adaptation of the myocardium to ischemic stress induced by repetitive short periods of ischemia and reperfusion. Meanwhile, it could also be demonstrated that IP prior to sustained warm ischemia can protect parenchymal and non-parenchymal liver cells by increasing the tolerance against I/R-related organ hypoxia under experimental as well as clinical conditions^[13-16]. An important observation was also that IP in human liver surgery was associated with better intraoperative hemodynamic stability, particularly on reperfusion of warm ischemic livers^[17].

Although the underlying protective mechanisms of IP are still not fully understood^[18], some studies have shown that the activation of Kupffer cells, leucocytes and the release of cytotoxic mediators on reperfusion may lead to a substantial breakdown of the hepatic microcirculation, an event which seems to play a key role following warm and cold ischemia^[19-21]. For example, Klar *et al.*^[22] observed an inverse correlation between the hepatic microvascular blood flow rate and the maximum postoperative enzyme release from the liver. On the other hand, in healthy livers, a balanced portal vein (PV) and hepatic artery (HA) inflow are significantly dependent on the arterial buffer response, an autoregulation system which influences the whole blood supply to the liver at the level of the hepatic arterioles and portal venules, and which is assumed to be predominantly the result of adenosine action^[23,24].

At this time, there is evidence that IP improves the hepatic microcirculation after warm as well as cold ischemia, but the influence of PM and IP on macrocirculatory parameters have not been elucidated as yet. In this study, we therefore investigated the hepatic inflow in patients undergoing liver resections with special regard to postischemic liver injury and patient outcome.

MATERIALS AND METHODS

Patients and randomization

The study was approved by the local Ethics committee

and written informed consent was obtained from each patient before randomization. We investigated 116 consecutive patients at our institution who were subjected to liver resection (time period 12 mo). Of these, only 68 patients could randomly be assigned according to the inclusion criteria. These were defined as “significant” hepatectomies, i.e. removal of at least one segment. A total of 48 patients were excluded from randomization for the following reasons: (1) extent of liver resection less than one segment according to Couinaud ($n = 16$); (2) anticipated necessity of total vascular exclusion ($n = 8$); (3) necessity of additional surgical procedures such as bilioenteric anastomosis or associated gastrointestinal procedures ($n = 3$); (4) laparoscopic liver resection ($n = 10$); (5) underlying liver cirrhosis ($n = 9$); and (6) emergency surgery ($n = 2$). Of the 68 randomized patients, 7 were withdrawn from the analysis because of intraoperative detection of inoperability. Finally, 61 patients were randomized to a control group (A, $n = 31$), receiving PM, and to a study group (B, $n = 30$), who received IP by cross-clamping the portal triad for 10 min followed by 10 min of reperfusion prior to PM. Determination of blood flow of the common HA and PV was carried out simultaneously before starting PM or IP (baseline) as well as 10 min after IP (only group B), and at 15 min of reperfusion as well as before abdominal closure (group A, 32 ± 4 min; group B, 29 ± 6 min after declamping the portal triad) using the transit-time flowmeter (CardioMed CM 2005; MediStern AS, Oslo, Norway). This device measures the difference in travel time between pulses transmitted in the direction of, and against, the flow. The blood flow velocity is directly proportional to the measured difference between upstream and downstream transit times. Because the cross-sectional area of the probe/vessel was known as the probes were individually adapted to the vessel diameter, the product of that area and the flow velocity provided a measure of volumetric flow. The calculations were easily performed by a microprocessor-based converter and displayed online on a computer during surgery.

Study design

The targeted endpoints were the occurrence of IP- and PM-related flow changes of the HA and PV at defined time points. Secondary endpoints were serum levels of alanine aminotransferase (ALT) on postoperative day 1 and complication rates. Operations were performed by 4 experienced abdominal surgeons in a routine clinical setting. Transection was started immediately after inducing PM which was maintained until the transection was finished. Parenchymal transection was performed using a water jet cutter (Saphir Medical, Lyon, France). The volume of the resected liver was determined by the quantity of displaced fluid in a pre-filled trough.

All anesthetic procedures were performed by the same team of experienced anesthesiologists ensuring a standardized protocol. To meet intraoperative fluid demand and to compensate for blood loss, crystalloids and colloidal solutions, respectively, were infused as described elsewhere^[17]. Adequate mean arterial pressure (MAP >

Table 1 Baseline data of study patients

Group	Age (yr)	Sex (n) M/F	Tumor (n) mal/non mal	Fibrosis (n) none/mod/sev	Steatosis (n) none/min/mod/sev
Control (A) (n = 30)	57 ± 14 (26-81)	18/12	26/4	10/15/5	8/16/5/1
IP (B) (n = 31)	55 ± 13 (28-77)	19/12	28/3	12/14/5	10/14/5/2
P-value	0.61	0.92	0.65	0.39	0.39

There were no significant differences between the control and ischemic preconditioning (IP) groups. Fibrosis: min ($\leq 10\%$); mod ($\leq 40\%$); liver steatosis: min ($\leq 25\%$); mod ($\leq 50\%$); sev ($\geq 50\%$); mal: malignant. Examination by pathologist of non-cancerous adjacent liver tissue; all patients included.

Table 2 No differences in the intraoperative data of the 2 groups of patients

Group	OP time (min) mean/range	PM time (min) mean/range	LR time (min) mean/range	LV resected (mL) mean/range	Segments resected (n) mean/range
Control (A) (n = 30)	260 ± 63 (170-420)	34 ± 14 (15-82)	30 ± 10 (10-50)	390 ± 303 (80-1400)	2.7 ± 1.3 (1-5)
IP (B) (n = 31)	271 ± 58 (180-420)	33 ± 12 (8-67)	31 ± 11 (15-56)	426 ± 453 (30-2000)	2.7 ± 1.1 (1-5)
P-value	0.36	0.70	0.83	0.77	0.69

OP: Operation; PM: Pringle maneuver; LR: Liver resection; LV: Liver volume.

65 mmHg), central venous pressure (CVP 9-14 mm Hg), and diuresis (> 100 mL/h) were maintained throughout the operation by fluid infusion and, when necessary, by administration of vasopressors (dopamine 2-3 μ g/kg per hour and/or norepinephrine) as appropriate.

Laboratory parameters of hepatocellular injury (ALT) and liver function (bilirubin) were obtained before surgery and on postoperative days 1, 2 and 7. Transient liver failure was defined as bilirubin levels > 5 mg/dL and/or prothrombin activity $< 40\%$ for at least 3 postoperative days. Fatal liver failure was defined as death from irreversible hepatic dysfunction in the absence of other causes.

Statistical analysis

Numerical values are presented as mean and standard deviation unless otherwise noted. All significance tests were 2-sided and a P -value < 0.05 was considered statistically significant. Data analysis was performed using SPSS 11.0 (SPSS Inc., Chicago, USA). Comparison between the 2 groups (with/without IP) was performed using the Mann-Whitney U test, the χ^2 test or the Fisher exact test, as appropriate. The association between flow parameters and peak levels of postoperative ALT (day 1) was evaluated by the Pearson Product Moment Correlation. Multivariate analysis of complications was performed by means of logistic regression (backward selection). A multivariate analysis was performed by entering parameters that appeared to be of significance on the univariate analysis into a Cox proportional hazard model to test for significant effects while adjusting for multiple factors.

RESULTS

Baseline data

The analysis of collected data was based on the criteria of the CONSORT group^[25]. There were no differences in demographic data and liver histology between the 2 groups (Table 1). Intraoperative parameters were also comparable between the controls and the study population (Table 2).

Flow characteristics

The perfusion data of the HA and PV prior to any intervention (baseline) did not differ between groups (Table 3). Patients who did not receive IP (controls), showed a markedly decreased PV flow by 29% at 15 min reperfusion and by 26% before abdominal closure (32 ± 4 min after declamping). Simultaneously, a slight increase in HA flow of 8% and 3.5% was observed after 15 and 32 min, respectively, of reperfusion of the liver remnants (Figure 1A). In contrast, patients who received IP (group B), maintained stable PV flow during the IP procedure as well as at 15 and 29 min after declamping the portal triad (Figure 1B). In addition, IP induced a more than 200% increase in HA perfusion immediately after IP and the significantly elevated arterial flow was maintained at 15 min (+56%) as well as at 29 min (+38%) after starting reperfusion of the liver remnants, demonstrating a continuing influence of IP on the postischemic blood supply (Table 3, Figure 1B). This results in a total increase in liver perfusion *via* HA and PV of 27% when patients underwent the IP procedure ($P < 0.01$, Table 3).

Laboratory parameters

Postischemic liver damage was measured by ALT levels during the postoperative course. In controls, we observed a significant ALT increase from 28 ± 12 U/L to 550 ± 659 U/L on day 1 when compared to preoperative values, which clearly suggests the PM as the cause of enzyme release. In contrast, IP-treated patients showed markedly reduced ALT levels of about 50% (247 ± 210 U/L, $P < 0.05$), indicating substantial hepatoprotection by this procedure (Table 4). In the further course, postischemic elevated ALT levels in both groups returned to normal within 7 d of hepatectomy. Serum bilirubin levels were determined as a parameter of hepatocellular function, but did not show notably different values at any time during the postoperative course (days 1-7, Table 4).

Intraoperative parameters and postoperative course

Blood loss as well as the need for autologous transfusion

Table 3 Flow characteristics of patients undergoing PM (control, *n* = 30) and IP + PM (study group, *n* = 31)

Group	Vessel	Baseline (mL/min)	After IP (mL/min)	15 min (mL/min)	Before abd. closure (mL/min)
Control	HA	141 ± 24	-	152 ± 29	146 ± 18
IP	HA	126 ± 19	263 ± 51	196 ± 38 ^a	174 ± 22 ^a
Control	PV	1023 ± 130	-	726 ± 121	757 ± 58
IP	PV	930 ± 94	920 ± 81	919 ± 75 ^b	949 ± 62 ^b
Total (Co)	HA + PV	1164 ± 72	-	878 ± 77	903 ± 37 ^c
Total (IP)	HA + PV	1056 ± 53	1183 ± 64	1115 ± 49 ^b	1123 ± 58 ^b

There were significantly enhanced PV, HA, and total flows in IP-treated patients. PM: Pringle maneuver; HA: Hepatic artery; PV: Portal vein; abd.: Abdomen; Co: Control. ^a*P* < 0.05, ^b*P* < 0.01 vs control; ^c*P* < 0.05 vs baseline.

Table 4 Outcome data of patients undergoing liver resection with PM (A) or with IP + PM (B)

Group	RPC (units) mean/range	ALT (U/L) mean/range	Bilirubin (mg/dL) mean/range	LF (<i>n</i>) transient/fatal ^a	Biliary compl. (<i>n</i>) major/minor
Control (A) (<i>n</i> = 30)	0.90 ± 1.24 (0-5)	550 ± 650 (54-2888)	1.73 (0.40-9.83)	2 (2)	2 ^b (4)
IP (B) (<i>n</i> = 31)	0.47 ± 1.31 (0-6)	247 ± 210 (45-852)	1.40 ± 1.26 (0.23-5.59)	0 (1)	1 (2)
<i>P</i> -value	0.014	0.04	0.69	0.04	0.04

^aDeath; ^bRequiring re-operation. Total complication rate was 14/30 (45%) in controls (A) and 6/31 (20%) in the IP group (B). One death in the study group (B) resulted from a perforated duodenal ulcer (postoperative day 21). RPC: Red packed cells; ALT: Alanine aminotransferase; LF: Liver failure; compl.: Complications.

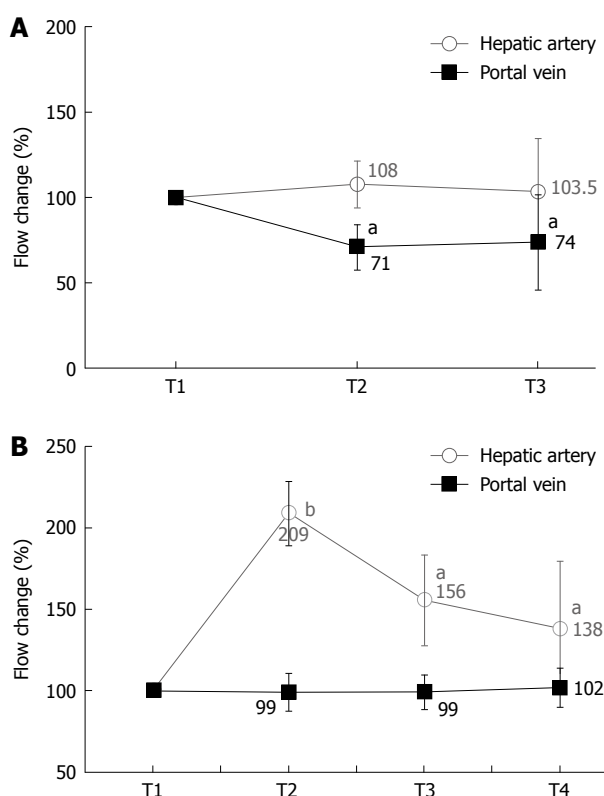


Figure 1 Changes in portal vein (PV) and hepatic artery (HA) inflow (100% = baseline) at operation (mean ± SD) in the control group (A) and study population (B). T1: Before starting the Pringle maneuver; T2: At the end of the IP procedure, i.e. 10 min ischemia and 10 min of reperfusion; T3: One minute of simultaneous reperfusion of PV and HA; T4: Immediately before abdominal closure (29 ± 6 min and 32 ± 4 min after stopping portal triad crossclamping in group A and B, respectively). ^a*P* < 0.05, ^b*P* < 0.01 vs baseline.

were significantly lower in the IP-treated group with 17% of patients receiving blood transfusion vs 48% in the control group (*P* < 0.05, Table 4). The postoperative

course was uneventful in 24/30 (80%) patients in group B but only in 17/31 (53%) patients in group A (*P* < 0.05). Liver dysfunction, as previously defined, occurred in 2 patients of group A, but only in one patient of the IP-treated group (Table 4). Biliary leakage ceased spontaneously in 4 of the 6 patients (67%) of controls, but the other two patients required re-operation and bilioenteric anastomosis. In the study group, 2 patients had transient bile secretion and one patient of this group needed re-operation (bilioenteric anastomosis) (Table 4).

Blood supply to the liver and hepatocellular injury

With regard to earlier work, demonstrating a strong correlation between microcirculatory failure and postischemic enzyme release^[22,26], it was of particular interest to determine whether there were changes in macrohemodynamic parameters, i.e. the PM and IP may have an impact on parenchymal cell damage. Firstly, we analyzed the correlation between PV flow and ALT levels on day 1. Interestingly, by applying the Pearson Product Moment Correlation we did not find a significant association between the amount of the hepatocellular injury and quality of PV perfusion, either in controls (*r* = -0.38, *P* = 0.3) or in IP-treated patients (*r* = -0.41, *P* = 0.2). In contrast, when the HA flow of patients with PM (controls) and the corresponding ALT values on day 1 were analyzed, we found a weak, but significant inverse correlation, indicating a substantial influence of the macrocirculation at reperfusion on post-ischemic liver injury (*r* = -0.62, *P* = 0.042, Figure 2A). This correlation was even more evident, when patients underwent IP prior to PM as shown in Figure 2B (*r* = -0.73, *P* = 0.024), suggesting the HA perfusion was more susceptible to the procedure of IP in warm liver I/R.

Predictors of postoperative morbidity

Parameters which were predictive for the development of

Table 5 Factors predicting occurrence of postoperative complications by multivariate logistic regression (backward selection) of factors of importance or significance on univariate analysis

Analysis (<i>P</i> -value)	IP treatment	Length of PM	Resected LV	HA flow	PV flow	Liver steatosis	Liver fibrosis
Univariate	0.038	0.043	0.067	0.044	0.72	0.81	0.94
Multivariate	0.047	0.022	NS	NS	NS	NS	NS

Treatment with IP and length of the PM are the only independent predictors of complications. NS: Not significant.

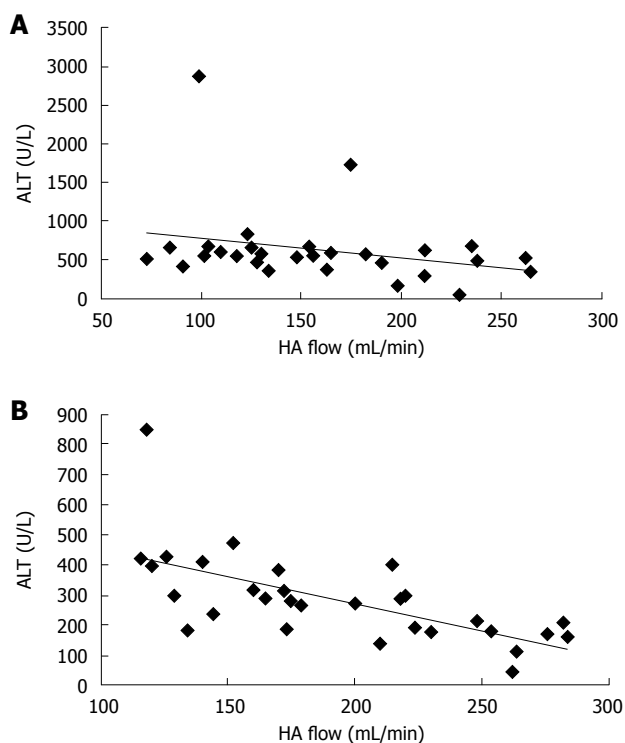


Figure 2 The Pearson product moment correlation between HA flow and alanine aminotransferase (ALT) levels. On day 1, there is an inverse correlation ($P < 0.05$) in the control group (A) undergoing PM ($r = -0.62$). In patients undergoing IP prior to PM (B), an even stronger correlation ($r = -0.73$, $P < 0.01$) was found. Straight lines represent regression analysis; data include values of all patients.

postoperative complications (ALT release, hepatic failure, biliary complications) are given in Table 5. Of 7 investigated parameters only the length of the PM and the procedure of IP were factors independently influencing the outcome of patients after hepatic resection. In particular, it was also found that the quality of HA perfusion was not predictive of complications, presumably reflecting the complex downstream mechanisms of liver I/R which might only in part be related to macrohemodynamic changes of blood flow to the postischemic liver.

DISCUSSION

This study presents the first assessment of human hepatic macrocirculation in response to I/R injury after the PM and IP in human hepatectomy. The main results obtained are: (1) that IP significantly improves hepatic macrocirculation upon reperfusion of liver remnants; (2) that the HA contributes most to the improved blood supply of the

liver; and (3) that the quality of HA flow exhibits a strong correlation with postischemic ALT release.

During liver surgery in humans clamping of the portal triad (PM) is practiced to minimize intraoperative blood loss, but can lead to serious liver dysfunction^[2,7,27]. Consequently, it has been generally accepted that periods of warm and cold ischemia of the liver should be shortened as much as possible^[4,28]. Some experimental and clinical studies suggested that intermittent clamping of the portal triad reduced the negative effects of prolonged continuous warm ischemia on hepatic reperfusion injury^[7,29]. However, the most important problems associated with that procedure are an increased blood loss during the episodes of reperfusion^[7,30] and a marked prolongation of the operation time^[30,31]. Ischemic preconditioning may combine the beneficial effects on reperfusion injury with the avoidance of additional blood loss during surgery^[30,32].

In animal experimental studies, hepatic I/R is associated with perfusion failure of sinusoids due to significant hemoconcentration, reduced perfusion pressure, pressure-related sinusoidal leukostasis, as well as sinusoidal narrowing caused by hypoxia-induced endothelial swelling^[33]. The impaired restoration of hepatic microvascular flow correlates also with the extent of liver injury after hemorrhagic shock and resuscitation^[34]. Prevention of microcirculatory failure may largely protect the liver from parenchymal cell necrosis after I/R, suggesting that the degree of microvascular failure determines the extent of lethal hepatocyte injury^[34]. Similar relationships between liver perfusion and injury could be observed in humans after warm and cold ischemia^[26,35]. Interestingly, Puhl *et al.*^[26] could demonstrate a significant inverse correlation of the quality of the microvascular perfusion with postoperative liver enzyme release as well as bilirubin elimination in human liver transplants. This indicates that the observed (relative) hyperemia in the sinusoids might confer protection against postischemic liver injury. Another study by Klar *et al.*^[22] could also demonstrate in patients an inverse correlation between the intraoperatively measured hepatic microvascular blood flow rate and the maximum postoperative enzyme release from the liver. However, neither study included data on macrohemodynamic parameters, i.e. flows in the HA and PV in these clinical settings. Therefore, it cannot be concluded that the preservation of the sinusoidal blood flow after I/R can be the result of preservation of the HA and PV inflow during the reperfusion period.

While changes in the nutritive sinusoidal blood flow are the result of complex humoral, cellular, and immunologic interactions, the mechanisms leading to microcirculatory shutdown after liver I/R are related, at least in part

also to upstream mechanisms, i.e. regional macrocirculation of the liver^[23,36]. Consequently, efforts should also focus on the preservation of an optimal blood supply to the liver under different pathological conditions at the levels of the HA and PV. In line with this, it was demonstrated that hepatosplanchnic blood flow was still reduced in humans at 60 min of reperfusion after severe hypovolemia, even though arterial blood pressure, cardiac output, and blood flow to other organs was already fully restored^[37].

In the present study, we observed a severely decreased portal blood flow and a minimal increase in the HA flow on reperfusion of livers undergoing more than 30 min of portal crossclamping, a time period which is known to induce significant hepatocellular injury in humans^[16]. We also found that IP could abolish the postischemic PV flow decrease and, *vice versa*, IP induced a significantly better HA flow throughout the reperfusion period, resulting in a markedly improved overall blood supply to the liver. This might be of substantial importance with regard to the nutritive sinusoidal perfusion, given that the autoregulatory capacity of the HA to maintain a constant flow rate in the presence of pathological conditions is limited, known as the hepatic arterial buffer response^[23,24]. Therefore, IP effectively restored the total hepatic flow to almost normal values during reperfusion whereas in the control group the minimal increase of the HA flow failed to compensate for the postischemic perfusion deficit at the PV (Table 3). Because hemodynamic parameters, like MAP, CVP, and heart rate were kept stable upon reperfusion, and IP-treated patients did need significantly lower norepinephrine administration to maintain an adequate MAP, this observation can only be explained by an IP-mediated effect^[17]. However, the exact mechanisms for that observation cannot be answered in the present study, although it was reported that both the hepatic arterial pressure-flow autoregulation and buffer response are mediated through changes in the wash-out of locally produced adenosine^[38], and that IP may effectively increase the formation of the vasodilator adenosine while the unwanted degradation of adenine nucleotides to purines caused by the PM can be attenuated by IP^[39].

Interestingly, we observed also a significant correlation between the postischemic ALT release on day 1 and the HA flow in controls, and this was even more pronounced in IP-treated patients, which clearly demonstrates that local macrohemodynamic changes at the HA may play an important role in the prevention of hepatic I/R injury. Because in our study hepatocellular damage was independent of postischemic PV flow changes, one can only speculate about the relevance of this observation, in particular with regard to the “small for size” problem in living related donor liver transplantation. This phenomenon, referred to as portal venous hyperperfusion of the partial liver allograft and associated with severe transplant dysfunction is thought to be the result of an imbalanced autoregulation of the arterial buffer response, resulting in a low concentration of adenosine-mediated HA branch constriction in the presence of increased portal perfusion^[40]. Because hepatectomies with the loss of up to 5 liver segments as in our study may be compared to the above-mentioned

situation, it was interesting that we found a substantial decrease of the PV flow upon reperfusion in controls which was not adequately compensated by an increase in the HA flow whereas in IP-treated patients the HA flow was significantly enhanced while the PV flow was kept stable during the reperfusion period, suggesting an impact of IP on the empirically observed reciprocal regulation between PV and HA inflow^[37]. However, the previously described substantial alterations of nutritive sinusoidal flow impairments may occur, even in the absence of overt macrohemodynamic changes, as a result of an I/R-induced heterogeneity of hepatic microvascular downstream mechanisms^[36,37]. In addition, despite postischemic ALT release being a well-established parameter for the estimation of hepatic injury following I/R, ALT levels alone are not predictive of the occurrence of postoperative liver failure following liver resection or transplantation^[14,41].

In summary, this study provides some new insights into macrohemodynamic changes during liver resection under inflow occlusion and on treatment with IP in humans. As we could not simultaneously investigate the hepatic microcirculation in this setting, the impact of HA and PV perfusion alterations on the nutritive blood supply in sinusoids remains speculative. Further studies are necessary to clarify this aspect, and in particular, the impact of macro- and microhemodynamic changes on postischemic liver function in humans.

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COMMENTS

Background

Liver surgery has become a safe procedure in the past years and is mainly done because of malignant tumors. A common strategy to reduce blood loss during surgery is to temporarily shut down the blood supply to the liver [pringle maneuver (PM)], which, however may be associated with severe hepatocellular injury and consequent enhanced morbidity.

Research frontiers

Many efforts were undertaken to overcome the deleterious effects of ischemia-reperfusion injury of the liver caused by the PM. A new method of hepatocellular protection comprises ischemic preconditioning (IP), i.e. an additional short ischemia and reperfusion period prior to sustained ischemia, as set by the PM. However, mechanisms of protection by IP are still largely unknown.

Innovations and breakthroughs

Recent reports have highlighted numerous mechanisms which are involved in the protection of ischemic livers, including humoral, cellular, and immunologic interactions. Furthermore, an improved hepatic microcirculation seems to play a key role in liver protection following IP. However, no data were available which comment on hepatic macroperfusion under different conditions, such as IP.

Applications

By understanding how changes of blood flows of the portal vein and hepatic artery under inflow occlusion or IP may influence hepatocellular damage, this

study may provide some strategies for therapeutic intervention during liver surgery, such as selective portal triad clamping.

Terminology

IP of the liver consists of a short ischemia-reperfusion period (e.g. 10 min/10 min) immediately prior to longer periods of liver ischemia, which are often necessary during extended liver resections. Although it seems paradoxical, this additional short ischemia-reperfusion period confers protection on the liver by different mechanisms.

Peer review

This is a good study from a well-known liver surgery group with excellent methodology and adequate data analysis. The group expanded on their previous investigations on the role of IP in liver protection following resection. In the current study, the authors intuitively studied the effect of IP on hepatic artery and portal vein blood flow and demonstrated that IP prevented postischemic flow reduction of the portal vein and significantly increased the arterial perfusion.

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Comparative analysis of clinicopathological correlations of cyclooxygenase-2 expression in resectable pancreatic cancer

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Abstract

AIM: To perform a comparative analysis of clinicopathological correlations of cyclooxygenase-2 (COX-2) expression in pancreatic cancer, examined by monoclonal and polyclonal antibodies.

METHODS: The COX-2 expression in 85 resection specimens of pancreatic ductal adenocarcinoma was immunohistochemically examined using both monoclonal and polyclonal antibodies. The final immunoscores were obtained by multiplying the percentage of positive cells with the numeric score reflecting the staining intensity.

COX-2 expression levels were classified into three categories (0, 1+, and 2+) and the clinicopathological correlations were statistically evaluated and analyzed.

RESULTS: The positive tumor expression rates of COX-2 were 80.5% using monoclonal antibody and 69.4% using polyclonal antibody. In the Kaplan-Meier analysis, no significant correlations were found between levels of COX-2 expression and overall survival (OS), but trends to longer OS were found in COX-2 negative cases using monoclonal antibody. Significantly longer disease free survival was revealed in COX-2 negative cases using monoclonal antibody ($P = 0.019$). No correlations between COX-2 expression levels and grade (G), tumor (T) status and nodal (N) status were demonstrated. Low histological grade showed a strong association with a longer OS ($P < 0.001$). Correlation of survival and T status revealed a shorter OS in T3 tumors, but the results reached only marginal statistical significance ($P = 0.070$). In the multivariate Cox proportional hazards regression model, histological grade, T and N status remained valuable predictors of a worse survival with borderline significance for T [hazards ratio (HR) = 4.18 for G (if G = 3, $P < 0.001$); HR = 1.64 for T (if T = 3, $P = 0.065$); HR = 2.53 for N (if N = 1, $P = 0.006$)]. Higher grade, T or N status was associated with a worse OS.

CONCLUSION: The immunohistochemically assessed level of COX-2 expression does not seem to represent a valuable independent prognostic factor and is not superior to the conventional prognostic factors.

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Key words: Pancreatic cancer; Cyclooxygenase-2; Immunohistochemistry; Monoclonal antibody; Polyclonal antibody

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INTRODUCTION

Invasive ductal adenocarcinoma is the most common neoplasm in the pancreas, constituting about 90% of all pancreatic tumors^[1]. Pancreatic cancer represents the fourth leading cause of cancer-related death among men and women in western countries^[2,3]. Pancreatic cancer has no early warning signs and symptoms, so most patients present with advanced disease. Despite improved diagnostic and therapeutic modalities, pancreatic cancer still has a very poor prognosis. The incidence of pancreatic cancer almost equals the mortality rate, and it has one of the lowest overall 5-year survival rates (under 5%) among the epithelial cancers^[4-6].

The currently accepted model of the development of pancreatic ductal adenocarcinoma (PDAC) understands the oncogenesis of this malignancy as a multistep process, characterized by the progression from the normal ductal epithelium through the spectrum of duct lesions known as pancreatic intraepithelial neoplasia (PanIN) to invasive ductal adenocarcinoma^[7,8]. PanINs represent precursor lesions of ductal adenocarcinoma and their classification system distinguishes three grades of PanINs, which harbor a number of well-established molecular events including activation of oncogenes, inactivation of tumor suppressor genes, inactivation of DNA mismatch repair genes, various epigenetic alterations, dysregulation of oncoproteins, and others^[9-11]. Mediators of inflammatory pathways [e.g. cyclooxygenase-2 (COX-2), nuclear factor- κ B (NF- κ B), 5-lipoxygenase (5-LOX), interleukin-8 (IL-8) *etc.*] are also known to play an important role in carcinogenesis of pancreatic cancer and represent a key link between chronic inflammation and cancer^[12-14]. Prostaglandin H2 synthase (COX) represents an enzyme which is involved in the conversion of arachidonic acid to prostaglandins. Two COX isoforms, COX-1 and COX-2, have been identified to date^[15]. COX-1 is constitutively expressed in many tissues, and is involved in prostaglandin synthesis under physiological conditions. COX-2 is expressed under certain extracellular or intracellular stimuli, including mitogens, growth factors, proinflammatory cytokines, hormones and infectious agents, and is a component of cellular responses to inflammation. COX-2 is overexpressed in many human solid tumors including pancreatic cancer^[16,17]. Several studies have suggested a potential involvement of COX-2 pathways in the regulation of tumor-associated angiogenesis and cell growth in pancreatic cancer. COX-2 has been demonstrated to inhibit apoptosis, promote cell proliferation and to induce the expression of vascular endothelial growth factor^[18-21]. COX-2 overexpression has been reported in 56% to 90%

of ductal adenocarcinomas^[22]. A high level of COX-2 overexpression has also been described in PanIN lesions^[23-25], and COX-2 was suggested as a potential therapeutic target for chemoprevention and therapy of pancreatic cancer^[22,26].

A significant inverse relationship between COX-2 overexpression and survival rates has been reported in retrospective studies of different types of malignancies^[27-32]. Conflicting results have been shown in pancreatic cancer^[33-39] and the possible role of primary antibody used for the detection of COX-2 expression has been suggested^[33,40]. Comparative analysis has not yet been performed. In our study the expression of COX-2 in resectable pancreatic ductal adenocarcinomas was immunohistochemically examined using both monoclonal and polyclonal antibodies, and analyzed and correlated with clinicopathological parameters.

MATERIALS AND METHODS

Study group and tissue specimens

The study group (summarized in Table 1) consisted of 85 patients [41 males (48.2%) and 44 females (51.8%); median age 61 ± 9.5 years (range 39-85 years)] with resectable pancreatic cancer who had undergone pancreatectomy at the Faculty Hospital Brno and the Masaryk Memorial Cancer Institute between 2000 and 2006. Seventy eight patients underwent hemipancreateoduodenectomy, 5 patients caudal pancreatectomy and 2 patients total pancreatectomy. No distant metastases were found at initial diagnosis (M0). Selected tumors were histologically confirmed to be invasive ductal adenocarcinomas of the pancreas, and in all patients the formalin fixed paraffin embedded tissues were available for immunohistochemistry. Grading of tumor differentiation was done based on WHO criteria combining the judgment of glandular differentiation including mucin production, mitotic count and nuclear features. Tumor staging was performed according to the International Union Against Cancer TNM System (the 6th edition). The follow-up was available for 75 patients, with five patients alive at the end of the study. Survival data for the patients were obtained from the National Oncological Register of Czech Republic. Resection specimens were fixed in 10% neutral buffered formalin for 24 h and then embedded in paraffin. Hematoxylin-eosin (HE) staining of tissue sections was used to identify representative samples with structures of pancreatic invasive ductal adenocarcinoma (up to three tissue blocks for sectioning were selected for each individual PDAC case). These were selected for immunohistochemical (IH) analysis.

Immunohistochemistry

The results of IH analysis of COX-2 expression using polyclonal anti-COX-2 antibody [rabbit polyclonal antibody against COX-2 (H-62), dilution 1:50; Santa Cruz Biotechnology, Inc., Santa Cruz, California, USA] were retrieved from our previous study^[38], in which the identical study population was examined.

Table 1 Patient, tumor and treatment characteristics, and levels of COX-2 expression (*n* = 85)

	<i>n</i>
Gender	
Male	41
Female	44
Mean age (range) (yr)	61 ± 9.5 (39-85)
Location of tumor	
Head, body	80
Tail	5
T category	
T1	6
T2	29
T3	50
N category	
N0	23
N1	62
M category	
M0	85
M1	0
Surgical procedure	
Pancreatoduodenectomy	78
Caudal pancreatectomy	5
Total pancreatectomy	2
Histological differentiation	
Well	8
Moderately	54
Poorly differentiated	23
No adjuvant therapy	30
Adjuvant therapy	33
Chemotherapy	23
Chemoradiotherapy	10
COX-2 expression with monoclonal antibody	
COX-2 negative	16
COX 1+	27
COX-2+	39
COX-2 expression with polyclonal antibody	
COX-2 negative	26
COX 1+	28
COX-2+	31

COX-2: Cyclooxygenase-2.

In the present study, the COX-2 expression was evaluated using mouse monoclonal anti-COX-2 antibody (dilution 1:50, clone CX229; Cayman Chemicals, Ann Arbor, MI, USA). IH detection of COX-2 was performed on 4 µm thick tissue sections applied to positively charged slides. The sections were deparaffinized in xylene and rehydrated through a series of alcohols. Antigen retrieval was performed in the lab microwave (Milestone, Sorisole, Italy) by heating in citrate buffer at pH 6.0 for 20 min at 98°C. The slides were incubated with anti-COX-2 antibodies overnight at 4°C. A streptavidin-biotin peroxidase detection system was used for COX-2 IH using monoclonal antibody (mouse IgG Vectastain Elite Kit, Vector Laboratories, Burlingame, California, USA). The visualization was performed using 3,3'-diaminobenzidine as a substrate (Fluca, Buchs, Switzerland). The slides were counterstained with Gill's hematoxylin.

Sections from COX-2 strongly positive colon carcinoma (immunoreactive 2 - see explanation below) were used as positive controls for COX-2 IH in each run. Additionally, COX-2 immunoreactivity in islets of endocrine pancreas served as an efficient internal positive control^[23]. Nega-

tive controls of COX-2 IH were performed by incubating samples without the primary antibody.

Evaluation of immunostaining

At least three different representative high-power (× 400) fields of tumor infiltration were examined. Cases with no stated minimal amount of the representative tumor tissue available were excluded. For both monoclonal and polyclonal antibody COX-2 IH, the percentage of positive cells was assessed, and the immunostaining intensity was classified into three categories: numeric score 0, no staining; numeric score 1, weak staining; numeric score 2, moderate and strong staining. The final immunoreactive score was obtained by multiplying the percentages of positive cells with the numeric score reflecting the staining intensity. Immunoreactive scores were categorized into three levels: 0 (immunoreactive < 20); 1 (immunoreactive 20-49); 2 (immunoreactive 50-200). In cases with heterogeneous expression of COX-2, the average score was counted.

Statistical analysis

Association of categorical parameters was analyzed and presented in contingency tables and tested using Pearson Chi-square test. Kaplan-Meier curves were constructed and median survival times were computed for survival data. Log-rank test was used to test differences between groups. Univariate and multivariate analyses of survival data were performed using Cox regression model to evaluate the predictive value of analyzed parameters (COX-2, histological grade, T status, N status). The level of significance was considered *P* < 0.05. All analyses were done using Statistica for Windows 8.0.

RESULTS

Patient, tumor and treatment characteristics, and levels of COX-2 expression are summarized in Table 1. The immunohistochemical analysis of COX-2 expression displayed considerable heterogeneity in staining intensity and percentage of positive cells between and within individual PDAC cases. In cases with heterogeneous COX-2 expression within a lesion, the average immunoreactive score for such a case was counted. Using both monoclonal and polyclonal antibodies, the PDAC cells showed diffuse cytoplasmic patterns of expression. Expression of COX-2 (Figure 1), examined using monoclonal anti-COX-2 antibody, was revealed in 66 cases (80.5%); 27 tumors (32.9%) expressed COX-2 at 1+ level, 39 at 2+ level (47.6%), and 16 cases (19.5%) were COX-2 negative (in 3 samples, a sufficient amount of neoplastic tissue was not available for IH analysis, and analysis of COX-2 expression using monoclonal antibody was not performed). Results of immunohistochemical analysis of COX-2 expression by polyclonal antibody were retrieved from our previous study^[38] and are included in Table 1 [28 tumors (32.9%) expressed COX-2 at 1+ level, 31 at 2+ level (36.5%), and 26 cases (30.6%) were COX-2 negative].

The median overall survival (OS) in the study population was 1.3 years. There was no significant difference between OS in males and females (median OS 1.1 years

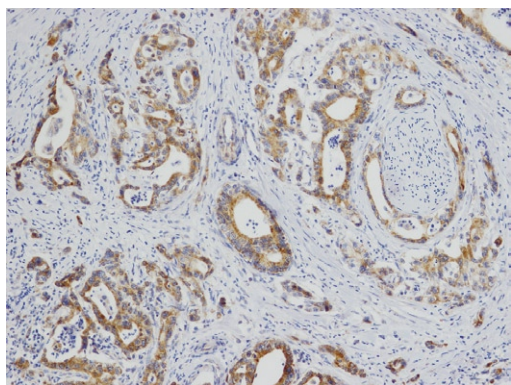


Figure 1 Overexpression of cyclooxygenase-2 (COX-2) in pancreatic invasive ductal adenocarcinoma with displayed perineural spreading of the tumor (COX-2 immunohistochemistry, original magnification, $\times 100$).

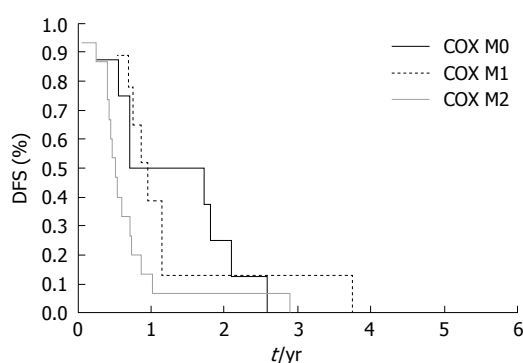


Figure 2 Kaplan-Meier disease free survival (DFS) curves for patients with resectable pancreatic cancer stratified by the level of COX-2 expression examined using monoclonal antibody. High expressors of COX-2 (2+, COX M2) had significantly lower survival curve than low expressors of COX-2 (1+, COX M1) or COX-2 negative cases (0, COX M0), $P = 0.019$.

in males *vs* 1.3 years in females; $P = 0.143$). Regarding nodal (N) status, N1 status was associated with shorter median OS (1.0 year in N1 group *vs* 1.5 years in N0 group; $P = 0.102$). Median OS for patients with adjuvant therapy was 1.4 years, and for patients without adjuvant therapy 1.3 years. The median disease free survival (DFS) for patients with adjuvant therapy was 0.8 years, and for patients without adjuvant therapy 0.7 years. Differences did not reach statistical significance.

No correlations between the levels of COX-2 expression (using both monoclonal and polyclonal antibodies) and the histological grade, T or nodal status were revealed.

In the Kaplan-Meier analysis, no significant correlations were found between the levels of COX-2 expression and OS (again using both polyclonal and monoclonal antibodies). However, trends to longer median OS were found in COX-2 negative cases (1.4 years in COX-2 negative *vs* 1.3 years in low expressors *vs* 1.0 in high expressors) using monoclonal antibody.

Correlating the levels of COX-2 expression and DFS; using polyclonal antibodies, trends to longer median DFS were found in COX-2 negative cases (1 year in COX-2 negative *vs* 0.7 years in low expressors *vs* 0.5 years in high expressors), but these values were not significant ($P = 0.211$); using monoclonal antibody, a statistically signifi-

cant correlation was found between COX-2 expression levels and DFS. Significantly longer DFS was revealed in COX-2 negative cases (median DFS 1.2 years in COX-2 negative *vs* 0.9 year in low expressors *vs* 0.5 year in high expressors, $P = 0.019$) (Figure 2).

Low histological grade showed a strong association with a longer OS ($P < 0.001$). Median OS for patients with grade 3 tumors was 0.7 years *vs* 1.6 years for grade 2 and 1.8 years for grade 1. Correlation of survival and T status revealed a shorter OS in T3 tumors, but the results reached only marginal statistical significance ($P = 0.070$). In a multivariate Cox proportional hazards regression model, histological grade (G), T, and nodal status remained valuable predictors of a worse survival with borderline significance for T [hazards ratio (HR) = 4.18 for G (if G = 3, $P < 0.001$); HR = 1.64 for T (if T = 3, $P = 0.065$); HR = 2.53 for N (if N = 1, $P = 0.006$)]. Higher grade, T or N status was associated with a worse OS.

DISCUSSION

Immunohistochemistry has become an integral part of histopathological diagnosis and can also provide important data to predict clinical course of the disease and potential therapeutic responsiveness. Thus, a validation of usefulness of different immunoprofiles and methodological approaches in predictive oncopathology is necessary. Based on a PubMed search, there are 7 papers^[33-39] reporting the correlations between COX-2 expression and selected clinicopathological parameters, including survival rates, in pancreatic cancer. The possible role of the COX-2 expression profile used as a prognostic factor for pancreatic ductal adenocarcinomas is largely discussed. Five studies did not show any trends or statistically significant correlations between COX-2 expression and survival rates^[35-39]. Our previous study^[38] also did not show significant correlations between these parameters, although trends to longer DFS in COX-2 negative cases were demonstrated. Recently, Matsubayashi *et al*^[34] reported that the COX-2 expression in patients with pancreatic ductal adenocarcinoma undergoing a potentially curative pancreaticoduodenectomy is predictive of survival, independent of other known prognostic markers, particularly in cancers ≥ 3 cm. These results were supported by Juuti *et al*^[33] who reported the expression of COX-2 to be associated with poor outcome for pancreatic ductal adenocarcinoma, which was independent of tumor stage, grade, or age in multivariate analysis. Moreover, these results were obtained, even if not only surgically resectable pancreatic cancers were included for analysis. Both Matsubayashi *et al*^[34] and Juuti *et al*^[33], who showed the significant correlation between COX-2 expression and clinical outcome, worked with mouse antihuman monoclonal antibodies. A discussion about the possible role of the selected antibody has been initiated. Antigenic blocking experiments showed higher sensitivity but lower specificity of polyclonal antibodies when compared with monoclonal antibodies^[40]. The presented comparative analysis only partially supported the possible prognostic role of COX-2 expression. Results retrieved from our previous study^[38], in which rabbit polyclonal antibody was

used, were completed with results obtained by examination of COX-2 expression using the same mouse antihuman monoclonal antibody that was used in the studies of Juuti *et al.*^[33] and Matsubayashi *et al.*^[34]. Using the monoclonal antibody, a significantly shorter DFS was found in patients with COX-2 positive tumors. Trends to shorter DFS in COX-2 expressors were only reported using the polyclonal antibody^[38]. No significant results were obtained regarding OS; the only trends to longer OS were demonstrated in COX-2 negative cases using monoclonal antibody. Our results do not sufficiently support the findings of Juuti and Matsubayashi, even when the standardized protocols for immunohistochemical evaluation of COX-2 expression, which should be preventive of known technical and interpretative pitfalls in immunohistochemistry, were used. Except for the mentioned type of antibody, the main factors potentially affecting immunohistochemical staining are the tissue processing, especially the type and length of fixation, and antigen retrieval. All tissues included in the study were routinely fixed in 10% neutral buffered formalin for 24 h, and antigen retrieval was performed using a standardized protocol. To avoid the misinterpretation of results, especially unspecific immunostaining, systems of positive (both external and internal) and negative controls were used in each run. Based on our results, COX-2 expression does not seem to represent a valuable independent prognostic factor, even if using the monoclonal antibody brought statistically significant results when correlating DFS and COX-2 expression levels and when trends to longer median OS were also demonstrated.

Correlating the COX-2 expression with the level of tumor differentiation, no significant relationship was revealed between the level of COX-2 expression and the histological grade of the pancreatic cancer independently of the antibody used. These findings are in agreement with previous studies^[23,35,41]. Merati *et al.*^[36] described increased expression of COX-2 in well differentiated ductal adenocarcinomas, but this trend was not observed in our study group, and the proposed role of COX-2 in carcinogenesis of pancreatic cancer remains puzzling.

The routinely used grading of the histological differentiation of tumors and evaluation of T and N status were proven to represent efficient prognostic factors. Higher grade of the tumor and N1 status were significantly associated with a shorter survival in patients with resectable pancreatic cancer. T3 status was associated with shorter survival only with marginal significance.

In conclusion, based on these presented results and the previously published data, an immunohistochemical assessment of COX-2 expression is not superior to the conventional prognostic factors such as tumor histological grade, stage, and nodal status. The possible role of COX-2 in potential targeted therapy and chemoprevention of pancreatic cancer using COX-2 inhibitors remain as unanswered questions and need further evaluation.

incidence of pancreatic cancer almost equals the mortality rate and the five-year survival rate does not reach 5%. Mediators of inflammatory pathways are known to play an important role in carcinogenesis and represent a key link between chronic inflammation and cancer.

Research frontiers

Cyclooxygenase-2 (COX-2) represents a key modulatory molecule in inflammation and carcinogenesis. COX-2 is known to have multiple tumorigenic effects. Increased expression of COX-2 has been observed in a variety of tumors including pancreatic cancer. In the literature, the prognostic significance of COX-2 expression including the role of antibody used for an evaluation of COX-2 expression profile have been discussed.

Innovations and breakthroughs

Recent reports have highlighted the role of COX-2 in carcinogenesis of pancreatic cancer and COX-2 has been suggested as a potential therapeutic target. This study confirmed the relationship of COX-2 to biological processes involved in pancreatic cancer, but its potential usefulness as a prognostic marker was not demonstrated.

Applications

The level of COX-2 expression does not seem to be a valuable independent prognostic factor, even if a significantly longer disease free survival and trends to longer overall survival were demonstrated in COX-2 negative cases using monoclonal antibodies. Immunohistochemical assessment of COX-2 expression is not superior to the conventional prognostic factors such as grade, stage and nodal status.

Terminology

COX-1 and COX-2 represent enzymes which are involved in the conversion of arachidonic acid to prostaglandins. COX-1 is expressed in many tissues under physiological conditions, while COX-2 is not constitutively expressed and several studies have suggested its positive role in regulation of growth and angiogenesis as well as inhibition of apoptosis in cancer.

Peer review

In this paper, Hermanova *et al* analyzed by immunohistochemistry COX-2 in pancreatic adenocarcinoma tissue samples. They concluded that the COX-2 expression does not seem to represent a valuable independent prognostic factor and is not superior to the conventional prognostic factors. Concerning the general view of the manuscript, it is characterized by a well done internal structure and acceptable quality of language. The data are clearly described and the statistical analysis seems to be correct.

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COMMENTS

Background

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Non-small-bowel lesions encountered during double-balloon enteroscopy performed for obscure gastrointestinal bleeding

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CONCLUSION: A significant proportion of patients (24.6%) had lesions within reach of conventional endoscopy. Careful repeat examination with gastroscopy and colonoscopy might be required.

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Key words: Bleeding; Obscure gastrointestinal bleeding; Endoscopy; Double-balloon enteroscopy

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Abstract

AIM: To report the incidence of non-small-bowel bleeding pathologies encountered during double-balloon enteroscopy (DBE) procedures and to analyse their significance.

METHODS: A retrospective study of a prospective DBE database conducted in a tertiary-referral center was conducted. A total of 179 patients with obscure gastrointestinal bleeding (OGIB) referred for DBE from June 2004 to November 2008 were analysed looking for the incidence of non-small-bowel lesions (NSBLs; all and newly diagnosed) encountered during DBE.

RESULTS: There were 228 (150 antegrade and 78 retrograde) DBE procedures performed in 179 patients. The mean number of DBE procedures was 1.27 per patient. The mean age (SD) of the patients was 62 ± 16 years old. There were 94 females (52.5%). The positive yield for a bleeding lesion was 65.9%. Of the 179 patients, 44 (24.6%) had NSBLs (19 of them had dual pathology with small-bowel lesions and NSBLs); 27 (15.1%) had lesions not detected by previous endoscopies. The most common type of missed lesions were vascular lesions.

Tee HP, Kaffes AJ. Non-small-bowel lesions encountered during double-balloon enteroscopy performed for obscure gastrointestinal bleeding. *World J Gastroenterol* 2010; 16(15): 1885-1889 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v16/i15/1885.htm> DOI: <http://dx.doi.org/10.3748/wjg.v16.i15.1885>

INTRODUCTION

Obscure gastrointestinal bleeding (OGIB) accounts for approximately 5% of all patients with gastrointestinal (GI) bleeding^[1], and in approximately 75% of these patients, lesions can be found in the small bowel^[2-4].

Double balloon enteroscopy (DBE) was introduced by Hironori Yamamoto in 2001^[5] and in the same year, the United States Food and Drug Administration approved the use of capsule endoscopy (CE)^[6]. With the advent of CE and DBE, the management of OGIB has been revolutionised^[7]. Mid-GI bleeding, previously considered almost inaccessible, is now effectively diagnosed and treated by a combination of CE and DBE as ambulatory procedures.

Often non-small-bowel lesions (NSBLs) are identified

during the search for pathology in the small bowel. This was a commonly reported phenomenon in push enteroscopy studies^[8,9] and has been re-confirmed in CE studies, showing an incidence of NSBL of between 6.4%^[10] and 38.8%^[11]. The only DBE series looking at NSBL in OGIB reported a missed rate of 24.3%^[12]; however, most of these patients had no prior CE. Our study investigated the incidence and clinical relevance of NSBL encountered during DBE performed for OGIB in patients who had prior CE and standard endoscopies.

MATERIALS AND METHODS

Study protocol

This is a single-center retrospective study in a tertiary referral teaching hospital in Sydney, Australia, conducted between June 2004 and November 2008. One endoscopist with experience in DBE and in therapeutic endoscopy performed the DBEs, with trainee registrars assisting with the outer tube. DBE was performed using the Fujinon enteroscope (Fujinon EN-450T5, Fujinon Corp., Saitama, Japan). DBE was performed *via* the antegrade (oral) or retrograde (anal) route, and the intention was to perform a targeted approach with the DBE. The approach was determined by the endoscopist, based on the time a lesion was seen in relation to the total small-bowel transit time on the CE study. If the lesion was within the proximal two thirds of the small bowel, then an antegrade DBE was used.

The DBE was performed with the patient either conscious or under deep sedation with a combination of intravenous midazolam (Pfizer, Bentley, Australia), fentanyl (Mayne Pharma Ltd., Mulgrave, Australia), and propofol (Fresofol 1%, Pharmatel Fresenius Kabi Pty Ltd, Hornby, Australia) administered by the assistant or attending anaesthetist. The preparation for the procedures included a fasting period of 8 h before the oral procedure and a routine sodium picosulphate-based bowel preparation (Picoprep, Pharmatel Fresenius Kabi Pty Ltd., Hornby, Australia), or sodium phosphate-based preparations (Fleet, Ferring Pharmaceuticals, Gordon, Australia) with a clear fluid diet the day before the procedure for the anal approach. The DBE technique was as previously described by the innovator H Yamamoto^[5].

A failed retrograde DBE was defined as failure to insert the tip of the scope beyond the terminal ileum (approximately 20cm beyond the ileocaecal valve), as previously defined by Fry *et al*^[12]. The antegrade DBE was considered to be a failure if the endoscopist failed to pass the duodeno-jejunal flexure.

Patients

All patients were referred by their specialist gastroenterologists or gastrointestinal surgeons to our tertiary referral service. All patients included in the study had OGIB as defined by the American Gastroenterological Association (AGA) criteria^[13]. Thus, all patients had their initial investigations (EGD and colonoscopy) performed by their

referring gastroenterologists within 6 mo of their CE. Information on patient demographics, previous investigations (endoscopic and radiological), findings and intervention with DBE, limitations of insertion, complication rates, and follow-up after therapy were all retrieved. Ethics board approval was obtained before data collection.

Patients were excluded if they had no prior CE, if the procedure could not be completed due to poor bowel preparation not allowing progress through the colon, procedures performed for colonic indications, sedation failure and technical/equipment failure.

NSBLs were defined as bleeding lesions proximal to the papilla of Vater or distal to the ileocecal valve (i.e. within reach of conventional upper and lower endoscopes). Small-bowel lesions (SBLs) were defined as bleeding lesions that lie between papilla of Vater and ileocaecal valve. Bleeding lesions were defined as lesions that definitely or probably explain the patient's bleeding or anaemia, such as active bleeding lesions, lesions with recent evidence of bleeding, or healed/healing lesions likely to have recently bled. Red marks and classical telangiectatic angioectasia were considered bleeding lesions whereas red spots were not.

Statistical analysis

The statistical software package SPSS for Windows Version 14 (SPSS Inc., Chicago, Ill) was used to analyse the data. mean \pm SD was used to summarise data for continuous variables, whereas percentages were used for categorical variables.

RESULTS

Demographics

We identified 179 patients with OGIB who were referred for DBE. Two hundred and twenty eight DBE procedures (150 antegrade; 78 retrograde) were performed. Twenty seven patients had both antegrade and retrograde procedures. The mean number of DBE procedures was 1.27 per patient. The mean age (SD) of the patients was 62 \pm 16 years. There were 94 females (52.5%).

Findings

DBE found a bleeding lesion in 118 (65.9%) patients and the distribution of bleeding lesions is summarised in Table 1. Ninety-three out of the total 179 patients (51.9%) had a positive finding localised to SBLs, with the most common being angioectasia ($n = 64$), followed by tumours/polyps ($n = 13$), and small bowel ulcers ($n = 11$) (Table 2). NSBLs were found in 44 (24.6%) patients (Table 3). Nineteen (10.6%) patients had dual bleeding pathologies in both the small bowel and outside the small bowel. Six (3.4%) were inconclusive due to failed procedures and all of them were of the retrograde group. Normal examinations were seen in 55 (30.7%) patients.

A total of 46 NSBLs were found in 44 patients. The majority of these 46 NSBLs were of vascular origin ($n = 27$), and the others were of peptic ($n = 13$) and neoplastic

Table 1 Findings by DBE performed for OGIB and anatomical distribution of bleeding lesions *n* (%)

Findings and distributions	Subjects
Positive for bleeding lesions	118 (65.9)
Small bowel only	74 (41.3)
Non-small bowel only	25 (14.0)
Small bowel and non-small bowel	19 (10.6)
Negative findings/normal	55 (30.7)
Inconclusive (failure of procedure)	6 (3.4)
Total	179 (100.0)

DBE: Double balloon enteroscopy; OGIB: Obscure gastrointestinal bleeding.

Table 2 Small-bowel bleeding lesions found in the study cohort

Small-bowel lesions	<i>n</i>
Angiectasia	64
Tumours/polyps	13
Ulcers	11
Bleeding mucosa	2
Bleeding diverticula	2
Meckel's diverticulum	1
Stricture	1
Total	94 ^a

^aNinety-four small bowel bleeding lesions were found in 93 patients. Nineteen patients also had NSBLs. NSBLs: Non-small-bowel lesions.

(*n* = 6) origins. Thirty-four of the lesions were found in the upper GIT; whereas 12 of the others were in the lower GIT. Of the 44 patients who had NSBL, 27 (15.1% from the total cohort) of their lesions were newly diagnosed during DBE, despite their prior investigations. These included angiectasia (*n* = 12), hiatus hernia with Cameron's erosions (*n* = 3), gastric ulcers (*n* = 2), and others (Table 3).

Interventions

Endoscopic interventions were performed in 96/118 (81.4%) patients who had positive bleeding lesions; 80 patients had therapeutic interventions (argon plasma coagulation in 75 and polypectomy in five), 14 patients had diagnostic biopsy, and two patients had tattoo placement for targeted surgery. Nineteen patients required medical treatment (18 were given proton pump inhibitors, one received chemotherapy for lymphoma), 11 patients were referred for surgery (eight neoplastic tumours, two bleeds that failed to be controlled endoscopically, and one Meckel's diverticulum).

Among the 44 patients who had NSBLs, endoscopic interventions were given in 23 patients; 20 of them had argon plasma coagulation and three had biopsies. Twenty one patients required medical treatments (18 required proton pump inhibitors and three were given treatment for variceal control). One patient had surgical resection for cecal carcinoma.

DISCUSSION

The AGA position paper concerning the evaluation and

Table 3 Classification of NSBLs according to the nature and site of the lesions with totals for all and newly diagnosed lesions by DBE

Classification of NSBLs encountered on DBE	NSBL (<i>n</i>)	Newly diagnosed NSBL (<i>n</i>)
Vascular	27	16
Upper		
Stomach/duodenal angiectasia	12	8
GAVE	3	1
Oesophageal varices	2	1
Gastric varices	1	1
Lower		
Colonic angiectasia	6	4
Haemorrhoids	3	1
Peptic	13	9
Upper		
Hiatus hernia with Cameron's ulcer	5	3
Gastric ulcers/erosions	3	2
GERD (Los Angeles grade 3 or 4)	2	1
Haemorrhagic gastritis	1	1
Duodenal ulcers	1	1
Lower		
Colonic ulcer	1	1
Neoplastic	6	3
Upper		
Gastric polyps	4	1
Lower		
Colonic polyp	1	1
Colorectal carcinoma	1	1
Total	46 ^a	28 ^b

^a46 NSBLs were found in 44 patients. Nineteen patients also had SBLs; ^b28 newly diagnosed NSBLs were found in 27 patients. GAVE: Gastric antral vascular ectasia; GERD: Gastro-esophageal reflux disease.

management of obscure GI bleeding was published in January 2000^[14] and revised in 2007^[13] following the availability of CE and DBE. The revised guidelines included CE as a pivotal investigation in OGIB. Depending of the findings on CE, DBE plays a key role in delivering targeted therapy or additional diagnostic clarification. One key element in the revised recommendations is for the inclusion of a repeat EGD and colonoscopy. The main point of contention is the timing of repeat procedures, as data is limited and hence the optimal timing of repeat procedures remains unclear.

Missed NSBLs have been a problem previously recorded in many published series. The literature for push enteroscopy has shown missed NSBL to account for up to 64.0% of all positive findings^[8]. In another push enteroscopy series, missed upper GIT lesions accounted for 10.2% of 233 patients who had OGIB^[9]. This issue has continued into the CE data, with several reports now showing missed NSBL in the order of 6.4%^[10] to 38.8%^[11]. Not surprisingly, Fry *et al*^[12] in their DBE series reported the missed NSBL rate to be 24.3%; however, in that series CE was not available for all of their patients. In our cohort, NSBLs were detected in 24.6% of patients, a number strikingly similar to Fry *et al*^[12]. However, all our patients had prior CE. In such circumstances, it stands to reason that CE would have identified some missed lesions and our figure should have been lower than that of Fry *et al*^[12]. There was one clear explanation

for this. Not all NSBLs in our series were in fact completely missed. A significant number of these NSBLs were suspected or known by the referring doctors. However, referrals were made to exclude dual pathology (SBL and NSBL). Excluding those NSBLs that were known by previous investigations, the true missed rate for NSBL in our cohort was actually 15.1%.

There are several reasons to explain why lesions could be missed on conventional endoscopies. Lesions such as Dieulafoy's can be very difficult to diagnose as they can "resolve" by the time patients have their endoscopy. Often small-bowel bleeding can be caused by similar types of lesions and hence are dependent on the "timing" of DBE. This is best illustrated in study by Pennazio *et al*^[15], in which the group of patients with ongoing overt bleeding had a 92.3% positive yield on CE when compared to groups with previous overt bleeding (12.9%) and guaiac-positive stool/iron deficiency anaemia (44.2%). If DBE could be performed during active bleeding then similar diagnostic rates with the addition of therapy should be achieved, limited only by the inferior total enteroscopy rates of DBE (77.5%^[16] in the hands of experts).

Lesions such as Cameron's erosions also depend on the timing of endoscopy to confirm them as the bleeding source. Often, gastroenterologists referred such patients to our service with a high index of suspicion for Cameron's lesions but with no successful endoscopic timing. Hypovolemia and anaemia could cause lesions to look less obvious and this is particularly the case with small angioectasia, and could account for some missed lesions. Inadequate endoscopic examination also remains an issue. Despite the ease of EGD, careful examination for small lesions placed in awkward or in blind spots should be considered, and a more diligent and thorough examination is warranted in patients with OGIB. Likewise, an excellent colon preparation is required with a careful mucosal examination to exclude small mucosal bleeding points.

Missed lesions are highly relevant in the management of OGIB. Hartmann *et al*^[17] studied 47 patients who had OGIB and were subjected to CE and intraoperative enteroscopy. Despite the accurate diagnosis from total small bowel examination and curative endoscopic and surgical treatments, on a mean follow up of 346 d, 25.5% of the patients had a re-bleed, and 19.1% of them required further interventions or blood transfusion. This suggests that despite the gold standard diagnostic and therapeutic approaches (in this case CE and IOE), many patients continue to bleed. Missed lesions could play a key role in these patients. In some series of patients with OGIB and a negative CE who continue to bleed, a repeat CE can find a lesion in up to 75% of patients^[18]. Hence, patients who re-bleed need repeat examination of their entire GI tract to look for missed lesions.

In our cohort, 19 patients with NSBL had dual bleeding pathologies with both NSBL and SBL involvement. This suggests that if a significant NSBL is found during DBE, the small bowel examination should be completed, as approximately half of these patients will

also have a significant small bowel finding. Conversely, if a definite bleeding site is found in the small bowel, then careful examination for NSBL is still warranted, even when no suspicion exists for NSBL due to the high true missed rate in our cohort of 15.1%.

The low diagnostic rates in our cohort reflect some real issues in managing patients with OGIB. Not all findings at CE are relevant and often the role of DBE is to exclude a lesion. One recurrent and classic situation is folds of bowel that appear like a polyp or a submucosal lesion. In addition, flecks of transported blood or mucous on the bowel surface could be mistaken as angioectasia or ulcers. Several studies, including our previous work^[19], found that many CE findings are false positives.

We recognised the limitation of this study as being a retrospective series. It was difficult to classify all lesions into bleeding and non-bleeding lesions. We had to include a group of lesions as probable bleeders due to the size, appearance, history, and behaviour of the lesions. For instance, large polyps, extensive ulcerative disease, and malignant-looking lesions were considered probable bleeders, even though no blood or altered blood was detected on endoscopy. Likewise, diverticular disease might be considered a bleeder or non-bleeder based on their number and appearance.

In conclusion, NSBLs are a common finding during DBE, despite prior endoscopic examinations. Importantly, 15.1% of patients had unsuspected findings and hence careful examination of the entire GI tract is essential in all patients.

COMMENTS

Background

The problem of "missed" lesions seen at subsequent endoscopic examinations has been well documented in patients with obscure gastrointestinal bleeding (OGIB). The exact degree of this problem in the new era of capsule endoscopy and double-balloon enteroscopy remains unclear.

Research frontiers

The degree of the problem, the type of lesions missed, and the timing of repeat endoscopic examinations require further evaluation.

Innovations and breakthroughs

This paper shows that in a tertiary referral practice, the rate of missed lesions can be as high as 1 in 4 patients and thus highlights the relevance of repeat examinations in patients with ongoing OGIB.

Applications

In patients with ongoing OGIB without a definite finding on endoscopic evaluation, repeat procedures are worthwhile.

Terminology

OGIB is defined as bleeding with a negative endoscopy and colonoscopy.

Peer review

This paper is well written and provides new information on missed sites of bleeding within reach of conventional endoscopy and colonoscopy following capsule and regular endoscopy. The value of double balloon enteroscopy is also highlighted in diagnostic and therapeutic terms.

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Prognosis of cancer with branch duct type IPMN of the pancreas

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pancreatic cancer in 25%, IPMN *per se* in 20%, and benign disease in 15% of the patients.

CONCLUSION: The prognosis for IPMN depends not on the IPMN *per se*, but on the presence of intra- or extra-pancreatic cancer.

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Key words: Intraductal papillary mucinous neoplasms of the pancreas; Long-term follow-up; Extra-pancreatic cancer; Pancreatic cancer; Prognosis

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Abstract

AIM: To examine the coexistence of metachronous and synchronous cancer in branch duct intraductal papillary mucinous neoplasms of the pancreas (IPMN).

METHODS: We reviewed the records of 145 patients with branch duct IPMN between January 1991 and April 2008 and assessed the relationship between IPMN and intra- or extra-pancreatic carcinoma and the outcome of IPMN.

RESULTS: The mean observation period was 55.9 ± 45.3 mo. Among the 145 patients, the frequency of extra-pancreatic cancer was 29.0%. The frequency of gastric cancer, colon cancer, breast cancer, and pancreatic cancer were 25.5%, 15.7%, 13.7%, and 9.8%, respectively. Twenty (13.8%) of the patients died. The cause of death was extra-pancreatic carcinoma in 40%,

INTRODUCTION

Intraductal papillary mucinous neoplasm of the pancreas (IPMN) shows a wide spectrum of histological presentations, ranging from adenoma with mild atypia to adenocarcinoma, and was first described by Ohashi *et al*^[1] in 1980. IPMN is divided into two types, the main duct type and the branch duct type. In general, branch duct IPMN develops slowly and has a comparatively good prognosis. However, in several studies, it became evident that IPMN is a disease that very frequently coexists with cancer. Therefore, the prognosis of IPMN is more closely related to the coexisting disease than to IPMN

per se^[2-8]. In the present study, the prognostic significance of the coexistence of metachronous and synchronous cancer in branch duct IPMN was investigated.

MATERIALS AND METHODS

The subjects were 145 patients with branch duct IPMN, including 33 resected and 112 non-resected cases, between January 1991 and April 2008, who had been periodically followed-up to date. The ratio of males to females was 79:66, the mean age was 66.3 ± 10.2 years (range: 29-89 years), and the mean observation period was 55.9 ± 45.3 mo (range: 1-217 mo). We classified IPMN into the main duct type without multi-locular dilatation of the branch duct, and the branch duct type. The Institutional Review Board of Tokyo Medical University reviewed and approved the entire study.

Indication of surgery for branch type IPMN

In our department, we consider that surgery for the branch type IPMN is indicated when the diameter of the cyst is 30 mm or more, the diameter of the mural nodule is 10 mm or more, and cytology findings of the pure pancreatic juice reveal suspected malignancy.

Strategy for branch type IPMN

For regular follow-up, we performed hematological tests, including those for tumor markers (carbohydrate antigen 19-9, and carcinoembryonic antigen) and pancreatic enzymes (amylase, lipase, and elastase-1), and several types of imaging (ultrasonography, endoscopic ultrasonography, computed tomography, or magnetic resonance cholangiopancreatography) every 6 to 12 mo.

Clinicopathological assessment of branch type IPMN

We assessed the clinicopathological findings as outlined below. (1) The relationship between IPMN and intra- or extra-pancreatic cancer; and (2) Prognosis of IPMN.

Statistical analysis

Normally distributed data are presented as mean \pm SD. Significance was analyzed by the χ^2 test or Fisher's exact probability test, and Aspin-Welch's *t*-test. Statistically significant differences were denoted by $P < 0.05$. Statistical analysis was performed using Stat Mate III (ATMS Co Ltd, Tokyo, Japan).

RESULTS

The resected patients had significantly greater mean cyst diameter, diameter of the main pancreatic duct, and height of node protrusion compared to those of non-resected patients ($P < 0.05$, $P < 0.01$, and $P < 0.01$, respectively, Table 1).

The relationship between IPMN and intra or extra-pancreatic cancer

There was no statistically significant difference regarding

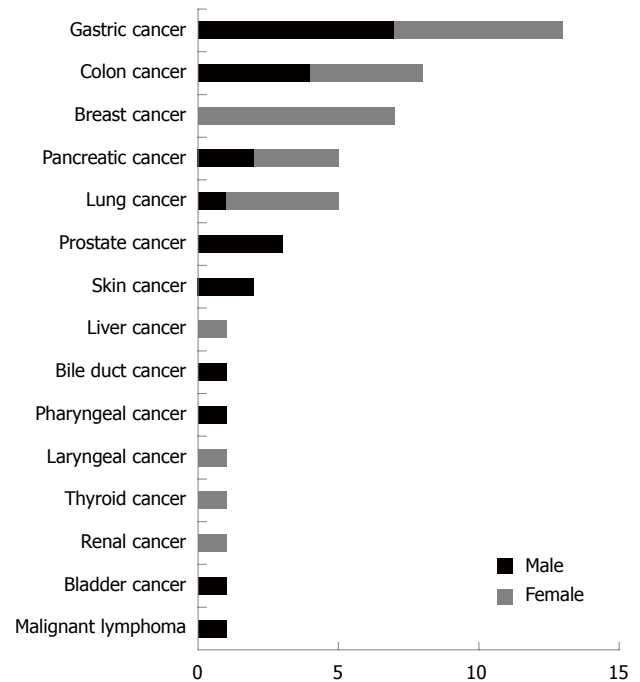


Figure 1 Details of intra- or extra-pancreatic cancers in patients with intraductal papillary mucinous neoplasms of the pancreas (IPMN).

Table 1 Characteristics of patients with branch duct type IPMN

	Total (n = 145)	Resection (n = 33)	Non-resection (n = 112)
Age (mean \pm SD) (yr)	66.3 \pm 10.2	63.9 \pm 10.5	67.0 \pm 10.1
Male:female	79:66	20:13	59:53
Median diameter of cystic lesion (mm)	20.1 \pm 13.0	23.8 \pm 11.5	19 \pm 13.3
Median diameter of MPD (mm)	2.2 \pm 2.3	3.6 \pm 3.3	1.7 \pm 1.8
Median diameter of mural nodule (mm)	1.6 \pm 5.3	5.8 \pm 9.8	0.3 \pm 1.6

P: P value (The presence of a statistically significant difference was denoted by $P < 0.05$). IPMN: Intraductal papillary mucinous neoplasms of the pancreas; SD: Standard deviation; MPD: Main pancreatic duct; NS: Not significant.

the diameter of the cystic lesion, main pancreatic duct, and mural nodule between the branch type IPMN in patients with or without intra or extra-pancreatic cancer, regardless of whether the IPMN had been resected or not (Table 2).

Details of intra- or extra-pancreatic cancer in patients with IPMN are shown in Figure 1. Four patients (one man, three women) had metachronous double cancer. Gastrointestinal cancer accounted for 41.2% (21/51).

Incidence of, and detection time for, intra- or extra-pancreatic cancer in patients with IPMN are shown in Table 3. Briefly, detection prior to IPMN was seen in 56.9% (29/51), simultaneous detection of cancer and IPMN was seen in 27.5% (14/51), five of which were

Table 2 Comparison of the branch type IPMN in patients with or without intra- or extra-pancreatic cancer

	IPMN with intra- or extra-pancreatic cancer (resection/non-resection)	IPMN without intra- or extra-pancreatic cancer (resection/non-resection)	P value
n	47 (16/31)	98 (19/79)	
Age	68.4 (65.0/70.0)	65.4 (63.2/66.4)	NS
Sex (male:female)	22:25 (7:9/15:16)	57:41 (13:6/44:35)	NS
Median diameter of cystic lesion (mm)	20.8 ± 11.8 (23.3 ± 10.4/19.5 ± 12.4)	19.7 ± 13.6 (23.0 ± 13.3/19.0 ± 13.7)	NS
Median diameter of MPD (mm)	2.2 ± 2.4 (3.5 ± 3.6/1.6 ± 1.2)	2.1 ± 2.3 (3.6 ± 2.9/1.7 ± 1.9)	NS
Median diameter of mural nodule (mm)	2.1 ± 5.7 (5.3 ± 8.7/0.5 ± 1.9)	1.3 ± 5.1 (5.6 ± 10.5/0.3 ± 1.5)	NS

Table 3 Incidence of, and detection time, for intra- or extra-pancreatic cancer in patients with IPMN (resected cases)

Neoplasm	Consciousness detection prior IPMN	Simultaneous with detection of carcinoma and IPMN	During follow- up for IPMN	Total
Gastric cancer	7 (1)	3 (3)	3 (0)	13 (4)
Colon cancer	4 (1)	4 (0)	0	8 (1)
Breast cancer	5 (2)	0	2 (0)	7 (2)
Pancreatic cancer	0	5 (4)	0	5 (4)
Lung cancer	3 (2)	1 (1)	1 (0)	5 (3)
Prostate cancer	2 (0)	0	1 (1)	3 (1)
Skin cancer	2 (1)	0	0	2 (1)
Liver cancer	1 (0)	0	0	1 (0)
Bile duct cancer	0	0	1 (0)	1 (0)
Pharyngeal cancer	1 (0)	0	0	1 (0)
Laryngeal cancer	1 (1)	0	0	1 (1)
Thyroid cancer	1 (0)	0	0	1 (0)
Renal cancer	0	1 (1)	0	1 (1)
Bladder cancer	1 (1)	0	0	1 (1)
Malignant lymphoma	1 (0)	0	0	1 (0)
Total	29 (9)	14 (9)	8 (1)	51 (19)

pancreatic cancer, and cancer was detected in 15.7% (8/51) during follow-up for IPMN.

Prognosis of IPMN, and clinicopathological findings in fatal cases of IPMN

Of the 145 patients with IPMN, 13.8% (20/145) died. As shown in Figure 2, the cause of death was extra-pancreatic cancer in 40% (8/20), pancreatic cancer in 25% (5/20), IPMN *per se* in 20% (4/20), and benign disease in 15% (3/20) of the patients. In particular, the cause of death of resected and non-resected cases is shown in Figure 2B and C.

The clinicopathological findings in four fatal cases of IPMN are shown in Table 4. The ratio of men to women was 3:1, the mean age was 64.5 years, two patients underwent surgery immediately after detection, and both had invasive adenocarcinoma accompanying IPMN. In one of the resected patients, the cyst diameter was 35.0 mm and the height of node protrusion was 19 mm. However, lymph node metastasis was observed post-operatively and the patient died of cancer 14 mo later. The other patient was found to have a nodular lesion with a diameter of 26.6 mm and pancreatic cancer was diagnosed. The patient underwent surgery, but hepatic metastasis occurred post-operatively and she died of cancer eight mo later. Of the

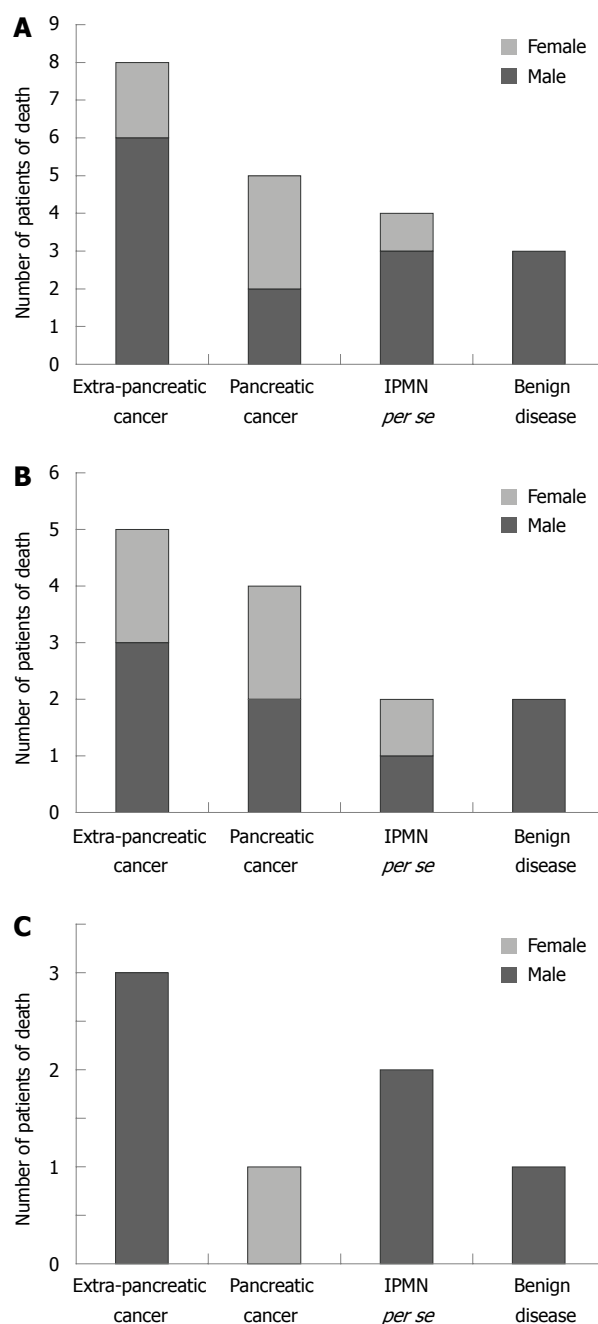


Figure 2 Number of patients of death. A: In patients with IPMN; B: In patients with resected IPMN; C: In patients with non-resected IPMN.

patients that died of cancer due to IPMN, two refused surgery and were therefore under observation only. They died

Table 4 Details of clinicopathological findings in fatal cases of IPMN

	Sex	Age (yr)	Region	Change of cystic lesion (mm)	Change of MPD (mm)	Change of mural nodule (mm)	Resection	Histopathological result	Period from diagnosis (operation) to death (mo)	Cause of death
Case 1	Male	89	Head	60→90	10→20	10→30	(-)	Adenocarcinoma (ch+, du+)	37	Cancer of death
Case 2	Male	58	Head	20→64	3→10	0→64	(-)	Adenocarcinoma (ch+, du+)	112	Cancer of death
Case 3	Male	53	Tail	35	1	19	(+)	Invasive ductal carcinoma derived from IPMN (INFα, mpd+)	15 (14)	Cancer of death
Case 4	Female	58	Head	0	1	26.6	(+)	Invasive ductal carcinoma derived from IPMN (INFβ, V2, ne1, du+, rp+)	9 (8)	Cancer of death

Table 5 Summary of the reports of concomitant extra-pancreatic cancers in patients with IPMN

Author (yr)	Number of IPMN patients	Extra-pancreatic cancers with IPMN patients	Age (mean ± SD)	Male: female	Follow-up period (mo)
Zhong (1996)	21	5	NA	NA	NA
Sugiyama (1999)	42	15	NA	NA	NA
Adsay (2002)	28	8	NA	NA	NA
Osanai (2003)	148	35	70.9	31:4	NA
Kamisawa (2005)	79	37	NA	NA	NA
Eguchi (2006)	69	32	NA	NA	NA
Choi (2006)	61	18	64.2 ± 8.4	13:5	65.8 ± 8.5
Yoon (2008)	210	77	65.3 ± 9.0	47:30	24.7
Ishida (2008)	61	15 ¹	67.0 ± 7.0	12:3	50.9
Present study	145	42 ²	68.3 ± 9.0	20:26	49.8 ± 42.4
Total	864	284 ³			

¹In those 2 patients who had metachronous double extra-pancreatic malignancies; ²In those 4 patients who had metachronous double extra-pancreatic malignancies; ³In those 6 patients who had metachronous double extra-pancreatic malignancies. NA: Not available.

at 37 and 112 mo, respectively, after detection. One non-resected patient initially had a 60 mm cyst and a 10 mm height mural nodule. When the patient died 37 mo later, the diameter of the cyst was 90 mm and the height of the mural nodule was 30 mm. The other non-resected patient initially had a cyst 20 mm in diameter with no mural nodule protrusion and when the patient died 112 mo later, the cyst had become a nodular lesion with a diameter of 64 mm.

DISCUSSION

We set out to determine the prognostic factors affecting cases of IPMN accompanied by cancer. In the present study, it became evident that cases complicated with metachronous and synchronous cancers in branch-type IPMN are quite common. Other studies that closely investigated such patients reported frequencies of extra-pancreatic cancer of 23.6%–46.8% and of a total of 864 IPMN patients, 32.9% (284/864) had extra-pancreatic cancer, which is almost the same as the subjects in the present study, where the frequency was 29.0% (42/145)^[2–10] (Table 5). In addition, the most frequently occurring complications were gastric cancer and colon cancer, which

was the same as in this study (Table 6).

On the other hand, other studies reported pancreatic cancer occurring in 3.8 to 9.2% of IPMN cases^[6,11,12]. In our study it was 3.5% (5/145), which was slightly lower. In general, the etiology of second primary tumors is complex, involving multiple factors such as: (1) shared risk factors for the primary and secondary tumors, (2) host susceptibility to the development of specific tumors, (3) altered immunity as a result of the primary tumor and/or treatment, and (4) treatment with cytotoxic agents (radiotherapy and chemotherapy)^[3]. In the present study, there was no clearly significant difference between cases complicated with other organ cancers and those without such complications in terms of cyst diameter, diameter of the main pancreatic duct, and presence or absence of mural nodules. This demonstrated that these data cannot be used as indices in the discovery of intra or extra-pancreatic cancer. Thus, further investigations, including follow-up studies, are necessary.

In the present study, most cases of IPMN were diagnosed after extra-pancreatic cancer was diagnosed. However, there are also reports stating that the time of IPMN diagnosis is unrelated to the onset of extra-pancreatic cancer^[3,5].

The follow-up period and imaging modality for follow-up vary according to reports, and no consensus has been obtained. In this study, no case of pancreatic cancer occurred during follow-up. However, several researchers have reported pancreatic cancer after diagnosis of IPMN^[6,11–15]. These findings demonstrate that periodic follow-up is necessary, even after IPMN is diagnosed.

The 5-year survival of patients with IPMN that undergo resection was reported to be for 36%–66%, even among those with invasive cancer. Thus, longer survival than that for pancreatic cancer can be expected^[16–20]. According to our data, 25% of deaths were due to pancreatic cancer, while 40% were due to cancer of other organs. On the other hand, the follow-up periods of two patients who refused surgery and opted for conservative treatment, but who then died due to IPMN, were 37 and 112 m, which was relatively long. Our data demonstrated that to determine the prognosis for IPMN, intra- or extra-pancreatic cancer is the key factor, rather than the IPMN *per se*.

In conclusion, to decide on the prognosis for IPMN, the presence of intra- or extra-pancreatic cancer is the main prognostic factor, rather than IPMN *per se*. Therefore,

Table 6 Types of cancers reported concomitant with extra-pancreatic cancers in patients with IPMN

Neoplasm	Zhong (1996)	Sugiyama (1999)	Adsay (2002)	Osanai (2003)	Kamisawa (2005)	Egichi (2006)	Choi (2006)	Yoon (2008)	Ishida (2008)	Present study	Total
Gastric cancer	0	4	0	8	12	4	8	29	6	13	84
Colon cancer	3	5	0	11	7	8	4	16	4	8	66
Lung cancer	0	1	1	5	4	5	0	3	1	5	25
Bile duct cancer	0	1	0	4	1	NS	2	8	1	1	18
Prostate cancer	0	1	1	2	1	2	0	1	0	3	11
Renal cancer	0	1	1	2	0	2	0	3	1	1	11
Breast cancer	0	1	0	0	2	NS	0	0	0	7	10
Esophageal cancer	2	0	0	2	4	NS	0	0	1	0	9
Malignant lymphoma	0	0	0	0	1	NS	2	4	1	1	9
Bladder cancer	0	1	1	0	0	2	1	1	0	1	7
Liver cancer	0	0	0	1	2	NS	0	1	1	1	6
Thyroid cancer	0	0	0	0	0	NS	1	3	0	1	5
Ampullary cancer	0	0	1	0	0	NS	0	3	1	0	5
Skin cancer	0	0	1	0	0	NS	0	1	0	2	4
Uterine cancer	0	0	0	0	1	NS	0	2	0	0	3
Laryngeal cancer	0	0	0	0	1	NS	0	0	0	1	2
Pharyngeal cancer	0	0	0	0	1	NS	0	0	0	1	2
Ovarian cancer	0	0	0	0	0	NS	0	1	0	0	1
Duodenal cancer	0	0	0	0	0	NS	0	1	0	0	1
Other cancer or unknown	0	0	2	0	0	9	0	0	0	0	11
Total	5	15	8	35	37	32	18	77	17	46	290

there is no fixed algorithm regarding the follow-up period or methodology, but in all cases, close follow-up, especially for the development of cancer, is very important.

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COMMENTS

Background

Several investigators have suggested that the prognosis of the intraductal papillary mucinous neoplasms of the pancreas (IPMN) is more closely related to coexisting diseases than IPMN *per se*.

Research frontiers

In reports on studies that closely investigated such patients, the frequency of extra-pancreatic cancer was 23.6%-46.8% in a total of 864 IPMN patients, and 32.9% (284/864) had extra-pancreatic cancer, which corresponds well with the data from the subjects in the present study, where the frequency was 29.0% (42/145). On the other hand, many studies reported pancreatic cancer to occur in 3.8% to 9.2% of IPMN cases.

Applications

It appears to be important to determine the presence of intra- or extra-pancreatic cancer when examining the patients with IPMN.

Terminology

IPMN including branch duct type and main pancreatic duct type, have high risk for intra- or extra-pancreatic cancer, leading to death of patients.

Peer review

This is an interesting paper.

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Gender differences of low-dose aspirin-associated gastroduodenal ulcer in Japanese patients

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Abstract

AIM: To clarify the gender differences about the clinical features and risk factors of low-dose aspirin (LDA) (81-100 mg daily)-associated peptic ulcer in Japanese patients.

METHODS: There were 453 patients under treatment with LDA (298 males, 155 females) who underwent esophagogastroduodenoscopy at the Department of Gastroenterology and Hepatology of Hiratsuka City Hospital between January 2003 and December 2007. They had kept taking the LDA or started treatment

during the study period and kept taking LDA during the whole period of observation. Of these, 119 patients (87 males, 32 females) were diagnosed as having LDA-associated peptic ulcer. We examined the clinical factors associated with LDA-associated peptic ulcer in both sexes.

RESULTS: A history of peptic ulcer was found to be the risk factor for LDA-associated peptic ulcer common to both sexes. In female patients, age greater than 70 years (prevalence ORs 8.441, 95% CI: 1.797-33.649, $P = 0.0069$) was found to be another significant risk factor, and the time to diagnosis as having LDA-associated peptic ulcer by endoscopy was significantly shorter than that in the male patients ($P = 0.0050$).

CONCLUSION: We demonstrated gender differences about the clinical features and risk factors of LDA-associated peptic ulcer. Special attention should be paid to aged female patients taking LDA.

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Key words: Low-dose aspirin; Gender; Peptic ulcer

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Okada K, Inamori M, Imajyo K, Chiba H, Nonaka T, Shiba T, Sakaguchi T, Atsukawa K, Takahashi H, Hoshino E, Nakajima A. Gender differences of low-dose aspirin-associated gastroduodenal ulcer in Japanese patients. *World J Gastroenterol* 2010; 16(15): 1896-1900 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v16/i15/1896.htm> DOI: <http://dx.doi.org/10.3748/wjg.v16.i15.1896>

INTRODUCTION

Low-dose aspirin (LDA) is one of the main agents used for the prevention of thromboembolic vascular events, and has the advantages of both low cost and a prolonged duration of antiplatelet action^[1]; however, it is associated with a doubling of the risk of gastrointestinal bleeding, even at doses as low as 75 mg daily^[2,3]. The gender differences in the clinical manifestations of LDA-associated gastroduodenal mucosal injury have not been well studied. The aim of this study was to clarify the clinical features and risk factors of LDA-associated gastroduodenal mucosal injury in both sexes.

MATERIALS AND METHODS

Patients

There were 453 patients under treatment with LDA (298 males; median age, 71 years; age range, 43-93 years and 155 females; median age, 73 years; age range, 47-95 years) who underwent esophagogastroduodenoscopy (EGD) at the Department of Gastroenterology and Hepatology of Hiratsuka City Hospital between January 2003 and December 2007. The study was conducted in accordance with the Declaration of Helsinki and the study protocol was approved by the Ethics Committee of Hiratsuka City Hospital. The exclusion criteria were: patients who were taking LDA for less than 7 d, patients who had been previously diagnosed or treated for LDA-associated gastroduodenal mucosal injury in the past, patients with gastric or duodenal cancer, history of gastric or esophageal surgery and any current active cancer. Detailed information about the drug treatment and other risk factors were obtained from the medical records and also from interviews with the patients when they underwent medical examination. Concomitant use of anti-ulcer drugs (proton-pump inhibitors (PPIs), Histamine type 2 receptor antagonists (H2RAs), muco-protective agents, anticoagulants and/or antiplatelets and antithrombotic agents was defined as starting with LDA or taken for more than 4 wk before receiving endoscopy.

Endoscopic assessments

Endoscopic examinations were performed in each patient. Only ulcers with whitish exudate that were over 5 mm in size^[4] and had perceptible depth were included in the determination of the ulcer, with ulcer scars being excluded.

Helicobacter pylori infection status

The patients diagnosed to have ulcer were checked for *Helicobacter pylori* (*H. pylori*) infection using the *H. pylori* serum antibody assay, urea breath test, rapid urease test and *H. pylori* culture of two biopsy specimens obtained from the gastric body and antrum. If one of the tests was positive, the patient was labeled as *H. pylori*-positive, and if more than two of the tests were negative, the patient was labeled as *H. pylori*-negative.

Table 1 Clinical characteristics of patients taking LDA enrolled in the present study

	Patients with ulcer (n = 119)	Patients without ulcer (n = 334)	P-value
Gender (male/female)	87/32	211/123	NS
Age (yr), median (range)	73.0 (47-92)	72.0 (45-95)	NS
Male (n = 298)	70.0 (47-92)	71.0 (43-93)	NS
Female (n = 155)	77.0 (54-92)	73.0 (47-95)	0.0338
Underlying disease (male/female)			
Ischemic heart disease	77 (61/16)	205 (131/74)	NS
Cerebral infarction	22 (10/12)	70 (39/31)	NS
Atrial fibrillation	16 (13/3)	43 (28/15)	NS
Others	4 (3/1)	16 (13/3)	NS
Symptoms (male/female)			
Melena, hematemesis	46 (29/17)	1 (0/1)	< 0.0001
GI symptoms	31 (21/10)	107 (72/35)	NS ¹
Anemia	17 (14/3)	40 (21/19)	0.0156
Body weight loss	1 (1/0)	7 (5/2)	0.0087
Appetite loss	1 (1/0)	8 (2/6)	0.0028
None (medical check)	18 (15/3)	146 (96/50)	0.0025
Others	5 (2/0)	25 (15/10)	> 0.9999
<i>Helicobacter pylori</i>	62.3% (43/69)	-	
Male	66.7% (30/45)	-	
Female	54.2% (13/24)	-	

¹Forty-six patients presented with melena and/or hematemesis and bleeding source in the stomach or duodenum identified by endoscopy and were excluded from GI symptoms in this analysis. LDA: Low-dose aspirin; GI: Gastrointestinal; NS: Not significant.

Statistical analysis

Statistical evaluation was carried out using Fisher's exact test, *t*-test or Mann-Whitney *U*-test and these variables were also evaluated by multivariate logistic regression model using a Wald statistic forward stepwise selection. The level of significance was set at *P* < 0.05. Statistical analyses were performed with Stat View software (SAS Institute, Cary, NC).

RESULTS

The baseline characteristics of the study population

The 453 patients under treatment with LDA who underwent EGD were included in this investigation. The baseline characteristics of the study population are summarized in Table 1. One hundred and nineteen (26.3%, 87 males, 32 females) of the 453 patients receiving LDA were diagnosed as having LDA-associated peptic ulcer, consisting of 92 patients with LDA-associated gastric ulcer (71 males, 21 females), 20 patients with duodenal ulcer (14 males, 6 females) and 7 patients with gastric and duodenal ulcers (2 males, 5 females).

The age of female patients with ulcer was greater than that of without ulcer (*P* = 0.0338). The most common indication for LDA was secondary prevention of ischemic heart disease (62.3%, 282/453). There were no significant differences in the distribution of underlying diseases between patients with ulcer and without ulcer (*P* = 0.8380). There were no significant differences in the presence of the gastrointestinal (GI) symptoms, defined as abdominal pain, nausea, reflux symptoms and/or the indigestion

Table 2 Univariate analysis to identify factors predictive of LDA-associated gastroduodenal ulcer in male and female patients

Factor	Male patients (<i>n</i> = 299)			Female patients (<i>n</i> = 155)		
	Patients with ulcer (<i>n</i> = 87)	Patients without ulcer (<i>n</i> = 211)	<i>P</i> -value	Patients with ulcer (<i>n</i> = 32)	Patients without ulcer (<i>n</i> = 123)	<i>P</i> -value
PPIs	1	22	0.0038	0	18	0.0253
H2RAs	16	53	NS	5	38	NS
Muco-protective agents	18	40	NS	6	18	NS
Hypertension	62	150	NS	21	88	NS
Hyperlipidemia	36	96	NS	14	51	NS
History of peptic ulcer	18	19	0.0109	9	9	0.0030
Diabetes mellitus	29	63	NS	11	30	NS
Insulin therapy	4	12	NS	3	3	NS
Anticoagulants	29	87	NS	11	35	NS
NSAIDs	3	25	NS	3	10	NS
Age ≥ 70 yr	45	123	NS	28	85	0.0443
Alcohol	28	63	NS	6	12	NS
Smoking	22	36	NS	1	8	NS

PPIs: Proton-pump inhibitors; H2RAs: Histamine type 2 receptor antagonists; NSAIDs: Nonsteroidal anti-inflammatory drugs.

Table 3 Age-stratified prevalence of LDA-associated peptic ulcer in males and females

Age (yr)	Patients with ulcer (<i>n</i> = 119) (M/F)	Patients without ulcer (<i>n</i> = 334) (M/F)
40-49	2/0	4/3
50-59	15/2	20/7
60-69	24/2	64/28
70-79	33/17	94/55
80-89	10/11	26/27
90-	2/1	3/3

syndrome, between the patients with ulcer and without ulcer ($P = 0.0761$). Forty-six patients presented with melena and/or hematemesis and bleeding source in the stomach or duodenum identified by endoscopy were excluded from GI symptoms in this analysis. Moreover, even in patients with peptic ulcer, about half were asymptomatic.

Univariate analyses to identify predicted factors of LDA-associated gastroduodenal ulcer in both sexes were shown in Table 2. A history of peptic ulcer was found to be the only significant risk factor common to both sexes (male, $P = 0.0109$, female $P = 0.0030$), and age greater than 70 years was another risk factor in female patients ($P = 0.0443$). As shown in Table 3, there were no significant differences in the prevalence of LDA-associated peptic ulcer among age brackets in either sex (male, $P = 0.3137$, female $P = 0.3612$).

H. pylori infection status

The *H. pylori* infection status was determined in 69 patients (58.0%, 69/119), but remained unknown in the remaining 50 patients who were therefore not included in the statistical analysis of this parameter. Of the 69 patients, 62.3% (43/69) were *H. pylori*-positive [gastric ulcer, 69.1% (38/55); duodenal ulcer, 25.0% (2/8); gastric and duodenal ulcer, 50.0% (3/6)] and 26 (37.7%) were *H. pylori*-negative. There were no significant differences in the *H. pylori* infection rate between both sexes [male: 66.7% (30/45), female: 54.2% (13/24)] ($P = 0.4344$). The

Table 4 Duration of LDA administration until diagnosis as having LDA-associated peptic ulcer in 106 patients

Month	Male (<i>n</i> = 78)	Female (<i>n</i> = 28)
-1	3	3
1-11	15	6
12-23	6	6
24-35	10	5
36-47	11	4
48-59	5	0
60-71	5	2
72-	23	2
Mean (min-max)	42 (1-223)	25.5 (1-130)

H. pylori infection rate was significantly lower in patients with LDA-associated ulcer as compared with that in patients with non-LDA-associated ulcer [including other nonsteroidal anti-inflammatory drugs (NSAIDs) associated ulcer] (77.0%, 419/544) diagnosed in the same study period (data are not shown in Tables) ($P = 0.0112$).

Time to diagnosis as having LDA-associated peptic ulcer by endoscopy

As shown in Table 4, we investigated the time from the start of LDA administration to the diagnosis as having LDA-associated peptic ulcer by endoscopy in 106 of the 119 patients (89.1%). LDA-associated peptic ulcer was diagnosed within the first month in 6 cases (5.7%) within the first 12 mo in 28 cases (26.4%), and within 5 years in 74 cases (69.8%). In female patients, the duration was significantly shorter than in male patients ($P = 0.0050$) (male: mean 42.0 mo; range, 1-223 mo, female: mean, 25.5 mo; range, 1-130 mo). The incidence of LDA-associated peptic ulcer seemed almost constant and did not decrease with time, at least over the follow-up period of 72 mo in this study.

Multivariate analysis to identify the risk factors of LDA-associated peptic ulcer in both sexes

Table 5 shows the results of multivariate analyses to identify the risk factors of LDA-associated peptic ulcer

Table 5 Multivariate analysis to identify risk factors of LDA-associated gastroduodenal ulcer in male and female patients

Factor	Odds ratio	95% CI	P-value
Male patients (n = 211)			
PPIs	0.069	0.009-0.555	0.0120
H2RAs	0.498	0.256-0.967	0.0396
History of peptic ulcer	3.745	1.734-8.088	0.0008
Female patients (n = 123)			
H2RAs	0.255	0.077-0.839	0.0245
History of peptic ulcer	6.439	1.874-22.131	0.0031
Age ≥ 70 yr	8.441	1.797-33.649	0.0069

Odds ratio: Prevalence odds ratio.

in both sexes. In male patients, a history of peptic ulcer (prevalence ORs 3.745, 95% CI: 1.734-8.088, $P = 0.0008$) was found to be associated with an increase in the risk, and a history of PPIs (prevalence ORs 0.069, 95% CI: 0.009-0.555, $P = 0.0120$) and/or H2RAs (prevalence ORs 0.498, 95% CI: 0.256-0.967, $P = 0.0396$) use was found to be associated with a decrease in the risk. In female patients, a history of peptic ulcer (prevalence ORs 6.439, 95% CI: 1.874-22.131, $P = 0.0031$) and age greater than 70 years (prevalence ORs 8.441, 95% CI: 1.797-33.649, $P = 0.0069$) were associated with an increase in the risk. Statistical analysis with respect to the use of PPIs could not be performed in female patients, because there was no female patient taking PPIs who was diagnosed as having LDA-associated peptic ulcer.

DISCUSSION

The present study demonstrated gender differences in the clinical features of LDA-associated gastroduodenal ulcer in Japanese patients. Previous reports have indicated age greater than 65 years, previous history of peptic ulcer^[4,5], use of other NSAIDs^[6] and *H. pylori* infection^[7] as some of the risk factors of LDA-associated peptic ulcer, and the concomitant use of antisecretory drugs as a factor reducing the risk of LDA-associated peptic ulcer^[5,8,9]. In this study, a history of peptic ulcer was found to be the risk factor of LDA-associated peptic ulcer common to both sexes. In female patients, advanced age or aging was found to be another significant factor. In elderly patients, gastric mucosal defenses are impaired because of the reduced mucus and bicarbonate secretion, and also the significant decrease in gastric mucosal perfusion. The reduced gastric mucosal defenses coupled with a reduced gastric prostaglandin biosynthesis may account for the higher susceptibility of the mucosa to damage-inducing agents^[10]. In general, female subjects are at a relatively reduced risk of developing gastric ulcers^[11]. In animal experimental models, female sex hormones have been demonstrated to show significant antiulcer activity and to promote healing of drug-associated ulcers (aspirin, indomethacin)^[12,13]. Estrogen has also been reported to reduce the incidence of *H. pylori*-associated gastric mucosal injuries, despite having no effect on the *H. pylori* infection status. Female sex hormones may exert a protective effect against LDA-

associated mucosal injury. In female patients of advanced age, the gradual decrease in the serum levels of female sex hormones after menopause and with advancing age, coupled with the reduced gastric mucosal defenses may be responsible for the increase in the risk of LDA-associated peptic ulcer.

In addition, time from the start of LDA until the diagnosis of LDA-associated peptic ulcer by endoscopy was shorter in the female patients. This gender difference, akin to the significantly higher relative risk and short time-to-onset of alcohol-related liver disease in women as compared to men for any given level of alcohol intake^[14], is yet to be well elucidated. The incidence of peptic ulcer seemed almost constant and does not decrease with time, at least over the follow-up period of 72 mo in this study (Table 3). It has been suggested that NSAID-associated gastrointestinal bleeding occurs mainly within the first few weeks of treatment^[15], which is a shorter period than that reported in LDA users. We should recognize that LDA-associated peptic ulcer may not occur for several years; therefore, it is important to continue antisecretory therapy, especially in high-risk patients. Future prospective cohort studies are needed to confirm this assumption.

COMMENTS

Background

The gender differences in the clinical manifestations of low-dose aspirin (LDA)-associated gastroduodenal mucosal injury have not been well-studied.

Research frontiers

The authors hypothesized that there were gender differences in clinical features of LDA-associated peptic ulcer, and it might be useful to identify the risk factors of LDA associated ulcer in both sexes.

Innovations and breakthroughs

In female patients, age greater than 70 years was found to be a significant risk factor of LDA associated peptic ulcer in addition to a history of peptic ulcer, and the time to diagnosis as having LDA-associated peptic ulcer by endoscopy was significantly shorter than that in the male patients.

Applications

By understanding the gender differences in clinical features of LDA-associated ulcer, this study may represent a future strategy for the prevention of LDA-associated peptic ulcer.

Terminology

Special attention should be paid to aged female patients taking LDA. People should also recognize that LDA-associated peptic ulcer may not occur for several years; therefore, it is important to continue antisecretory therapy, especially in high-risk patients.

Peer review

This study is adequately designed and performed. It means a contribution in a topic of high relevance.

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Chemotherapy with laparoscope-assisted continuous circulatory hyperthermic intraperitoneal perfusion for malignant ascites

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Abstract

AIM: To investigate the procedure, feasibility and effects of laparoscope-assisted continuous circulatory hyperthermic intraperitoneal perfusion chemotherapy (CHIPC) in treatment of malignant ascites induced by peritoneal carcinomatosis from gastric cancers.

METHODS: From August 2006 to March 2008, the laparoscopic approach was used to perform CHIPC on 16 patients with malignant ascites induced by gastric cancer or postoperative intraperitoneal seeding. Each patient underwent CHIPC three times after laparoscope-assisted perfusion catheters placing. The first session was completed in operative room under general anesthesia, 5% glucose solution was selected as perfusion liquid, and 1500 mg 5-fluorouracil (5-FU) and 200 mg oxaliplatin were added in the perfusion solution. The second and

third sessions were performed in intensive care unit, 0.9% sodium chloride solution was selected as perfusion liquid, and 1500 mg 5-FU was added in the perfusion solution alone. CHIPC was performed for 90 min at a velocity of 450-600 mL/min and an inflow temperature of $43 \pm 0.2^\circ\text{C}$.

RESULTS: The intraoperative course was uneventful in all cases, and the mean operative period for laparoscope-assisted perfusion catheters placing was 80 min for each case. No postoperative deaths or complications related to laparoscope-assisted CHIPC occurred in this study. Clinically complete remission of ascites and related symptoms were achieved in 14 patients, and partial remission was achieved in 2 patients. During the follow-up, 13 patients died 2-9 mo after CHIPC, with a median survival time of 5 mo. Two patients with partial remission suffered from port site seeding and tumor metastasis, and died 2 and 3 mo after treatment. Three patients who are still alive today survived 4, 6 and 7 mo, respectively. The Karnofsky marks of patients (50-90) increased significantly ($P < 0.01$) and the general status improved after CHIPC. Thus satisfactory clinical efficacy has been achieved in these patients treated by laparoscopic CHIPC.

CONCLUSION: Laparoscope-assisted CHIPC is a safe, feasible and effective procedure in the treatment of debilitating malignant ascites induced by unresectable gastric cancers.

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Key words: Intraperitoneal hyperthermic perfusion; Laparoscopy; Chemotherapy; Gastric cancer; Malignant ascites

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INTRODUCTION

Malignant ascites are a common complication induced mainly by advanced ovarian cancer and also by gastrointestinal cancers (e.g. gastric cancer, colorectal cancer, and liver cancer). Once vast malignant ascites occur in patients with advanced gastric carcinoma or postoperative peritoneal seeding, disseminated seeding metastasis can be observed. Patients would lose the opportunity to undergo surgery, and nothing can be done except treating the pyloric obstruction or other complications. In addition, the prognosis is extremely poor and the survival time is generally as low as less than 2 mo^[1].

In recent years, continuous circulatory hyperthermic intraperitoneal perfusion chemotherapy (CHIPC) has appeared as a new auxiliary chemotherapy for abdominal malignant tumors. Preliminary clinical efficacy has been achieved in the prevention and treatment of malignant tumor metastasis^[2-4].

Currently, laparoscope-assisted CHIPC has been used clinically to treat malignant ascites in a few medical institutions in the United States, France and Italy. In comparison with traditional CHIPC, laparoscope-assisted CHIPC has a good clinical profile and carries the benefits of definitive palliation of ascites with a minimal invasion, fewer traumas, less pain, shorter recovery time and more stable efficacy, thus avoiding the abdominal laparotomy and large incision necessary in patients with unresectable tumors^[5-8]. However, little information related to the facilities, therapeutic temperature, velocity of drug perfusion or complications involved in laparoscope-assisted CHIPC has been available. This limits the widespread use of laparoscope-assisted CHIPC in clinical chemotherapy. Therefore, in this study, we present a detailed report on the use of laparoscope-assisted CHIPC in treatment of patients with malignant ascites induced by advanced gastric cancers.

MATERIALS AND METHODS

Inclusion criteria and clinical data

This protocol was approved by Cancer Hospital of Guangzhou Medical College. From August 2006 to March 2008, 16 patients with malignant ascites induced by advanced gastric cancers were treated *via* laparoscope-assisted CHIPC.

The diagnosis of primary gastric cancer complicated by malignant ascites was established and existence of peritoneal diffusive seeding was verified by laparoscopic examination. Thus these patients were not well suited for the tumor resection. They had no serious cardiopul-

monary diseases. Informed consent of CHIPC and laparoscopic surgery was obtained from all the patients.

As shown in Table 1, of the 16 patients in this study, there were 7 men and 9 women aged 43-74 years (median age, 56 years). Karnofsky (KPS) marks ranged from 40 to 70 points and the course of disease ranged from 5-30 d (15 d on average). There were 7 primary cases of gastric cancer and 9 cases of postoperative gastric cancer, which were diagnosed by laparotomy, gastric fiberoptic endoscopy, serum tumor markers (CEA and CA199) and ascite cytology. Ultrasonic B and laparoscopic examinations displayed 4000-9000 mL seroperitoneum in all the patients. Five patients had obvious abdominal distention, abdominal pain and oliguria or anuria with high intraperitoneal pressure caused by ascites, and no apparent palliation was found after repeated abdominal puncture drainage, 11 patients had numerous free cancer cells within the ascites, 2 cases presented obvious bloody ascites, and 1 case presented chyle-like ascites.

Laparoscopic examination and chemotherapeutic catheter placement

After endotracheal anesthesia, a transverse cut (10 mm long) was performed at the belly, 5 mm below the umbilicus. The seroperitoneum was extracted as completely as possible; artificial pneumoperitoneum was established *via* an open procedure with a pressure of 13 mmHg (1 mmHg = 0.133 kPa); a 10-mm Trocar was inserted into the abdominal cavity *via* the working port. Thereafter, the laparoscope (10 mm and 30°) was inserted *via* the 10 mm Trocar to examine the abdominal viscera and tumors. The site, size and clinical stages of tumors were examined laparoscopically. Patients with peritoneal diffusive seeding and unresectable tumors were advised to receive laparoscope-assisted CHIPC.

In the process of laparoscope-assisted CHIPC, three new ports were prepared under the guidance of a laparoscope. On the right side, the second and third ports (both 10 mm long) were prepared at the cross-point of the mid-clavicular line and transverse surfaces, with two finger spaces above and below the umbilicus, respectively. On the left side, the fourth port (10 mm long) was prepared at the cross-point of the mid-clavicular line and transverse surface, with two finger spaces below the umbilicus. Thereafter, under the guidance of laparoscope, a 10 mm Trocar was inserted into the abdominal cavity *via* the working port. Two perfusion catheters were placed in the right superior abdominal cavity *via* the third and fourth working ports, respectively. One drainage catheter was placed in the Douglas' cavity of the lowest place in the pelvic cavity *via* the second working port. Then, the laparoscope was placed in the inferior abdomen and the Trocar was inserted. Subsequently, the laparoscope was pulled out, and the perfusion catheter was placed in the Douglas' cavity of the lowest place in the pelvic cavity under the guidance of the Trocar.

CHIPC procedures

CHIPC was performed by our self-developed "BR TRG

Table 1 Clinical data of patients undergoing laparoscope-assisted CHIPC in gastric cancer complicated by malignant ascites

Case No.	Gender	Age (yr)	Disease course (d)	Ascite volume (mL) [free cancer cells (+/-)]	Diagnosis	Examination	Pathology
1	F	46	17	7000 (+)	GC	GSB	MAC
2	M	58	21	4500 (+)	PGC	PP	M-LDAC
3	F	73	18	6000 (-)	GC	GSB	MDAC
4	F	58	12	4000 (+)	PGC	PP	MAC
5	M	76	7	4800 (+)	PGC	GSB	LDAC
6	M	58	9	9000 (+)	PGC	PP	SRCC
7	F	45	12	7000 (-)	GC	GSB	LDAC
8	F	59	80	4000 (+)	PGC	PP	SRCC
9	M	60	21	4500 (+)	PGC	PP	LDAC
10	M	67	13	4800 (-)	PGC	PP	M-LDAC
11	F	61	15	5000 (+)	GC	GSB	LDAC
12	M	52	14	6000 (+)	PGC	PP	MAC
13	F	68	9	5500 (+)	PGC	PP	LDAC
14	F	45	5	5800 (+)	GC	GSB	SRCC
15	M	59	12	6000 (-)	PGC	PP	LDAC
16	F	72	16	7200 (-)	PGC	GSB	M-LDAC

CHIPC: Circulatory hyperthermic intraperitoneal perfusion chemotherapy; GC: Gastric cancer; PGC: Postoperative gastric cancer; GSB: Gastroscopic biopsy; PP: Postoperative pathology; MAC: Mucinous adenocarcinoma; M-LDAC: Medium-low differentiated adenocarcinoma; SRCC: Signet ring cell cancer.

II type high-precision hyperthermic perfusion intraperitoneal treatment system” (authorized by two patents from China: Intraperitoneal Hyperthermic Perfusion Treatment System with patent number ZL2006200613779 and Continuous Intraperitoneal Hyperthermic Perfusion Treatment Apparatus with patent number ZL2006200613764) with precise $\pm 0.2^{\circ}\text{C}$ temperature control, $\pm 5\%$ flow control accuracy, and an automatic cooling function.

Patients completed the first session of CHIPC in the operating room under general anesthesia. The second and third sessions were performed in the intensive care unit on the first and second day after the operation. At the first session, 5-fluorine (1500 mg) and oxaliplatin (200 mg) were added into the 5% glucose solution (4500–6000 mL) as perfusion liquid. Then CHIPC was performed for 90 min with a velocity of 450–600 mL/min and an inflow temperature of $43 \pm 0.2^{\circ}\text{C}$.

At the second and third sessions, pethidine hydrochloride (75 mg) and promethazine hydrochloride (25 mg) were administered by intramuscular injection less than 10 min before CHIPC. Propofol (3–8 mL/h, iv) as an anesthetic agent with a continuous vein pump. The dose was continuously adjusted according to patient status. The temperature and duration of CHIPC were the same as for the first session; 5-fluorine (1500 mg) was added to the 0.9% saline solution (4500–6000 mL) as perfusion liquid. When the third session of CHIPC was completed, the intra-abdominal perfusate and ascites were drained out. Two perfusion catheters were then pulled out, and a drainage catheter was remained most frequently in the body.

Evaluation and determination of efficacy

Two weeks later, malignant ascites were collected for the last time; the remained drainage catheter was withdrawn; and ultrasonic B reexamination was performed to evaluate ascite remission status. Thereafter, ultrasonic B or computed tomography (CT) was performed for follow-up, at least once a month. Ascite status and tumor progression

were assessed. Karnofsky marks were used to evaluate ascite remission and patient quality of life before CHIPC and one and two weeks after the last CHIPC, respectively. All data were statistically analyzed using SPSS13.0 statistical software.

According to the modified WHO criteria of efficacy assessment in malignant tumors^[1], clinical efficacy was divided into three grades: (1) complete remission (CR): ascites are completely absorbed after treatment sustained over 4 wk; (2) partial remission (PR): ascites are reduced by 50% and sustained over 4 wk; and (3) no consequence (NC): ascites reduced no obvious or increased after treatment.

RESULTS

Clinical efficacy

The intraoperative course was uneventful in all cases; mean operative period for laparoscope-assisted perfusion catheters placing was 80 min; no postoperative deaths or complications related to the laparoscope-assisted CHIPC procedure occurred in this study. After the first laparoscope-assisted CHIPC, the daily amount of ascite outflow was 100–300 mL. After the first day, ascite outflow gradually decreased. A week later, daily outflow was 0–30 mL. During the period from two weeks after laparoscope-assisted CHIPC to the end of follow-up, clinical CR of ascites and related symptoms were achieved in 14 of the 16 treated patients (90.5%), and PR was achieved in 2 patients (9.5%). Thus the objective remission rate (ORR = CR + PR) was 100%. The Karnofsky mark indicating patient quality of life was 50–90, which was increased significantly in comparison with before laparoscope-assisted CHIPC ($P < 0.01$).

Before laparoscope-assisted CHIPC, free cancer cells were found in the ascites of 11 patients (Figure 1A). However, free cancer cells degenerated and necrotized after the first CHIPC (Figure 1B). No intact free cancer cells were found in ascites after the second laparoscope-assisted CHIPC, but degenerated and necrotized cancer cells were

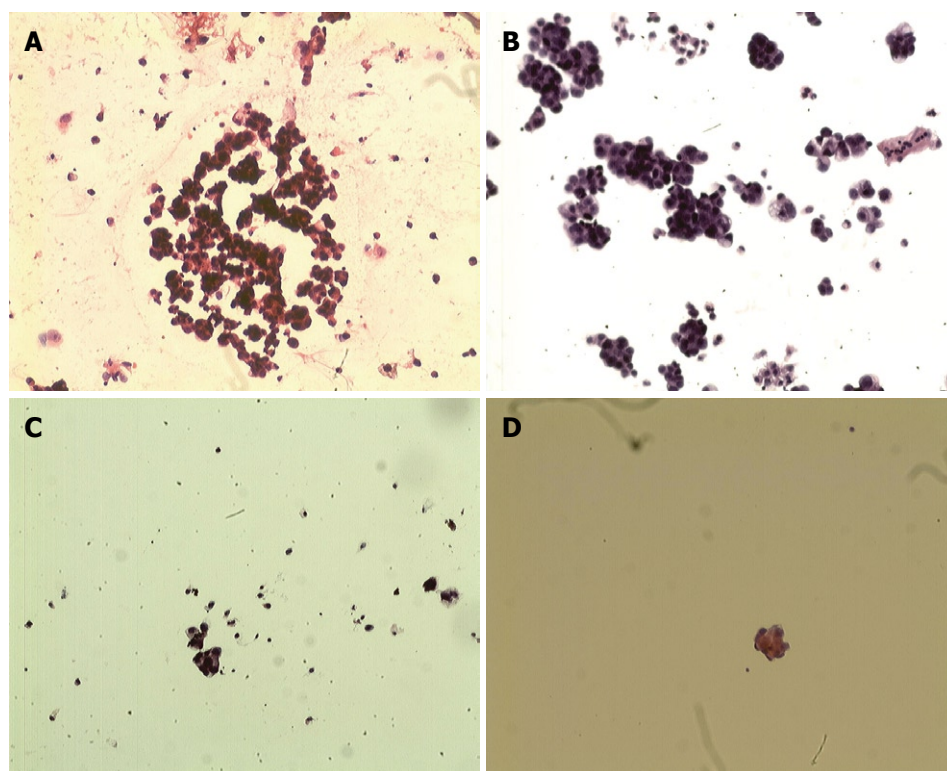


Figure 1 Cancer cells ascites. A: Numerous free cancer cells were found in ascites before circulatory hyperthermic intraperitoneal perfusion chemotherapy (CHIPC) (HE, $\times 100$); B: Intact free cancer cells in ascites clearly reduced in number, and some degenerated and necrotized after the first CHIPC (HE, $\times 100$); C: No intact free cancer cells but rather degenerated and necrotized cells were found in ascites after the second CHIPC; D: No free cancer cells were found in ascites after the third CHIPC.

observed (Figure 1C). No intact free cancer cells were found in the abdomen after the third CHIPC (Figure 1D). In addition, bloody ascites in two cases and chyle-like ascites in 1 case turned clear after the first laparoscope-assisted CHIPC. In addition, the general status of patients improved after the third laparoscope-assisted CHIPC. Mental status, appetite and body weight improved, and symptoms of anemia were obviously alleviated. Thus, satisfactory initial clinical efficacy has been achieved in these patients treated by laparoscope-assisted CHIPC.

Side effects

In the course of laparoscope-assisted CHIPC, patients had no obviously abnormal vital signs except for transient fever, abdominal distension and bellyache. Dynamic re-examination in the anterior, median and posterior phases of laparoscope-assisted CHIPC revealed no anomalies in hepatorenal functions. Bone marrow suppression of I - II degrees was observed in three cases and slight gastrointestinal reaction in one case; and symptoms were remitted after symptomatic treatment. In addition, there were no complications, such as abdominal incision infection or adhesive ileus, after the laparoscope-assisted CHIPC operation.

Follow-up and prognosis

Clinical efficacies of laparoscope-assisted CHIPC in patients with gastric cancer complicated by malignant ascites are summarized in Table 2. All patients had complete follow-up data. During the follow-up (2-9 mo), 13 patients survived from 2 to 9 mo with a median survival time of 5 mo. The causes of death were intestinal obstruction (7 cases), gastrointestinal bleeding (3 cases), or cachexia (3 cases). Two patients with partial remission suffered from port-site seeding of the tumor and died within 2 and 3 mo

after treatment, respectively. Three patients still alive today survived 4, 6 and 7 mo, respectively. Five patients failed in resection of the primary lesions in the re-laparotomy, and cecal fistula or ileal fistula was made in 4 patients for treatment of intestinal obstruction induced by tumor infiltration. The diagramed survival curves are shown in Figure 2.

DISCUSSION

Malignant ascites induced by malignant tumors in the abdominal cavity (e.g. ovarian cancer, gastric cancer, colorectal cancer, and other intra-abdominal cavity) are a common complication of intraperitoneal malignant tumors. The prognosis is extremely poor and the survival time is very short^[1]. Gastric cancer is one of the most common intraperitoneal malignant tumors complicated by malignant ascites. Patients with gastric cancer complicated by malignant ascites suffer mostly from the rapid increase in ascites in addition to the clinical symptoms, vital signs and cachexia of primary diseases. However, diuretic treatment is not effective; single-puncture drainage of ascites leads to rapid re-growth; and multiple puncture drainage of ascites can cause severe complications such as hypoalbuminemia and hydrogen electrolyte disturbance. The ascites cause abdominal distention, abdominal pain and oliguria or anuria, which strongly impact the quality of life and render clinical treatment difficult. Therefore, suppressing or eliminating the rapid growth of ascites has great significance in the comprehensive treatment of patients with advanced gastric carcinoma or postoperative celiac diffusive seeding^[1].

In recent years, CHIPC has appeared as a new auxiliary chemotherapy for intraperitoneal malignant tumors. Hyperthermic chemotherapeutic liquid at large doses fa-

Table 2 Clinical efficacy of laparoscope-assisted CHIPC in patients with gastric cancer complicated by malignant ascites

Case No.	Therapeutic outcome	Port-site seeding (+/-)	KPS mark		Survival time (mo)	Death/living
			Before treatment	After treatment		
1	CR	-	40	80	9	Death
2	CR	-	60	90	7	Death
3	CR	-	60	70	5	Death
4	CR	-	50	80	5	Death
5	CR	-	70	90	7	Death
6	CR	-	50	70	4	Death
7	PR	+	40	60	2	Death
8	CR	-	50	70	4	Death
9	CR	-	50	90	5	Death
10	CR	-	60	80	5	Death
11	CR	-	60	80	4	Death
12	PR	+	50	70	3	Death
13	CR	-	40	80	7	Death
14	CR	-	70	80	7	Living
15	CR	-	60	70	4	Living
16	CR	-	60	80	6	Living

KPS: Karnofsky; CR: Complete remission; PR: Partial remission.

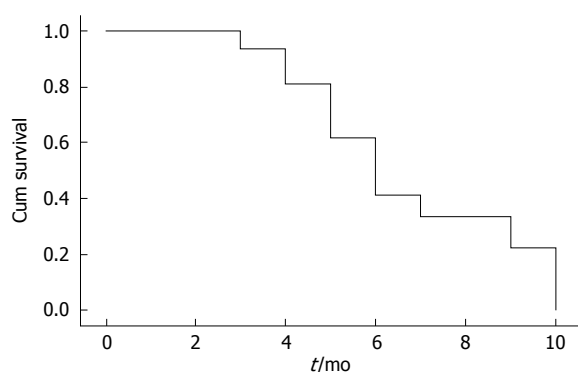


Figure 2 Diagrammed survival curves of patients undergoing laparoscopic CHIPC in gastric cancer complicated by malignant ascites.

cilitates a full and effective reach of the drugs to the tiny celiac carcinoma metastases. Perfusion chemotherapy affects mechanical removal of intra-abdominal free cancer cells. During the perfusion, chemotherapeutic drugs are locally administered to the abdominal cavity, which leads to a high, constant and sustained local drug concentration. However, fewer drugs enter into the systemic circulation, which reduces the systemic side-effects. Therefore, CHIPC has obvious advantages in comparison with simple intraperitoneal chemotherapy in the treatment of malignant ascites. Preliminary efficacy has been achieved in the prevention and treatment of the peritoneal seeding of malignant tumors^[5-8].

In the process of laparoscope-assisted CHIPC, the site, size and clinical stages of tumors are laparoscopically examined. Patients with peritoneal diffusive seeding and unresectable tumors are advised to undergo laparoscope-assisted CHIPC. Laparoscope-assisted CHIPC integrates the benefits of a definitive palliation of ascites with a minimal invasion, which leads to fewer traumas, less pain, shorter recovery time and solid efficacy, thus avoiding the abdominal laparotomy and large incision necessary in patients with unresectable tumors. There-

fore, laparoscope-assisted CHIPC is a potential therapy for malignant ascites^[9-12].

Ferron *et al*^[13] performed laparoscope-assisted CHIPC on five adult pigs in France. Trocar was placed in the four celiac corners; omental total resection was performed. The final Trocar below the umbilicus was positioned using Dapdics, which is helpful in operating upon the ansa intestinalis. The abdominal cavity was filled with hot saline for 30 min at 43°C. As a result, the liquid was well distributed in the intra-abdomen with the appropriate temperature, and no surgical complications occurred. Therefore, the authors suggest that laparoscope-assisted CHIPC is safe and feasible for chemotherapy of malignant ascites.

Gesson-Paute *et al*^[14] performed laparoscope-assisted CHIPC on ten adult pigs to investigate the changes in pharmacokinetics. In the experiment, perfusion and drainage catheters were laparoscopically placed in the upper and lower abdominal quadrants. In control animals, drainage catheters were placed laparotomically. CHIPC was performed with perfusion liquid containing oxaliplatin (460 mg/m²) for 30 min at 41-43°C. The perfusion liquid was sampled every minute to determine the drug concentration. The results showed that the operation was performed smoothly without complications. Drug absorption rate was 41.5% in the experimental group but 33.4% in the control. This difference was not statistically significant ($P = 0.0543$, $n = 5$). The half-life of oxaliplatin in laparoscope-assisted CHIPC was significantly shorter than that in laparotomic CHIPC (37.5 min *vs* 59.3 min, $P = 0.02$), which indicates that the adsorption of oxaliplatin in laparotomic CHIPC is significantly faster than that in laparotomic CHIPC, leading to increased clinical efficacy. In addition, the celiac drug kinetic curvature for laparoscopic and laparotomic CHIPC was 16.3 *vs* 28.1, $P = 0.02$, respectively, which reflects intraperitoneal barrier penetration of oxaliplatin. Therefore, the authors believed that laparoscope-assisted CHIPC-associated pharmacokinetics elucidate intraperitoneal drug adsorption and support the viewpoint that laparoscope-assisted CHIPC is safe, with robust clinical efficacy.

Facchiano *et al*^[15] treated five patients with malignant ascites after palliative resection of gastric cancer by laparoscope-assisted CHIPC in a hospital affiliated to the Paris University. Celiac perfusion and drainage catheters were laparoscopically placed in the upper and lower abdominal quadrants, and laparoscope-assisted CHIPC was performed with perfusion liquid containing mitomycin plus platinum for 60-90 min at an inflow temperature of 45°C. The results showed that the operation went on smoothly without related complications for a mean 181 min; malignant ascites were eliminated in all 5 cases. The authors suggest that laparoscope-assisted CHIPC in patients with malignant ascites after palliative resection of gastric cancer is safe and feasible, with robust clinical effect.

Garofalo *et al*^[16] performed laparoscope-assisted CHIPC on 14 cases of malignant ascites and five cases of gastric cancer, three cases of colorectal cancer, three cases of ovarian cancer, two cases of breast cancer and one case of intraperitoneal pseudomyxoma, respectively. Laparoscope-assisted CHIPC was performed with chemotherapeutic drugs (e.g. carboplatin, cisplatin and mitomycin according to the primary tumor type) for 90 min at inflow temperature of 42°C. Four drainage catheters were laparoscopically placed in the upper and lower abdominal quadrants and were pulled out after perfusion. The results indicated that malignant ascites had been well controlled in all 14 cases, and postoperative CT scanning confirmed pelvic effusion in one case. The authors conclude that laparoscope-assisted CHIPC is safe and efficient for chemotherapy of patients who suffer from advanced malignant tumors complicated by ascites and peritoneal seeding and who are not suited for cytoreductive surgery. However, the prerequisites are surgeon's experience with laparoscopic tumor staging and patient's capacity to undergo laparoscope-assisted CHIPC.

Knutsen *et al*^[17] performed laparoscope-assisted CHIPC on five patients who underwent laparotomic cytoreductive surgery three to eight weeks earlier. The primary diseases included adenocarcinoma of the cecum in one case, appendiceal adenocarcinoma in three cases and gallbladder adenocarcinoma in one case. The inflow temperature was 42.1°C and outflow temperature was 40.5°C. After the operation, one patient exhibited port-site seeding, one patient died of tumor progression, and three patients survived without tumor progression and enjoyed a normal quality of life within four months after laparoscope-assisted CHIPC. The author suggests that laparoscope-assisted CHIPC after laparotomic cytoreductive surgery can guarantee the constant velocity of perfusion and maintain a stable intra-abdominal temperature.

In the process of CHIPC, drainage tubes tend to be plugged up by the great omentum or intestinal canals, so unobstructed drainage is the key to successful CHIPC. Through the long-term observation of clinical practice, we have mastered reliable means to place laparoscope-assisted CHIPC tubes and prevent jamming of the drainage tube. Drainage tubes which pass through the perforations of the right upper quadrant or left upper quadrant are placed in the lowest pelvic cavity (Douglas' cavity), and perfusion tubes are placed in the abdominal

hepatorenal recess and the splenic recess through the perforations of the right lower quadrant or left lower quadrant, respectively. When undergoing laparoscope-assisted CHIPC, the patient is placed in the supine or head-high-foot-low (15°) positions. After the abdominal cavity is full of liquid, drainage tubes are open to drain the liquid, with help of the body position. The great omentum and intestinal canals are difficult to position within the pelvic cavity and therefore unlikely to plug up the drainage tube, thus unobstructed circulatory perfusion is needed. If pelvic drainage tubes are plugged up, smooth drainage can be ensured by changing infusion tube from the right upper quadrant (hepatorenal recess) into drainage tube to the lower quadrant (Douglas' cavity) and changing the patient's position from head low to supine position. In this study, all the patients suffered from gastric cancer-induced malignant ascites and their great omentums were all excised or contracted into clumps. The abdominal cavity volume was big enough to ensure smooth drainage; there was no failure in the drainage tube jam in laparoscope-assisted CHIPC.

We have successfully developed a High Precision Intraperitoneal Hyperthermic Perfusion Treatment system with independent intellectual property rights. In this study, we used this machine for laparoscope-assisted CHIPC to treat 16 patients with malignant ascites induced by peritoneal carcinomatosis from gastric cancers. All patients with ascites have remitted which indicates a 100% effective rate as reported by other authors^[15,17]. Bloody ascites in two cases and chyle-like malignant ascites in one case turned clear very quickly after the first laparoscope-assisted CHIPC. The median survival time was 5 mo, which is prolonged as compared with the traditional therapy, and other reports as well^[16,17]. General status, mental status, appetite and body weight improved, symptoms of anemia were alleviated, and initial clinical efficacy was satisfactory in the patients. All these results imply that laparoscope-assisted CHIPC has good clinical efficacy.

In addition, our clinical observations indicate that the primary tumors remain or even that peritoneal seeding foci are aggravated after laparoscope-assisted CHIPC. However, the symptoms associated with malignant ascites decreased or disappeared completely and ultrasonic B examination has not revealed ascite recurrence. The theory of this system remains to be elucidated. At the same time, bloody and chylous ascites became clear very quickly after laparoscope-assisted CHIPC, a process which also requires further studies.

Port-site seeding is a frequent problem for laparoscopic doctors. Patients who undergo laparoscope-assisted CHIPC have many free cancer cells in the abdominal cavity and more severe port-site seeding. Two patients with port-site seeding are included in this study and exhibited only partial remission after laparoscope-assisted CHIPC, which indicates poor clinical efficacy. However, this study includes only a limited number of cases, and the influence of laparoscope-assisted CHIPC on the quality of life and long-term survival of patients requires further researches.

In conclusion, laparoscope-assisted continuous circulatory intraperitoneal hyperthermic perfusion chemotherapy

(CHIPC) in gastric cancer complicated by malignant ascites guarantees the uniform velocity of perfusion liquid, maintains the stable intra-abdominal temperature, facilitates a full and effective reach of the drugs to the celiac cancers, and has advantages such as minimal invasion, less pain and short recovery time. These factors contribute to achieving a good clinical efficacy, including improved quality of life and prolonged survival. Therefore, laparoscope-assisted CHIPC is safe and feasible for a widespread clinical use.

COMMENTS

Background

Malignant ascites is a common complication of advanced peritoneal malignant tumors, and treatment of this malady is very difficult clinically. Continuous circulatory hyperthermic intraperitoneal perfusion chemotherapy (CHIPC) has achieved satisfactory therapeutic effects in treatment of malignant ascites.

Research frontiers

Laparoscope-assisted CHIPC integrates the benefits of definitive palliation of gastric cancer complicated by malignant ascites with fewer traumas, less pain, shorter recovery time and more stable efficacy, thus avoiding the abdominal laparotomy and large incision necessary in patients with unrespectable tumors.

Innovations and breakthroughs

Laparoscope-assisted CHIPC is a safe, feasible and effective procedure to treat debilitating malignant ascites induced by unresectable intraperitoneal carcinomatosis of gastric cancers.

Applications

Laparoscope-assisted CHIPC for gastric cancer complicated by malignant ascites has advantages such as minimal invasion, less pain and short recovery time. These factors contribute to achieving a good clinical efficacy, including improved quality of life and prolonged survival.

Terminology

CHIPC is a novel adjuvant therapy for intra-abdominal malignancies. High-doses of warm chemotherapy liquid are administered intra-abdominally so that chemotherapy drugs can have a full contact with the tumor metastases. Chemotherapeutic agents administered into the abdominal cavity have a higher, constant, and persistent drug concentration but less drugs entering the circulatory system, and few systemic toxic side effects.

Peer review

The scientific and innovative contents as well as readability of this paper can reflect the advanced levels of the clinical research in gastroenterology both at home and abroad.

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Remission induction and maintenance effect of probiotics on ulcerative colitis: A meta-analysis

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Abstract

AIM: To evaluate the induction of remission and maintenance effects of probiotics for ulcerative colitis.

METHODS: Information was retrieved from MEDLINE, EMBASE, and the Cochrane Controlled Trials Register. The induction of remission and promotion of maintenance were compared between probiotics treatment and non-probiotics treatment in ulcerative colitis.

RESULTS: Thirteen randomized controlled studies met the selection criteria. Seven studies evaluated the remission rate, and eight studies estimated the recurrence rate; two studies evaluated both remission and recurrence rates. Compared with the non-probiotics group, the remission rate for ulcerative colitis patients who received probiotics was 1.35 (95% CI: 0.98-1.85). Compared with the placebo group, the remission rate of ulcerative colitis who received probiotics was 2.00 (95% CI: 1.35-2.96). During the course of treatment, in patients who received probiotics for less than 12 mo compared with the group treated by non-probiotics, the remission rate of ulcerative colitis was 1.36 (95% CI: 1.07-1.73). Compared with the non-probiotics

group, the recurrence rate of ulcerative colitis patients who received probiotics was 0.69 (95% CI: 2.47-1.01). In the mild to moderate group who received probiotics, compared to the group who did not receive probiotics, the recurrence rate was 0.25 (95% CI: 0.12-0.51). The group who received *Bifidobacterium bifidum* treatment had a recurrence rate of 0.25 (95% CI: 0.12-0.50) compared with the non-probiotics group.

CONCLUSION: Probiotic treatment was more effective than placebo in maintaining remission in ulcerative colitis.

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Key words: Inflammatory bowel disease; Ulcerative colitis; Probiotics; Meta-analysis

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Sang LX, Chang B, Zhang WL, Wu XM, Li XH, Jiang M. Remission induction and maintenance effect of probiotics on ulcerative colitis: A meta-analysis. *World J Gastroenterol* 2010; 16(15): 1908-1915 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v16/i15/1908.htm> DOI: <http://dx.doi.org/10.3748/wjg.v16.i15.1908>

INTRODUCTION

Inflammatory bowel disease is a chronic recurrent disease, which mainly consists of ulcerative colitis and Crohn's disease, and whose causes are as yet unclear. It is still assumed that inflammatory bowel disease is caused by integrative factors, such as genetic susceptibility, an imbalance between gastrointestinal probiotics and pathogens, an impairment of intestinal epithelial cells, and

host immune dysfunctions. Many clinical and research studies have indicated that intestinal flora dysbacteriosis contributes to the pathogenesis of ulcerative colitis. Probiotics are non-pathogenic beneficial flora, which have important effects on maintaining the balance of intestinal flora^[1]. Probiotics can adjust the metabolic activity of the intestinal flora and their components by preventing bacterial overgrowth and by maintaining the integrity of the intestinal mucosal barrier, thereby adjusting and stabilizing the intestinal environment^[2,3].

Inducing remission and preventing recurrence and complications are the primary goals of inflammatory bowel disease treatment^[4]. Many ulcerative colitis patients experience a short period of remission after induction but then must be treated by surgery after recurrent attacks. Postoperative pouchitis is always caused by the reduction of *Lactobacillus lactis* and *Bifidobacterium*. Many studies have discussed the positive effects of probiotics for treating stomach and intestine diseases, including ulcerative colitis^[5-7]. However, the sample size has been relatively small, such that there is no definitive evidence as to whether probiotics are helpful. Thus, this paper systematically evaluates probiotics' curative effects for treating ulcerative colitis based on existing random control trials.

MATERIALS AND METHODS

Retrieval strategy

Data was retrieved from the databases: We searched the data from MEDLINE (1966 to August 2009), EMBASE (1980 to August 2009), and the Cochrane Controlled Trials Register (1995 to August 2009). The keywords were used below: probiotics, inflammatory bowel disease, ulcerative colitis, *Escherichia coli*, *Lactobacillus*, *Bifidobacterium*, and yeasts. Language was limited to studies published in English. Moreover, abstracts presented at main international gastrointestinal disease meetings (including Digestive Disease Week of the American Gastroenterological Association, the World Congress of Gastroenterology) for the past five years were analyzed by joint manual retrieval. The retrieval results were reviewed by two evaluators, and a third person was consulted if the two evaluators' opinions were different.

Selection criteria: All the random control experiments that compared probiotics with ulcerative colitis treatment medicine or placebo were collected, including abstracts and full texts. The selection criteria were as follows: (1) adult and pediatric studies were included; (2) the experiments compared the curative effect of probiotics with standard therapy for ulcerative colitis or placebo; (3) all were random control tests; (4) abstracts and meeting presentations were selected; and (5) patients had a definite diagnosis of ulcerative colitis using definite diagnosis standards. Reviews and case reports were excluded.

Data retrieval and quality assessment

Two researchers selected the papers after reading the titles, abstracts, and full texts. They evaluated the quality of each

selected paper and retrieved the data. If they had different opinions, a third researcher helped them to determine the applicability of the paper in question. The quality of the methods used by the selected random control experiments was assessed by the Cochrane Reviewer Handbook 5.0 random control experiment quality assessment standard. The following methods were evaluated: (1) Random method: random method right, random method not described, random method error; (2) Allocation concealment: concealment method right, concealment method not described, concealment method error, concealment method not used; (3) Blind method: whether the evaluator was blinded to the conditions of the experiment; and (4) Whether lost or exit: if there are lost or exit conditions, the reasons are indicated clearly or not, and whether intention-to-treat analysis was used. According to these four quality criteria, all answers that were "right or enough", where the possibilities for bias are the least; the assessment was level "A". If one or more criteria were not described, it means that the assessment was partially satisfied. In this condition, there is a possibility of relative bias, and the assessment was designated as level "B". If one or more criteria were erroneous or not used, there was a high possibility of relative bias, and the assessment was designated as level "C".

Statistical analysis

The relative risk and 95% confidence interval (95% CI) was calculated based on the data. Statistical analysis was performed with Cochrane Collaboration's Revman 5.0. Heterogeneity analyses were performed on the experimental results. If there was obvious heterogeneity, the random effects model was chosen, if not, the fixed effects model was chosen.

Whether there was publication bias could be observed by an inverted funnel plot, and the bias level was assessed by the formula:

$$\sum_{j=1}^m \ln(OR_j) = 0$$

$$m = \left[\frac{k \ln(OR)}{1.96} \right]^2 \bar{\omega} / k - k$$

Where k is the number of selected papers; m is the least unpublished number of reports that yield a combined effect size with no statistical significance; $\bar{\omega}$ is the average weight of k published reports (reciprocal of variance); and $\ln(OR)$ is the estimate value logarithm of the combined effect size.

RESULTS

A total of 286 papers were retrieved. After a thorough screening of the titles, abstracts, or full texts and excluding reviews, uncontrolled tests, and basic research, 13 papers were ultimately selected^[5-11] (Figure 1). One of the 13 papers was published as an abstract, 12 of were published as full text; seven of them evaluated the remission rate, eight papers assessed the recurrence rate, and two papers evaluated both the remission rate and recurrence rate. The span of the research or the duration of

Table 1 Characteristics of included studies

Study	Disease severity	Probiotic	Control group	Dose of pro-biotic/day	Treatment duration	N (probiotic/control group)	Induction or maintenance of remission N (probiotic/control group)
Miele <i>et al</i> ^[13] , 2009	Mild-to-moderate	VSL#3	Placebo	450-1800 × 10 ⁹	12 mo	14/15	Induction of remission 13/4; Maintenance of remission 11/4
Sood <i>et al</i> ^[12] , 2009	Mild-to-moderate	VSL#3	Placebo	3.6 × 10 ¹²	12 wk	77/70	Induction of remission 33/11
Henker <i>et al</i> ^[15] , 2008	Inactive UC	<i>E. coli</i> Nissle 1917	5-ASA	5 × 10 ¹⁰	12 mo	24/10	Maintenance of remission 18/7
Zocco <i>et al</i> ^[20] , 2006	Inactive UC	Lactobacillus GG	Mesalazine	18 × 10 ⁹	12 mo	65/60/62	Maintenance of remission 55/48/52
Matthes <i>et al</i> ^[11] , 2006	Mild-to-moderate	<i>E. coli</i> Nissle 1917	Placebo	10-40 × 10 ⁸	4 wk	46/11	Induction of remission 20/2
Furrie <i>et al</i> ^[9] , 2005	Active	Synbiotic (<i>Bifidobacterium longum</i> + inulin-oligofructose)	Potato starch and sachet of 6 g powdered maltodextrose	4 × 10 ¹¹	4 wk	9/9	Induction of remission 5/3
Tursi <i>et al</i> ^[8] , 2004	Mild-to-moderate	Balsalazide/VSL#3	Mesalazine/balsalazide	900 × 10 ⁸	8 wk	30/30/30	Induction of remission 24/21/16
Kruis <i>et al</i> ^[18] , 2004	Inactive	<i>E. coli</i> Nissle 1917	Mesalazine	2.5-25 × 10 ⁹	12 mo	162/165	Maintenance of remission 89/104
Kato <i>et al</i> ^[10] , 2004	Mild-to-moderate	<i>Bifidobacterium breve</i> strain Yakult <i>Bifidobacterium bifidum</i> strain Yakult <i>Lactobacillus acidophilus</i> <i>Bifidobacteria</i>	BFM without <i>B. bifidum</i> and <i>L. acidophilus</i> Starch	10 ⁹	12 wk	10/10	Induction of remission 4/3
Cui <i>et al</i> ^[16] , 2004	Active	<i>Bifidobacterium Breve</i> <i>Bifidobacterium Bifidum</i> <i>Lactobacillus acidophilus</i> YIT 0168	BFM without these <i>Bifidobacteria</i>	1.26 g/d	8 wk	15/15	Maintenance of remission 12/1
Ishikawa <i>et al</i> ^[17] , 2003	Mild-to-moderate	<i>Bifidobacterium Breve</i> <i>Bifidobacterium Bifidum</i> <i>Lactobacillus acidophilus</i> YIT 0168	BFM without these <i>Bifidobacteria</i>	10 × 10 ⁸	12 mo	11/10	Maintenance of remission 8/1
Rembacken <i>et al</i> ^[14] , 1999	Active	<i>E. coli</i> Nissle 1917 serotype O6: K5: H1	Mesalazine	5 × 10 ¹⁰	12 mo	57/59	Induction of remission 39/44; Maintenance of remission 31/27
Kruis <i>et al</i> ^[19] , 1997	Inactive UC	<i>E. coli</i> Nissle 1917 serotype O6: K5: H1	Mesalazine	50 × 10 ⁹	12 wk	50/53	Maintenance of remission 42/51

E. coli: *Escherichia coli*.

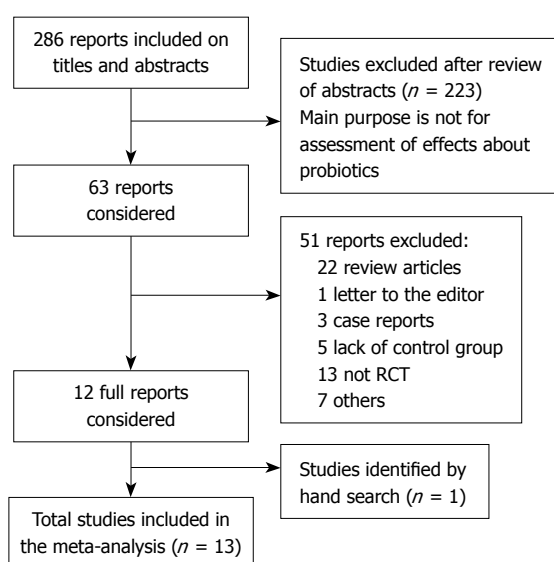


Figure 1 Study selection process. A flowchart was present in this figure and the flowchart summarizes the selection of studies including numbers and reasons of studies excluded.

the follow-up visits was 1 to 12 mo. All the papers were published in English. The general conditions of the selected research are shown in Table 1^[8-20].

Quality assessment of selected papers

The quality assessment of the 13 selected papers is shown in Table 2. Three reports were level A, eight were level B, and two papers were level C.

Induction of remission of ulcerative colitis by probiotics

Seven reports evaluated the remission rate, which involved a total of 399 patients. Among the 399 patients, probiotics were used as an auxiliary therapy in 219 patients, and 180 patients were treated by standard therapy or placebo. Comparing the probiotics auxiliary therapy group with non-probiotics auxiliary therapy group, the remission rates were not significantly different (remission rate: 1.35, 95% CI: 0.98-1.85, $P = 0.07$), but there was an obvious heterogeneity in the results ($P = 0.02$, $I^2 = 62\%$). The total remission rate of the probiotics auxiliary therapy group was 68.2%, and for the non-probiotics auxiliary therapy group it was 60.4% (Figure 2A).

Sub-group analysis

Due to the heterogeneity of the total remission rate, sub-groups were analyzed based on the severity of disease, placebo or not, different kinds of probiotics, and the span of probiotics therapy. The selected reports were first separated into a mild to middle subgroup and an active

Table 2 Methodological quality of the 13 RCTs

Study	Random method	Allocation concealment	Blind method	Lost or exit	Quality assessment	Inclusion/exclusion criteria	Outcome measurement
Miele <i>et al</i> ^[13] , 2009	Right	Right	Double blind	Yes, use ITT analysis	A	Both	CAI by Lichtiger; EI score; HI score
Sood <i>et al</i> ^[12] , 2009	Right	Right	Double blind	Yes, use ITT analysis	A	Both	DAI by Sutherland
Henker <i>et al</i> ^[15] , 2008	Right	Non-described	Non-described	No	B	Inclusion criteria	CAI by Rachmilewitz
Zocco <i>et al</i> ^[20] , 2006	Right	Non-described	Non-described	No	B	Both	CAI by Rachmilewitz; EI by Baron; HI by Truelove-Richard
Matthes <i>et al</i> ^[11] , 2006	Right	Not used	Double blind	Yes, use ITT analysis	C	Not mentioned	DAI by Sutherland
Furrie <i>et al</i> ^[9] , 2005	Right	Non-described	Double blind	No	B	Inclusion criteria	CAI by Walmsley; SI by Baron
Tursi <i>et al</i> ^[8] , 2004	Right	Non-described	Non-described	Yes, use ITT analysis	B	Both	CAI by Lennard; EI score; HI score
Kruis <i>et al</i> ^[18] , 2004	Right	Right	Double blind	Yes, use ITT analysis	A	Both	Scores according to Rachmilewitz
Kato <i>et al</i> ^[10] , 2004	Right	Non-described	Non-described	No	B	Both	CAI by Lichtiger; EI by Harig, Scheppach; HI by Matts
Cui <i>et al</i> ^[16] , 2004	Right	Not used	Non-described	No	C	Not mentioned	Not mentioned
Ishikawa <i>et al</i> ^[17] , 2003	Right	Non-described	Non-described	No	B	Inclusion criteria	Exacerbation of clinical symptoms
Rembacken <i>et al</i> ^[14] , 1999	Right	Non-described	Double blind	Yes, use ITT analysis	B	Inclusion criteria	Scores according to Rachmilewitz
Kruis <i>et al</i> ^[19] , 1997	Right	Non-described	Double blind	Yes, use ITT analysis	B	Both	The same of the CAI score under the <i>E. coli</i> and mesalazine; Scores according to Rachmilewitz

CAI: Clinical activity index; SI: Sigmoidoscopy index; EI: Endoscopy; HI: Histology index.

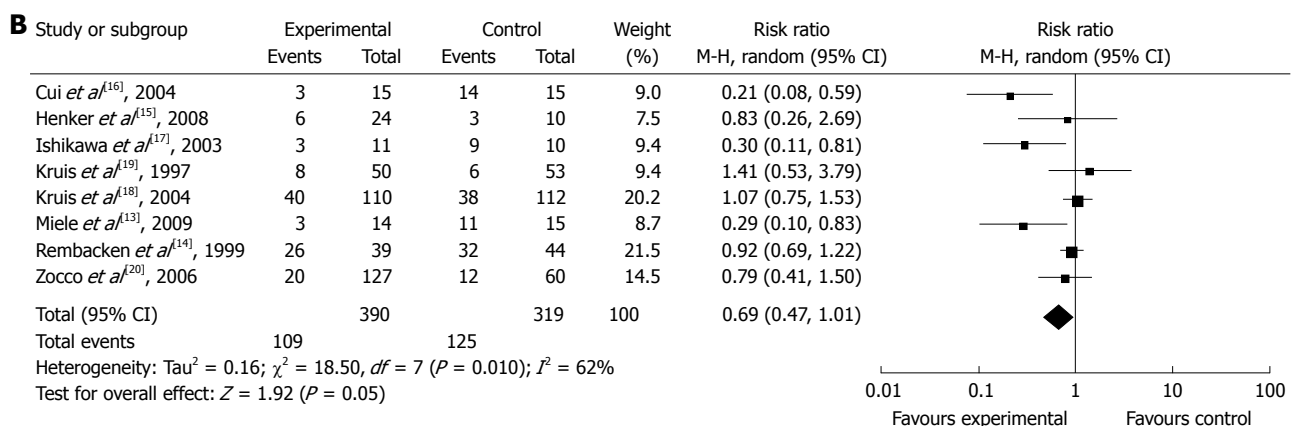
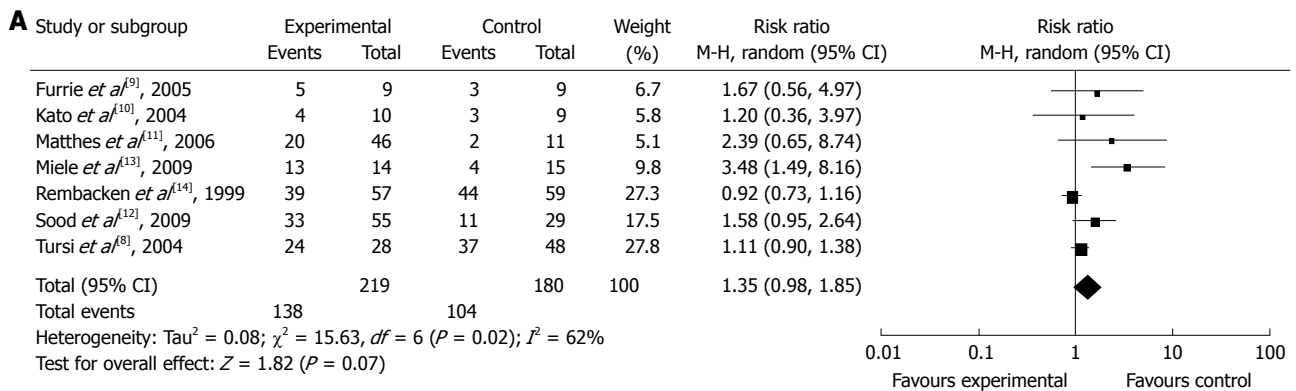


Figure 2 Remission rate (A) and relapse rate (B) of probiotic group vs control group.

phase subgroup. The remission rate of the mild to middle sub-group was 1.64 (95% CI: 0.98-2.72) and heterogeneity was obvious ($P = 0.02$, $I^2 = 66\%$). The remission rate for the active phase subgroup was 0.97 (95% CI: 0.77-1.22), and heterogeneity existed ($P = 0.28$, $I^2 = 13\%$). Secondly, the selected reports were divided into a placebo controlled sub-group and a non-placebo controlled sub-group. The remission rates of these two sub-groups were 2.00 (95% CI: 1.35-2.96) and 1.00 (95% CI: 0.85-1.18), respectively. The remission rate of the probiotics auxiliary therapy sub-group was significantly better than the placebo sub-group, and there were obvious heterogeneities in these two sub-groups ($P = 0.46$, $I^2 = 0\%$). The selected reports were then separated into a VSL#3 subgroup, an *E. coli* subgroup, and a *Bifidobacterium longum* subgroup. The remission rate of the VSL#3 subgroup was 1.66 (95% CI: 0.87-3.15), and heterogeneity was obvious ($P = 0.006$, $I^2 = 80\%$). The remission rate of the *E. coli* subgroup was 1.02 (95% CI: 0.80-1.30), and heterogeneity was obvious ($P = 0.12$, $I^2 = 59\%$). The remission rate of the *Bifidobacterium longum* subgroup was 1.43 (95% CI: 0.64-3.19), and heterogeneity was not apparent ($P = 0.69$, $I^2 = 0\%$). Finally, the selected research was divided into groups that received treatment for at least 12 mo or less than 12 mo, based on the course of treatment. The remission rate for the 12 mo sub-group was 1.69 (95% CI: 0.43-6.67), and heterogeneity was not apparent ($P = 0.002$, $I^2 = 90\%$). The remission rate of the sub-group that received treatment for less than 12 mo was 1.36 (95% CI: 1.07-1.73). In the subgroup that received treatment for less than 12 mo and was treated by probiotics auxiliary therapy, the remission rate was obviously better than the control group, and heterogeneity existed ($P = 0.33$, $I^2 = 14\%$).

Sensitivity analysis

Seven reports evaluated the remission rate. In one of the studies, the patients were treated by probiotic enema formulations but not by humatin, so this research was excluded. Sensitivity analysis indicated that the total remission rate was 1.30 (95% CI: 0.94-1.78), and that the statistical heterogeneity was significant ($P = 0.02$, $I^2 = 63\%$). There was no significant differences in the remission rates between the groups that received probiotics or non-probiotics.

Effect of probiotics on the recurrence rate in maintenance treatment of ulcerative colitis

Eight reports assessed the recurrence rate, which involved a total of 709 patients, 390 of them used probiotics for maintenance treatment, and 319 patients used control medicine or a placebo. There was a significant difference between the total recurrence rates between the probiotics maintenance therapy group and placebo maintenance therapy group (recurrence rate: 0.69, 95% CI: 0.47-1.01, $P = 0.05$). The total recurrence rate for the probiotics maintenance therapy group was 27.9%, while the recurrence of the control group was 39.2%. The heterogeneity of the total recurrence rate was found to be significant by meta-analysis ($P = 0.01$, $I^2 = 62\%$).

Subgroup analysis

Due to the significant heterogeneity of the total recurrence rate, it was necessary to analyze the subgroups to explain and/or reduce the heterogeneity. The selected studies were first divided into an active phase subgroup, a remission stage subgroup, and a mild to middle subgroup. The remission rate of the active phase subgroup was 0.48 (95% CI: 0.10-2.26) with obvious heterogeneity ($P = 0.003$, $I^2 = 89\%$). The remission rate of the remission stage subgroup was 1.01 (95% CI: 0.14-0.61) without heterogeneity ($P = 0.96$, $I^2 = 0\%$). The recurrence rate of the probiotics therapy group was much lower than the non-probiotics therapy group. The selected research was then separated into a placebo control subgroup and a non-placebo control subgroup. The remission rate of the placebo control sub-group was 0.25 (95% CI: 0.12-0.51) without obvious heterogeneity ($P = 0.68$, $I^2 = 0\%$). The non-placebo control subgroup's remission rate was 0.92 (95% CI: 0.75-1.14) with visible heterogeneity ($P = 0.26$, $I^2 = 24\%$), which was obviously higher than the placebo control sub-group. The remission rate of the probiotics therapy group was not significantly different from the non-placebo control sub-group.

The selected research was then grouped into an *E. coli* subgroup, a *Bifidobacterium* subgroup, and a VSL#3 *Lactobacillus* subgroup. The remission rate of the *E. coli* subgroup was 1.02 (95% CI: 0.81-1.29), and there was no obvious heterogeneity ($P = 0.76$, $I^2 = 0\%$). The remission rate of the *Bifidobacterium* subgroup was 0.25 (95% CI: 0.12-0.51) without visible heterogeneity ($P = 0.63$, $I^2 = 0\%$). The VSL#3 *Lactobacillus* subgroup remission rate was 0.59 (95% CI: 0.35-1.01) with significant heterogeneity ($P = 0.11$, $I^2 = 60\%$). Finally, the selected studies were divided into patients that had received treatment for 12 mo and those that had received treatment for less than 12 mo. The remission rate of the 12 mo subgroup was 0.83 (95% CI: 0.68-1.03) with significant heterogeneity ($P = 0.07$, $I^2 = 51\%$), while the remission rate of the group that had received treatment for less than 12 mo was 0.55 (95% CI: 0.09-3.51) with significant heterogeneity ($P = 0.009$, $I^2 = 85\%$) (Figure 2B).

Publication bias assessment

The publication bias could not only be qualitatively analyzed using a funnel plot to observe whether a bias existed, but we could also quantitatively count the bias level using a formula to determine the extent of the publication bias.

Quantification of publication bias: In the above analysis concerning probiotic treatment and its effect on the remission rate and recurrence rate of ulcerative colitis, the publication bias was analyzed quantitatively; however, quantification of the publication bias requires positive results, thus the total recurrence rate of ulcerative colitis affected by probiotics only was assessed.

To compare the recurrence rate between the probiotics group and non-probiotics group, the values used were as follows: $\ln(R) = -0.3711$, $W = 101.59$, $m = 8 \times (0.3711/1.96)^2 \times (101.59/8) - 8 = 21$. The result showed

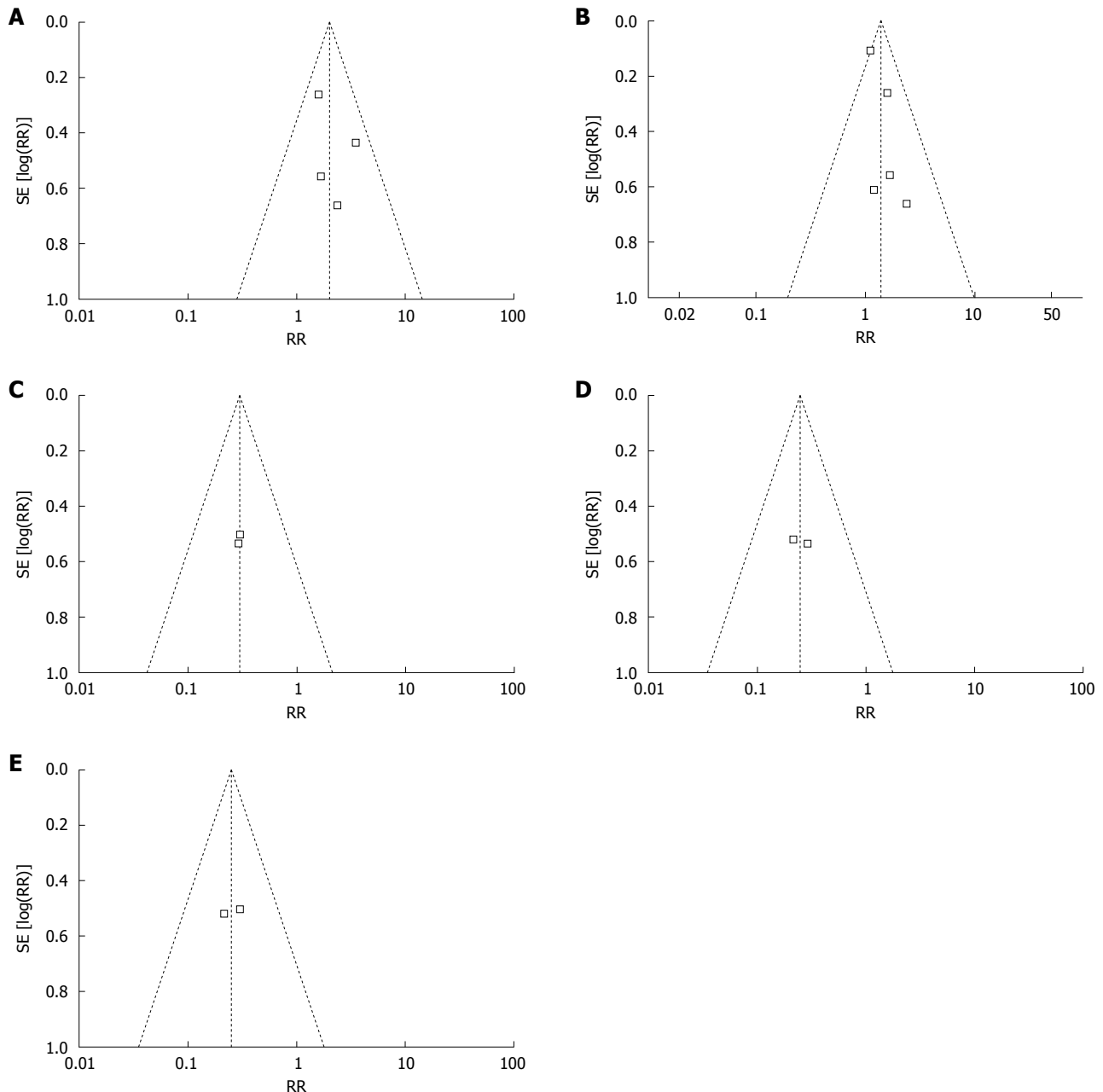


Figure 3 Inverted funnel plot analysis of the relative of remission rate. A: Inverted funnel plot analysis of the relative of remission rate between probiotics group and placebo group; B: Less than 12 mo group: Inverted funnel plot analysis of the relative of remission rate between probiotics group and non-probiotics group; C: Mild and middle UC group: Inverted funnel plot analysis of the relative of remission rate between probiotics group and non-probiotics group; D: Inverted funnel plot analysis of the relative of remission rate between probiotics group and placebo group; E: Inverted funnel plot analysis of the relative of remission rate between *Bifidobacterium longum* therapy group and non-probiotics therapy group.

the analysis results could not be reversed unless 21 non-statistically significant papers were added. This indicated that publication bias had little effect on the results in the analysis of recurrence rate of probiotics group and non-probiotics group. Therefore, the results were reliable.

Qualitative evaluation of publication bias: Inverted funnel plot analysis can analyze the results from a fixed effect model. Figure 3 shows that the inverted funnel plot had an uneven distribution and apparent asymmetry. This meant that in the patients who were treated for less than 12 mo, there was a publication bias in the relative of re-

mission rate between the probiotics group and the non-probiotics group. The inverted funnel plots that were symmetrical indicated that there was no visible publication bias.

DISCUSSION

This study shows that using probiotics provides no additional benefit in inducing remission of ulcerative colitis, but probiotics auxiliary therapy is much better than non-probiotics therapy for maintenance therapy. Research has discussed the various effects of probiotics as an ulcerative colitis therapy. The mechanism of probiotics in ulcerative

colitis therapy mainly involves the following: (1) to prevent pathogen infiltration by restraining bacterial adherence and bacteria translocation, or to produce anti-bacterial substances that inhibit pathogenic bacteria; (2) to improve the intestinal mucosal barrier function, and the permeability and stability of the mucosal barrier; and (3) to regulate the mucosal immune response. Probiotics cause modifications of the mucosal immune response, improve activities of macrophages and NK cells, stimulate the production of antibodies, regulate the nuclear factor- κ B (NF- κ B) pathway, induce the apoptosis of T cells, and reduce the secretion of proinflammatory factors^[21]. However, most of the studies on the effects of probiotics in ulcerative colitis therapy were performed *in vitro* or in animal models. Although probiotic effects are closely related to applied clinical conditions, there are some disputes between the results from basic research and clinical research. In addition, the inconsistency of research baselines has also caused disagreement among clinical researchers.

Although this study shows that using probiotics provides no additional benefit in the induction of remission for ulcerative colitis, there was a visible heterogeneity in the total remission rate. This effect might be related to the different methods used by the selected papers, such as the kinds of probiotics used, the treatment time, the types of ulcerative colitis, medication compliance of patients, whether patients were treated by antibiotics simultaneously, difference of the control groups, the analysis of the results, and the sample size. Some research shows that there are significantly different effects yielded by different kinds of bacteria and yeasts used as therapies^[22,23]. Subgroup analysis demonstrated that the probiotics group was more likely to be in remission than the placebo group of patients with ulcerative colitis. In the subgroup that received treatment for less than 12 mo, the remission rate of the probiotics group was obviously higher than the non-probiotics group. The results suggested that probiotics auxiliary therapy could be used as another choice to induce the remission of ulcerative colitis, which has a better remission effect than the non-probiotics therapy group after 1 year. Some research has shown that the protective effects of probiotics act during the initial stage of ulcerative colitis impairment, but not during the refractory period. This result still needs to be confirmed using additional random control tests.

In the maintenance therapy stage, the probiotics auxiliary therapy was obviously better than non-probiotic therapy; that is, the recurrence rate of probiotics auxiliary therapy group was significantly lower than non-probiotics therapy group during the maintenance therapy stage, and this difference was heterogeneous. Subgroup analysis showed that the effect of probiotic maintenance therapy was obviously better than non-probiotic therapy in the mild to middle subgroup, placebo subgroup, and *Bifidobacterium* subgroup, and this difference was statistically significant. The results demonstrated that probiotics auxiliary therapy was more suitable in ulcerative colitis patients. Compared with standard treatments, such as 5-ASA or mesalazine, the effect of probiotics auxiliary therapy was

not significantly different, but was obviously better than placebo therapy. The recurrence rate of the *Bifidobacterium* group was much lower than the non-probiotics therapy group, and the difference was statistically significant.

The main advantage of probiotics therapy is that it does not affect the regeneration of normal mucosa during active therapy^[23]. To overcome gastrointestinal tract infections, probiotics must be non-pathogenic and resistant to antibiotics. In addition, probiotics can resist stomach acid, bile, and antibiotics and can modify immune processes to destroy invading microorganisms^[24].

In this meta-analysis, the evaluation criteria of the results were not totally consistent. To a certain extent, this condition added to the heterogeneity of the systemic review.

In conclusion, whether the use of probiotics is better than non-probiotics therapy during the remission induction stage and maintenance stage of ulcerative colitis still needs further clinical trials, including variations of bacterial species, applied dosage, treatment timing, and the course of treatment.

COMMENTS

Background

Ulcerative colitis is a chronic recurrent colon disease whose pathogenesis is not clear. Whether probiotics should be used in treatment remains in dispute. This paper compares the effects of probiotics and drug treatment of ulcerative colitis or placebo in remission induction and maintenance of ulcerative colitis.

Research frontiers

Many studies indicated probiotics have positive effects in treating gastrointestinal diseases, including ulcerative colitis. However, the data comes from small sample studies and does not provide enough evidence of probiotics' advantage.

Innovations and breakthroughs

Thirteen randomized controlled experiments with relatively larger sample sizes, systematically evaluated the probiotics' remission induction and maintenance effects in treating ulcerative colitis.

Applications

Through meta-analysis, probiotics should be considered as an auxiliary medicine in the remission induction stage and maintenance stage of ulcerative colitis.

Peer review

This is a meta-analysis of the effect of probiotics on ulcerative colitis, which might provide some useful information. It is a nice, well-conducted study.

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Natural taurine promotes apoptosis of human hepatic stellate cells in proteomics analysis

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Abstract

AIM: To study the differential expression of proteins between natural taurine treated hepatic stellate cells and controls, and investigate the underlying regulatory mechanism of natural taurine in inhibiting hepatic fibrosis.

METHODS: A proteomic strategy combining two-dimensional gel electrophoresis and ultra-performance liquid chromatography-electrospray ionization-tandem mass spectrometry (UPLC-ESI-MS/MS) was used to study the differential expression of proteins and Western blotting was used to validate the results. Gene ontology (GO) method was utilized to analyze the functional enrichment of differentially expressed proteins. Flow cytometry was performed to compare the apoptosis rate between taurine-treated and untreated hepatic stellate cells (HSCs).

RESULTS: Nineteen differentially expressed proteins (11 up-regulated and 8 down-regulated) were identified

by 2D/MS, and the expression profiles of GLO1 and ANXA1 were validated by Western blotting. GO analysis found that these differentially expressed proteins were enriched within biological processes such as "cellular apoptosis", "oxidation reaction" and "metabolic process" in clusters. Flow cytometric analysis showed that taurine-treated HSCs had a significantly increased apoptosis rate when compared with the control group.

CONCLUSION: Natural taurine can promote HSC apoptosis so as to inhibit hepatic fibrosis.

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Key words: Natural taurine; Proteomics; Hepatic stellate cell; Hepatic fibrosis; Apoptosis

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INTRODUCTION

Hepatic fibrosis (HF) is the excessive accumulation of extracellular matrix (ECM) through which the liver repairs injury^[1,2]. HF occurs in most types of chronic liver diseases due to various causes, and approximately 25%-40% of HF cases may ultimately progress to hepatic cirrhosis or even hepatocellular carcinoma. Therefore, prevention of the initiation of HF and interference with or reversal

of the fibrotic process is important for controlling the development of chronic liver diseases, which has become one of the research focuses in China and other countries^[3,4].

Hepatic stellate cells (HSCs) are nonparenchymal cells found in the perisinusoidal space of the liver also known as the space of Disse^[5]. HSCs contribute significantly to the occurrence of HF because activated HSCs have been found to secrete a great amount of ECM when migrating, proliferating and synthesizing in the injured site of the liver^[6,7]. It is generally believed that reversal of the fibrotic process is difficult once HSCs are activated. Some studies, however, have shown that HF may be inhibited to a certain extent by reducing the number of HSCs *via* apoptosis induction^[8,9].

Taurine, or 2-aminoethanesulfonic acid (C₂H₇NO₃S), is an important anti-injury substance in the body^[10]. Taurine has a protective effect on various types of liver injury^[11,12] and may mitigate HF by inhibiting the deposition of ECM^[13]. The antifibrotic mechanism of taurine may involve its inhibition of the activation and proliferation of HSCs^[9,14]. Because the molecular mechanism of taurine-mediated antifibrotic activity has not been fully unveiled and is little studied, it is imperative to use “omics” methods to systematically investigate the molecular mechanism by which taurine inhibits HF.

Therefore, in the present study, we analyzed the differential expression of proteins between taurine-treated HSCs and controls using two-dimensional electrophoresis (2-DE), ultra-performance liquid chromatography-electrospray ionization-tandem mass spectrometry (UPLC-ESI-MS/MS) and bioinformatics method to identify proteins associated with the inhibition of HF by taurine. Moreover, we aimed to understand better the molecular mechanism underlying taurine's protection against HF and thereby provide new targets for the management of HF and drug development.

MATERIALS AND METHODS

Materials

Human HSCs (LX-2) were purchased from XiangYa Central Experiment Laboratory, Central South University, Changsha, Hunan Province, China. Natural taurine was provided by Yuanlong Pearl Co. Ltd., Beihai, Guangxi Zhuang Autonomous Region, China. Acrylamide, bisacrylamide, glycine and HRP-ECL luminescent substrate were purchased from Sigma (USA). Immobilized pH gradient (IPG) strips (immobiline™ DryStrip, pH3-10, nonlinear, 18 cm) and image analysis software (ImageMaster 2D platinum) were purchased from Amersham Pharmacia. Bradford protein assay kit was provided by Beyotime Institute of Biotechnology (China). Primary and secondary antibodies were purchased from Santacruz; Annexin V apoptosis assay kit, from Nan Jing KeyGen Biotech Co., Ltd. (China); vertical electrophoresis apparatus and Trans-Blot system, from Bio-Rad (USA); Epics XL II flow cytometer, from Beckman Coulter (USA); inverted light microscope, from Olym-

pus (Japan); IPGphor IEF System and ImageScanner, from Amersham Pharmacia. Vertical slab electrophoresis apparatus (PROTEAN II xi Cell) was bought from Bio-Rad (USA) and UPLC-ESI-MS/MS system was provided by Micromass (UK).

Methods

Incubation and treatment of HSCs (LX-2): The cells were incubated in DMEM containing 100 U/mL penicillin, 100 U/mL streptomycin and 10% fetal bovine serum at 37°C, 50 mL/L CO₂ and saturated humidity. The medium was replaced every other day. When the cell density achieved 80%-90%, trypsin was used for digestion and the cell concentration was adjusted to be 1 × 10⁵/mL. The supernatant was discarded and then reaction was maintained for 48 h after the addition of 2% DMEM containing 40 mmol/L taurine. HSCs were randomized equally to receive treatment with or without taurine.

Protein extraction and quantification: The cells were collected after centrifugation and washed in PBS twice. After 10 µL of RNAase and 10 µL of DNAase, were added respectively, the solution was placed in ice bath at 4°C for 20 min and then centrifuged at 14000 × *g* for 30 min and the supernatant was collected. Protein quantification was performed by the Bradford method. In the same way, 1 µL of the sample was collected and 99 µL of water was added followed by supplementation with 1 mL of Bradford working solution. After shaking for 5 min, measurement was conducted. The absorbance of the test sample was measured at 595 nm.

2-DE: The first-dimension isoelectric focusing (IEF) electrophoresis was performed on an IPGphor IEF System. Proteins were centrifuged for 2 min before loading. A loading amount of 150 µg was dissolved in rehydration solution containing 8 mol/L urea, 0.02% Chaps, 0.02 mol/L dithiothreitol (DTT) and 0.05% IPG buffer and then 800 µL of cover fluid was added. The focused gel strips were equilibrated twice in sodium dodecyl sulfate (SDS) equilibration buffer (1.5 mol/L tris-Cl, pH 8.8, 50 mmol/L, 30% Glycerol, 6 mol/L Urea, 2% SDS, Bromophenol blue trace) and rotated on a shaking table for 15 min × 2. The first equilibration buffer was supplemented with 20 mmol/L DTT and the second equilibration buffer was added with 100 mmol/L iodoacetamide. Gel strips were taken out and then the vertical second-dimension sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was run on a PROTEIN II xi Cell system. Polyacrylamide gels (13%) were used for separation. Electrophoresis was run at a constant current of 40 mA for 40 min and at 60 mA for 5 h until the bromophenol blue front reached the bottom of the gel. Rapid silver staining was performed in accordance with the manufacturer's manual. Gels were stained with 0.1% Coomassie brilliant blue R350 at 100°C for 10 min and then de-stained in 10% acetic acid overnight.

Gel image analysis: Stained 2-DE gels were scanned in transmission scan mode using an Image-scanner™. Then,

matching of protein spots and differential expression analyses between the treatment group and the control group were performed using ImageMaster 2D Platinum 5.0 software.

Mass spectrometry and database query: In-gel digestion was done for 19 differentially expressed protein spots whose concentrations met the requirement of mass spectrometry (MS). In-gel digested products were analyzed by UPLC-ESI-MS/MS (Micromass Company). The mass spectral data obtained after processing with the data analysis software PLGS v2.3 were used to search through the NCBI database with a MS/MS ion strategy using Mascot software (<http://www.matrixscience.co.uk>). The search parameters included trypsin digestion, M oxidation and iodoacetamide alkylation (variable modifications), and 1 missed cleavage. The mass error tolerance in both the MS and MSMS data was 0.2-Da.

GO functional analysis: GO is a stratified tree structure for analysis of gene and protein functions^[15]. We used the BinGO plugin in the Cytoscape platform to analyze functions of 19 differentially expressed proteins, with special focus on the enrichment of these proteins in biological process^[16,17]. Then, we classified the significantly enriched GO terms according to functional similarity. Finally, we visualized the significantly enriched GO terms and their parent/child items in a tree structure in the Cytoscape platform and indicated the classification of the GO terms in the diagram.

Apoptosis detection: HSCs were incubated in the presence of 40 mmol/L taurine for 48 h, washed in PBS twice and then re-suspended in 100 μ L of buffer containing calcium ion. After this, staining was carried out for 20–30 min by adding 5 μ L of Annexin V-FITC dye and then for 5 min by supplementation with 5 μ L of PI dye. The cell concentration was adjusted to be about 1×10^5 /mL by adding a proper amount of calcium ion-containing buffer. The cells were detected on a flow cytometer within 1 h, with the excitation wavelength and the emission wavelength of 488 nm and 530 nm, respectively. The experiment was repeated in triplicate for each sample.

Western blotting: Total proteins in HSCs were extracted as described in the section of protein extraction and quantification, quantified by the Bradford method, isolated by SDS-PAGE and then transferred onto a nitrocellulose membrane with a Trans-Blot SD apparatus. The membrane was blocked in a blocking buffer (TBST solution containing 5% skim milk) at room temperature (RT) for 2 h. Primary antibodies were added at a dilution of 1:500 and the membrane facing down was incubated at RT for 1 h with slight shaking. After incubation with primary antibodies, the membrane was washed and incubated at RT for 1 h with mild shaking in the presence of horseradish peroxidase (HRP) conjugated goat anti-

mouse secondary antibodies (1:5000). After this, the membrane was washed. *Visualization of protein bands* was achieved by the chemiluminescence method and the films were developed and fixed. GAPDH was used as an internal reference. The experiment was repeated in quadruple for each protein.

Statistical analysis

Data were expressed as mean \pm SD. Difference in the relative content of differentially expressed protein spots was analyzed by *t* test for statistical significance ($P < 0.05$) and *t* test was also performed to determine whether treatment with taurine caused a statistically significant change in apoptosis of HSCs ($P < 0.05$). Hypergeometric test was used to test the enrichment of GO terms. A multiple testing procedure controlling false discovery rate (FDR) was performed as described by Benjamini and Hochberg, with the threshold for enrichment significance set to be $FDR < 0.01$ ^[18]. All statistics were performed on R software.

RESULTS

2-DE and image analysis

Under the same experimental conditions, the 2-DE experiment was repeated 3 times separately for 2 groups of protein samples. Three pieces of parallel gels with high resolution and good reproducibility were obtained for each group. The mean number of protein spots from 3 pieces of gels was 745 ± 32 in the taurine treatment group *vs* 748 ± 25 in the control group, with a match rate of 86.08% between groups. Six hundred and forty-three differential protein spots were screened from both groups. Of these protein spots, 19 spots (11 up-regulated and 8 down-regulated) showed more than three-fold differential expression (Figure 1) following treatment with taurine. Figure 2 is a local amplification of 4 pairs of up-regulated or down-regulated protein spots, which clearly demonstrates changes in protein expression between taurine-treated HSCs and controls.

Identification of differentially expressed proteins by UPLC-ESI-MS/MS

Identification by UPLC-ESI-MS/MS was performed for the 19 differentially expressed proteins following in-gel digestion and peptide extraction. The resulting peptide fragments were used to search through the NCBI database using Mascot software and these 19 differentially expressed proteins were successfully identified (ions scores > 38 , $P < 0.05$). Table 1 summarizes the code, name, relative molecular weight, isoelectric point and peptide fragment coverage for the differentially expressed proteins. The most significantly up-regulated expression was observed in GLO1, a glyoxylase capable of catalyzing and forming lactoylglutathione, which has been reported to be linked to inflammatory reactions and tumors^[19,20]. In contrast, AKR1A1, an aldehyde reductase important for both glucose metabolism and oxidative reactions^[21], showed the most markedly down-regulated expression.

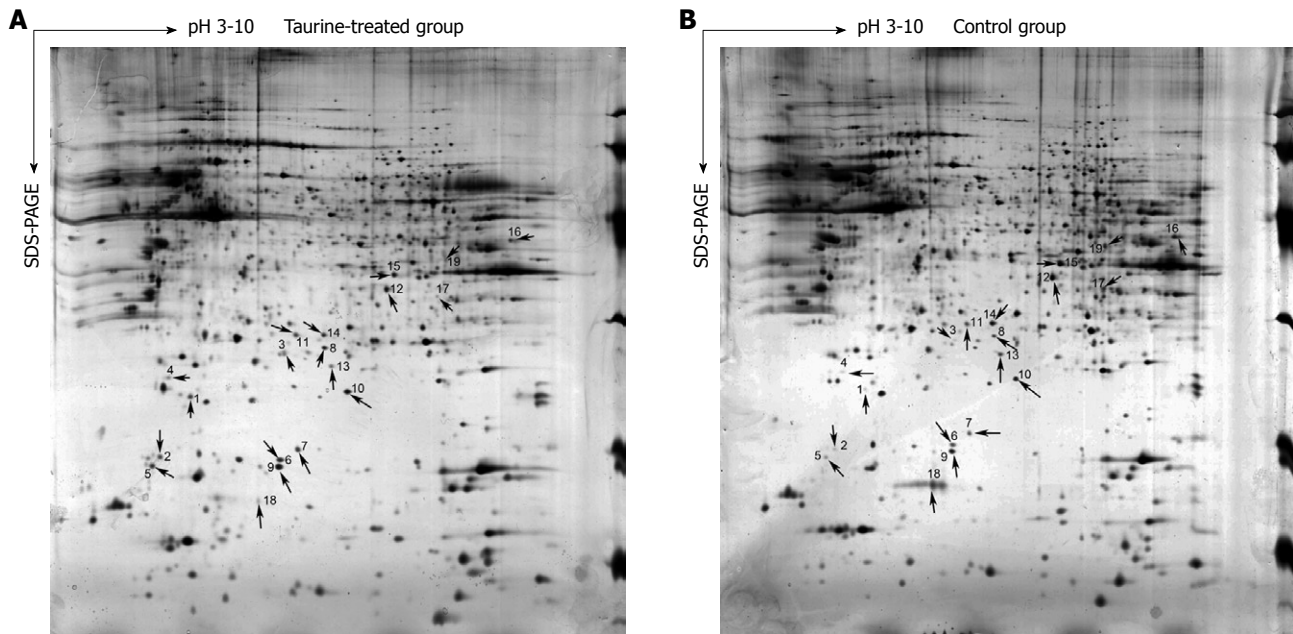
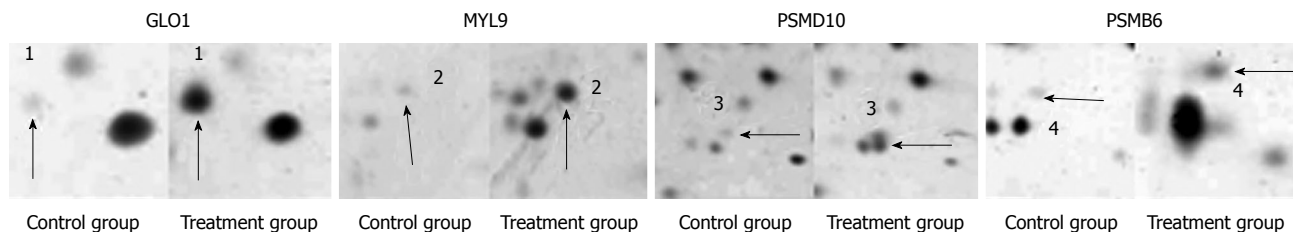


Figure 1 Two-dimensional gel electrophoresis (2-DE) images of taurine-treated hepatic stellate cells (HSCs) and controls. A: Taurine-treated group; B: Control group. In both images, transverse arrows represent isoelectric focusing and longitudinal ones represent sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Up-regulated proteins



Down-regulated proteins

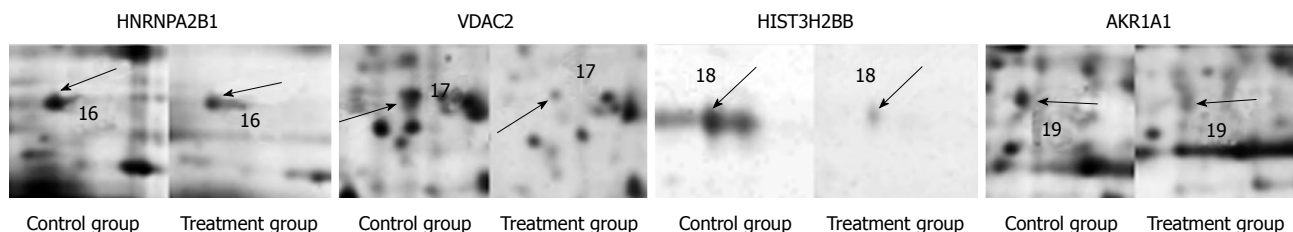


Figure 2 Local amplification of some differentially expressed protein spots (arrows). Up-regulated: GLO1, MYL9, PSMD10 and PSMB6, and down-regulated: HNRNPA2B1, VDAC2, HIST3H2BB and AKR1A1.

Validation of differentially expressed proteins by Western blotting

Among the differentially expressed proteins identified successfully by mass spectrometry, the protein GLO1 and ANXA1 were selected and subjected to Western blotting detection. Taurine treatment caused a significantly up-regulated expression of GLO1 and ANXA1 in HSCs (Figure 3). These expression profiles were completely consistent with the locally amplified 2-D electrophoretogram in Figure 2. Therefore, Western blotting detection of the differentially expressed proteins confirmed the reliability and validity of the proteomic high throughput experiments.

GO functional analysis of differentially expressed proteins

We entered the names of the differentially expressed proteins into the BinGO plugin in the Cytoscape platform to perform GO functional enrichment analysis, with special focus on the changes in the biological process of HSCs after taurine treatment. Table 2 lists 9 significantly enriched GO terms and relevant proteins identified after screening with a threshold of FDR < 0.01. Functional categorization of the GO terms revealed that these differentially expressed proteins were enriched within biological processes such as “cellular apoptosis”, “oxidation reaction” and “metabolic process” in clusters. Figure 4 provides

Table 1 MS identification of differentially expressed proteins in taurine-treated and control groups

Code	Protein spot ID	Record number of protein	Name of protein	Description	log2 (differential ratio)	Relative molecular weight (kDa)	Isoelectric point	Score	Coverage (%)
Up-regulated proteins									
1	1394	gi 2392338	GLO1	Chain a, human glyoxalase i with benzyl-glutathione inhibitor	4.25	20.6	5.12	408	51
2	1431	gi 29568111	MYL9	Myosin regulatory light chain 9 isoform a	3.30	19.8	4.80	631	62
3	1330	gi 4506217	PSMD10	Proteasome 26S non-ATPase subunit 10 isoform 1	3.24	24.4	5.71	279	35
4	1380	gi 558528	PSMB6	Proteasome subunit Y	2.62	25.3	4.80	403	26
5	1438	gi 4507669	TPT1	Tumor protein, translationally-controlled 1	2.48	19.6	4.84	375	61
6	1428	gi 4502101	ANXA1	Annexin I	1.48	38.7	6.57	1069	53
7	1419	gi 5174539	MDH1	Cytosolic malate dehydrogenase	1.18	36.4	6.91	223	17
8	1321	gi 4504517	HSPB1	Heat shock protein beta-1	0.99	22.8	5.98	567	49
9	1432	gi 5453710	LASP1	LIM and SH3 protein 1	0.95	29.7	6.61	362	34
10	1385	gi 31543380	PARK7	Parkinson disease protein 7	0.70	19.9	6.33	128	50
11	1285	gi 2982080	SOD1	Chain a, familial als mutant g37r cuznsod	0.50	15.9	5.87	292	45
Down-regulated proteins									
12	1130	gi 5453559	ATP5H	ATP synthase, H+ transporting, Mitochondrial F0 complex, subunit d isoform a	-0.66	18.5	5.21	277	47
13	1355	gi 5802974	PRDX3	Peroxioredoxin 3 isoform a precursor	-0.70	27.7	7.67	128	16
14	1284	gi 194097323	ECHS1	Mitochondrial short-chain enoyl-coenzyme A hydratase 1 precursor	-0.83	31.4	8.34	481	30
15	1067	gi 9955007	PRDX2	Chain a, thioredoxin peroxidase b from red blood cells	-1.14	21.8	5.44	446	38
16	953	gi 14043072	HNRNPA2B1	Heterogeneous nuclear ribonucleoprotein A2/B1 isoform B1	-1.86	37.4	8.97	181	15
17	1165	gi 190200	VDAC2	Porin	-2.81	38.1	6.32	281	22
18	1464	gi 1568551	HIST3H2BB	Histone H2B	-3.19	13.9	10.31	270	35
19	980	gi 5174391	AKR1A1	Aldo-keto reductase family 1, member A1	-3.55	36.6	6.32	306	22

MS: Mass spectrometry.

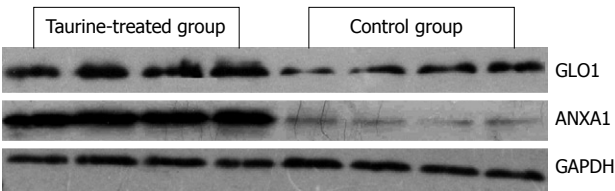


Figure 3 Results of validation by Western blotting for differentially expressed proteins. GAPDH was used as an internal reference. The experiment was repeated 4 times for each protein. Four columns in the right side represent the control group and the other 4 columns in the left side are from the taurine-treated group.

a visualized network of the 9 significantly enriched GO terms and their parent/children items. We can see that Cluster 1, 2 and 3 correspond to the category of “cellular apoptosis”, “oxidation reaction” and “metabolic process” in Table 2, respectively. The consistency between these two dimensions suggests an important role of these biological processes in taurine-treated HSCs.

Results of apoptosis detection

To further confirm the functional findings from proteome data, we detected apoptosis of HSCs treated with or without taurine. Figure 5 shows that taurine treatment resulted in a significant increase in the apoptosis rate of HSCs (13.6% ± 3.3% *vs* 4.65% ± 1.1% in the control group, *P* < 0.05). Thus, proteome data and results of apoptosis detection

consistently suggest that taurine may have an inhibitory effect on HF by promoting HSC apoptosis.

DISCUSSION

In the present study, we employed a proteomics strategy combining 2-DE and UPLC-ESI-MS/MS to investigate difference in protein expression between natural taurine-treated and untreated HSCs. Nineteen differentially expressed proteins (11 up-regulated and 8 down-regulated) were identified. The protein GLO1 and ANXA1 were then selected and subjected to validation by Western blotting, yielding expression profiles in full agreement with results obtained by 2-DE. GO functional analysis showed that these differentially expressed proteins were enriched within biological processes - “cellular apoptosis”, “oxidation reaction” and “metabolic process” in clusters. Flow cytometric analysis showed that taurine-treated HSCs had a significantly increased apoptosis rate compared with the control group. Our comparative proteomic analysis revealed that taurine may inhibit HF by promoting HSC apoptosis.

The apoptosis mechanism of HSCs is complex where various apoptotic pathways are not only inter-independent but also interconnected. For instance, the iron chelator deferoxamine, which may increase the activity of caspase 3 when inducing the release of mitochondrial cytochrome C (cyt-c), induces HSC apoptosis *via* both the mitoch-

Table 2 Functional categorization of differentially expressed proteins

GO number	Functional description	Proportion (%)	Protein	P value	FDR value
Apoptosis-related					
51093	Negative regulation of developmental process	32	TPT1 ANXA1 HSPB1 GLO1 PRDX2 SOD1	2.20E-6	2.61E-4
42981	Regulation of apoptosis	32	TPT1 ANXA1 HSPB1 GLO1 PRDX2 SOD1	3.19E-5	1.49E-3
43067	Regulation of programmed cell death	32	TPT1 ANXA1 HSPB1 GLO1 PRDX2 SOD1	3.45E-5	1.49E-3
Oxidation reaction-related					
42542	Response to hydrogen peroxide	16	PRDX3 SOD1 PARK7	4.28E-6	4.07E-4
6979	Response to oxidative stress	21	PRDX2 PRDX3 SOD1 PARK7	8.18E-6	5.65E-4
302	Response to reactive oxygen species	16	PRDX3 SOD1 PARK7	8.33E-6	5.65E-4
Metabolism-related					
44248	Cellular catabolic process	37	PSMB6 AKR1A1 PSMD10 ECHS1 PRDX3 SO	9.67E-6	5.74E-4
9056	Catabolic process	37	PSMB6 AKR1A1 PSMD10 ECHS1 PRDX3 SO	3.12E-5	1.49E-3
42743	Hydrogen peroxide metabolic process	11	PRDX3 SOD1	2.45E-4	9.69E-3

Hypergeometric test, FDR < 0.01. GO: Gene ontology.

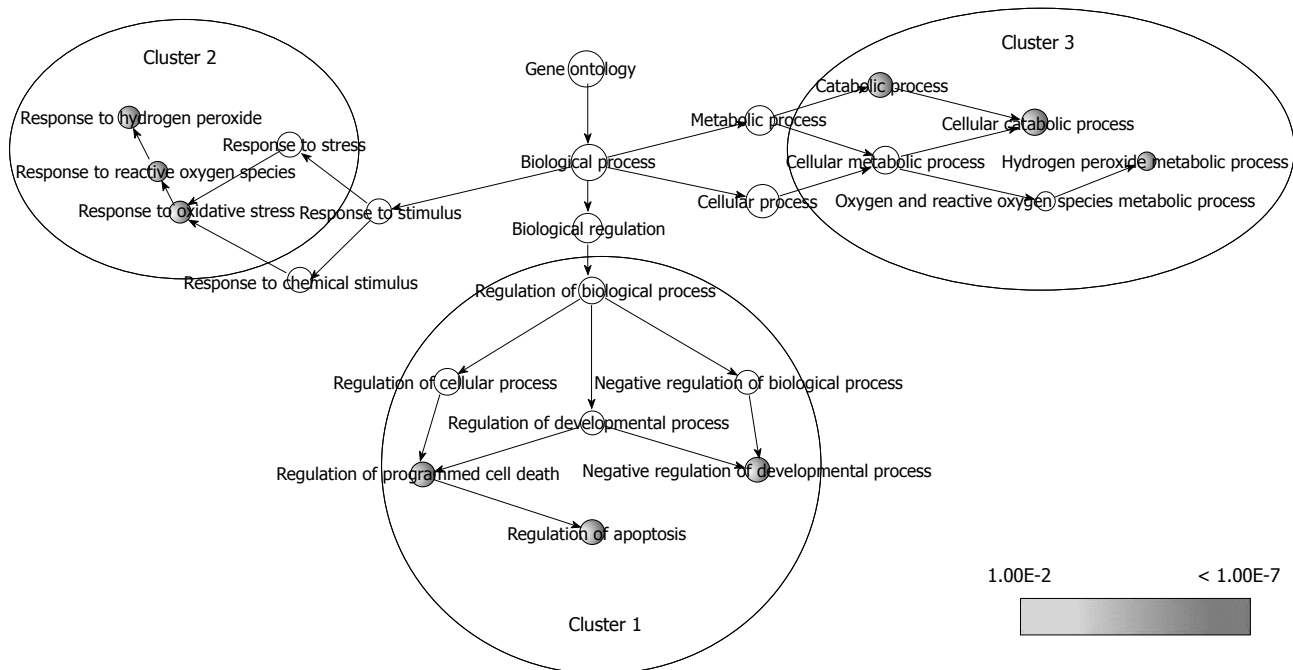


Figure 4 Network diagram of functional categorization of differentially expressed proteins. The categories in this tree structure fully correspond to the 3 categories in Table 2 and the depth of color ranging from light to dark gray indicates the significance level of FDR in Table 2. Gray nodes represent significantly enriched GO terms and white nodes signify GO terms that have a parent/child relationship with gray nodes. Connecting lines between nodes construct a directional hierarchy of the GO terms.

ondrial pathway and the death receptor pathway^[22]. Our previous study has also found that natural taurine may regulate the TGF- β 1/smad signaling pathway and has an activity against HF^[23]. To date, however, the molecular mechanism and relevant pathways responsible for taurine's ability to promote HSC apoptosis have not been well understood. During confirmation of this phenomenon, we found differential expression of proteins including TPT1, ANXA1, HSPB1, GLO1, PRDX2 and SOD1 in taurine-treated HSCs, which was tightly associated with cellular apoptosis. Therefore, we presume that TPT1, ANXA1, HSPB1, GLO1, PRDX2 and SOD1 are important proteins in the taurine-mediated pathway of HSC

apoptosis. These proteins may play a role in classical apoptotic pathways. Or they may constitute, on their own, a specific apoptotic pathway for HSCs by interacting with each other and regulating other proteins. We need to analyze taurine-mediated HSC apoptosis based on findings. Our another previous work has shown that natural taurine may protect mitochondria in HF rats by regulating liver lipid peroxidation, indicating an anti-HF effect^[24]. Functional analysis of the differentially expressed protein demonstrated the ability of taurine to regulate oxidation reaction in HSCs. In fact, oxidation-related biological processes, such as reactive oxygen species (ROS) in Table 2, are closely associated with apoptosis induction^[25]. Among

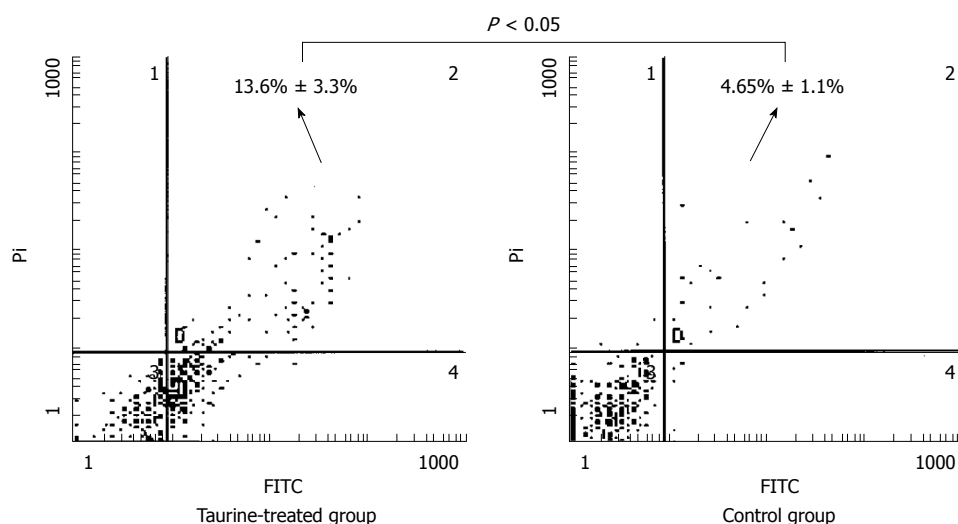


Figure 5 Apoptosis profiles of HSCs treated or untreated with taurine. The experiment was repeated 3 times for each sample (*t* test, $P < 0.05$). Cell count in the second quadrant was used to calculate the apoptosis rate.

the proteins involved in oxidation reaction, some may be engaged in HSC apoptosis as well (SOD1), and some belong to the same protein family (PRDX3 and PRDX2). Moreover, the hydrogen peroxide metabolic process listed in Table 2 is actually one of the basic steps of oxidation reaction^[26,27] and this metabolism-related process also involves SOD1 and PRDX3. Thus, it is possible that, in the interconnected process of HSC apoptosis, SOD1 and PRDX3 play a critical mechanism-specific role. For the reasons given above, we presume that natural taurine may facilitate the release of cyt-c from mitochondria and then promote HSC apoptosis by directly or indirectly inducing activation of caspase *via* ROS^[25,28] and that SOD1 and PRDX3 are two significant functional proteins in the process.

The hepatic parenchyma consists of parenchymal liver cells (PLCs) including hepatocytes and non-parenchymal liver cells (NPLCs) associated with sinusoids including HSCs. It has been demonstrated that the activation of HSCs is the key issue in the pathogenesis of HF^[7]. Changes of hepatocytes proliferation may stimulate hepatocyte interaction with HSCs possibly *via* cell adhesion molecules leading to HSC activation and HSC clustering^[29,30]. It is obvious that HSCs is the most important HF target cell line in the liver, however, HSC-HSC and hepatocyte-HSC cell adhesion may play a combined role in HF. Therefore, study of the activation and apoptosis of HSC can extensively survey the possible pathological mechanisms of HF, but further investigations on animal models or clinical patients *in vivo* will obtain more exact results for HF in living organisms.

Taurine can either be synthesized chemically or extracted from natural sources. However, natural taurine is more superior to synthetic taurine in promoting HSC apoptosis^[31]. The advantage and innovativeness of our present study are reflected in the selection of natural taurine for investigation of its mechanism in regulating HSC apoptosis. In addition, since no studies using proteomic high throughput technologies to investigate the inhibition of HF by taurine have been published in China and other countries, our findings are useful for

future systematic studies on biological mechanism.

Life is a vast and complex dynamic network system and the therapeutic mechanism of drug treatment mimics an integrated modulatory chain effect involving a variety of pathways in regulating biological networks^[32]. The present study, which systematically investigated how natural taurine may regulate HF-associated biological networks, will help to cast a new light on the action mechanism and targets of taurine.

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COMMENTS

Background

Hepatic fibrosis (HF) occurs in most types of chronic liver diseases and approximately 25%-40% of HF cases may ultimately progress to hepatic cirrhosis. Hepatic stellate cells (HSCs) contribute significantly to the occurrence of HF and the activation of HSC is the key issue in the pathogenesis of HF. It is expected that HSCs are targets for pharmacological or molecular interventions for the treatment of HF.

Research frontiers

Taurine is a kind of important anti-injury substance in the body. Taurine has a protective effect on various types of liver injury. It has been clear that the antifibrotic mechanism of taurine may involve its inhibition of the activation and proliferation of HSCs. However, the molecular mechanism of taurine-mediated antifibrotic activity is largely unknown. Now the research hotspot is to unveil the underlying regulatory mechanisms and accumulate data for drug development.

Innovations and breakthroughs

In the present study, the authors analyzed the differential expression of proteins between taurine-treated HSCs and controls by comparative proteomics technologies. It is the first investigation to study the natural taurine mediated protection effects against HF. Nineteen differentially expressed proteins were identified and functional analysis provided confident evidence of HSC apoptosis promotion by taurine treatment.

Applications

The study results are very helpful to understand better the molecular mechanism underlying taurine's protection against HF and thereby provide new targets for the management of HF and drug development.

Peer review

The authors investigated the effect of taurine on the HSCs by proteomic analysis. They identified 19 differently expressed proteins belonging to the

biological process of apoptosis, cellular oxidation and metabolic processes. They also found an increased rate of apoptosis in taurine exposed cells. Overall, this work is of interest in relation to taurine induced protection in HF and in the underlying alterations in specific proteins in HSC.

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Cystic fibrosis and Crohn's disease: Successful treatment and long term remission with infliximab

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INTRODUCTION

The association of cystic fibrosis (CF) and Crohn's disease (CD) is well known, but, to date, there are very few cases in the literature of patients suffering from mucoviscidosis who have required treatment with infliximab.

CF is the most common life-threatening autosomal recessive disease in Caucasian children; it has an incidence of 1 case in every 2500 children born alive^[1]. CF involves an anomalous function of the exocrine glands, caused by a mutation of a gene (cystic fibrosis transmembrane conductance regulator, *CFTR*) located on chromosome 7, which codes for a protein involved in ion transport through the cell membrane^[1]. More than 1000 mutations of *CFTR* have been identified, the most common of which is ΔF 508, found in 50% of CF patients. Pulmonary complications are the most common causes of mortality, but the presenting symptoms are very often linked to gastrointestinal and pancreatic biliary diseases. These are mainly caused by the unusual viscosity of the secretions in hollow organs and in the ducts of solid organs^[1].

Meconium ileus, intussusception, appendicitis, rectal prolapse, gastro-esophageal reflux, CD and fibrosing colonopathy are the gastrointestinal diseases observed in patients suffering from CF^[1,2].

CD is a chronic inflammatory bowel disease which may be localized throughout the gastrointestinal tract. The association between CD and CF is known; there are reports of a prevalence of CD in patients suffering from CF 17 times higher than in controls^[3].

CD in patients suffering from CF is, therefore, not an exceptional event; the use of an immunosuppressant such as infliximab in patients suffering from CF may, however, be considered as uncommon, as seen in the lack of data available in the literature. Probably, underlying this lack of

Abstract

The association of cystic fibrosis and Crohn's disease (CD) is well known, but to date, there are very few cases in the literature of patients suffering from mucoviscidosis who have required treatment with infliximab. We report the case of a 23-year-old patient suffering from cystic fibrosis and severe CD treated successfully with infliximab without any infective complications or worsening of the pulmonary disease and with a long term (2 years) complete remission.

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Key words: Cystic fibrosis; Crohn's disease; Infliximab

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data is the fear that the immunosuppressive properties of such a biological treatment would be contraindicated by the characteristic infections in CF, especially of the lung.

We report the case of a 23-year-old patient suffering from CF and severe CD who was treated successfully with infliximab and who is in long term remission.

CASE REPORT

This is a report of the case of a 23-year-old female suffering from CF who, from the age of 16 years, started having recurrent abdominal pain associated with weight loss. She was regularly followed up by a centre for CF.

At the age of 14 years, she underwent explorative laparotomy with appendectomy for suspected acute abdomen. The operation was complicated by the appearance of a cutaneous fistula at the site of the surgical wound. Because of persistence of symptoms associated with severe deterioration of nutritional condition, she was sent to our centre. A low digestive endoscopy carried out at our unit showed a condition of acute pancolitis compatible with chronic inflammatory bowel disease. The histological examination of the multiple biopsies taken confirmed the suspected diagnosis of CD.

An induction treatment cycle with prednisone, full dose for 4 wk, with mesalazine and metronidazole was initiated. Having obtained clinical and endoscopic histological remission, we reduced the prednisone dose, stopped metronidazole and continued maintenance treatment with mesalazine. The patient continued treatment with mesalazine alone with good clinical progress for about four years during which she did not come to us for check-ups but preferred to go to the hospital in her city of residence.

In 2003, she underwent emergency laparotomy at another centre following the appearance of acute abdomen. Surgery showed a pericolic abscess collection in the cecum and ascending colon; she was then subjected to ileocecal resection and ileotransverse colonic anastomosis. At two months following the surgery, the girl was treated with steroids, azathioprine and mesalazine for severe flare-up of disease. She continued with low dose steroid treatment (5-10 mg/d) for about two years; treatment with azathioprine was suspended owing to poor efficacy and was replaced with methotrexate (MTX).

During this period of time the patient presented stable respiratory function (spirometry, clinical outcome of exacerbation), but she developed chronic *Pseudomonas aeruginosa* airway infection. Therefore, ciprofloxacin or trimethoprim sulfamethoxazole or doxycycline was administered for 2 wk twice every 3 mo.

In August 2006, the patient returned to us spontaneously following recurrent episodes of abdominal pain and considerable weight loss (about 10 kg in the previous 2 mo). An ileocolonoscopy carried out under sedoanalgesia showed hyperacute disease of the sigmoid-rectum (Figure 1A and B). Multiple "tags" were present in the anal site with a big "knife-cut" lesion at the level of the posterior commissure. The histological examination confirmed a severe reactivation of her disease. The

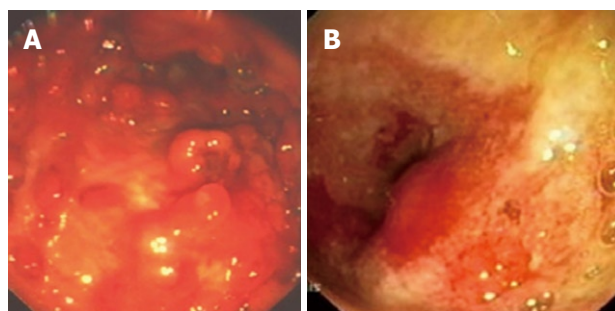


Figure 1 Ileocolonoscopy findings. A: Multiple inflammatory pseudopolyps; B: Hyperacute Crohn's disease of the sigmoid-rectum.

CD activity index (CDAI) was 390. Index values of 150 and below are associated with quiescent or non-active disease (i.e. "remission"); values over 150 are indicative of active disease, and over 450, extremely severe disease^[4]. Total parenteral nutrition was therefore started with complete suspension of fractionated feeding by oral administration and medical treatment was resumed (cortisone, metronidazole as antibiotic and mesalazine as anti-inflammatory).

Due to the steroid therapy, fasting blood glucose pathologically increased (320 mg/dL) and therefore subcutaneous insulin therapy was started with an improvement of glucose intolerance.

Endoscopic histological examination performed after a short time (4 wk), showed only modest improvement of the lesions found earlier during the endoscopic examination, despite improvement of the general condition.

Because of the severity of the lesions, particularly of the anus, and after informing the patient and the pneumologists, we decided to start a biological treatment cycle with infliximab in addition to treatment with azathioprine (AZA) (2.5 mg/kg per day) in a single administration in the morning, associated with antibiotic treatment with 3rd generation cephalosporin and glycopeptide. The infusions of infliximab can be superimposed with this treatment regime, which is used in our centre, with an initial treatment at 0, 2, and 6 wk, followed by a maintenance phase with infusions every 8 wk.

At the discontinuation of steroids, insulin therapy was no longer necessary.

After the first three intravenous infusions of infliximab, endoscopic examination showed complete regression of anal lesions and distinct improvement of the lesions of the sigmoid rectum, with healing appearance of the mucosa and no continuous lesions up to the ileocolic anastomosis.

The appearance of the ileum, explored to about 40 cm, was within acceptable limits. Nine months after beginning the treatment and after 7 infusions of infliximab, the patient showed distinct improvement in her general condition and body weight increased by 13 kg.

Twelve months after commencement of the treatment, condition continues to be optimal and there has been no complication involving infection in the lung or septic episodes. The working of the lungs was kept stable during

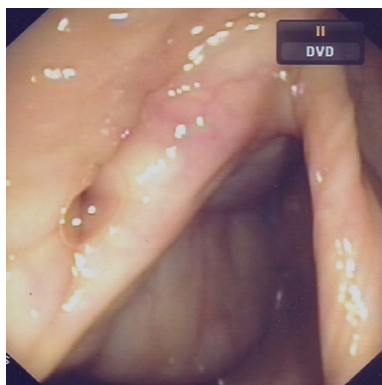


Figure 2 Endoscopic remission.

the course of treatment with infliximab (forced expiratory volume in 1 s (FEV1) 85% in April 2006 and FEV1 80% in July 2007), without any pulmonary exacerbations.

Suspension of the biological treatment was therefore programmed after the 10th infusion, while treatment with AZA and mesalazine as maintenance continued. Since the last infliximab infusion two years have passed and a complete remission of CD has been maintained both clinically and endoscopically (Figure 2).

DISCUSSION

This report deals with the case of a patient suffering from CF who, at the age of 16 years, was diagnosed as suffering from CD. The literature reports frequent cases of CD during the course of CF. The prevalence is about 17 times greater than that of controls^[3]; this shows that there is a pathogenic relationship between the two diseases. The etiopathogenesis has not yet been identified but a number of mechanisms have been proposed, the most probable of which is an altered immune response to a chronic infection^[4]. Furthermore, patients who are carriers of the ΔF 508 mutation seem to have an increased risk of developing gastrointestinal problems^[5].

The literature contains case reports of diagnosis of CD in patients suffering from CF who complain of long periods of abdominal pain and most of all, loss of weight, in spite of appropriate nutritional and therapeutic support^[6,7]. As in the case of our patient, protracted gastrointestinal symptoms and lack of response to basic treatment make it necessary to conduct instrumental examinations, in particular endoscopic examinations such as gastroscopy and colonoscopy, associated with perendoscopic biopsies, which allow a differential diagnosis between CD and fibrosing colonopathy^[7,8]. This has led, in the last few years, to an increase in the diagnosis of CD during the course of CF^[7], which was not suspected in the past. The aim of treatment during the course of CD is to induce and maintain remission of the disease. Corticosteroids have demonstrated a high efficiency in inducing clinical remission in patients with moderate to severe forms of disease^[9,10]. In order to maintain the remission and to reduce the risk of dependence on corticosteroids, there is general agreement regarding the early introduction of im-

munosuppressants such as AZA, MTX, 6 mercaptopurine (MP) and also infliximab^[11-13]. In the case of our patient, given the modest clinical and endoscopic response after one month of treatment with steroids, this therapeutic opportunity was also discussed with the pneumologists. Biological treatment with infliximab associated with AZA was therefore started, because of the presence of significant anal lesions, although the literature currently contains few case reports of patients suffering from CF treated with infliximab. However, numerous studies have shown the efficacy of infliximab in inducing or maintaining remission of disease in adults suffering from CD who have moderate-severe forms, with perianal localization or with fistulizing forms^[9,10,14-17]. In patients with CF the presence of bronchiectasis and colonization with *Pseudomonas*, as in our patient, would normally represent a relative contraindication to the administration of infliximab, but numerous studies of bronchoalveolar lavage fluid from patients with CF have shown a high concentration of inflammatory mediators such as tumor necrosis factor α (TNF α)^[18]. Indeed, drugs such as azithromycin ameliorate lung function in cystic fibrosis patients while also reducing the levels of TNF α ^[19].

In our experience with this patient, we observed a significant clinical improvement even after the first dose. This improvement was endorsed by the resolving of endoscopic and histological lesions, as is also reported in the literature^[9,20,21]. The association of infliximab with AZA was justified by the fact that concurrent treatment with AZA, 6 MP or MTX may be helpful in maintaining the clinical response to infliximab, reducing the amount of circulating antibodies directed towards the latter; which are thought to be responsible for some cases of non response^[22-25].

As shown in the literature^[18,19], as well as in the case of our patient, the moment a treatment focused for CD was introduced, although not free of risks, a distinct improvement of the general condition was observed with weight increase of about 13 kg. After over 12 mo of treatment with infliximab and AZA, no pulmonary complication which would have compelled suspension of treatment was observed. In addition, the literature shows that no severe adverse events have occurred and that there was no reported increase in the prevalence of respiratory tract infections during infliximab administration in patients with chronic obstructive pulmonary disease^[26].

The main questions arising from treatment with infliximab in this patient were, firstly, the possibility that the biological treatment could increase susceptibility to infections by opportunistic pathogens given the concurrent basic lung disease, since patients with cystic fibrosis already spontaneously suffer from such infections. Moreover, the infections most frequently reported during infliximab infusions are respiratory tract infections and urinary tract infections (35% infliximab-treated patients vs 25% placebo recipients)^[27].

Secondly, given the excellent clinical, endoscopic, and histological progress of the patient, there was doubt regarding the best moment for suspension of the drug,

especially taking into consideration the possibility of causing allergies or reduced efficacy in case of re-treatment because of the possible presence of antibodies against infliximab^[9,22,23,25,28,29].

As regards the first challenge, the constant cover with antibiotic treatment was found to be effective in keeping the lung disease under control, as it did not worsen but rather remained stable as is shown by the spirometry tests taken during the course of immunosuppressive treatment.

As regards suspension of treatment, in the absence of precise bibliographic guidance, we believed that it was appropriate to suspend treatment in accordance with previously reported data after a period of not less than one year^[30].

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Use of biological meshes for abdominal wall reconstruction in highly contaminated fields

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Abstract

Abdominal wall defects and incisional hernias represent a challenging problem. In particular, when a synthetic mesh is applied to contaminated wounds, its removal is required in 50%-90% of cases. Biosynthetic meshes are the newest tool available to surgeons and they could have a role in ventral hernia repair in a potentially contaminated field. We describe the use of a sheet of bovine pericardium graft in the reconstruction of abdominal wall defect in two patients. Bovine pericardium graft was placed in the retrorectus space and secured to the anterior abdominal wall using polypropylene sutures in a tension-free manner. We experienced no evidence of recurrence at 4 and 5 years follow-up.

INTRODUCTION

Abdominal wall defects and incisional hernias represent a challenging problem. The risk of developing an incisional hernia after a midline laparotomy is up to 11%^[1]. The size of the abdominal wall defect and the potential presence of contamination of the site can complicate this commonly performed surgical repair. Several techniques have been adopted over time. Primary repairs often lead to unacceptable high tension, and their recurrence rate has been reported as high as 12%-50%^[1,2]. In patients whose fascial defect is significant, mesh repair is preferable in order to obtain a tension-free abdominal wall closure. In the latter case, the recurrence rates are reported between 3% and 24%^[2-4]. According to the literature, the use of prosthetic mesh reduces the recurrence rate but is also associated with serious complications in 10%-15% of cases. Some of these complications, such as infection, fistula and skin erosion, often lead to mesh removal^[4]. In particular, when

a synthetic mesh is applied to contaminated wounds, its removal is required in 50%-90% of the cases^[5]. Strength, flexibility, host tissue compatibility and ability to avoid infections should characterize an ideal mesh. Many synthetic and biological mesh tissues have been proposed over time but no single material, nor newer biosynthetic mesh, has fulfilled these requirements and gained universal acceptance. We present two cases in which biological meshes were used successfully in contaminated fields.

CASE REPORT

Case 1

A 62-year-old white woman presented to the General Surgery and Senology Unit of our University with a 6-mo history of abdominal pain after right hemicolectomy for carcinoma through a midline laparotomy at another institution. Her medical history was also significant for obesity, hypertension and coronary artery disease. She stated that pain occurred soon after the surgical procedure, together with random fever. Six months later, she developed an abdominal wall abscess that spontaneously drained externally through a small incisional hernia in the right-upper abdomen, and formed a persistently draining sinus. Following this, she underwent incisional hernia repair with pre-peritoneal prolene mesh placement. After 4 mo, she developed a small fistula again (Figure 1A), therefore, she was admitted to our division for further care. On physical examination, we observed a well-healed midline incision with mild tenderness along the incision itself. Computed tomography (CT) of the abdomen showed a multiloculated collection around the mesh, which measured approximately 12 cm × 15 cm. A course of antibiotic therapy (imipenem) was initiated before bacterial cultures were obtained. The first culture was positive for *Staphylococcus aureus* and *Pseudomonas aeruginosa*. The second, after 1 wk of appropriate antibiotic administration, remained negative for any microorganism.

We scheduled the patient for exploration and mesh removal. We entered the abdominal cavity through her previous midline laparotomy. The cavity was then exposed and the fluid collection aspirated along with debris. The inflammatory process extended to the periostium of the tenth rib. Removal of the mesh from the anterior abdominal wall required extensive and tedious dissection. The abdomen was entered and all adhesions to abdominal organs (bowel and omentum) were removed. We noticed an anomaly of the rib profile, therefore, after further investigation, we discovered a bone fracture with surrounding tissue rearrangement. We excised the rib for histological examination and it was consistent with osteomyelitis (Figure 1B and C). After removal of the contracted mesh, we created skin flaps and re-positioned the posterior rectus sheath. We placed a sheet of bovine pericardium graft (Tutomes[®]; Tutogen Medical, Germany) in the retrorectus space and secured the mesh in a tension-free manner to the abdominal wall, using polypropylene sutures. The anterior rectus sheath was then

re-positioned with Vicryl, and the skin was closed with staples (Figure 1D). The patient had no significant postoperative complications, and she was discharged on postoperative day 6. We performed CT at 5 mo after surgery, which showed no seroma and excellent remodeling of the host tissue. There was no clinical evidence of recurrence at her 5-year follow-up visit.

Case 2

A 69-year-old man with a previous history of left hemicolectomy for diverticular disease underwent elective incisional hernia repair with intraperitoneal polytetrafluoroethylene (PTFE) mesh placement 8 mo later. Postoperatively, the patient developed a persistent infected seroma, which was unresponsive to antibiotic treatment. The patient underwent surgery again to drain the collection, debride the area, and substitute the contracted Goretex mesh with a polypropylene mesh in the retrorectus space. This attempt proved unsuccessful and led to abscess formation after only 1 mo. The patient was scheduled for elective removal of the polypropylene mesh. After removal of the contracted mesh (Figure 2A-C), the posterior rectus sheath was re-positioned to the midline, and a sheet of bovine pericardium mesh (Tutomes[®]) was implanted in the retrorectus space and secured to the anterior abdominal wall using polypropylene sutures in a tension-free manner. The anterior abdominal wall was then re-positioned with Vicryl interrupted sutures and the skin was closed with staples (Figure 2D). The patient had no significant postoperative complications, and he was discharged on postoperative day 3. At his 4-year follow-up visit, there was no evidence of recurrence.

DISCUSSION

Treatment of incisional hernias

Incisional hernias are still a challenging problem for the surgeon. Their primary repair leads to high recurrence rates (up to 50%) because of the tension created and myocutaneous flap necrosis^[1,2].

Many techniques have been proposed over time to reduce tension, such as relaxing incisions, and compartment release. Unfortunately, the results are far from being optimal^[6,7]. Furthermore, large, full-thickness, abdominal wall defects secondary to wide resection of malignant tumors, traumatic injuries, or congenital abnormalities, cannot be closed primarily. The use of prosthetic meshes has then become necessary. Along with the traditional open techniques of mesh implantation, the more recent laparoscopic approach has gained popularity because of the decrease in wound infection, recurrence rates and recovery time.

Unfortunately the intrinsic property of tissue ingrowth of the prosthetic meshes currently used, such as polypropylene, polyester and PTFE, is also the cause of unwanted adhesions, chronic sinus (2%-6%), fistula formation (0%-2%) and wound infections (2%-17%)^[8]. Other potential complications of synthetic meshes in-

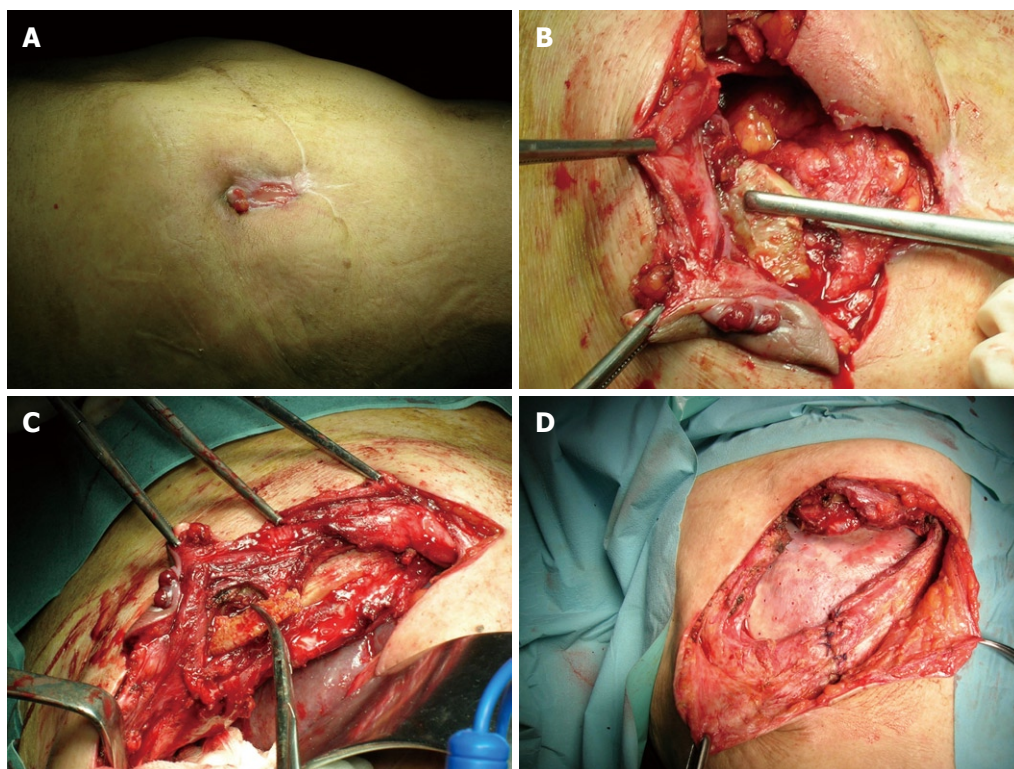


Figure 1 Case 1. A: Abdominal wall with subcutaneous fistula; B: Rib anomaly; C: Bone fracture with tissue rearrangement; D: Sheet of bovine pericardium mesh; anterior rectus sheath re-positioned with Vicryl.

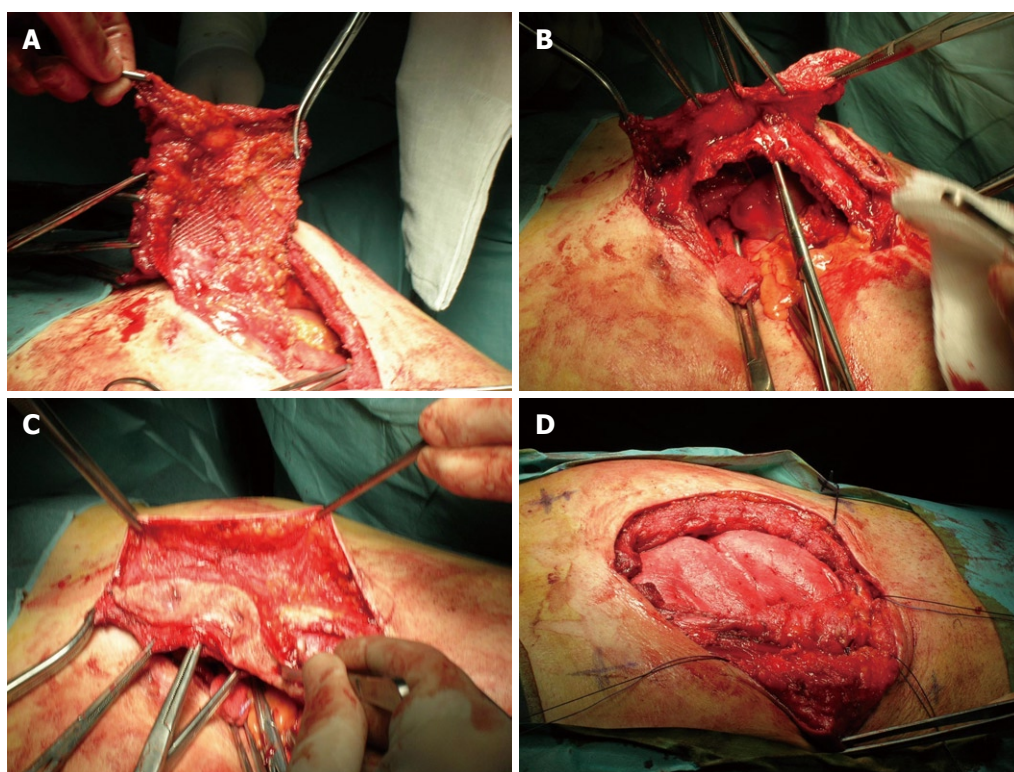


Figure 2 Case 2. A-C: Preexisting contracted mesh; D: Sheet of bovine pericardium mesh in the retrorectus space.

clude mesh contraction, migration, folding of the edges with visceral contact, recurrence, inflammation, seroma, and chronic pain due to inflammatory response and/or

nerve entrapment. As a result, complications are common following ventral hernia repair, and occur in 5%-30% of laparoscopic cases and 27%-34% after open cases^[9].

Table 1 Classification of biological grafts

Species of origin	Allogenic	Xenogenic
Type of collagen matrix	Dermis	Intestinal submucosa
Decellularization process	Yes	Yes
Cross-linking	Yes	No
Sterilization method	Salt solution, patented non-denaturing agents	H ₂ O ₂ , NaOH γ irradiation
Storage (need for refrigeration)	Yes	No
Rehydration requirement	Yes	No

Contaminated abdominal wall defect: role of biological prostheses

Even more challenging is the repair of complex contaminated abdominal wall defects, since no ideal operative technique or material for fascial reconstruction is currently available. The fear of fibrosis, erosions, infection and fistulas with the commonly used prosthetic meshes, has led engineers and surgeons to investigate alternative materials in order to achieve tension-free repair in a single-stage operation, even in highly contaminated fields. Bio-synthetic grafts seem to offer a solution to this challenging problem. Their concept is to provide a collagen and other extracellular matrix scaffold, in which the host fibroblasts can create angiogenesis and deposit new collagen. The non-synthetic nature of these products allows them to be more resistant to infections. Several biological grafts are available on the market. Their classification is based on the species of origin (allogenic, xenogenic), type of collagen matrix utilized (dermis, pericardium, intestinal submucosa), decellularization process, presence or absence of cross-linking, storage requirements (need for refrigeration or not), and the need for rehydration (Table 1).

Bovine pericardium (TUTOMESH®) is a cadaveric bovine pericardium surgical mesh that is processed by solvent dehydration followed by gamma irradiation. It has no risk for transmission of viruses or prions. The graft consists of collagenous connective tissue with three-dimensional intertwined fibers, it has multidirectional mechanical strength, and can be implanted regardless of the direction of the graft. Collagenous connective tissue with multidirectional fibers retains the mechanical strength and elasticity of the native tissue, while providing the basic structure to support replacement by new endogenous tissue (known as remodeling). It is indicated for use in general, gynecological, cardiac and plastic surgery, such as repair of pericardial structures, soft tissue deficiencies, rectal and vaginal prolapse, and hernias (including diaphragmatic, femoral, incisional, inguinal, lumbar, paracolostomy, and umbilical hernias), gastric banding, muscle flap reinforcement, and reconstruction of the pelvic floor.

A bioprosthesis derived from human acellular dermal matrix (Alloderm®; Lifecell Corporation, Brachburg, NJ, USA) has been used as a tissue substitute for skin

grafting over the past 20 years, but its use for abdominal wall reconstruction is relatively recent. At present, there are not enough data or long-term reports to establish universal consensus for its use over other bioprotheses, but it is the material with the most published literature. Some authors have described concerns about the laxity of the material and its ability to stretch over time, with the development of eventration or pseudo-recurrences^[9].

Another available product is derived from porcine dermal collagen (Permacol™; Tissue Science Laboratories plc, Covington, GA, USA). It is a flexible acellular cross-linked porcine dermal collagen that is approved by the US Food and Drug Administration for use as a tissue substitute. It has been used in the United Kingdom since 1998. In addition to its three-dimensional collagen architecture that is similar to human dermis, it supports fibroblast infiltration and neovascularization. As a result of its manufacturing peculiarity of cross-linking, its remodeling process is delayed in the host tissue, which provides additional strength, but also possible incomplete remodeling. The porcine dermal collagen graft is easy to handle and has demonstrated comparable tensile strength to polypropylene. Integration into host tissue and neovascularization allow for antibiotic diffusion and in turn, greater resistance to infections, as in other similar biological grafts^[10].

Surgical techniques: overview

Four types of incisional hernia repair with mesh have been commonly described.

In the inlay technique, the fascial defect cannot be approximated, so the prosthetic material is secured to the edges of the fascia to achieve abdominal wall closure. The onlay technique places the mesh above a primary fascial closure. The more recently described sublay technique (Rives Stoppa repair) applies the concepts of a tension-free technique in which the prosthetic mesh is secured in the retrorectus space after closure of the posterior rectus sheath/peritoneum. The aim is to prevent contact between the mesh and abdominal viscera. The coverage of the mesh provided by the muscle decreases the complications related to the mesh proximity to the subcutaneous tissue (seroma, wound infection)^[11,12]. Finally, with the advent of meshes designed with an anti-adhesive barrier on one side, the mesh position has been moved one layer deeper, into the peritoneal cavity; the so-called intraperitoneal technique. In the latter, the mesh is secured to the abdominal wall with stitches, staples or tacks, and this is commonly performed laparoscopically.

Review of the literature

The data available on biological mesh implants for ventral hernia repair are very recent, and mostly in the form of case series. Lindermann *et al.*^[13] have published their experience using bovine pericardium graft in 37 incisional and two parastomal hernias. They have described four recurrences in the incisional group treated with the inlay technique (fascial defect cannot be approximated). They have suggested that the high elasticity of the mesh during the

remodeling phase could be the likely cause of recurrence. Post *et al*^[14], in their series of 13 successful incisional hernias with sublay bovine pericardium, have described one case in which the mesh had to be re-attached after it had pulled off the suture line during a coughing spell. No infection, pain, foreign body feeling or recurrence was seen. Parker *et al*^[10] have reported their experience using Permacol in nine patients affected by complicated abdominal defects with either a contaminated wound or a history of a hernia mesh infection. In all cases, the mesh was placed in the retromuscular underlay position, and in spite of the presence of active infection or gross contamination, no infectious complications occurred. Baghai *et al*^[15] have evaluated 20 biological mesh implants in 17 patients who required recurrent ventral hernia repair following synthetic mesh explantation due to infection. Different biological grafts were utilized in the study (including porcine intestinal submucosa, human and porcine dermis). Also, all the implants were carried out with an open technique in the inlay, onlay or retrorectus position. All the patients treated with the inlay or onlay technique ($n = 17$) experienced wound infection and/or dehiscence within 2 wk of surgery. The three remaining biological implants, placed in the retrorectus space with closure of the anterior fascia, were successful with no infection, dehiscence or recurrences at 6 mo follow-up. Therefore, Baghai *et al*^[15] have suggested that biological mesh implants are not effective when used with an inlay or onlay technique. According to these preliminary results, the mesh position, regardless of the type of biological mesh composition and texture, may play a key role in the incidence of hernia recurrence. Patients whose defect is corrected by a bridging technique (inlay technique) seem to have an increased tendency to develop eventration and a higher recurrence rate, compared with those whose defect is reinforced after fascia closure (sublay technique)^[9]. According to some authors, the use of biological meshes in a bridging manner (inlay technique) provides only a temporary means for restoration of abdominal wall integrity in highly contaminated fields, which leads to future implantation of a more durable prosthesis to reestablish the integrity of the abdominal wall. Conversely, when the biological mesh is used to reinforce a primary fascia closure (sublay or underlay technique), the rate of recurrence seems comparable with other prosthesis^[16,17]. In the setting of a contaminated wound, delayed closure has been proposed to prevent a closed environment infection and limit recurrence, but its routine use has not gained wide support^[18].

Choice of biological prosthesis

The scarcity of literature comparing the different types of biological grafts precludes an evidenced-based decision about which one to use. Therefore, we have to decide according to the characteristics of the grafts to choose the appropriate product.

Permacol is available in larger sizes than cadaveric graft (up to 18 cm × 28 cm), it is less expensive than human cadaveric dermal graft, and does not require rehy-

dration before being placed^[10].

Bovine pericardium meshes have been used safely even for the repair of inguinal or paraesophageal hernias, in which the constant movement of structures is a risk factor for recurrence^[19]. In our opinion, one of the main advantages of bovine pericardium graft resides in the relative lack of elastin compared to dermal products (both allogenic and xenogenic). This, in turn, results in a higher ratio of mature collagen/elastin at the end of the remodeling, possibly minimizing eventration and pseudo-recurrence^[9]. Also, these products are easy to handle, do not require refrigeration, have a long shelf life, and can be used immediately after opening without long rehydration processes. Finally, they tend to be less expensive than their human tissue counterparts. However, further studies with larger numbers of patients and longer follow-up are required to guide better the right choice of material^[9,20].

In conclusion, complex reconstruction of the abdominal wall can be challenging. Biosynthetic meshes are the newest tool available in the surgeon's armamentarium and they could have a role in ventral hernia repair in clean and contaminated fields. Their use is based on the concept of providing a collagen and extracellular matrix scaffold in which the host fibroblasts can create angiogenesis and deposit new collagen. In reviewing our results, the bovine pericardium as a xenogenic implant is a useful alternative to allogenic materials in incisional hernia repair. Further experience and longer follow-up is necessary to determine the results of bioprosthesis when used for abdominal wall reconstruction.

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Gastric adenomyoma presenting as melena: A case report and literature review

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INTRODUCTION

Gastric adenomyoma (AM) is a rare, benign tumor, characteristically composed of glands and cysts, lined by columnar, flattened epithelia and a prominent smooth muscle stroma. The entity was first described by Magnus-Alsleben^[1] in 1903. Patients with gastric AM may be asymptomatic, or have nonspecific gastrointestinal symptoms such as epigastric pain, vomiting^[2-5]. We describe, herein, an extremely rare case of gastric AM presenting as melena.

CASE REPORT

A 68-year-old man was referred to our hospital with a 1-week history of melena. He had no remarkable past medical history and alcohol consumption or history of smoking or drugs. Upon hospitalization, physical examination, routine laboratory parameters and tumor markers including carcinoembryonic antigen, cancer antigen 19-9, and alpha fetoprotein were normal, except for markers of hypochromic anemia including hemoglobin 89 g/L (normal range: 120-160 g/L), hematocrit 28.6% (normal range: 40%-50%), mean corpuscular hemoglobin concentration 311 g/L (normal range: 320-360 g/L), serum ferritin 8.31 µg/L (normal range: 21.81-274.66 µg/L).

Abdominal computed tomography (CT) scanning showed a heterogeneous mass in the gastric antrum. Tumor tissue was slightly enhanced after injection of a

Abstract

Gastric adenomyoma (AM) is a rare benign tumor characterized by gland-like structures embedded within a smooth muscle stroma. We report a case of a 68-year-old man with gastric AM admitted to our hospital for melena. Endoscopic examination revealed a gastric mass of about 4 cm in diameter, located in the antrum. Histologic examination of the excised specimen showed irregularly arranged glands and interlacing smooth muscle bundles surrounding the glandular elements. Although gastric AM is rare, it should be considered in differential diagnosis of extramucosal gastric tumor.

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Key words: Adenomyoma; Stomach; Melena; Histopathology; Endoscopic examination

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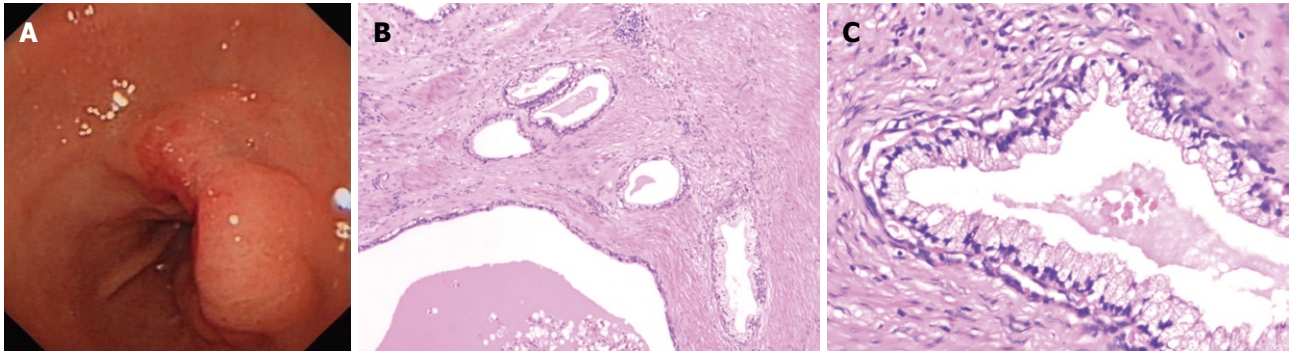


Figure 1 Gastric adenomyoma. A: Upper gastrointestinal (GI) endoscopy showing an irregular lesion with central ulcer in the gastric antrum; B: Microscopy showing several glands and cyst surrounded by smooth muscle (HE, $\times 50$); C: High-power view showing glands lined by columnar mucinous epithelium (HE, $\times 200$).

contrast medium. Neither further invasion beyond the gastric wall, nor visible metastatic lesions in the liver or enlarged lymph nodes were observed.

Endoscopic examination revealed an ulcerated mass of about 4 cm in diameter, located on the lesser curvature and posterior wall of the gastric antrum, with the duodenum partly involved (Figure 1A). The esophagus and the remaining parts of stomach were normal. Histopathological examination of mass biopsies showed several dilated glands with mild atypia in an inflamed stroma and necrosis. A decision was made to proceed with surgery in order to relieve the symptoms. As frozen sections revealed the possibility of a gastric AM, a distal subtotal gastrectomy was performed. Postoperative recovery was uneventful.

Macroscopic examination revealed a 50 mm \times 43 mm \times 23 mm ulcerated mass with sharp margins and a greyish-white cut surface. It was solid and cystic with clear, mucinous fluid in the lumens. The cysts ranged up to 8 mm. Microscopic examination showed that the tumor was located mainly in submucosa and muscularis propria of the stomach, composed of glands and cysts surrounded by bundles of smooth muscle tissue (Figure 1B). Epithelial cells lining the glands and cysts were columnar and flattened mucinous cells (Figure 1C). Cellular stratification was minimal and nuclei were basally located with only slight atypia. Mitotic activity was absent. Complete sectioning of this mass failed to demonstrate exocrine or endocrine pancreatic structures. Accumulations of inflammatory mononuclear cells were noted near the glands.

DISCUSSION

AM of the gastrointestinal tract is rare, most frequently observed in the duodenum, gall bladder and stomach. Most cases of gastric AM occur in adults with no symptom or have nonspecific gastrointestinal symptoms. Scarc clinical manifestations such as localized peritonitis have been reported^[6]. To the best of our knowledge, this is the first case of gastric AM presented with melena. We ascribe the hemorrhage to mucosal ulcer.

In spite of the availability of newer diagnostic techniques, including CT and endoscopic ultrasonography^[7], it is still difficult to diagnose gastric AM before operation.

Endoscopic examination cannot differentiate gastric AM from other extramucosal lesions, such as gastrointestinal stromal tumor, lipoma, neurilemmoma, hemangioma, gastrointestinal autonomic nerve tumor, carcinoid, lymphoma or even gastric carcinoma. In the majority of cases, endoscopic biopsies are superficial and fail to obtain representative tumor tissue. Therefore, frozen section is very helpful to establish the intraoperative diagnosis and to avoid unnecessary extensive operations. In the present case, two biopsies were not diagnostic, frozen section revealed the possibility of gastric AM.

Histologically, gastric AM should be differentiated from high-grade adenocarcinoma and gastritis cystica profunda. Gastric adenocarcinoma associated with gastric AM has been reported^[8]. The common features that favor an adenocarcinoma are epithelial atypia, mitotic figures, and fibrous stromal reaction. Gastritis cystica profunda (GCP) is characterized by elongation of gastric foveola with hyperplasia and cystic dilatation of gastric glands, extending into the gastric submucosal layer. The absence of smooth muscle bundles around the cysts helps to differentiate GCP from AM.

Based on the similarities of epithelial component of AM to pancreatic ducts, several authors consider gastric AM as a variant of ectopic pancreas without exocrine or endocrine components^[9,10]. Yamagiwa *et al.*^[11] divided ectopic pancreas into type I (with all elements of normal pancreatic tissues), type II (with pancreatic tissues but no islets), and type III (with pancreatic ducts only). Gastric AM represents type III. However, Takeyama *et al.*^[6] considered AM as a hamartoma based on the disordered proliferation of smooth muscle.

In summary, although gastric AM is rare, it should be always considered in the differential diagnosis of extramucosal gastric lesions. Despite the development of modern diagnostic techniques, its diagnosis remains challenging. If in doubt, frozen section can help to avoid unnecessary radical operation.

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Hepatoprotective effects of antioxidants in chronic hepatitis C

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Abstract

We have read with interest the paper published in issue 2, volume 16 of *World Journal of Gastroenterology* 2010 by Nakamura *et al*, demonstrating that the antioxidant resveratrol (RVT) enhances hepatitis C virus (HCV) replication, consequently, they conclude that RVT is not a suitable antioxidant therapy for HCV chronic infection. The data raise some concern regarding the use of complementary and alternative medicine since the most frequent supplements taken by these patients are antioxidants or agents that may be beneficial for different chronic liver diseases. A recent study by Vidali *et al* on oxidative stress and steatosis in the progression of chronic hepatitis C concludes that oxidative stress and insulin resistance contribute to steatosis, thus accelerating the progression of fibrosis. We are particularly interested in investigating how the oxidative and nitrosative stress mechanisms are involved in the pathogenesis of different chronic liver diseases.

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Key words: Antioxidant; Chronic hepatitis C; Resveratrol; S-adenosyl-methionine; Nitric oxide; N-acetyl-cysteine

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TO THE EDITOR

We read with interest the paper by Nakamura *et al*^[1] demonstrating that the antioxidant resveratrol (RVT) enhances the hepatitis C virus (HCV) replication, consequently, they conclude that RVT is not a suitable antioxidant therapy for HCV chronic infection. The data raise some concern regarding the use of complementary and alternative medicine since the most frequent supplements taken by these patients are antioxidants or agents that may be beneficial for different chronic liver diseases. A recent study by Vidali *et al*^[2] on oxidative stress and steatosis in the progression of chronic hepatitis C (CHC) concludes that oxidative stress and insulin resistance contribute to steatosis, thus accelerating the progression of fibrosis. They speculate that therapeutic regimens including anti-oxidant agents could be of clinical relevance in CHC patients infected with genotype non-3, according to their own results^[2]. There is evidence that antioxidant therapies may ameliorate the necro-inflammatory activity in CHC patients^[3].

We are particularly interested in investigating how the oxidative and nitrosative stress mechanisms are involved in the pathogenesis of different chronic liver diseases^[4]. Based on this rationale, we would like to support the hypothesis that antioxidant compounds might be proven beneficial for acute and chronic liver diseases. According to ours and other author experience, nitric oxide (NO)

has deleterious consequences in the presence of reactive oxygen species, since peroxynitrites are formed. NO participates in the pathophysiology of viral (chronic hepatitis B and CHC) and autoimmune (primary biliary cirrhosis and autoimmune hepatitis) liver diseases^[5,6], as well as in acute liver allograft rejection^[7]. Moreover, NO contributes to liver ischemia-reperfusion injury and inhibition of inducible nitric oxide synthase (iNOS) shows beneficial effects^[8]. Consistently, we found that N-acetyl-cysteine (NAC) modulates the expression of iNOS in human hepatocytes stimulated by proinflammatory cytokines^[9]. The effect occurs by blocking the activation of the iNOS promoter, and is associated with modulation of NF- κ B activity, a central transcription factor for induction of iNOS expression. The biological phenomenon might well be the basis of the therapeutic effects of NAC on chronic liver diseases different from those caused by acetaminophen intoxication.

Further insights into the hepatoprotective mechanisms of antioxidants might be learnt by analysing the glutathione precursor S-adenosyl-methionine (SAME) actions. Administration of SAME to patients with alcoholic liver cirrhosis showed beneficial effects^[10]. Its possible mechanisms of action include: (1) acting as a methyl donor compound and contributing to restoration of mitochondrial glutathione content, which is necessary to counterbalance the oxidant environment in cirrhotic liver, and (2) attenuating the hepatic production of NO, through the modulation of nitric oxide synthase-2. SAME exerts these effects by accelerating re-synthesis of inhibitor κ B alpha and blunting the activation of nuclear factor κ B, thereby reducing the transactivation of NO synthase-2 promoter^[11].

Taking all these data together, abundant evidence suggests that antioxidants can effectively attenuate the oxidative and nitrosative stress in liver injury, ultimately improving inflammation and fibrosis progression. It is worth testing these drugs in future clinical trials including CHC patients, mainly those who present negative predictive factors of sustained virological response to standard antiviral regimens. However, controversies raise from the results of the study by Nakamura *et al*^[1] promoting the possible need of investigations on the effects of different antioxidants on HCV replication before its use as a supplement in treatment of CHC patients.

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Events Calendar 2010

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Negligence and Litigation in Medical
Practice

January 25-29
Waikoloa, HI, United States
Selected Topics in Internal Medicine

January 26-27
Dubai, United Arab Emirates
2nd Middle East Gastroenterology
Conference

January 28-30
Hong Kong, China
The 1st International Congress on
Abdominal Obesity

February 11-13
Fort Lauderdale, FL, United States
21th Annual International Colorectal
Disease Symposium

February 26-28
Carolina, United States
First Symposium of GI Oncology at
The Caribbean

March 04-06
Bethesda, MD, United States
8th International Symposium on
Targeted Anticancer Therapies

March 05-07
Peshawar, Pakistan
26th Pakistan Society of
Gastroenterology & Endoscopy
Meeting

March 09-12
Brussels, Belgium
30th International Symposium on
Intensive Care and Emergency
Medicine

March 12-14
Bhubaneswar, India
18th Annual Meeting of Indian
National Association for Study of
the Liver

March 23-26
Cairo, Egypt
14th Pan Arab Conference on
Diabetes PACD14

March 25-28
Beijing, China
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the Liver

March 27-28
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25th Annual New Treatments in
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April 07-09
Dubai, United Arab Emirates
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2010

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12th World Congress of Endoscopic
Surgery

April 14-18
Vienna, Austria
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2010

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Dubrovnik, Croatia
3rd Central European Congress
of surgery and the 5th Croatian
Congress of Surgery

May 01-05
New Orleans, LA, United States
Digestive Disease Week Annual
Meeting

May 06-08
Munich, Germany
The Power of Programming:
International Conference on
Developmental Origins of Health
and Disease

May 15-19
Minneapolis, MN, United States
American Society of Colon and
Rectal Surgeons Annual Meeting

June 04-06
Chicago, IL, United States
American Society of Clinical
Oncologists Annual Meeting

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Singapore, Singapore
13th International Conference on
Emergency Medicine

June 14
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Gastro-intestinal Models in
the Research of Probiotics and
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September 11-12
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Chinese journal article (list all authors and include the PMID where applicable)

- 2 **Lin GZ**, Wang XZ, Wang P, Lin J, Yang FD. Immunologic effect of Jianpi Yishen decoction in treatment of Pixu-diarrhoea. *Shijie Huaren Xiaohua Zazhi* 1999; **7**: 285-287

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- 3 **Tian D**, Araki H, Stahl E, Bergelson J, Kreitman M. Signature of balancing selection in Arabidopsis. *Proc Natl Acad Sci USA* 2006; In press

Organization as author

- 4 **Diabetes Prevention Program Research Group**. Hypertension, insulin, and proinsulin in participants with impaired glucose tolerance. *Hypertension* 2002; **40**: 679-686 [PMID: 12411462 PMCID:2516377 DOI:10.1161/01.HYP.0000035706.28494.09]

Both personal authors and an organization as author

- 5 **Vallancien G**, Emberton M, Harving N, van Moorselaar RJ; Alf-One Study Group. Sexual dysfunction in 1, 274 European men suffering from lower urinary tract symptoms. *J Urol* 2003; **169**: 2257-2261 [PMID: 12771764 DOI:10.1097/01.ju.0000067940.76090.73]

No author given

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

Volume with supplement

- 7 **Geraud G**, Spierings EL, Keywood C. Tolerability and safety of frovatriptan with short- and long-term use for treatment of migraine and in comparison with sumatriptan. *Headache* 2002; **42** Suppl 2: S93-99 [PMID: 12028325 DOI:10.1046/j.1526-4610.42.s2.7.x]

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- 8 **Banit DM**, Kaufer H, Hartford JM. Intraoperative frozen section analysis in revision total joint arthroplasty. *Clin Orthop Relat Res* 2002; **(401)**: 230-238 [PMID: 12151900 DOI:10.1097/00003086-200208000-00026]

No volume or issue

- 9 Outreach: Bringing HIV-positive individuals into care. *HRS-A Careaction* 2002; 1-6 [PMID: 12154804]

Instructions to authors

Books

Personal author(s)

- 10 **Sherlock S**, Dooley J. Diseases of the liver and biliary system. 9th ed. Oxford: Blackwell Sci Pub, 1993: 258-296

Chapter in a book (list all authors)

- 11 **Lam SK**. Academic investigator's perspectives of medical treatment for peptic ulcer. In: Swabb EA, Azabo S. Ulcer disease: investigation and basis for therapy. New York: Marcel Dekker, 1991: 431-450

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- 12 **Breedlove GK**, Schorfheide AM. Adolescent pregnancy. 2nd ed. Wiczorek RR, editor. White Plains (NY): March of Dimes Education Services, 2001: 20-34

Conference proceedings

- 13 **Harnden P**, Joffe JK, Jones WG, editors. Germ cell tumours V. Proceedings of the 5th Germ cell tumours Conference; 2001 Sep 13-15; Leeds, UK. New York: Springer, 2002: 30-56

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- 14 **Christensen S**, Oppacher F. An analysis of Koza's computational effort statistic for genetic programming. In: Foster JA, Lutton E, Miller J, Ryan C, Tettamanzi AG, editors. Genetic programming. EuroGP 2002: Proceedings of the 5th European Conference on Genetic Programming; 2002 Apr 3-5; Kinsdale, Ireland. Berlin: Springer, 2002: 182-191

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- 15 Morse SS. Factors in the emergence of infectious diseases. Emerg Infect Dis serial online, 1995-01-03, cited 1996-06-05; 1(1): 24 screens. Available from: URL: <http://www.cdc.gov/ncidod/EID/eid.htm>

Patent (list all authors)

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

Statistical data

Write as mean \pm SD or mean \pm SE.

Statistical expression

Express *t* test as *t* (in italics), *F* test as *F* (in italics), chi square test as χ^2 (in Greek), related coefficient as *r* (in italics), degree of freedom as *v* (in Greek), sample number as *n* (in italics), and probability as *P* (in italics).

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Use SI units. For example: body mass, *m* (B) = 78 kg; blood pressure, *p* (B) = 16.2/12.3 kPa; incubation time, *t* (incubation) = 96 h, blood glucose concentration, *c* (glucose) 6.4 ± 2.1 mmol/L; blood CEA mass concentration, *p* (CEA) = 8.6 $24.5 \mu\text{g/L}$; CO₂ volume fraction, 50 mL/L CO₂, not 5% CO₂; likewise for 40 g/L formaldehyde, not 10% formalin; and mass fraction, 8 ng/g, etc. Arabic numerals such as 23, 243, 641 should be read 23 243 641.

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Quantities: *t* time or temperature, *c* concentration, *A* area, *l* length, *m* mass, *V* volume.

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Effect of the timing of gluten introduction on the development of celiac disease

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subsequent development of CD. Here, we present and review the most recent evidences regarding the effect of timing of gluten introduction during weaning, the amount of gluten introduced and simultaneous breast-feeding, on the development of CD.

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Key words: Celiac disease; Gluten; Weaning; Breast-feeding; Prevention; Autoimmunity; Anti-transglutaminase antibody; Duodenal biopsy

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Abstract

Celiac disease (CD) is a permanent auto-immune enteropathy, triggered in genetically predisposed individuals by the ingestion of dietary gluten. Gluten is the alcohol-soluble protein component of the cereals wheat, rye and barley. CD is a multifactorial condition, originating from the interplay of genetic and environmental factors. The necessary environmental trigger is gluten, while the genetic predisposition has been identified in the major histocompatibility complex region on chromosome 6p21, with over 90% of CD patients expressing HLA DQ2 and the remaining celiac patients express DQ8. The fact that only about 4% of DQ2/8-positive individuals exposed to gluten develop CD, has led to the recognition that other genetic and environmental factors are also necessary. In the last few years, several epidemiological studies have suggested that the timing of the introduction of gluten, as well as the pattern of breastfeeding, may play an important role in the

INTRODUCTION

Celiac disease (CD) is a permanent auto-immune enteropathy, triggered in genetically predisposed individuals by the ingestion of dietary gluten. Gluten is the alcohol-soluble protein component of the cereals wheat, rye and barley. It is composed of 2 major protein fractions: glutenin and gliadin; most of the toxic activity exerted by gluten in CD is due to gliadin.

CD is an increasingly recognized condition; its true prevalence is, however, difficult to ascertain, because many (arguably the majority) of affected people are asymptomatic or show mild symptoms and signs^[1,2].

CD is a multifactorial condition, originating from the interplay of genetic and environmental factors. The necessary environmental trigger is gluten, while the genetic predisposition has been identified in the major his-

to compatibility complex region on chromosome 6p21, with over 90% of CD patients expressing HLA DQ2 (DQA1*05/DQB1*02) or in the trans position in HLA-DR5/DR-7 heterozygous patients. The remaining celiac patients express DQ8 (DQA1*0301/DQB1*0302). While these haplotypes confer the highest genetic risk for CD, the fact that only about 4% of DQ2/8 positive individuals exposed to gluten develop CD, has led to the recognition that other genetic factors are also necessary^[3]. In the last few years, significant advances have been made leading to the identification of 6 regions that harbor genes controlling immune responses in relevant biological pathways. So far, 7 additional candidate genes have been uncovered that are considered as plausible contributors to the development of CD^[4,5].

In the last few years, several epidemiological studies have suggested that the timing of the introduction of gluten into the diet, as well as the pattern of breastfeeding, may play an important role in the subsequent development of CD.

THE ROLE OF GLUTEN-WHEN

The attractive hypothesis that the age at first introduction of gluten in predisposed individuals could influence the onset of CD relies upon the fact that at some point in time during development, humans appear to lose their ability to develop oral tolerance to newly introduced antigens^[6]. On the other hand, early introduction of solid foods (i.e. before the intestinal immune system reaches a certain level of maturation) may lead to development of intolerance^[7].

In reality, the results of most older studies had suggested that the age at first introduction of gluten would not affect the development of CD, while it would modify the onset of symptoms^[8].

A decrease of the incidence of biopsy-proven CD following a change in weaning practices was first reported at the end of 1970s among the pediatric population of West Somerset, England^[9]. The authors noticed that the incidence of CD declined from 1:1228 to 1:4168 following the recommendations to avoid both the addition of cereals to bottle feeds and the introduction of gluten before 4 mo of age. The cohort of children considered in this study was quite small and it was a retrospective analysis; additionally, the overall incidence of CD reported appears markedly low compared to the present knowledge of the epidemiology of CD. In spite of these limitations, that work gave the first support to the role of age at first exposure to gluten in the development of CD.

Much stronger epidemiological evidence about the role of timing of solid introduction on the development of CD came from a recent 10-year observational study that investigated the age at first introduction (before 3 mo of age, between 3 and 7 mo and at 7 mo or later) of gluten-containing cereals in a group of 1560 children at risk of CD or type 1 diabetes (positivity to

HLA-DR3, -DR4 or a first-degree relative with type 1 diabetes) in relation to the subsequent risk of developing CD autoimmunity (CDA)^[10]. CDA was defined as positive tissue transglutaminase antibody (tTG Ab) on 2 or more consecutive visits or a positive tTG Ab and a small bowel biopsy consistent with CD. The results, generated as part of a larger effort within the so-called DAISY project, showed that, out of 51 children who developed CDA, those exposed to gluten in the first 3 mo of age had a 5-fold increased risk of CDA compared to those exposed at 4 to 6 mo [hazard ratio (HR), 5.17; 95% confidence interval (CI): 1.44-18.57]; and those who received gluten for the first time at 7 mo of age or after showed a slightly increased hazard ratio compared with those exposed at 4-6 mo (HR, 1.87; 95% CI: 0.97-3.60). These results seem to confirm the existence of a “window” period, during which the first exposure to gluten should occur in order to minimize the risk of subsequent development of CD. Perhaps the biggest limitation of the DAISY study is the lack of data on the amount of gluten ingested. Although the authors speculated that the children that received gluten after 7 mo consumed a higher amount of gluten on its introduction, this remains speculative.

THE ROLE OF GLUTEN-HOW MUCH

The epidemic of CD among Swedish children observed in the mid 80s suggests that the amount of gluten ingested during weaning can play a pivotal role in the development of CD. After 1982, Sweden experienced a quite unique epidemic of CD in children under 2 years of age, with the annual incidence increasing 4-fold to 200-240 cases/100 000 inhabitants per year, followed from 1995 by a sharp decline to the previous level of 50-60 cases^[11,12]. Such a trend was not observed in nearby countries (i.e. Denmark and England), where, in contrast, a decline in the incidence of CD was noticed in the same period. This epidemic pattern was later related to new dietetic guidelines (later changed for this reason) that resulted in gluten being introduced to infants after they had been weaned from the breast, and in addition (due to concomitant, unrelated reasons) larger amounts of gluten were also given at that time.

To make the story even more complex, came the observation from analysis of the data of the Swedish CD epidemic, that the overall prevalence of CD diagnosed by serological screening (EMA) was not different in the group of children born in 1996-1997 compared with those born in 1992-1993^[13]. This finding indicated that the amount of gluten introduced during weaning might affect the development of symptomatic CD, but does not protect the children from being affected by sub-clinical or silent forms of the disease.

Finally, follow-up studies of that same cohort of children born during the epidemic^[14] showed that the increased risk of developing CD carries on with time, as the prevalence of this condition in 12-year-old children

(thus born during the epidemic) has been reported to be as high as 3%^[14].

The regional differences in the epidemiology of CD in India also give support to the hypothesis that the amount of gluten plays an important role in the onset of CD. CD is reported frequently in high wheat-consuming states in Northern India and quite rarely in the Southern States, where rice is the staple food^[15].

THE ROLE OF BREASTFEEDING

The protective effect of breastfeeding on the development of food allergy has been long suspected. Indeed, the majority of the studies on the specific protective effect of breastfeeding on the development of CD, although having very different methodologies and population selection, found a negative correlation between the duration of breastfeeding and the development of CD^[16-18]. A rigorous meta-analysis reviewed all the observational epidemiological studies dealing with the effect of breastfeeding on development of CD and found that children being breastfed at the time of gluten introduction had a 52% reduction in risk of developing CD, compared to their peers who were not breastfed at the time of gluten introduction [pooled odds ratio (OR), 0.48; 95% CI: 0.40-0.59]^[19].

The same author proceeded to estimate how many cases of CD could be prevented in the UK by assuming higher rates of breastfeeding, to conclude that if all babies were breastfed at the time of gluten introduction, this would result in the prevention of more than 2500 cases of CD per year^[20].

The observation of the Swedish experience also gave some clues about the protective role of breastfeeding on CD. The Swedish children that were breastfed at the first exposure to gluten, even with high amounts, showed a lower risk of developing CD than those who were formula fed (OR, 0.59; 95% CI: 0.42-0.83), and the risk was reduced further if they continued to be breastfed afterwards (OR, 0.36; 95% CI: 0.26-0.51)^[21,22].

Surprisingly, the data generated in the context of the DAISY project previously mentioned^[8] did not provide evidence on the protective role of prolonged breastfeeding. The authors themselves pointed out this discrepancy, ascribing it to the different methodology - the DAISY study was prospective and focused on high risk children, whereas all the previous studies were retrospective and included the general pediatric population.

In spite of all the evidence outlined, some crucial issues remain unanswered: (1) Does breastfeeding at the first exposure to gluten offer a permanent protection against CD or does it only delay its appearance? (2) Are those children breastfed during the introduction of gluten more likely to develop extraintestinal ("atypical") CD? A series of 162 celiac children enrolled at the University of Chicago showed that children breastfed at the time of gluten introduction were just as likely to develop intestinal as extra-intestinal symptoms, whereas children

who were not breastfed when weaned with gluten had a much higher chance of showing intestinal symptoms^[6]; and (3) What is the mechanism underlying the protective role of breastfeeding towards the development of CD? Presently one can only speculate on 4 possible ways: (a) breast milk contains substances with immunomodulatory activity on the intestinal mucosa, within the idiopathic network; (b) children that are breastfed at gluten introduction during weaning receive lower amounts of gluten, even on a pro/kg body weight basis; (c) breastfeeding prevents gastrointestinal infections, a known contributing factor in the pathogenesis of CD; or (d) the absence of possible multiple contemporary associations with other solids and nutrients.

ESPGHAN RECOMMENDATIONS

On the basis of the above described epidemiological data, the European Society for Paediatric Gastroenterology, Hepatology and Nutrition (ESPGHAN) Committee recommended avoidance of both early (< 4 mo) and late (> 7 mo) introduction of gluten and introduction of small amounts of gluten gradually while the child is still breastfed^[23]. This temporary window allows the possibility of modulating the mucosal immune response, with progressively decreasing breastfeeding and maturation of the gastro-intestinal system^[22].

CONCLUSION

There remain several open questions about the role of early feeding practices on the development of CD, including the role of additional genetic factors, the type of gluten introduced, and, certainly not least, the role played by the microbiota. It appears that celiac children, even in remission, have a composition of microflora that differs from healthy controls^[24]. It is likely that CD may be the end result of an intricate interplay by all of these factors. The challenge is for us to tease out their roles and understand the specific mechanisms that ultimately lead to CD, in order to achieve the ambitious goal of its prevention.

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Importance of hepatitis C virus-associated insulin resistance: Therapeutic strategies for insulin sensitization

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documented, therapeutic guidelines for preventing the distinctive complications of HCV-associated insulin resistance have not yet been established. In addition, mechanisms for the development of HCV-associated insulin resistance differ from lifestyle-associated insulin resistance. In order to ameliorate HCV-associated insulin resistance and its complications, the efficacy of the following interventions is discussed: a late evening snack, coffee consumption, dietary iron restriction, phlebotomy, and zinc supplements. Little is known regarding the effect of anti-diabetic agents on HCV infection, however, a possible association between use of exogenous insulin or a sulfonylurea agent and the development of HCC has recently been reported. On the other hand, insulin-sensitizing agents are reported to improve sustained virologic response rates. In this review, we summarize distinctive complications of, and therapeutic strategies for, HCV-associated insulin resistance. Furthermore, we discuss supplementation with branched-chain amino acids as a unique insulin-sensitizing strategy for patients with HCV-associated insulin resistance.

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Abstract

Insulin resistance is one of the pathological features in patients with hepatitis C virus (HCV) infection. Generally, persistence of insulin resistance leads to an increase in the risk of life-threatening complications such as cardiovascular diseases. However, these complications are not major causes of death in patients with HCV-associated insulin resistance. Indeed, insulin resistance plays a crucial role in the development of various complications and events associated with HCV infection. Mounting evidence indicates that HCV-associated insulin resistance may cause (1) hepatic steatosis; (2) resistance to anti-viral treatment; (3) hepatic fibrosis and esophageal varices; (4) hepatocarcinogenesis and proliferation of hepatocellular carcinoma; and (5) extrahepatic manifestations. Thus, HCV-associated insulin resistance is a therapeutic target at any stage of HCV infection. Although the risk of insulin resistance in HCV-infected patients has been

Key words: Hepatitis C virus; Diabetes mellitus; Insulin resistance; Complications; Treatments; Branched-chain amino acid

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INTRODUCTION

Insulin resistance is frequently seen in patients with

hepatitis C virus (HCV) infection^[1,2]. Although in the general population, lack of exercise and overeating are major causes of insulin resistance, in patients with HCV infection, hepatic inflammation, activated inflammatory cytokines, and HCV-induced impairments of insulin and lipid signaling molecules are also important factors for the development of insulin resistance^[3-14]. Therefore, the prevalence of insulin resistance is higher in patients with HCV infection compared to that in the general population and patients with other hepatobiliary disorders^[6,15].

Generally, insulin resistance results in the development of type 2 diabetes mellitus and increases the risk of life-threatening complications such as cardiovascular diseases, renal failure, and infections. However, these complications are not major causes of death in cirrhotic patients with insulin resistance^[16]. On the other hand, the development of intrahepatic complications, including hepatocellular carcinoma (HCC), is known to be associated with insulin resistance^[17-21]. Insulin resistance is also reported to be involved in the development of extrahepatic manifestations of HCV infection including gastric cancer^[22-24].

Reduction of fasting blood glucose and hemoglobin A1c (HbA1c) is a well-established therapeutic strategy for prevention of complications in diabetic patients^[25,26]. However, in patients with chronic liver diseases, fasting blood glucose and HbA1c are not always available for evaluation of glucose metabolism because of decreased hepatic glycogen content^[27] and increased turnover of hemoglobin^[28]. Furthermore, an association between the use of exogenous insulin or sulfonylurea agents and the development of HCC has recently been reported^[29,30]. Although therapeutic guidelines for inhibiting the distinctive complications of HCV-associated insulin resistance are not yet available, amelioration of insulin resistance is considered to inhibit complications and improve prognosis. Here, we summarize treatments that could reduce HCV-associated insulin resistance.

In this review, we summarize distinctive complications of, and therapeutic strategies for, HCV-associated insulin resistance. In addition, we discuss the merits of branched-chain amino acid (BCAA) supplementation as a unique insulin-sensitizing strategy for patients with HCV-associated insulin resistance.

DISTINCTIVE COMPLICATIONS OF HCV-ASSOCIATED INSULIN RESISTANCE

Complications of HCV-associated insulin resistance are different from those of lifestyle-associated insulin resistance^[16]. Cardiovascular diseases are major causes of death in patients with lifestyle-associated insulin resistance^[31]. However, these complications are not major causes of death in patients with HCV-associated insulin resistance^[16]. In contrast, HCV-associated insulin resistance is involved in the development of various complications associated with HCV infection. Here, we summarize events associated with insulin resistance that are distinctive complications of HCV-associated insulin resistance (Figure 1).

Hepatic steatosis

Hepatic steatosis is commonly observed^[32,33] and is an independent risk factor for disease progression in patients with HCV infection^[34]. Various mechanisms are operative in the development of hepatic steatosis. HCV core protein induces production of reactive oxygen species and lipid peroxidation^[35]. HCV core protein also regulates secretion of very low-density lipoprotein, triglycerides, and apolipoprotein B through regulation of fatty acid synthase, microsomal triglyceride transport protein, peroxisome proliferator-activated receptor γ (PPAR γ), and sterol regulatory element binding protein-1c^[9,36-38]. Thus, HCV itself is directly involved in the development of hepatic steatosis. In addition, insulin is an anabolic hormone and promotes hepatic lipogenesis through activation of hydroxymethylglutaryl-CoA reductase and acetyl-CoA carboxylase^[39]. In addition, insulin inhibits lipolysis through regulation of phosphodiesterase type 3B^[19]. In HCV core gene transgenic mice, the development of insulin resistance precedes the development of hepatic steatosis, suggesting that insulin resistance may induce hepatic steatosis^[8,40]. However, hepatic steatosis could also cause insulin resistance^[41,42], and therefore, the initial step in HCV-related metabolic disorders remains unclear in patients with HCV infection.

Resistance to anti-viral treatment

Insulin resistance is associated with a poor response to anti-viral treatment in patients with HCV genotype 1, 2, and 3 infections^[10,43-46]. Although the reason for an association between insulin resistance and resistance to anti-viral treatment is largely unknown, the following are possibilities. Insulin resistance is known to increase hepatic lipid synthesis^[47]. Since the lipid droplet is an important organelle for HCV replication^[48], accumulation of hepatic lipid droplets may increase HCV replication and result in poor responses to anti-viral treatment, even in patients with HCV genotype 2 and 3^[45].

Alternatively, HCV core protein is reported to up-regulate suppressor of cytokine signaling (SOCS) 3^[6,49-52], which acts as an adaptor to facilitate the ubiquitination of signaling proteins, leading to subsequent proteasomal degradation of SOCS3^[19]. HCV core protein-induced SOCS3 upregulation promotes proteasomal degradation of insulin receptor substrate (IRS) 1 and IRS2, resulting in the development of insulin resistance in patients with HCV infection^[6,19,44]. Simultaneously, SOCS3 is also known to inhibit interferon- α -induced expression of the anti-viral proteins 2',5'-oligoadenylate synthetase and myxovirus resistance A through inactivation of Janus kinase, a signal transducer and activator of the transcription pathway^[49]. Thus, SOCS3 seems to be a key molecule for a cross-talk between insulin resistance and resistance in patients with HCV infection. In fact, hepatic expression of SOCS3 has predictive value for the outcome of anti-viral therapy in patients with HCV infection^[53,54].

Hepatic fibrosis and esophageal varices

Insulin resistance is closely associated with progression

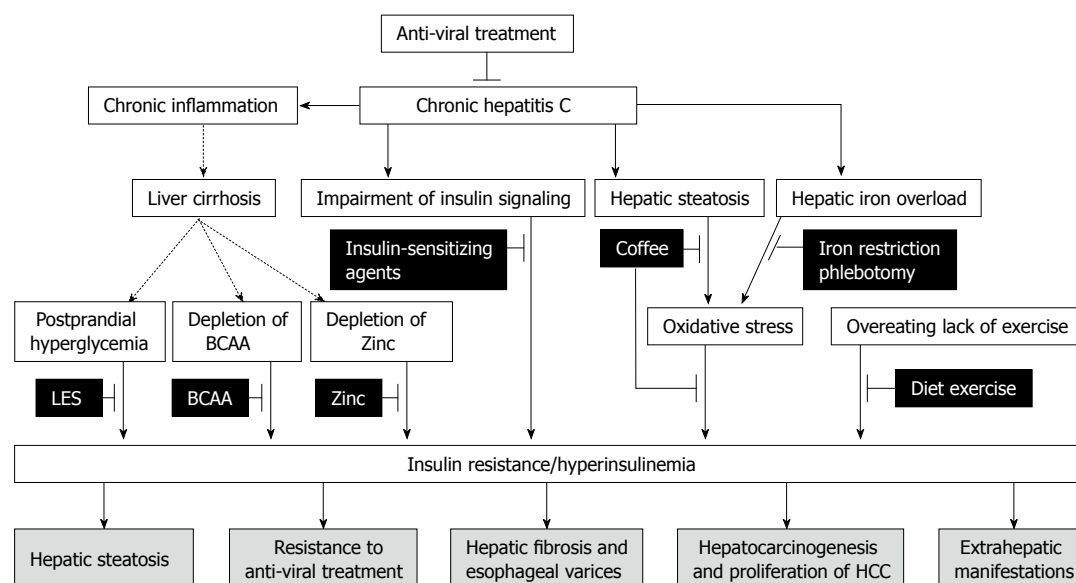


Figure 1 Pathogenic mechanisms and therapeutic strategies for hepatitis C virus (HCV)-associated insulin resistance. Black squares indicate therapeutic strategies for HCV-associated insulin resistance. Proper diet, exercise, iron restriction, phlebotomy, and coffee intake are recommended to any stage of liver disease. In cirrhotic patients, a late evening snack (LES), branched-chain amino acid (BCAA) supplementation, and a zinc supplement are also recommended. Insulin-sensitizing agents can be used in patients with chronic hepatitis C, however, the agents are not always recommended for patients with liver cirrhosis because of severe adverse effects.

of hepatic fibrosis in patients with HCV infection^[6,11,55]. The hepatocyte is known to degrade circulating insulin, and, therefore, hepatic fibrosis may reduce insulin clearance, resulting in increased serum insulin levels regardless of the presence of insulin resistance^[56]. However, insulin resistance is seen in early stages of chronic hepatitis C^[6]. Furthermore, even in patients that have received a liver transplantation for HCV-related liver cirrhosis, insulin resistance is a risk factor for rapid progression of hepatic fibrosis^[57]. These findings suggest that insulin resistance promotes hepatic fibrosis. Insulin resistance may directly affect hepatic stellate cells and increase connective tissue growth factor (CTGF), which causes production of extracellular matrix^[58]. Alternatively, insulin resistance-induced hepatic lipid accumulation may increase oxidative stress, resulting in progression of hepatic fibrosis^[32].

Insulin resistance is also a risk factor for esophageal varices in cirrhotic patients with HCV infection^[59]. As the hepatic fibrosis is correlated with the development of esophageal varices, insulin resistance may be associated with the development of esophageal varices through progression of hepatic fibrosis^[60]. In addition, insulin modulates the endothelial synthesis of nitric oxide and endothelin^[61], regulators of sinusoidal blood flow^[62]. Thus, insulin-induced hepatic fibrosis and vasoconstriction may be possible mechanisms for the development of esophageal varices.

Hepatocarcinogenesis and proliferation of HCC

Liver cirrhosis, aging, and being a male are well-known risk factors for the development of HCC in patients with HCV infection^[18,63]. In addition, insulin resistance is now recognized as an independent risk factor for the devel-

opment of HCC worldwide^[18,63]. Diabetes is reported as the only independent risk factor for HCC in patients with chronic hepatitis C^[21]. Moreover, development of diabetes-related HCC is reported to be independent of viral hepatitis and alcoholism^[64]. These findings suggest that insulin resistance has direct effects on hepatocarcinogenesis. Although precise mechanisms for this effect remain unclear, the following explanations may be put forward. Insulin resistance causes lipid accumulation^[19]. Visceral adiposity results in changes in serum adipocytokine levels, including reduction of adiponectin, which suppresses effects for hepatocarcinogenesis^[65]. Hepatic lipid accumulation also increases oxidative stress, which may be responsible for the development of HCC^[18,63]. Besides these possibilities, insulin has a mitogenic effect^[19,30], suggesting that insulin may be directly linked to hepatocarcinogenesis^[19].

Insulin resistance may be associated not only with hepatocarcinogenesis, but also with proliferation of HCC. We have examined the significance of insulin resistance on the prognosis in patients with HCV-associated HCC and found that insulin resistance is an independent risk factor for poor prognosis^[20]. As no significant difference was seen in disease-free survival between patients with and without insulin resistance, these findings indicate that insulin resistance accelerates the proliferation of HCC^[20]. In good accordance with our results, Saito *et al.*^[66] reported that reduction of serum insulin levels by continuous infusion of octreotide significantly suppressed proliferation of HCC. Although the mechanisms for insulin-induced proliferation of HCC remain obscure, insulin exerts growth-promoting activity through activation of a mitogen-activated protein kinase pathway^[19]. In addition, overexpression of transducing molecules for insulin signaling, IRS1^[67] and IRS2^[68],

and downregulation of suppressing molecules for insulin signaling, phosphatase and tensin homologue^[69], and SH2 domain-containing inositol phosphatase-2^[20] occur in HCC. Thus, HCC may be sensitive to insulin stimulation.

Extrahepatic manifestations

HCV causes extrahepatic manifestations including mixed cryoglobulinemia, Sjögren's syndrome, and non-Hodgkin lymphoma, oral lichen planus, oral squamous cell carcinoma, and malignancies other than HCC^[22-24,70-73]. In patients with extrahepatic manifestations of HCV, fasting insulin levels and homeostasis model assessment for insulin resistance are significantly higher than for patients without extrahepatic manifestations^[22]. Among various extrahepatic manifestations, insulin resistance is associated with oral lichen planus^[23], oral squamous cell carcinoma^[24], and multiple primary cancers including gastric cancer^[24]. Although reasons for this association remain unclear, a high prevalence of precancerous lesions and cancers are seen in patients with type 2 diabetes mellitus^[74,75], suggesting that insulin resistance or hyperinsulinemia may enhance carcinogenic activities.

DISTINCTIVE THERAPEUTIC STRATEGY FOR HCV-ASSOCIATED INSULIN RESISTANCE

Despite awareness of the increased risk of insulin resistance, therapeutic guidelines to inhibit distinctive complications of HCV-associated insulin resistance have not yet been established. HCV itself has a significant impact on the development of insulin resistance, and eradication of HCV improves insulin resistance^[44,46,76]. Thus, anti-viral therapy is a fundamental therapeutic strategy for patients with HCV infection. In addition, amelioration of insulin resistance is considered to inhibit complications and improve prognosis. Here, we summarize treatments which could improve HCV-associated insulin resistance as therapeutic strategies (Figure 1).

Late evening snack

Proper diet and exercise are fundamental for patients with lifestyle-associated insulin resistance as well as patients with HCV-associated insulin resistance^[77-80]. As a nutritional treatment for liver cirrhosis, divided energy intake (4 to 6 meals/d) has been recommended^[77,79]. As postprandial hyperglycemia is characteristic of HCV-associated insulin resistance^[77-80], a decrease in energy intake per meal reduces postprandial hyperglycemia and hyperinsulinemia. In particular, a late evening snack is reported not only to improve glucose intolerance^[81-84], but also to suppress hepatocarcinogenesis in cirrhotic patients^[85].

Coffee consumption

Coffee consumption reduces the risk of elevated serum alanine aminotransferase activity^[86], hepatic fibrosis^[87], and disease progression in chronic hepatitis C^[88]. Coffee

consumption also reduces the risk of HCC independent of HCC etiology^[89]. Caffeine is metabolized by hepatic cytochrome P450 1A2 into 3 metabolites, the dimethylxanthines paraxanthine, theobromine, and theophylline. Of these metabolites, theophylline inhibits transforming growth factor- β -stimulated CTGF expression through PPAR γ and Smad 2/3-dependent pathways. Since CTGF and transforming growth factor- β are important factors associated with progression of hepatic fibrosis and hepatocarcinogenesis, a metabolite of caffeine, theophylline, may have an inhibitory effect on the development of complications associated with HCV infection. In addition, coffee has significant effects on glucose metabolism^[90]. In an animal experiment, the insulin-sensitizing effects of coffee have been demonstrated^[91]. Similarly, in a human study, coffee consumption reduced fasting glucose and insulin levels^[90,92]. Although the mechanisms for the coffee-induced insulin-sensitizing effect remain unclear, some possibilities exist. Chlorogenic acids, a constituent of coffee, inhibits hepatic glucose-6-phosphate translocation^[90,93], limits glucose absorption from the gut by inhibiting Na⁺-dependent transport^[94], and increases the secretion of glucose regulating hormone, glucagon-like peptide (GLP)-1, from the gut^[90,95,96]. These findings suggest that a constituent of coffee, chlorogenic acid, directly ameliorates HCV-associated insulin resistance. Furthermore, coffee modulates lipid metabolism^[97,98] and lowers body weight^[90], indicating that coffee may suppress the lipid-induced increase in oxidative stress and ameliorates HCV-associated insulin resistance.

Phlebotomy

Hepatic iron overload produces oxidative stress and is a factor responsible for the development of HCV-associated insulin resistance^[4,99-101]. Although the pathogenesis of hepatic iron overload remains unclear, recent studies showed that iron-regulating molecules are modulated by HCV infection. Hepcidin is a negative regulator of duodenal iron absorption and macrophage iron release^[100] and decreased hepatic expression of hepcidin is seen in both HCV polyprotein transgenic mice^[102] and patients with HCV infection^[103-105]. In addition, upregulation of hepatic expression of transferrin receptor 2, a mediator of iron uptake, is responsible for hepatic iron overload^[106].

In order to reduce hepatic iron deposition, dietary iron restriction and phlebotomy are effective. Dietary iron restriction (less than 7 mg/d) decreases serum alanine aminotransferase levels in patients with HCV infection^[107]. Phlebotomy reduces oxidative stress as well as insulin resistance in patients with HCV infection^[101,108,109]. A long-term combination treatment with phlebotomy and dietary iron restriction reduces the risk of development of HCC in patients with HCV infection^[110].

Supplementation of zinc

Zinc plays a crucial role in the metabolism of protein, carbohydrate, lipid, nucleic acid, and ammonia^[111-113]. In fact, zinc supplementation improves glucose disposal in patients

with cirrhosis^[114]. Zinc also inhibits hepatic inflammation^[115] and hepatic fibrosis^[116]. More recently, zinc supplementation was shown to lower the cumulative incidence of HCC in patients with HCV infection^[117]. It is unclear whether these inhibitory effects of zinc on progression of liver disease are mediated by amelioration of insulin resistance. However, zinc participates in the synthesis, storage and secretion of insulin^[118] and regulates the binding ability of insulin to bind to its receptor^[113]. As the serum zinc level is decreased in patients with HCV infection^[115,117], supplementation of zinc could be a therapeutic option.

Anti-diabetic agents

Exogenous insulin and sulfonylurea agents: Anti-diabetic agents are effective for decreasing plasma glucose and HbA1c levels, leading to prevention of diabetes mellitus-associated complications including cardiovascular diseases^[119,120]. However, it has never been determined whether anti-diabetic agents prevent complications or improve prognosis in patients with HCV infection. Use of exogenous insulin or sulfonylurea agents may worsen hyperinsulinemia. In fact, we, along with others, recently reported an association between exogenous insulin or sulphonylurea treatment and the development of HCC in patients with HCV infection^[29,30,121]. Use of exogenous insulin is also reported to be associated with the development of colon cancer^[122] and other malignancies^[123]. Although a causal relationship between exogenous insulin and the development of HCC remains controversial^[124], the reduction of serum insulin levels is a first line therapeutic strategy for insulin resistance^[125-128].

Insulin-sensitizing agents: Insulin resistance is associated with a poor response to anti-viral treatment in patients with HCV infection^[10,43-46]. Amelioration of insulin resistance may improve the response to anti-viral treatment. However, the impact of insulin-sensitizing agents, biguanides and thiazolidinediones, on sustained virologic response (SVR) rates has not yet been established. Recently, metformin, a biguanide agent, has been reported to ameliorate HCV-associated insulin resistance, and increase the SVR rate in HCV genotype 1 infected patients with normalization of homeostasis model assessment for insulin resistance at week 24 of therapy^[129]. Pioglitazone, a thiazolidinedione agent, has also been reported to ameliorate insulin resistance and increase SVR rates in patients with HCV genotype 4 infection^[130]. Although the insulin-sensitizing mechanisms of metformin and pioglitazone are different, both agents are known to upregulate IRS^[131,132], which is the molecule responsible for HCV-associated insulin resistance^[3,6,50], and to improve HCV-associated insulin resistance. Because both agents have severe adverse effects, neither is recommended for patients with liver cirrhosis. Biguanides predispose cirrhotic patients to lactic acidosis^[133]. Thiazolidinediones cause overproduction of hydrogen peroxide leading to severe hepatotoxicity^[134]. Thus, further validation for safety is required.

Dipeptidyl peptidase IV (DPPIV) inhibitor is a new

therapeutic agent^[135] and its clinical efficacy in type 2 diabetes has been shown^[136]. Although no study has examined the effect of DPPIV inhibitor on HCV-associated insulin resistance, we found that activation of DPPIV is a factor responsible for HCV-associated insulin resistance^[27]. Thus, a DPPIV inhibitor may be suited for ameliorating HCV-associated insulin resistance.

BCAA supplementation, a possible insulin-sensitizing agent

BCAA are constituents of proteins and are required for protein synthesis^[19,78,137,138]. In addition, BCAA are reported to modulate glucose metabolism. Leucine and isoleucine induce glucose transporter 1 and 4 translocation to the plasma membrane of muscle cells and improve glucose metabolism in a carbon tetrachloride-induced cirrhotic rat model^[139]. In addition, leucine enhances the insulin-induced activation of the Akt/mammalian target of the rapamycin pathway in adipocytes of db/db mice^[140]. Moreover, isoleucine increases hepatic phosphatidylinositol 3-kinase activity and improves insulin resistance in Zucker fa/fa rats, a model of severe insulin resistance^[141]. Recently, knockout of the mitochondrial BCAA aminotransferase gene in mice, in which results in elevated plasma BCAA levels, was found to ameliorate insulin resistance^[142]. Thus, BCAA improve insulin signaling in various animal models *via* several pathways. In good agreement with these results in animals, in human studies, we have recently shown that BCAA-enriched supplementation reduces insulin resistance in patients with HCV infection^[143,144]. In a multicenter, randomized, controlled trial, BCAA supplementation led to a reduction in the risk of HCC in cirrhotic patients^[145]. This suppressive effect on hepatocarcinogenesis was more evident in obese patients with HCV infection^[145]. Both obesity and HCV induce the development of insulin resistance. Thus, BCAA may improve insulin resistance and subsequently inhibit insulin resistance-induced hepatocarcinogenesis^[19,145].

CONCLUSION

In this review, we summarize the distinctive complications of, and therapeutic strategies for, HCV-associated insulin resistance. Although cardiovascular diseases, renal failure, and infections are well-known complications of lifestyle-associated insulin resistance, these complications are not major causes of death in cirrhotic patients with insulin resistance. HCV-associated insulin resistance rather causes (1) hepatic steatosis, (2) resistance to anti-viral treatment, (3) hepatic fibrosis and esophageal varices, (4) hepatocarcinogenesis and proliferation of HCC, and (5) extrahepatic manifestations. These complications are life-threatening, and therapeutic strategies for HCV-associated insulin resistance have to be considered on the basis of its pathogenic mechanisms.

Pathogenic mechanisms for HCV-associated insulin resistance differ from those for lifestyle-associated insulin resistance. Postprandial hyperglycemia, lipid-induced oxi-

ductive stress, hepatic iron overload, and depletion of zinc are responsible for the development of HCV-associated insulin resistance. Therefore, a late evening snack, coffee consumption, dietary iron restriction, phlebotomy, and supplementation of zinc are recommended therapeutic strategies. No clinical guidelines for the use of anti-diabetic agents are available for patients with HCV-associated insulin resistance. However, use of exogenous insulin or sulphonylurea may increase the risk for HCC. On the other hand, insulin-sensitizing agents may improve the SVR rate of anti-viral treatment. In addition, BCAA supplementation has an insulin-sensitizing effect as well as a suppressive effect on hepatocarcinogenesis. Thus, in order to ameliorate HCV-associated insulin resistance, various therapeutic approaches are required.

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Natural epitope variants of the hepatitis C virus impair cytotoxic T lymphocyte activity

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Abstract

AIM: To understand how interactions between hepatitis C virus (HCV) and the host's immune system might lead to viral persistence or effective elimination of HCV.

METHODS: Nucleotides 3519-3935 of the non-structural 3 (NS3) region were amplified by using reverse transcription polymerase chain reaction (PCR). PCR products of the HCV NS3 regions were integrated into a PCR[®] T7TOPO[®] TA vector and then sequenced in both directions using an automated DNA sequencer. Relative major histocompatibility complex binding levels of wild-type and variant peptides were performed by fluorescence polarization-based peptide competition assays. Peptides with wild type and variant sequences of NS3 were synthesized locally using F-moc chemistry

and purified by high-performance liquid chromatography. Specific cytotoxic T lymphocytes (CTLs) clones toward HCV NS3 wild-type peptides were generated through limiting dilution cloning. The CTL clones specifically recognizing HCV NS3 wild-type peptides were tested by tetramer staining and flow cytometry. Cytolytic activity of CTL clones was measured using target cells labeled with the fluorescence enhancing ligand, DELFIA EuTDA.

RESULTS: The pattern of natural variants within three human leukocyte antigen (HLA)-A2-restricted NS3 epitopes has been examined in one patient with chronic HCV infection at 12, 28 and 63 mo post-infection. Results obtained may provide convincing evidence of immune selection pressure for all epitopes investigated. Statistical analysis of the extensive sequence variation found within these NS3 epitopes favors a Darwinian selection model of variant viruses. Mutations within the epitopes coincided with the decline of CTL responses, and peptide-binding studies suggested a significant impact of the mutation on T cell recognition rather than peptide presentation by HLA molecules. While most variants were either not recognized or elicited low responses, such could antagonize CTL responses to target cells pulsed with wild-type peptides.

CONCLUSION: Cross-recognition of CTL epitopes from wild-type and naturally-occurring HCV variants may lead to impaired immune responses and ultimately contribute to viral persistence.

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Key words: Epitopes; Human; T Cells; Cytotoxic; Anergy; Viral

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INTRODUCTION

Hepatitis C virus (HCV) is one of the leading causes of chronic liver disease^[1] arising from persistent infection that lasts decades despite evidence of humoral and cellular immune responses^[2]. Chronic infection occurs in up to 85% of patients^[3]. The mechanisms responsible for high rates of HCV persistence are unknown, but are thought to involve a complex interplay between the host immune system and viral diversity^[4], which may lead to viral escape through the mutation of epitopes recognized as targets of the immune response^[4,5]. The combination of a very high rate of HCV replication, estimated at 10^{12} virions per day^[6], and an RNA-dependent RNA polymerase that lacks proofreading ability^[7] sets the stage for Darwinian selection of variant or mutant viruses *via* pressure mediated by humoral and cellular immune responses^[8].

Selective pressure appears to be applied by all elements of the immune response including antibody-producing B-cells and both CD4⁺ and CD8⁺ T cells. Such persistence of HCV infection has been particularly associated with mutations in epitopes encompassed within the hypervariable region 1 of HCV envelope glycoprotein 2, recognized by both antibodies and CD4⁺ helper T cells^[9,10]. Studying a class II restricted immunodominant epitope within the non-structural 3 (NS3) protein region of HCV, we have identified a highly significant variation that correlated with escape from CD4⁺ T cell responses^[11-14]. Other sequence variations in epitopes recognized by CD8⁺ cytotoxic T lymphocytes (CTLs) have been identified in chimpanzees^[4,15] and humans^[16-22] with chronic HCV infections.

CTLs recognize peptide fragments of cellular or viral proteins in the form of short peptides comprising 8-11 amino acids presented in association with major histocompatibility complex (MHC) class I molecules on the surface of infected cells^[23-27]. These peptides are usually derived from intracellular viral protein pools and associated in the lumen of the endoplasmic reticulum with MHC class I molecules, after which the MHC-peptide complex is transported to the cell surface and recognized by a specific T cell receptor (TCR) located on the surface of the CD8⁺ killer T cell.

Variation within a viral epitope can lead to a total or partial loss of functional recognition by CTL. Substitutions occurring at key anchor residues may alter peptide affinity for MHC class I molecules and thereby interfere with antigen presentation and effector T-cell mediated clearance of infected cells. Other variations, primarily in solvent-accessible residues, may abrogate TCR recogni-

tion altogether or alter it in such a way that critical activation signals are not transmitted to the cytotoxic T cells resulting in attenuated responses or even anergy^[10,16-18]. Examples of attenuated responses have also been found with HIV and HBV^[28-32]. It is thought that by antagonizing T-cell responses to native epitopes, viruses expressing mutant epitopes might aid in the survival of infected cells producing wild-type viruses, which would otherwise be recognized and destroyed by CTL. Although parts of this issue have been examined in chronic HCV infection^[15-18,33], unresolved questions remain, including whether naturally occurring variants antagonize CTL responses to wild-type epitopes that are found within the same host. To address this, viral sequences were examined by assessing CTL activity in three epitopes that were previously identified by Koziel *et al.*^[34]. Comprising amino acids 1073-1081, 1131-1139, and 1169-1177, these 9-mer epitopes are restricted by human leukocyte antigen (HLA)-A*02 and recognized by specific CTL clones. Initially, we examined epitope heterogeneity in the viral sequences from an A*02-positive patient applying polymerase chain reaction (PCR) technology. This was followed by the synthesis of peptides corresponding to observed variations in these epitopes and subsequently used to sensitize A*02-expressing target cells. Results illustrate allele-specific viral evolution and escape from a dominant CD8⁺ CTL response. While most variants were either not recognized or elicited low responses, such could antagonize CTL responses to target cells pulsed with wild-type peptides. The ability to interfere with CTL function was independent of the ability of variant peptides to bind MHC molecules. Overall, we view this study as a bridge to understanding how interactions between HCV and the host's immune system might lead to viral persistence or effective elimination of HCV.

MATERIALS AND METHODS

Human subjects

Peripheral blood samples were collected from a patient B3019 with chronic HCV at approximately 12 mo (B3019.1), 28 mo (B3019.3), and 63 mo (B3019.5) after infection. This patient never received any therapeutic intervention during the 5 years chronic HCV infection. The presence of HCV-specific antibodies and HCV RNA in the patient's serum was determined as described previously^[11]. Blood was collected in acid citrate dextrose anticoagulant, centrifuged at $400 \times g$ for 15 min, and divided into plasma and buffy coat fractions. After isolation of peripheral blood mononuclear cells (PBMC) over Lymphocyte Separation Medium (Organon/Teknika), plasma and PBMC were stored at -70°C or in liquid nitrogen, respectively.

Amplification of the HCV NS3 region using reverse transcription PCR

Total HCV genomic RNA was isolated from 140 µL of B3019.1, 3019.3 and 3019.5 sera using the QIAamp® Viral RNA Mini Kit (QIAGEN, Inc.). The cDNA

was synthesized using reverse transcriptase (RT) from Moloney's murine leukemia virus and random hexadecoxynucleotide primers (Invitrogen). HCV cDNA was then amplified using nested primer sets ("outer" sense prime: 5'-GGCCTCCTAGGGTGTATAATCACC-3'; "outer" antisense primer: 5'-GAGGAGTTGTCCGTGAACAC-3'; "inner" sense primer: 5'-CAGATCGTGTCAACTGCTAC-3'; "inner" antisense primer 5'-CCA-CAGGGATAAAGTCCACC-3') specific for nucleotides 3519-3935 of the NS3 region. Primers were created based on the previously reported HCV sequence from genotypes 1a, (isolate H77), which generated a final PCR product of 417 bp.

Initial PCR was performed using the outer primer set starting with heat-activation of the ProofStart DNA Polymerase at 95°C for 5 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s and extension at 72°C for 1 min. A final extension step at 72°C for 10 min was introduced to increase the pool of full-length products. Two microliters of the first amplification product was transferred into the second nested PCR reaction mixture containing the "inner" primer pair. The second round of amplification was performed for 35 cycles under equal conditions. For all PCR amplifications, ProofStart DNA Polymerase (QIAGEN) with proofreading capabilities was used. PCR products were analyzed on a 1.5% agarose gel stained with ethidium bromide. As described elsewhere^[35], all measures were taken to avoid contamination of PCR samples.

As a control strategy for polymerase errors, two different experimental approaches were applied. In a first experiment, the plasmid pT7 TOPO-TA/NS3 from the Hutchinson strain (1a) of HCV^[36] was diluted to 10⁻¹⁴ g/mL and amplified using the same PCR procedure described above. In an alternative experiment, reverse transcription PCR (RT-PCR) was used to amplify an RNA template derived from the pT7 TOPO-TA/NS3 plasmid. The RNA template was obtained utilizing a T7 RNA polymerase (USB) according to the supplier's instructions. The resulting transcript was treated with DNase I for 15 min at room temperature and RNA was extracted using the QIAamp® Viral RNA Mini Kit (QIAGEN, Inc.). Reverse transcription and amplification of this control RNA was carried out as described above.

Cloning and sequencing

PCR products of the HCV NS3 regions were integrated into a PCR® T7TOPO® TA vector (Invitrogen, Carlsbad, CA). Ligations and transformations were executed according to the manufacturer's instructions. Recombinant clones were then screened for positive PCR product integration by using a PCR amplification procedure detecting HCV NS3 inserted fragments. Plasmid DNAs with confirmed inserts were purified with QIAprep® spin miniprep kit (QIAGEN, Inc.) according to the standard protocol from Qiagen and further analyzed. Thirty independent clones for each sample of B3019.1, B3019.3 and B3019.5, as well as 23 and 17 independent clones

for each control strategy, respectively, were sequenced in both directions using an automated DNA sequencer (373A, Applied Biosystems). The sequencing results were analyzed using GCG SeqWeb package (V2.0.2). The polymerase error rate under applied conditions was calculated as [(No. of sporadic changes)/[(No. of clones) × (sequence length) × (PCR cycles)] as described by Smith *et al*^[37].

Peptide synthesis

Peptides with wild type and variant sequences of NS3 1073-1081, NS3 1131-1139 and NS3 1169-1177 were synthesized locally using F-moc chemistry and purified by high-performance liquid chromatography (HPLC). Peptide powder was dissolved in a drop of DMSO and adjusted to approximately 1 mg/mL with RPMI 1640 tissue culture medium before being used to stimulate PBMC in CTL cloning, cytotoxicity and antagonist assays. For the competition assay procedure, the FITC-labeled peptide was commercially synthesized by Synpep (Dublin, CA) using solid-phase strategies and purified with reverse-phase HPLC. For this procedure, NS3 peptides were originally dissolved in 100% DMSO at a concentration of 10 mmol/L. Subsequent dilutions were done in 1 × bovine γ globulin in PBS (BGG/PBS; 0.5 mg/mL; 0.05%; Sigma; St. Louis, MO).

Fluorescence polarization-based peptide competition assay

To determine relative MHC binding levels of wild-type and variant peptides, fluorescence polarization (FP)-based peptide competition assays were performed as described^[38,39]. Initially, the four components of the binding reaction (competitor peptide, tracer, sHLA and β2m) were prepared as concentrates. The fluorescent-labeled tracer peptide (pFITC P5), ALMDKVL-K(FITC)-V, and the sHLA-A*0201 component of the reaction were diluted to appropriate 8 × and 2 × solutions, respectively. The β2m component (Fitzgerald Industries International; Concord, MA) was prepared as an 8 × mix and always added in a 2 × molar excess of the used sHLA concentration. Each competitor peptide was prepared at various dilutions and added as 4 × solutions. For all preparations, 1 × BGG/PBS was used as buffer. Next, each individual well of a black 96-well LJJL HE PS microplate (Molecular Devices) was loaded with 5 μL of the prepared 8 × β2m, 10 μL of each competitor solution, and 5 μL of 8 × pFITC. To start the peptide exchange procedure, the 2 × sHLA mixes was activated by incubating at 53°C for 15 min before adding 20 μL to the previously loaded wells reaching a final volume of 40 μL. All reagents were added to the wells of the microtiter plate sequentially using manual pipettors. The plates were then read at room temperature using an Analyst AD (Molecular Devices; Sunnyvale, CA) until no further increase in polarization was observed indicating that equilibrium was reached (24-48 h). Data analysis was performed using the software package Prism (GraphPad), by direct fit to the

appropriate models by computer-aided, nonlinear regression analysis.

CTL cloning

Specific CTL clones toward HCV NS3 wild-type peptides 1073-1081 (wt1073), 1131-1139 (wt1131) and 1169 (wt1169) were generated. Briefly, PBMCs were thawed, diluted rapidly at 4°C, washed twice by centrifugation at $400 \times g$ for 10 min, resuspended and plated into wells of 96 well flat bottom plates (200 μ L) at a density of 2×10^6 cells/mL. Cells were then stimulated individually with wild-type NS3 peptides 10 nmol/L at 37°C in a 5% CO₂ incubator. After 7 d of incubation, 20 U/mL rhIL-2 (ENDOGEN) was added to the cultures. On day 14, cells were screened for the ability to lyse target cells pulsed with NS3 wild-type peptide. Functional cells were further subcloned by limiting dilution (at cell densities of 10, 3, 1 and 0.3 cells/well) in 96 well round bottom plates which contained 10^5 irradiated, autologous PBMCs, 20 U/mL rhIL2, and 10 nmol/L HCV NS3 wild-type peptide. Positive clones were further expanded and re-stimulated in 24-well plates with 10^5 irradiated (3000 rad) autologous PBMCs, in the presence of 10 nmol/L wild-type peptide and 20 U/mL rhIL-2 in RPMI 1640 medium supplemented with 25 mmol/L HEPES buffer, 2.0 mmol/L L-glutamine, 50 U/mL penicillin, and 100 mg/mL streptomycin, 5.0 mg/mL gentamicin, 10 U/mL sodium heparin, 1.0 mmol/L sodium-pyruvate, and 10% pooled AB human serum (complete RPMI-10 AB). Finally, cells were tested for cytolytic recognition of B-LCL targets pulsed separately with wild-type peptides. 14 d after the last stimulation, specific CTL clones were maintained in a long term culture in T-25 flasks by re-stimulating 2×10^6 cells every 2 wk with 1×10^6 irradiated (3000 rad) allogeneic PBMC feeders and 50 U/mL rIL-2 in complete RPMI-10 AB media. Not immediately used clones were frozen in liquid nitrogen for later usage.

Tetramer staining and flow cytometry

The CTL clones specifically recognizing HCV NS3 wild-type peptides were washed with 10% FCS in PBS followed by staining with the HCV NS3 1073-1081 peptide MHC class I tetramer complexes as described previously^[40]. The following antibodies and tetramer complexes were used: Anti-CD8-FITC, anti-mouse IgG FITC (Pharmingen, San Diego, CA, USA), HLA-A*02-restricted HIV p17 epitope tetramer PE, HLA-A*02-restricted HCV NS3 1073-1081 tetramer PE. Specific CTL clones were incubated with the antibodies and tetramer reagents for 45 min at room temperature in the dark, then washed with $1 \times$ PBS and resuspended in 500 μ L of $1 \times$ PBS. Samples were analyzed on a FACScan flow cytometer (Becton-Dickinson, Mountain View, CA, USA). The data were analyzed by WinMDI 2.8 software, kindly provided by Dr. Joel Trotter. Both the HCV NS3 1073-1081 peptide-MHC class I tetramer complex and the negative control tetramer complex HIV p17 peptide SLYNTVATL were made by the NIAID tet-

ramer Facility, Emory University Vaccine Center (Emory University, Atlanta, GA, USA).

Cytotoxicity assay

Cytolytic activity of CTL clones was measured using target cells labeled with the fluorescence enhancing ligand, DELFIA EuTDA (Perkin-Elmer Life Sciences, Norwalk, CT) according to the manufacturer's instructions. As target cells, the HLA-A*02 positive Epstein-Barr virus (EBV) transformed B-cell line (L.B3019) was labeled with DELFIA BATDA reagents at 37°C for 20 min, washed three times, and incubated with the indicated concentration of peptide for 1 h. After three additional washes, effector cells were added at various concentrations and incubated for 2 h in 96-well round-bottom plates (5000 target cells per well) at 37°C in 5% CO₂. After centrifugation at $500 \times g$ for 5 min, 20 μ L of supernatant was transferred to corresponding wells of a flat bottom plate and 200 μ L of europium solution was added. Fluorescence was measured using a Wallace Victor2 Multilabel Counter (Perkin-Elmer Life Sciences). Percent specific release was calculated according to the following formula: Percent specific lysis = $100 \times [(experimental\ release - spontaneous\ release) / (maximum\ release - spontaneous\ release)]$. Results were reported as the means of duplicate wells.

Antagonist assay

To measure the ability of each variant peptide to antagonize CTL responsiveness against wild-type peptide, an antagonist assay was performed using the method described by Jameson *et al.*^[41] under slightly modified conditions. Briefly, target cells were labeled with DELFIA BATDA reagents at 37°C for 20 min, washed three times with RPMI 1640 medium, and then pulsed for 1 h with 10 nmol/L wild-type peptide. After removal of the wild-type peptide by another wash step ($3 \times$), the cells were pulsed a second time using varying concentrations of variant or control peptide for another hour. After a final wash step, specific CTL effector cells were added at various concentrations and incubated for 2 h. After incubation, the cytolytic activity of CTL was measured as described above. Percent inhibition of lysis was calculated as $\{\% \text{ inhibition} = 100 \times [(A-B)/A]\}$ where A is the percent specific lysis in the absence and B is the percent lysis in the presence of the variant peptide under investigation. Each point represents the mean of duplicate wells.

RESULTS

Mutations in the HCV NS3 region

The majority of RNA viruses produce RNA polymerases that lack proofreading activity, and thus introduce mutations into the viral genome. In the presence of immune selection pressure exerted by CTLs against wild-type virus, this genomic diversity could facilitate preferential expansion of mutant progeny encoding altered

	Epitope 1073-1081	Epitope 1131-1139	Epitope 1169-1177
	Wt QIVSTATQTFLAT CINGVCWTV YHAGTRTIA SPKGPVIQMYTNVDQDLVGWPAPOGSRSLTPCTCGSSDL YLVTRHADV IPVRRRGDSRGSLLSPRPISYLKGS SGGP LLCPAGHAV GLFRAAVCTRGVAKAVDFIPV		
12- 1	-----	-----T-----	-----
12- 2	-----	-----	-----y-----
12- 3	-----	-----H-----	-----P-----
12- 4	-----	-----Δ-----	-----
12- 5	-----	-----Δ-----	-----
12- 6	-----	-----	-----R-----
12- 7	-----S-----	-----	-----
12- 8	-----A-----	-----	-----
12- 9	-----	-----A-----	-----
12-10	-----A-----	-----V-----	-----
12-11	-----S-----	-----	-----
28-12	-----	-----	C-----
28-13	-----	H-----	-----R-----
28-14	-----	-----	-----C-----
28-15	-----	-----	-----P-----
28-16	-----	-----Δ-----	-----
28-17	-----	-----	-----V-----
28-18	-----Δ-----	-----	-----S-----
28-19	-----R-----	-----	-----
28-20	-----	-----	-----R-----
28-21	-----Y-----	-----V-----	-----
28-22	-----Δ-----	-----	-----
28-23	-----	-----G-----	-----
28-24	-----	-----D-----	-----M-----
28-25	-----	-----	-----Δ-----
63-26	-----A-----	-----	-----
63-27	-----	-----T-----	-----
63-28	-----R-----	-----	-----
63-29	-----	-----D-----	-----M-----
63-30	-----Δ-----	-----	-----T-----
63-31	-----	-----	-----G-----
63-32	-----	-----D-----	-----
63-33	-----	-----A-----	-----
63-34	-----	-----D-----	-----
63-35	-----	-----	-----S-----
63-36	-----	-----Δ-----	-----
63-37	-----	-----	-----Δ-----
63-38	-----	-----Δ-----	-----
63-39	-----Δ-----	-----	-----
63-40	-----Δ-----	-----A-----	-----

Figure 1 Summary of sequence changes detected over time within the hepatitis C virus (HCV) non-structural 3 (NS3) fragment of a single patient. HCV RNA extracted from patient sera at months 12, 28 and 63 was amplified by reverse transcription polymerase chain reaction (RT-PCR). A panel of molecular clones spanning the NS3 fragment was aligned with the wild-type consensus sequence which is shown on top (underlined). From a total of 51 nucleotide substitutions found, 39 were nonsynonymous, causing amino acid alterations (capital letters). The amplified NS3 fragment encompasses epitopes NS3 1073-1081, 1131-1139 and 1169-1177. Dashed lines indicate identity to the consensus sequences, whereas positions of synonymous mutations not causing amino acid alterations are marked with the greek delta sign (Δ).

epitopes that evade recognition by effector T cells. To monitor HCV genetic diversity within a fragment of the NS3 region, three different sera samples of a single subject with chronic HCV, collected at approximately 12, 28, and 63 mo after infection, were investigated by sequence analysis. In particular, the region 1060-1198 was selected for analysis because of the inclusion of previously identified HLA-A*02 restricted epitopes comprising amino acids 1073-1081, 1131-1139, and 1169-1177. An overall sequence comparison to the wild-type sequence revealed a set of 40 out of 90 individually sequenced clones (44.4%) carrying one or more mutations (Table 1). Within the 40 alternative sequences, a total of 51 nucleotide

substitutions were detected of which 12 (23.5%) were synonymous (SYN) silent mutations and 39 (76.5%) were nonsynonymous (NSY) mutations, respectively, leading to specific amino acid alterations within this NS3 fragment (Figure 1). The frequency of sporadic substitutions was calculated as 1.9×10^{-5} according to the formula described by Smith *et al.*^[37]. Furthermore, the frequency of mutations observed at 3 different time points of disease progression for the same patient was not significantly different.

To determine if observed mutations are consistent with a positive Darwinian selection model as described in other reports^[13,14,20], we compared the ratios of NSY/

Table 1 Mutations in the HCV NS3 region

Sample	Month 12	Month 28	Month 63	Total
No. of clones tested	30	30	30	90
Clones with mutations	11	14	15	40
Total mutations	13	20	18	51
SYN mutations	2	4	6	12
NSY mutations	11	16	12	39

SYN: Synonymous; NSY: Nonsynonymous; HCV: Hepatitis C virus; NS3: Non-structural 3.

SYN mutation for the NS3 fragment covering amino acid 1060-1198. As defined earlier for positive Darwinian selection, the rate of NSY substitution usually exceeds the SYN substitution rate and heterogeneity increases more quickly, whereas at sites subject to negative selection, the NSY/SYN ratio is < 1 and the heterogeneity will be much lower^[37,42]. Among the total 51 nucleotide substitutions, 18 occurred in the first codon position, 18 at the second and 15 at the third position. According to the methods used by Nei *et al.*^[43] and Wang *et al.*^[44], theoretical values for SYN and NSY mutations were calculated as 11.7 and 39.3, respectively, which closely match our observed values of 12 for SYN and 39 for NSY. The total NSY/SYN ratio was 3.3, which is consistent with the positive Darwinian selection theory. Furthermore, we also compared our observed mutation values from different sections of the amplified region within NS3 with theoretical values for NSY mutations (Table 2). As seen in Figure 2, we found a significantly higher frequency of NSY mutations for section 1070-1119, 1130-1139, 1150-1159 and 1170-1189, earlier described as variable regions and also harboring the known HLA-A*02 epitopes. As expected, these findings are in contrast to the frequencies found within the so-called conservative regions covering sections 1060-1069 and 1120-1129 and showing a much lower mutation rate. It is notable that the 10-fold higher mutation rate within the epitope region 1073-1081, 1131-1139 and 1169-1177 compared to the conservative regions, with a NSY/SYN ratio of 2.8, suggests a high level of positive selective pressure on these immunogenic regions. No mutations were found in sections 1140-1149, 1160-1169 and 1190-1198 potentially carrying sequences not under immunological pressure within this patient. Variants do not seem to accumulate within CTL epitopes and most NSY are transient.

To ensure that the observed mutations were not due to nucleotide misincorporations introduced by the RT or polymerase during the amplification reaction, two control experiments were carried out: In the first experiment, 0.01 pg of a known NS3 plasmid template was subjected to PCR amplification in the usual two step protocol and subcloned. Sequence analysis of 23 independent clones showed absolute identity compared to the parental clone. In a second experiment, 0.01 pg of the same NS3 plasmid template was linearized and *in vitro* transcribed with T7 RNA polymerase. Limiting amounts of the RNA transcript was subjected to the

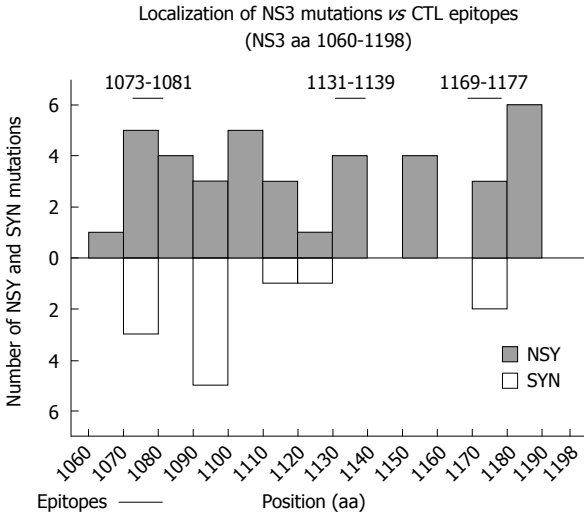


Figure 2 Localization of NS3 mutations in the proximity of three cytotoxic T lymphocyte (CTL) epitopes. Sequence alterations within the NS3 region between amino acid (aa) 1060-1198 are graphically visualized. Nonsynonymous (top) and synonymous (bottom) mutations are shown separately. NS3 CTL epitope regions 1073-1081, 1131-1139 and 1169-1177 are indicated.

complete RT-PCR amplification procedure, and the amplified product was subcloned and sequenced. Out of 17 independent clones sequenced, only one demonstrated a single nucleotide change. An error rate of 2.02×10^{-6} was calculated according to the formula described by Smith *et al.*^[37] and found to be well in line with the Pfu DNA polymerase error rate reported by others^[44,45]. Conclusively, these findings not only highlight the fidelity of the polymerase used for our viral genome amplification procedure, but also confirm the necessity of utilizing proofreading DNA polymerases to prevent false interpretation of mutations in genetic diversity studies of RNA viruses. Taken together, our results suggest that the mutations identified by our approach primarily represent naturally occurring mutations in the HCV RNA genome, rather than artificial PCR errors.

In addition, particular attention was given to the analysis of sequence changes encompassing epitopes recognized by CTL responses^[34]. Changes that emerged within stretches of sequence containing CTL epitopes are shown in Figure 1. As seen, sequence data from the epitopes defined within the NS3 region clearly reveal evolution in these target regions. By month 63 after transmission, bulk sequence data from the single targeted regions illustrates the development of mixed viral populations while still maintaining the original dominant sequence. Complete replacement of the initial virus population with viruses bearing nonsynonymous sequence changes within one or more epitope-containing regions could not be observed. For epitope CINGVCWTV (1073-1081), three mutations developed at residues 1, 6 and 7, whereas 4 different mutations were noted within epitope YLVTRHADV (1131-1139) with changes at residues 1, 4, and 6. Only 2 alterations were observed for epitope LLCPAGHAV at residues 3 and 8, which may indicate a less immunologically pressured epitope. These

Table 2 Nonsynonymous and synonymous mutations in different regions of HCV NS3

Region (aa)	Length (bp)	PCR cycles	No. of clones	Mutation		P	Mutation	
				Expected sporadic	NSY		SYN	NSY/ SYN
1060-1069	30	70	90	0.25	1	> 0.050	0	1/0
1070-1079	30	70	90	0.25	5	< 0.001	3	5/3
1080-1089	30	70	90	0.25	4	< 0.001	0	4/0
1090-1099	30	70	90	0.25	3	< 0.001	5	3/5
1100-1109	30	70	90	0.25	5	< 0.001	0	5/0
1110-1119	30	70	90	0.25	3	< 0.001	1	3/1
1120-1129	30	70	90	0.25	1	> 0.050	1	1/1
1130-1139	30	70	90	0.25	4	< 0.001	0	4/0
1140-1149	30	70	90	0.25	0	NS	0	0
1150-1159	30	70	90	0.25	4	< 0.001	0	4/0
1160-1169	30	70	90	0.25	0	NS	0	0
1170-1179	30	70	90	0.25	3	< 0.001	2	3/2
1180-1189	30	70	90	0.25	6	< 0.001	0	6/0
1190-1198	27	70	90	0.23	0	NS	0	0
1073-1081 ¹	27	70	90	0.23	4	< 0.001	2	4/2
1131-1139 ¹	27	70	90	0.23	4	< 0.001	0	4/0
1169-1177 ¹	27	70	90	0.23	3	< 0.001	2	3/2

¹Epitope region. Expected sporadic mutations = $ER \times L \times Nc \times N \times P/Ns$, where ER = error rate of polymerases (2.02×10^{-6}), L: Nucleotide length of compared region; Nc: Number of PCR cycles; N: Number of clones sequenced; P: Proportion of sporadic mutations expected to produce amino acid substitutions (2/3), Ns: Number of sample (1) according to Smith *et al.*^[37] (1997). PCR: Polymerase chain reaction; NS: No significant.

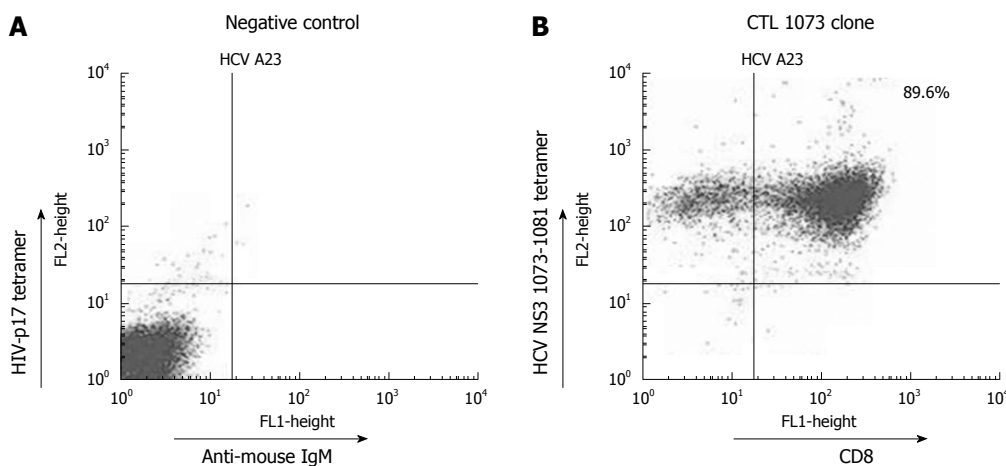


Figure 3 HCV NS3 1073-1081 tetramer staining using a CTL clone specific for wt1073. A: As a negative control, CTL clone 1073 was stained with the HIV-p17 tetramer reagent and anti-mouse IgM FITC. As expected, no cell shift was observed; B: The same CTL clone was stained with the HCV NS3 1073-1081 tetramer and anti-CD8⁺-FITC. A positive HCV NS3 1073-1081 tetramer staining is shown with tetramer/CD8⁺ cells located in the upper right quadrant. The origin of cells seen in the upper left quadrant could not be identified and need further investigation. All staining experiments were repeated three times with similar results. Identical results were obtained with HCV NS3 1073-1081 tetramer staining using other CTL clones for wt1073 (data not shown).

numbers are small, but consistent with the possibility of CTL-mediated selection.

Cytotoxicity of wt-specific CTL clones against various natural epitope variants

Next, we examined the phenotypic effects of these changes upon CTL recognition. For this reason, we initially produced CTL clones specifically recognizing target cells pulsed with wild-type peptides. CTL clone 1073 was generated recognizing MHC complexes loaded with wt1073, whereas CTL 1131 and CTL1169 were made to bind complexes harboring wt1131 and wt1169, respectively. Their specificity was confirmed in a separate set of experiments, where CTL clones were stained with

MHC class I tetramers loaded with the corresponding HCV NS3 wild-type peptide. Figure 3 shows the result for the CTL clone 1073.3 containing tetramer positive CD8⁺ cells in the upper-right quadrant. Additional staining experiments were performed using clones 1073.2 and 1073.4 with similar outcome (data not shown).

Subsequently, the cytolytic activity of these CTL clones was determined by incubating them with target cells pulsed with either variant or wild type peptides as reference. Results obtained for CTL 1073 (Figure 4A) showed a more than 80% cytolytic activity, confirming the capability of this CTL clone to recognize wild-type peptide wt1073 presented by the HLA-A*02 positive EBV transformed target B-cell line (LB3019). In all instances,

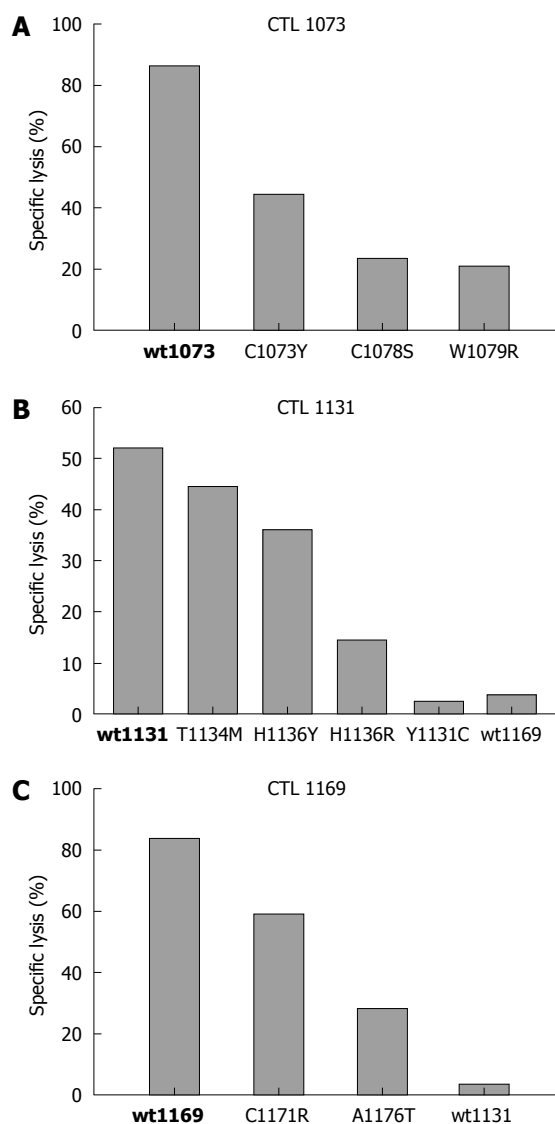


Figure 4 Effect of amino acid changes on epitope-specific CD8⁺ CTL responses. The relative ability of synthetic peptides corresponding to the indicated wild type (bold) and variant epitope sequences to sensitize autologous target cell lines for lysis by CTL clone 1073 specific for wt1073 (A), clone 1131 specific for wt1131 (B) and clone 1169 specific for wt1169 peptide (C) was determined using *in vitro* cytotoxicity assays. The results shown are the specific lysis (%) of target cells incubated with each wild type (bold) and variant peptide at a concentration of 10 nmol/L. As expected, the highest cytotoxicity was found against wild-type peptides followed by various degrees of lysis for epitope variants. Target cells used in these assays were prepared from the B cell line (L.B3019) with an effector to target ratio of 20:1.

the amino acid changes found within this epitope region (1073-1081) showed reduced ability of the variant peptides to sensitize target cells for CTL lysis. Changes from cysteine (C) to serine (S) at position 6 and tryptophan (W) to arginine (R) at position 7 were found to be more effective than changes from a cysteine (C) to tyrosine (Y) at position 1 thus showing only a 50% reduction of specific lysis. Similar results were found for the epitope region 1169-1177 using CTL1169 (Figure 4C). The cytolytic activity for the parental peptide wt1169 again was over 80%. Variant C1171R, containing an amino acid change from cysteine (C) to an arginine (R) at position 3 could

still be recognized with a 60% lytic activity, whereas only a minor activity level was noted for A1176T, showing a change from alanine (A) to threonine (T) at position 8. The control experiment using unrelated wild-type peptide wt1131 did not produce any cytolytic response confirming the specificity of CTL recognition. Finally, CTL clone 1131 (covering epitope region 1131-1139) (Figure 4B) was also, as expected, able to sensitize target cells for CTL lysis when using the wt peptide (wt1131). Target cells loaded with variant peptide T1134M and H1136Y with mutations at position 4 [threonine (T) to methionine (M)] and position 6 [histidine (H) to tyrosine (Y)], respectively, seemed nearly unaffected, showing only minor loss of cytolytic activity compared to the wild-type response, suggesting that the mutations seen in these particular cases did not abolish CTL recognition. In contrast, CTL 1131 completely failed to respond to variant peptide Y1131C and showed only minor lytic activity using target cells loaded with peptide H1136R harboring an amino acid substitution at position 6 [histidine (H) to arginine (R)]. This result suggests that the mutations within variant peptide Y1131C from a tyrosine (Y) to a cysteine (C) at position 1 was highly efficient to abrogate CTL recognition in contrast to the observation made for CTL 1073, where the opposite mutation at the same position from a cysteine (C) to tyrosine (Y) had much less impact on the lytic capability of the CTL clone. Overall, a decrease in specific lysis could be demonstrated for the majority of variant peptides compared to wild-type responses indicating that most of our identified HCV NS3 epitope variants were able to escape or lower specific CTL recognition to various degrees.

Impact of variant peptides on MHC class I binding

To investigate directly whether these amino acid changes within the variant peptides were due to impairment of peptide binding to MHC and/or to alteration of recognition of the peptide-MHC complex by T cells, we performed a series of peptide binding assays to determine if our identified viral peptide epitope variants lost their capacity to form stable class I major histocompatibility complexes compared to their wild-type counterpart. After peptides representing putative escape variants were synthesized, binding affinities of both wild-type and variant peptide epitopes were assessed using serial dilutions of the peptides in FP-based peptide competition assays utilizing soluble HLA (sHLA)-A*0201 molecules as the binding entity. Each peptide tested generated its own binding isotherm from which IC₅₀ values were extracted. Figure 5A-C present multiple reaction curves obtained from the competition experiments, whereas Table 3 summarizes assessed IC₅₀ values for the peptides along with their exact amino acid sequences. Using previously obtained results^[39], we used other assay systems as guidelines to define an FP-based classification system, where peptides with an FP-based IC₅₀ value of 5000 nmol/L and lower were considered high affinity binding, 5000-50 000 nmol/L IC₅₀ values were considered medium-affinity binding, 50 000-1 000 000 nmol/L

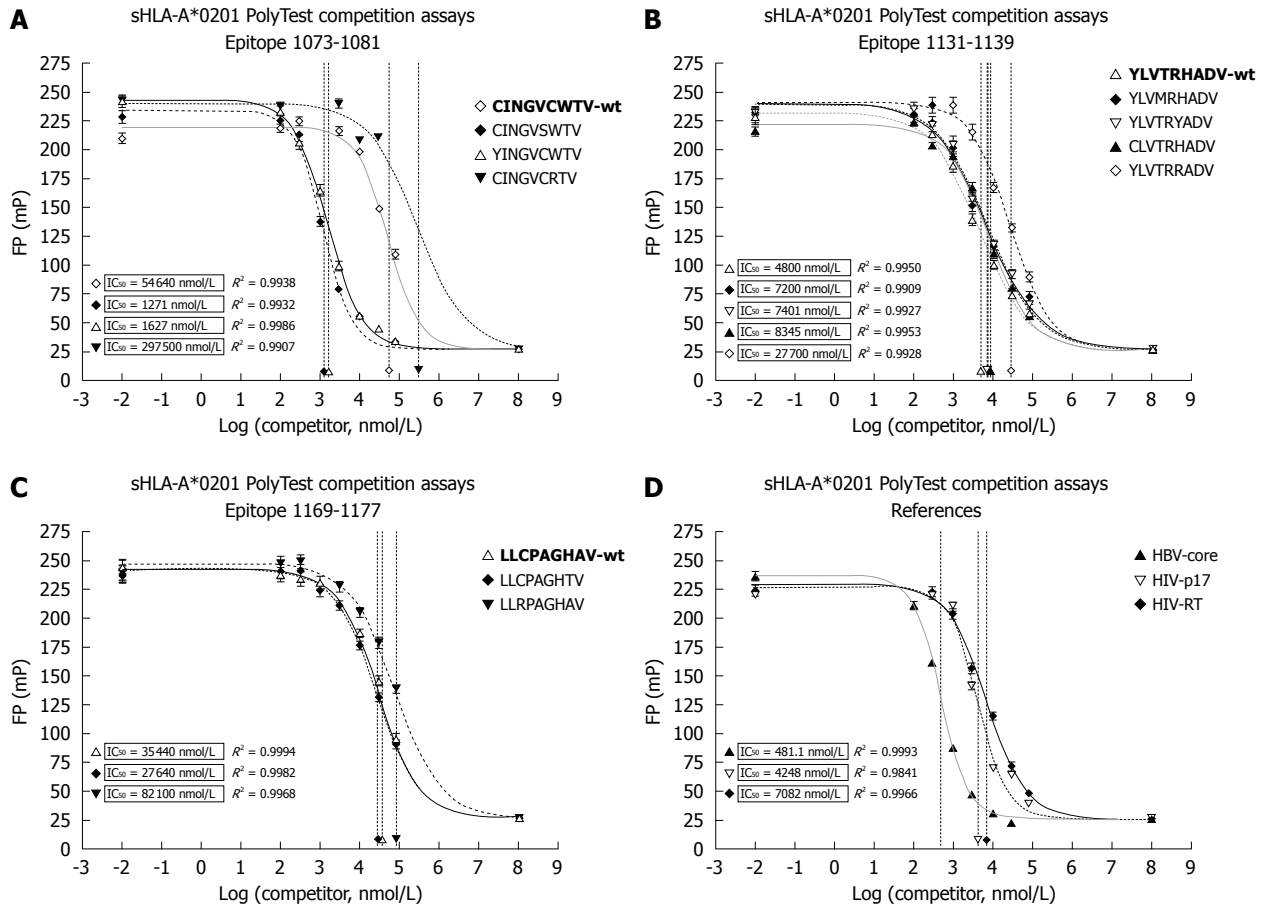


Figure 5 Soluble human leukocyte antigen (HLA)-A*0201 competition assays determining the binding capacity (IC_{50}) of natural HCV epitope variants. The affinity of three wild type HCV peptide epitopes (bold) wt1073 (A), wt1131 (B), wt1169 (C) derived from three locations within the NS3 region and their variants C1073Y, C1078S and W1079R (A), Y1131C, T1134M, H 1136Y and H1136R (B), C1171R and A1176T (C) was determined. Control peptides HBV-Core, HIV-p17 and HIV-reverse transcriptase (RT) were included to provide reference values to other viral systems. Their affinities are presented in graph (D). For each experiment, 8 serial dilutions (100 nmol/L–80 μ mol/L) of the unlabeled viral peptide were used to compete against a FITC-labeled tracer peptide. After reaching equilibrium, IC_{50} values for all peptides were determined by fitting the data to a dose-response model using the software Prism. R^2 values indicate the goodness of fit.

Table 3 Binding capacity, cytotoxicity and antagonicity of HCV NS3 peptides

Sequence	Name	Peptide origin	Position	Peptide length (aa)	IC_{50} (nmol/L)	R^2	Cytotoxicity 10 nmol/L (%) ¹	Inhibition 1:1 (%)
CINGVCWTV	Wt1073	HCV NS3	1073-1081	9	54 640	0.994	86.74	-
CINGVSWTV	C1078S	HCV NS3	wt1073 (C6S)	9	1271	0.993	24.57	59.30
YINGVCWTV	C1073Y	HCV NS3	wt1073 (C1Y)	9	1627	0.999	45.21	44.70
CINGVCRTV	W1079R	HCV NS3	wt1073 (W7R)	9	297 500	0.991	21.02	54.50
YLVTRHADV	Wt1131	HCV NS3	1131-1139	9	4800	0.995	52.03	-
YLVMRHADV	T1134M	HCV NS3	wt1131 (T4M)	9	7200	0.991	44.53	36.75
YLVTRYADV	H1136Y	HCV NS3	wt1131 (H6Y)	9	7401	0.993	35.56	92.54
CLVTRHADV	Y1131C	HCV NS3	wt1131 (Y1C)	9	8345	0.995	2.01	67.89
YLVTRRADV	H1136R	HCV NS3	wt1131 (H6R)	9	27 700	0.993	14.53	47.46
LLCPAGHAV	Wt1169	HCV NS3	1169-1177	9	35 440	0.999	84.56	-
LLCPAGHTV	A1176T	HCV NS3	wt1169 (A8T)	9	27 640	0.998	27.75	61.42
LLRPAGHAV	C1171R	HCV NS3	wt1169 (C3R)	9	82 100	0.997	59.98	44.39
FLPSDFFPV	HBV-Core	HBV Core	(18-27) ^[99]	10	481.1	0.999	NS	NS
SLYNTVATL	HIV-p17	HIV-1 p17	(77-85) ^[100]	9	4248	0.984	NS	NS
ILKEPVHGV	HIV-RT	HIV-1 Pol	(476-484) ^[47]	9	7082	0.997	NS	NS

¹Means of two experiments. Variant substitutions are expressed in underlined letters.

IC_{50} values were judged low-affinity binding, and IC_{50} values above 1 mmol/L were regarded as no binders. Additionally, low affinity binders were further subdi-

vided into a low (50 000–350 000 nmol/L) and very low affinity category (350 000–1 000 000 nmol/L). To provide better correlation between peptide binding affinity and

immunogenicity, binding results from peptides known for their ability to induce potent and specific CTL responses were presented (Figure 5A). Among our viral controls, the HBV-derived epitope FLPSDFFPSV was found to display very high affinity values. As one of the more referenced peptides found in literature, FLPSDFFPSV is known for high affinity binding to A*0201^[46-48] as well as for inducing potent CTL responses^[49]. Additional controls were the peptides SLYNTVATL and ILKEPVHGV, two other well-studied HIV-derived CTL epitopes^[50-52].

According to our FP-based classification system, wild-type peptides wt1131 and wt1169 were found to be of high and medium affinity, respectively, matching with the high cytolytic activity level of these CTL clones (Figure 4B and C). Somewhat unexpected was the low affinity binding observed for wild-type peptide wt1073, showing a very high cytolytic response when used in combination with CTL clone 1073 (Figure 4A). However, it has to be noted that this wild-type peptide has two cysteine residues within its sequence, allowing for disulfide bond formation. Potentially, this characteristic could lead to a reduced availability of intact peptides during the assay procedure directly affecting IC₅₀ determination, ultimately causing an underestimation of its binding capacity. As the two variant peptides within this group, C1078S and C1073Y, have only a single cysteine in their sequence and demonstrate very high affinity thus strongly supporting our hypothesis of a cys-cys interference.

In reviewing the binding results found for each variant peptide, none of the amino acid changes identified within each epitope region abolished peptide binding. Motif analysis of these variants showed that none of the anchor positions (defined at position 2 and 9 for nonameric peptides and critical for high affinity A*0201-related binding) contained any amino acid alterations, consistent with an escape mechanism affecting mainly peptide regions responsible for TCR recognition rather than MHC binding. More specifically, variants T1134M, H1136Y and Y1131C covering epitope 1131-1139 remained in close affinity range of the wild-type peptide indicating that mutations at positions 1, 4, and 6 had practically no effect on their binding capacity. An exception within this epitope region is variant peptide H1136R, whose binding capacity dropped 5.8 fold compared to the wild-type peptide probably caused by the introduction of a positive charge derived from the arginine (R) residue replacing the original histidine (H) residue at position 6. Similar observations were made in variant peptides W1079R and C1171R, in which the introduction of an arginine (R) at position 7 and 3 resulted in a more dramatic decrease in affinity. W1079R shifted to a 5.4 and C1171R to a 2.3 fold lower binding capacity. It is noteworthy that variant W1079R, like parental peptide Wt1073, possesses a disulfide bond potentially causing interference in binding thus also causing an underestimation of its binding capacity. Nevertheless, these arginine substituted variant peptides were still able

to bind A*0201. Interestingly, in some cases such as C1078S, C1073Y and A1176T, the binding capacity actually increased compared to the wild-type peptide.

CTL antagonism

Because peptide variants of class I -restricted epitopes potentially could antagonize naturally occurring epitopes^[31], we explored this possibility by using different ratios of variant to wild-type peptide concentrations (V:W). In these antagonist assays, target cells were pre-pulsed with wt1073 (Figure 6A), wt1131 (Figure 6B) or wt1169 (Figure 6C) peptides for 1 h and pulsed a second time after excessive washing with variant or control peptide for another hour. Notably, results showed that all of the specific clones were antagonized by their corresponding variant peptides generally providing inhibition values above 35% for the V:W ratio 1:1 (Figure 6). This observation is in good agreement with the obtained IC₅₀ data suggesting that all variant peptides are capable of binding HLA-A*0201. In more detail, NS3 variant peptides C1073Y, C1078S and W1079R behaved as strong antagonists for CTL 1073 and inhibited lysis of target cells at all three ratios tested (Figure 6A). An exception is variant peptide W1079R, which could not significantly inhibit the lysis of target cells at the low V:W ratio 0.1:1. This is most likely due to its much lower affinity compared to the other variant peptides within this epitope region. However, the fact that W1079R performed well as antagonist above the 1:1 ratio supports our earlier concern of reduced activity within the peptide binding assay which seems not to influence this cell-based antagonist assay. Equal results were obtained for variant peptides Y1131C, T1134M, H1136R and H1136Y testing antagonistic effects using CTL 1131 (Figure 6B). However, because of an insufficient amount of cell material obtained for CTL clone 1131, experiments were only conducted at a single V:W ratio of 1:1. Nevertheless, all of the variant peptides acted as antagonists for this CTL with highest inhibition of target lysis seen for H1136Y (92%), followed by Y1131C (68%), H1136R (47%), and T1134M (37%). The low inhibition results obtained for T1134M was somewhat unexpected considering its much higher binding capacity and cytotoxicity compared to the other variants within this group. This observation seems to indicate the presence of other factors involved to successfully antagonize CTL responses within this experimental setup such as peptide stability towards degradation and also uptake and transport mechanisms of the target cell potentially influencing peptide availability within the cell. Furthermore, both variant peptides C1171R and A1176T also demonstrated strong capabilities to antagonize CTL 1169 responses as shown in Figure 6C. Moreover, it was found that control peptide wt1131 (specific to CTL clone 1131), with high HLA-A*0201 binding affinity, was unable to inhibit CTL recognition of the wild-type peptides for CTL 1073 and 1169, respectively (Figure 6A and C). A similar result was obtained for wt1169 together with CTL 1131, suggesting that those natural variant peptides do not

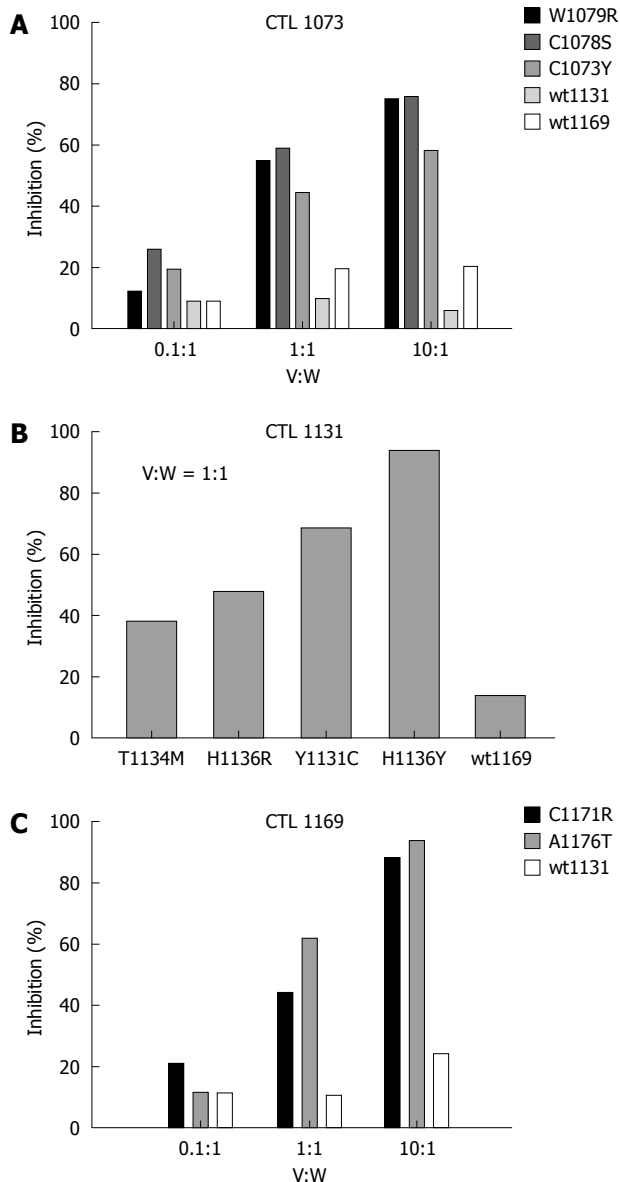


Figure 6 Antagonistic effect of NS3 peptide variants inhibiting specific cytolytic activity of specific CTL clones. A detailed inhibition profile is shown for CTL clones 1073 (A) and 1169 (C) in which target cells were pre-pulsed with 10 nmol/L of wild-type peptide, and then incubated at indicated ratios of variant to wild-type peptide concentrations (V:W). Wild-type peptides wt1131 and wt1169 were used as negative controls for experiments using CTL clone 1073, where only a single control (wt1131) was used for tests involving CTL clone 1169. Both data sets were determined at effector to target ratios of 20:1. Because of lack of material, a more simplified profile is shown for CTL clone 1131 (B) testing only a V:W ratio of 1:1 with wt1169 as negative control. All experiments were performed in duplicates.

simply exert their inhibitory activity by competing with wild-type peptides for HLA binding as recently described for hepatitis B epitopes by Bertoletti *et al.*^[28]

DISCUSSION

In recent years, there has been an increasing interest in HCV vaccine approaches that elicit CTL, which recognize and eliminate cells infected with HCV. Unlike antibodies, effective CTL responses can be directed against epitopes

derived from any viral protein, raising the possibility that CTLs can be targeted to regions that are more conserved than the viral envelope. Current vaccine modalities can elicit potent CTL responses against multiple viral epitopes. Indeed, many lines of evidence indicate that cell-mediated immunity plays a major role in restraining HCV infections. Several studies have suggested an association between certain MHC class I and class II alleles in the control of viral replication. Strong HCV-specific CD4⁺ and CD8⁺ T cell responses against multiple viral epitopes have been associated with clearance of HCV during acute infection^[1,15,53-60], and thought to be important contributors to protective immunity^[58,61,62]. A typical example of replication control by CTL was recently presented for HIV, showing antibody-mediated depletion of CD8⁺ cells in infected macaques, which resulted in dramatically increased virus loads in both acute and chronic infections^[63-65]. However, despite the presence of specific CD8⁺ CTL responses, more than 80% of individuals develop a persistent HCV infection. Although there is increasing evidence for the importance of HCV-specific T cell responses in the resolution of HCV infection, reasons for the failure of the immune system to eradicate the virus are less clear^[66]. Functional impairment of the antigen-specific CTL responses has been observed by several investigators and is thought to be possible reasons for viral persistence despite measurable T cell responses in HCV^[66-69]. Under normal circumstances CD8⁺ T-cells contribute to the control of viral infections by recognizing peptides of viral proteins presented by MHC class I molecules on infected cells. If HCV peptides are presented to the immune system, why does the virus persist? One potential explanation for this phenomenon is that HCV seems to accumulate mutations in both its structural and NS proteins^[36,70], and escape mutants may emerge under the presence of immune selection pressure exerted by CTLs against wild-type virus. Despite the importance of the CTL epitope viral mutation for immune evasion, in HCV infection many highly targeted epitopes have a low mutation frequency. Epitopes such as HLA-A2 restricted NS3 1073-1081 are consistently targeted by CD8⁺ T cells, but amino acid mutations facilitating immune evasion are rarely observed^[16,71]. Since the NS3 protein shares both protease and NTPase-dependent helicase functions, it has been proposed that mutations in these epitopes may be lethal to the virus^[72]. Additionally, another study indicates that CTL escape mutations emerging early in infection are not necessarily stable, but are eventually replaced with variants that achieve a balance between immune evasion and fitness for replication^[73].

CTLs contribute to the control of viral infections by recognizing peptides of viral proteins presented by MHC class I molecules on infected cells. Some viruses have developed strategies to evade recognition by CTL and one of these strategies involves antigenic variation in CTL epitopes. The emergence of CD8⁺ escape variants has been demonstrated in numerous other viral infections chronically infecting their host like HBV, HIV, or SIV^[74-78]. In HCV infection, a strong association between viral persistence and the development of escape mutations has

been demonstrated in the chimpanzee model^[4,15,62]. In this study, animals with persistent infections developed mutations in multiple regions of the viral genome encoding known epitopes and were largely confined to the MHC class I restriction element expressed by these animals. Further, such mutations correlated with abrogated CTL function. In addition to studies in acute hepatitis, other sequence variations in epitopes recognized by CD8⁺ CTLs have been identified in humans with chronic HCV infections^[16-20,22]. By focusing on single MHC class I alleles, Tsai *et al.*^[18] observed variant epitope sequences with CTL antagonist activity within an A*02-restricted HCV E1 epitope in two patients who developed chronic infections, whereas Timm *et al.*^[20] described the development of CTL responses against a single HLA-B*08-restricted epitope within the NS3. Furthermore, Ray *et al.*^[22] used the unique approach of comparing the sequences of viruses from 22 humans with chronic hepatitis C with the sequence of the single common virus. The expression of HLA-B*07, HLA-B*35, or HLA-B*37 alleles were found to be linked to the presence of mutations in epitopes presented by these alleles, indicating a likely role for CTL-mediated pressure in driving viral evolution. All these manuscripts constitute a critical mass of evidence for CTL mutations in MHC class I-restricted epitopes of HCV, which may play an important role in evasion of the antiviral CTL response. CTL activities *in vivo* may be impacted by cross-recognition with HCV-related or unrelated epitope sequences found in humans. For example, there exists partial sequence homology between the NS3 1131-1139 and NS4 1585-1593 epitopes and between NS 1073-1081 epitope and influenza-A neuraminidase, a common human pathogen.

Study of viral evolution throughout the course of HCV infection has hence proved extremely difficult in the past. Much effort has therefore recently been directed to the monitoring of HCV evolution. Such analyses allow definitive assessment of changes within the viral genome, which are critical in determining the role of immune selection pressure in viral evolution. Since the high rate of chronicity after acute HCV infection is difficult to explain in the presence of a multi-specific CTL response^[18], we sought to identify mechanisms favoring viral persistence. As noted earlier, we have previously described an immunodominant T-cell epitope restricted by HLA-DR15 in HCV NS3, NS3 358-375, for which epitope variants evolve through immune selection and stimulate not only attenuated levels of proliferation and IL-2 production, but also higher levels of type 2 cytokines^[13,79]. We reasoned that if CD8⁺ CTL exerts selection pressure on the virus, then the frequency of amino acid replacement should be higher in class I MHC restricted epitopes as well, potentially altering the outcome of infection by preventing or delaying clearance of infected hepatocytes by T lymphocytes and thus contribute to persistent HCV infection.

In order to identify escape mutations in a single patient with chronic infection, we monitored the genetic diversity

in a region of the HCV NS3 protein that contains HLA-A*02-restricted CTL epitopes, NS3 1073-1081^[34,54,80], NS3 1131-1139^[81,82], and NS3 1169-1177^[60,83]. Sequence analysis presented extensive variations in this region along with significant substitutions in segments encoding the class I restricted epitopes. Furthermore, results showed that the ratio of nonsynonymous base substitution (which changed the amino acid encoded) to synonymous base substitution (which left encoded amino acids unchanged) in these NS3 epitopes was 10-fold higher than in flanking sequences. This is comparable with our previous observations and consistent with the model for a positive Darwinian selection pressure expected for immune-mediated selection of escape variants at the epitope level^[13,14,21]. Genetic variation is inherent to all RNA viruses but has been best characterized for HIV-1, which is the result of a high number of errors made by the RT enzyme^[84], the absence of an RT proofreading mechanism during replication^[85,86], the fast turnover of virions in HIV infected individuals^[87-89] and selective immunological pressure from the host. Since HCV replication is directed by an error-prone RNA-dependent RNA polymerase encoded by the viral NS5b gene, which due to its propensity to introduce mutations into the viral genome, seems to provide the same selective advantage enjoyed by HIV, facilitating preferential expansion of the mutant progeny that potentially evade immune recognition. As a result, the virus population in an infected patient does not consist of a single uniform sequence but rather a distribution of different variants or quasispecies. The generation of new antigenic variants that escape the current immunological attack may lead to a persistent infection that culminates in the development of chronic infections. However, the massive heterogeneity observed in the worldwide epidemic of HIV-1 originated from a rapid viral turnover in HIV infected individuals, and seems to be much less extensive in chronic HCV infected individuals where the initial highly homogeneous virus population changes with much slower kinetics towards a mixed viral population.

Indeed, this process of immune evasion through mutation that characterizes infection with HCV viruses is a substantial barrier to the development of successful vaccines and therapeutic interventions based on manipulation of the T cell response. From this perspective, it is essential to gain a more integrated picture of the controlling influences that underlie the complex relationship between HCV and CD8⁺ T cell immunity. Due to the observed viral evolution in the NS3 region, we hypothesized that the newly discovered variant sequences may resemble mutations capable of escaping from the original CTL response against the wt epitope. For this reason, we examined the relationship between cloned CTL responses and variant viral peptide sequences derived from the three NS3 epitopes. In total, 5 out of the 9 CTL responses studied here were not recognized by specific CTL and another four variant peptides dramatically reduced the cytolytic activity of CTL. Nonetheless, comparison of features of CTL suggested that both

quantitative and qualitative factors may play a role in determining the pressure exerted by individual epitope-specific CTL responses on *in vivo* viral replication. Considering that HLA-A*02 represents the most frequent allele in the Northern American population with a gene frequency of 27.2% in Caucasians, 23.0% in Hispanics, 22% in Natives and 12.3% in African Americans^[90], we assume that, in the absence of reversion, certain variants can be expected to be present at least in some HLA-A*02-negative subjects with chronic infection. Another factor that likely has an important impact on the extent and kinetics of viral escape from epitope-specific CTL responses is the cost of escape to intrinsic viral fitness. Several papers^[91,92] provide examples of high costs to intrinsic viral fitness preventing a lasting impact on overall viral evolution of particular epitopes. If CTL escape constitutes a common and significant means of immune evasion in HCV infection, vaccination strategies should be designed to elicit a response that will have the minimal chance of being escaped after infection. One way in which escape can be reduced is by induction of a T cell response that exerts balanced pressure against multiple viral epitopes (e.g. composed of multiple epitope-specific responses of similar magnitude and efficacy). A vaccine-generated host immune response that attacks the primary viral strain and subsequent mutants that arise during replication would possibly circumvent persistence through elimination or drive viral evolution towards defective mutants with high fitness cost and which lack the ability to infect new host cells or replicate in infected cells.

With respect to cellular immune responses, mutations can have effects other than loss of binding to MHC or TCR molecules. Studies in HLA class I restricted systems have demonstrated that altered peptide ligands (APLs) may antagonize the immune response or lead to antigen-specific anergy not only in CTL responses to HCV^[10,16-18] but also to the inhibition of CTL responses to native antigens in other viral systems^[28,31,32,93,94]. Based on our previous work with the class II restricted epitope NS3 358-375^[13,14], we were interested to know whether class I restricted variant epitopes for NS3 could also act as APLs and thus antagonize CTL function. Indeed, most variant peptides for each of the three CTL epitopes were capable of acting as antagonists and suppressed CTL recognition of wild-type peptide epitopes. For some of the variant peptides, inhibition was detectable at antagonist concentrations as low as 1 nmol/L, which is similar to physiological levels of natural peptides within infected cells according to Christinck *et al.*^[95]. Thus, it is interesting to speculate that escape and antagonism may together serve to blunt the CTL response to multiple HCV epitopes. Ultimately, antagonism may play an important role in the persistence of HCV and other viral infections, where mutant viruses harboring antagonist APL epitopes may aid in the survival of wild-type viruses which otherwise would be recognized and destroyed by CTL. Furthermore, these observations are likely due to TCR

antagonism as opposed to MHC blockade, which is supported by the fact that experiments using wild-type control peptides with high HLA-A*02 binding affinity failed to show inhibition of CTL recognition of targets pulsed with a CTL corresponding wild-type peptide. This represents a different mechanism than that reported earlier by Bertolotti *et al.*^[28]. In terms of the practical implications of this phenomenon, reported antagonism of T lymphocyte activity in a vaccine study is of particular concern because the antagonism was identified in a patient that became infected following vaccination^[96]; antagonism may therefore represent a potential mechanism for vaccine failure and requires further careful consideration.

Overall, our data are correlative and it is important to emphasize that the coexistence of virus encoding wild type and variant epitopes does not prove that such are selected for by *in vivo* CTL responses. In the absence of a convenient animal model for HCV infection, the causal relationship between blunted CTL responses and a variant viral peptide sequence in HCV infection cannot be tested directly^[97,98]. Nonetheless, the presence of variant peptides that are not recognized by or are able to antagonize specific CTL in the same patient is consistent with the notion that CTL pressure on a mutable virus such as HCV can result in the selection of escape or APL variants, similar to proposals by others with respect to HCV^[16,18] and other viral infections^[31,74]. The fact that all variant peptides in the NS3 1073-1081 epitope were able to antagonize two CTL clones specific for the wild-type sequence suggests that immune selection for variants, if it exists, may be very strong. In line with this is the fact that similar results were obtained with variant peptides located in epitopes NS3 1131-1139 and NS3 1169-1177 although clones were differentially susceptible to the inhibitory activity of certain variant peptides. Alternatively, if such variants arise from quasispecies variations present in the initial inoculum, they would need to be maintained without sacrificing viral fitness in a significant way. This raises a question, currently unanswered, as to whether mutation of the HCV genome recapitulates quasispecies diversity within a single patient. To our knowledge this has not been investigated, but the answer would have important ramifications for understanding the immuno-pathogenesis of an HCV infection.

In summary, the findings presented here illuminate the potential mechanisms that underlie observed patterns of mutational immune escape. Analyses of MHC binding data suggest that amino acid substitutions in the bound peptide preferably impact TCR recognition, rather than MHC binding, as a consequence of continuous shifts in antigen topography that exemplify adaptive viral evolution to the individual host environment. In addition, the ability of naturally occurring variant forms to antagonize CTL clones, as suggested within this study, is increasingly recognized in chronic infections of other viruses. In this light, persistence of HCV seems to be facilitated by viral evolution not only enabling the escape from prominent CTL responses but also through antagonistic effects triggered by

viral variants. It can be imagined that various variant peptides, which were found to have similar binding affinities to the MHC, compete against the wild type and therefore help to maintain wild-type virus by lowering the number of wild-type peptides to be recognized by patient CTLs. In any event, simultaneous analysis of the viral nucleotide sequence and the CTL response to multiple CTL epitopes in the same individual is needed to determine the potential contribution of CTL escape variants to HCV persistence. Information on heterogeneity in a single carrier seems very important in understanding immunopathogenic processes that may be influenced by viral genomic changes; such goes beyond a simple paradigm of viral escape from strong and multi-specific CTL responses against various immunodominant epitopes and should be considered as a potential determinant of HCV persistence. However, variants do not seem to accumulate within CTL epitopes but occur in early infection. Other minor species may be present but not detected and/or arise at time points other than those examined. We suggested that HCV escape mutants occurring are transient, but are eventually replaced with variants/or wild type that may seek a balance between avoiding recognition by host immune cells and reducing fitness for replication. Ultimately, viral mutants that escape immune recognition are a formidable challenge to the design of an effective HCV vaccine.

COMMENTS

Background

The mechanisms responsible for high rates of hepatitis C virus (HCV) persistence are unknown, but may involve viral escape through the mutation of epitopes recognized as targets of the immune response. It is thought that by antagonizing T-cell responses to native epitopes, viruses expressing mutant epitopes might aid in the survival of infected cells producing wild-type viruses, which would otherwise be recognized and destroyed by cytotoxic T lymphocytes (CTLs). Although parts of this issue have been examined in chronic HCV infection unresolved questions remain, including whether naturally occurring variants antagonize CTL responses to wild-type epitopes that are found within the same host.

Research frontiers

Variation within a viral epitope can lead to a total or partial loss of functional recognition by CTL. Substitutions occurring at key anchor residues may alter peptide affinity for major histocompatibility complex (MHC) class I molecules and thereby interfere with antigen presentation and effector T-cell mediated clearance of infected cells. Overall, the authors' study showed the way to understanding how interactions between HCV and the host's immune system might lead to viral persistence or effective elimination of HCV.

Innovations and breakthroughs

Mutations in MHC class I-restricted HCV epitopes that affect T-cell receptor recognition by CD8⁺ CTLs have also been correlated with the severity and persistence of HCV infection. The authors examined viral evolution in three immunodominant HLA-A*02-restricted NS3 epitopes in a patient chronically infected with HCV. Results obtained provide convincing evidence of immune selection pressure for all epitopes investigated. Mutations within the epitopes coincided with the decline of CTL responses, and peptide-binding studies suggested a significant impact of the mutation on T cell recognition. Results illustrate allele-specific viral evolution and escape from a dominant CD8⁺ CTL response.

Peer review

The authors have examined the pattern of natural variants within three HLA-A2-restricted NS3 epitopes in one patient with chronic HCV infection at 12, 28 and 63 mo post-infection.

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Molecular determinants of the antitumor effects of trichostatin A in pancreatic cancer cells

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Abstract

AIM: To gain molecular insights into the action of the histone deacetylase inhibitor (HDACI) trichostatin-A (TSA) in pancreatic cancer (PC) cells.

METHODS: Three PC cell lines, BxPC-3, AsPC-1 and CAPAN-1, were treated with various concentrations of TSA for defined periods of time. DNA synthesis was assessed by measuring the incorporation of 5-bromo-2'-deoxyuridine. Gene expression at the level of mRNA was quantified by real-time polymerase chain reaction. Expression and phosphorylation of proteins was monitored by immunoblotting, applying an infrared imaging technology. To study the role of p38 MAP kinase, the specific enzyme inhibitor SB202190 and an inactive control substance, SB202474, were employed.

RESULTS: TSA most efficiently inhibited BrdU incorporation in BxPC-3 cells, while CAPAN-1 cells displayed the lowest and AsPC-1 cells an intermediate sensitivity. The biological response of the cell lines correlated with the increase of histone H3 acetylation after TSA application. In BxPC-3 cells (which are wild-type for *KRAS*), TSA strongly inhibited phosphorylation of ERK 1/2 and AKT.

In contrast, activities of ERK and AKT in AsPC-1 and CAPAN-1 cells (both expressing oncogenic *KRAS*) were not or were only modestly affected by TSA treatment. In all three cell lines, but most pronounced in BxPC-3 cells, TSA exposure induced an activation of the MAP kinase p38. Inhibition of p38 by SB202190 slightly but significantly diminished the antiproliferative effect of TSA in BxPC-3 cells. Interestingly, only BxPC-3 cells responded to TSA treatment by a significant increase of the mRNA levels of bax, a pro-apoptotic member of the *BCL* gene family. Finally, in BxPC-3 and AsPC-1 cells, but not in the cell line CAPAN-1, significantly higher levels of the cell cycle inhibitor protein p21^{Waf1} were observed after TSA application.

CONCLUSION: The biological effect of TSA in PC cells correlates with the increase of acetyl-H3, p21^{Waf1}, phospho-p38 and bax levels, and the decrease of phospho-ERK 1/2 and phospho-AKT.

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Key words: Pancreatic cancer; Histone deacetylase inhibitor; Trichostatin-A; *KRAS*; MAP kinases; p21^{Waf1}; AKT

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INTRODUCTION

Pancreatic cancer (PC) constitutes the fourth to fifth leading cause of cancer deaths in Western countries. Despite many scientific efforts in recent years, PC still has

the worst survival rate (< 5%) of all common human tumors^[1,2], and therapeutic progress has been very slow. Important reasons for this dissatisfying situation include the lack of markers for early diagnosis, and the limited efficiency of radio- and chemotherapy.

In the development of ductal adenocarcinoma, the most common form of PC, accumulation of somatic gene mutations in pancreatic progenitor cells plays a key role^[3]. For example, oncogenic mutations of the *KRAS* gene are detectable in approximately 90% of pancreatic adenocarcinomas. Other frequent genetic alterations in PC include loss or inactivation of the anti-oncogenes *p53*, *p16/CDKN2A* and *DPC4*^[3].

While the essential contribution of somatic gene mutations is well established, recent studies have implicated epigenetic alterations in pancreatic carcinogenesis as well^[4]. Thus, several genes with tumor suppressor properties, such as *p16/CDKN2A* (if not genetically inactivated)^[5], *p57^{KIP2}/CDKN1C*^[6] and *BNIP3*^[7], were shown to frequently undergo epigenetic promoter silencing by aberrant methylation of CpG islands. Hypomethylation of CpG's, in contrast, has been described for several genes overexpressed in PC, including *claudin4*, *S100A4*, and *prostate stem cell antigen*^[8].

Besides promoter methylation, histone modification by acetylation and further post-translational modifications represents a key principle of epigenetic regulation. Histone acetylation is associated with a repressed chromatin state, and tightly controlled by two classes of enzymes, histone acetyltransferases and histone deacetylases^[9-11]. Inhibitors of histone deacetylases (HDACI) display anti-cancer activities and are, therefore, of growing clinical interest^[12]. With respect to PC, recent experimental studies have suggested that HDACI may efficiently inhibit growth and induce apoptosis even of drug resistant cell lines^[13-15]. Furthermore, a synergistic action with classic cytostatic drugs, such as gemcitabine, has been demonstrated^[16-18]. We have recently shown that HDACI also exert antifibrotic efficiency by inhibiting functions of pancreatic stellate cells^[19]. Since fibrosis is considered a progression factor of PC, this "side effect" might enhance antitumor activities of these drugs.

The molecular targets of HDACI in PC are incompletely characterized. Although expression of cell-cycle inhibitors (e.g. *p21^{Waf1}/CDKN1A*^[14,17]) and modulation of pro-apoptotic pathways^[13,15,17] have been implicated in HDACI action, the molecular determinants of HDACI efficacy or ineffectiveness are currently unclear. Here, we have chosen three human PC cell lines that differ in their biological sensitivity to the HDACI trichostatin A (TSA) for a comparison of TSA effects at the molecular level. We have focussed on pathways that are considered to be important both for PC cell growth/survival as well as HDACI action. By this approach, we have identified molecular correlates of TSA effectiveness in PC cells.

MATERIALS AND METHODS

Materials

Media and supplements for cell culture were obtained from Biochrom (Berlin, Germany), the 5-bromo-2'-deoxyuridine

(BrdU) labelling and detection enzyme-linked immunosorbent assay kit from Roche Diagnostics (Mannheim, Germany), and SB202190 and SB202474 from Merck Chemicals/Calbiochem (Darmstadt, Germany). TRI reagents and all chemicals used for reverse transcription and TaqmanTM real-time polymerase chain reaction (PCR) were from Applied Biosystems (Foster City, CA, USA). PVDF membrane was supplied by Millipore (Schwalbach, Germany), anti-p21^{Waf1} immunoglobulin (mouse monoclonal) by Becton Dickinson Biosciences Pharmingen (Heidelberg, Germany), the other primary antibodies (all raised in rabbits) by New England BioLabs (Frankfurt, Germany), and Odyssey[®] blocking buffer, stripping buffer and secondary antibodies for immunoblotting by LI-COR (Bad Homburg, Germany). Tissue culture dishes (Corning plasticware), standard laboratory chemicals and TSA were from Sigma-Aldrich (St. Louis, MO, USA). The HDACI was dissolved in ethanol and stored at -20°C as a stock solution (3.3×10^{-3} mol/L).

Cell culture

All cells used in this study represent human pancreatic carcinoma cell lines. CAPAN-1 cells were grown in IMDM supplemented with 17% foetal calf serum (FCS) and 10 mL/L non-essential amino acids (dilution of a $100 \times$ stock solution). BxPC-3 cells were cultured in RPMI with 10% FCS. The culture medium for AsPC-1 cells was DMEM containing 10% FCS. All culture media were supplemented with 10^5 U/L penicillin and 100 mg/L streptomycin. The cell lines were grown at 37°C in a 5% CO₂ humidified atmosphere. When reaching subconfluency, the cells were harvested by trypsinization, and recultured according to the experimental requirements.

Quantification of DNA synthesis

DNA synthesis was assessed by measuring incorporation of BrdU into newly synthesized molecules. Therefore, PC cells growing in 96-well plates were pretreated with drugs as indicated. 24 h after drug application, BrdU labelling was initiated by adding labelling solution at a final concentration of 1×10^{-5} mol/L (in the culture medium). After an overnight incubation, labelling was stopped, and BrdU uptake was measured according to the manufacturer's instructions.

Immunoblotting

Cells were harvested by medium aspiration and addition of boiling lysis buffer^[20] directly to the cell monolayer. Cellular proteins received from equal numbers of cells were separated by SDS-polyacrylamide gel electrophoresis (10%-12% gels, depending on the experimental requirements) and transferred onto PVDF membrane by semi-dry blotting. After blotting, the filters were blocked and processed by incubation with the indicated primary antibody as previously described^[20,21]. The secondary antibody was IRDye[®] 800CW conjugated goat anti-mouse IgG and goat anti-rabbit IgG, respectively. All immunoblots were scanned at a wavelength of 800 nm, using an Odyssey[®]

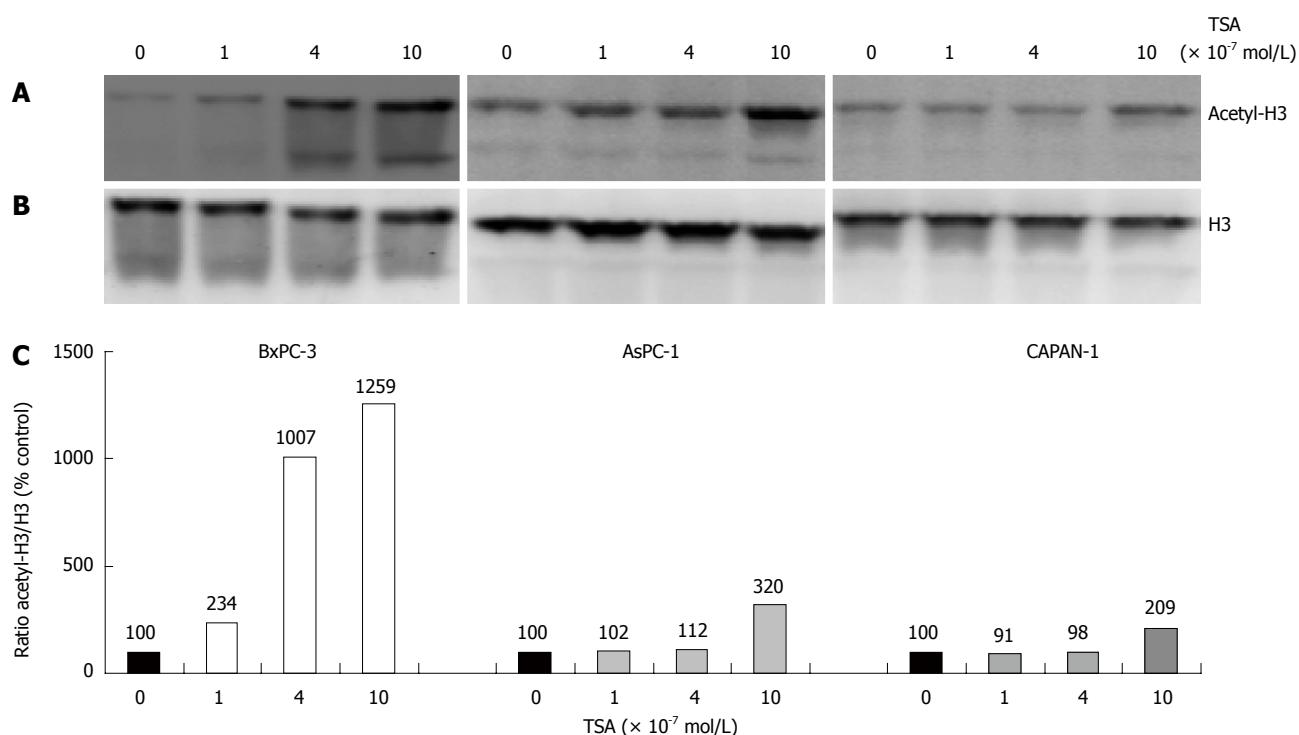


Figure 1 Enhancement of histone H3 acetylation by trichostatin-A (TSA). The indicated pancreatic cancer (PC) cell lines were treated with various concentrations of TSA for 24 h. A: Histone H3 acetylation was analyzed by immunoblotting; B: Reprobing of the blot with an anti-H3 protein-specific antibody revealed no systematic differences of the histone H3 amount among the samples; C: Acetyl-histone H3 levels were further investigated using image analysis software and related to the histone H3 protein level. Therefore, acetyl-histone H3 and H3 protein signal intensities were determined, and the ratio acetyl-histone H3/H3 protein was calculated. A ratio of 100% corresponds to control cells cultured without TSA. The data shown are representative of three independent experiments.

Infrared Imaging System. Signal intensities were quantified by means of the Odyssey[®] software version 3.0. Prior to reprobing with additional primary antibodies, the blots were treated with stripping buffer according to the instructions of the manufacturer.

Quantitative reverse transcriptase-PCR using real-time TaqMan[™] technology

The pancreatic cancer cell lines were challenged with TSA as indicated. Afterwards, total RNA was isolated with TRI reagent according to the manufacturer's instructions. The RNA was reverse transcribed into cDNA by means of TaqMan[™] Reverse Transcription Reagents and random hexamer priming. Relative quantification of target cDNA levels by real-time PCR was performed in an ABI Prism 7000 sequence detection system (Applied Biosystems) using TaqMan[™] Universal PCR Master Mix and the following Assay-on-Demand[™] human gene-specific fluorescently labelled TaqMan[™] MGB probes: Hs00180269_m1 (bax), Hs00355782_m1 (p21^{Waf1}), and Hs99999905_m1 [glyceraldehyde-3-phosphate dehydrogenase (GAPDH), used as house-keeping control gene]. Following the guidelines of the manufacturer, PCR was performed under the following conditions: 95°C for 10 min, 50 cycles of 15 s at 95°C, 1 min at 60°C. The reactions were run at least in duplicate, and repeated 6 times with independent samples. Relative expression of each mRNA compared with GAPDH was calculated according to the equation $\Delta Ct = Ct_{\text{target}} - Ct_{\text{GAPDH}}$. The relative amount of target mRNA in

control cells and samples treated with drugs as indicated was expressed as $2^{-(\Delta\Delta Ct)}$, where $\Delta\Delta Ct_{\text{treatment}} = \Delta Ct_{\text{sample}} - \Delta Ct_{\text{control}}$.

Statistical analysis

Results are expressed as mean \pm SE for the indicated number of separate cultures per experimental protocol. Statistical significance was analyzed using the indicated statistical test. $P < 0.05$ was considered to be statistically significant.

RESULTS

TSA enhances histone acetylation in PC cell lines

In initial experiments, we compared the effects of TSA on the acetylation of histone H3 in the three different pancreatic cancer cell lines used in this study (Figure 1). In all cell lines, a dose-dependent increase of H3 acetylation was observed, suggesting an inhibition of histone deacetylase activity. The effect of TSA was stronger in BxPC-3 cells than in the other two cell lines, and AsPC-1 cells were somewhat more sensitive to TSA treatment than CAPAN-1 cells. The functional consequences of TSA action were investigated in subsequent experiments.

TSA inhibits DNA synthesis of pancreatic cancer cells

TSA significantly inhibited the incorporation of BrdU into newly synthesized DNA in all cell lines tested, but with remarkably different efficiency (Figure 2): While BxPC-3 cells showed a significant response at a TSA con-

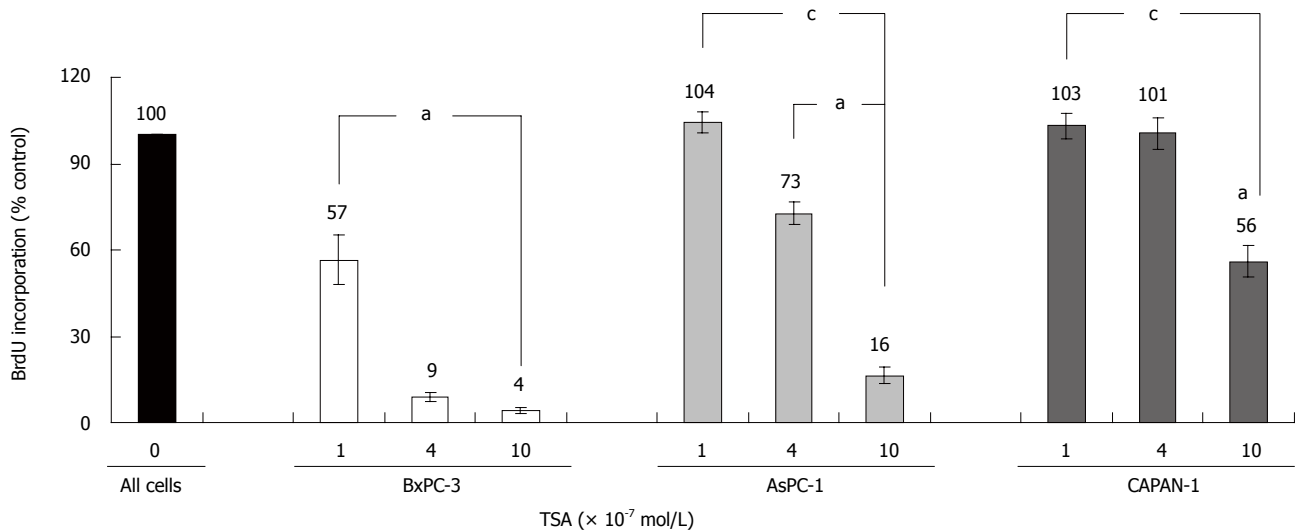


Figure 2 Effects of TSA on the BrdU incorporation of PC cell lines. BxPC-3, AsPC-1 and CAPAN-1 cells were treated with TSA as indicated for 24 h, before DNA synthesis was assessed with the BrdU incorporation assay. 100% BrdU incorporation corresponds to cells cultured without TSA. Data are presented as mean \pm SE ($n = 6$ separate cultures); $^aP < 0.05$ vs control cultures, $^cP < 0.05$ vs BxPC-3 cells (Wilcoxon's rank sum test).

centration of 1×10^{-7} mol/L, 10 times higher doses were required to reduce the DNA synthesis of CAPAN-1 cells. AsPC-1 cells displayed an intermediate sensitivity. Furthermore, at any concentration tested, BrdU incorporation was significantly stronger inhibited in BxPC-3 cells than in the other two cell lines. Action of HDACI has previously been linked to the suppression of cell proliferation and induction of apoptosis^[13-15], and diminished incorporation of BrdU might be an indicator of both. Although a differentiation between these processes was not our main focus, we noticed that at TSA concentrations up to 4×10^{-7} mol/L the rate of cell death did not increase in any cell line over a treatment period of 48 h (data not shown).

Effects of TSA at the level of signal transduction

In the next experiments, the molecular basis of the different TSA responsiveness of our PC cell lines was studied. Therefore, we chose the approach to focus on intracellular proteins that have previously been implicated both in HDACI action and stimulation/inhibition of PC cell growth. As shown in Figure 3, a cell line-specific pattern of the TSA response was observed.

In BxPC-3 cells, but not in the other two cell lines, treatment with TSA at 10×10^{-7} mol/L significantly diminished phosphorylation of the kinases ERK 1 and 2, which are key elements of the Ras-Raf-MEK-ERK pathway^[22] (Figure 3A, panel 1 and 2, and Figure 3B). Furthermore, only in BxPC-3 cells TSA at 10×10^{-7} mol/L almost completely blocked phosphorylation of AKT (Figure 3A, panel 3 and 4, and Figure 3C), which acts downstream of the phosphatidylinositol (PI) 3-kinase^[23]. A further evaluation of the PI 3-kinase/AKT pathway revealed that its best-characterized negative regulator, the PI 3-kinase phosphatase PTEN^[23], was expressed and phosphorylated in all three cell lines in a TSA-independent manner (Figure 3A, panel 5 and 6; quantification data not shown). These data suggest that PTEN was not involved in the mediation of

TSA effects on AKT.

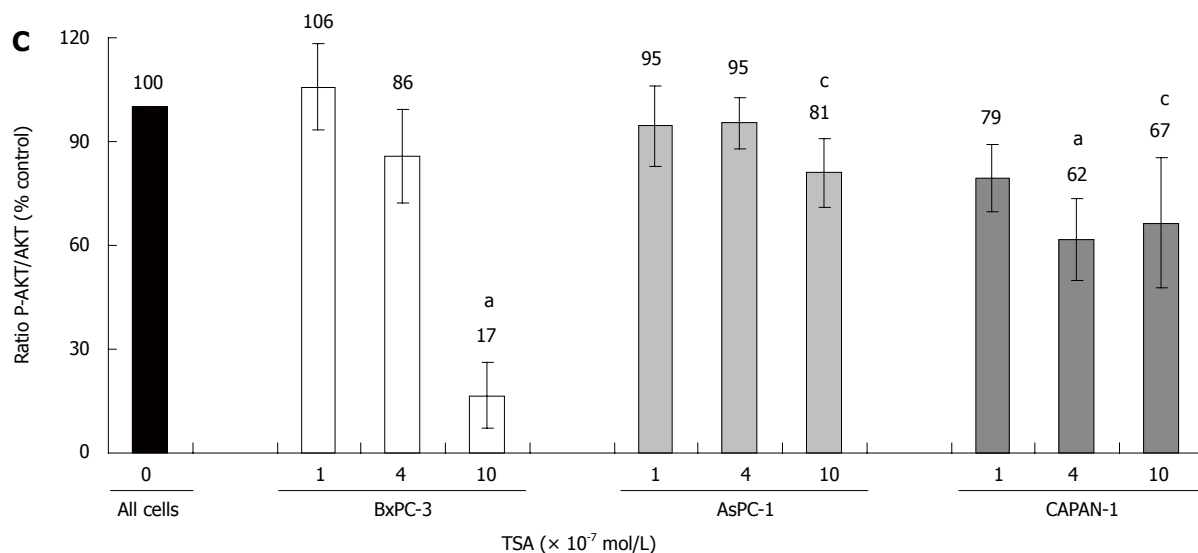
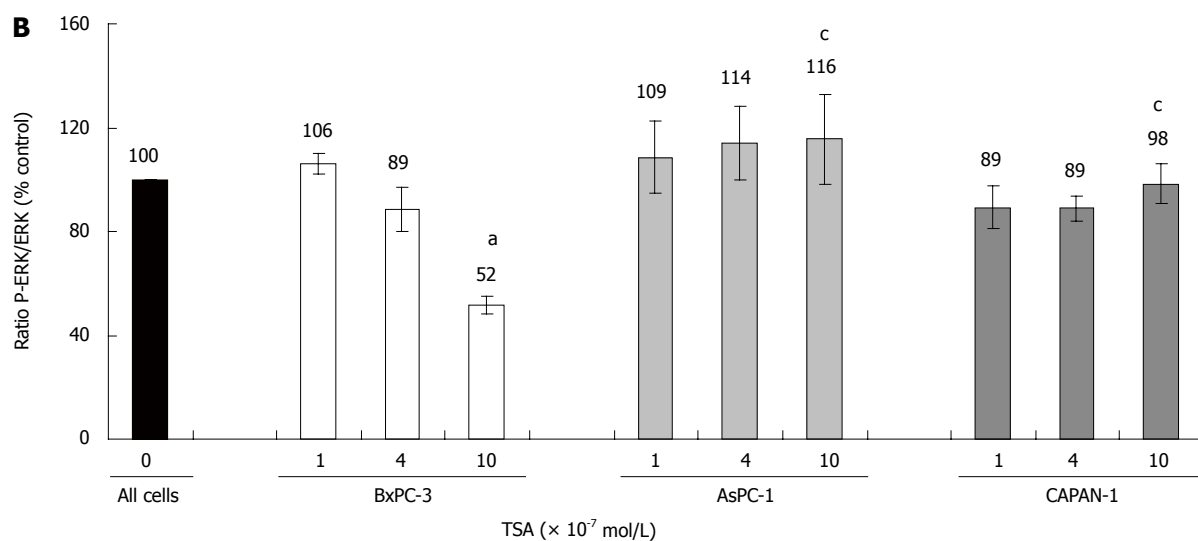
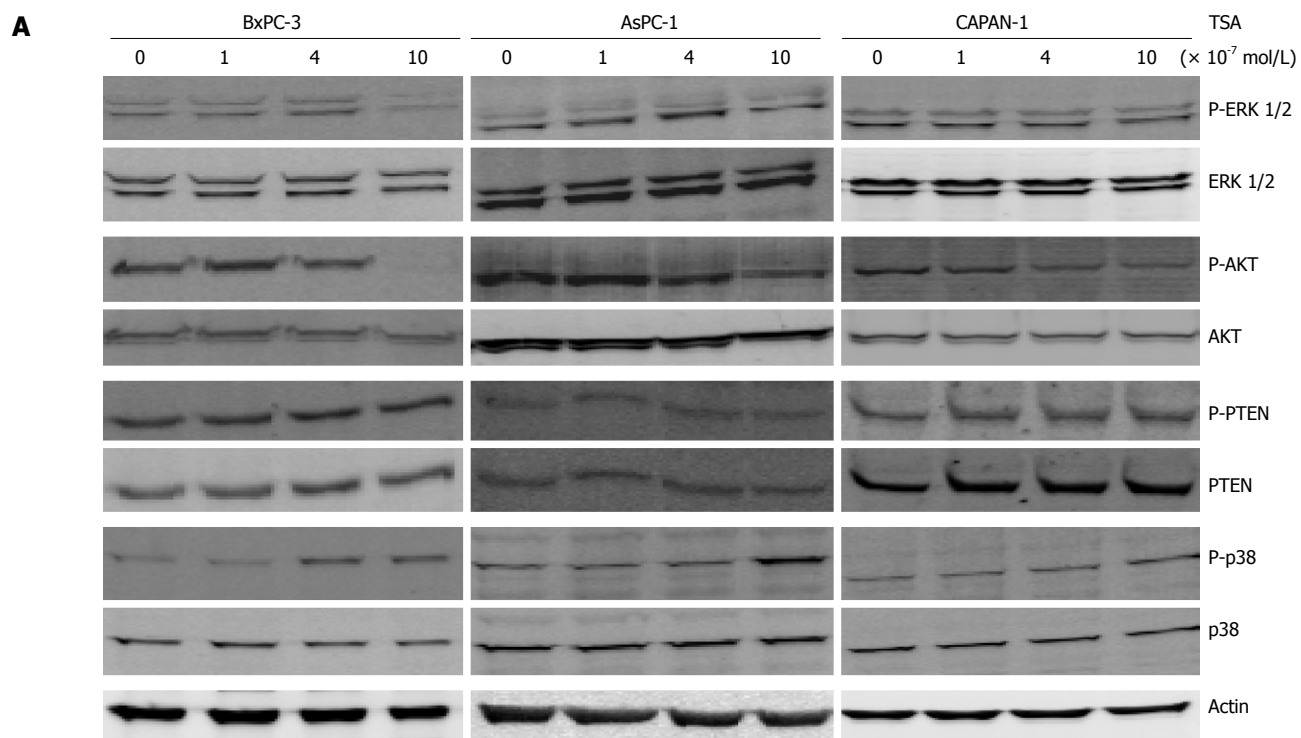
Finally, we studied how TSA treatment affected phosphorylation of the MAP kinase p38, which like ERK plays a profound role in tumorigenesis^[24]. Here, in all three cell lines a dose-dependent enhancement of phosphorylation was observed (Figure 3A, panel 7 and 8, and Figure 3D). The effect was most pronounced in BxPC-3, followed by AsPC-1 and CAPAN-1 cells.

Effects of p38 MAP kinase inhibition on DNA synthesis of PC cells

To study the biological consequences of p38 MAP kinase activation by TSA, the specific inhibitor SB202190 and an inactive control substance, SB202474, were employed. In the absence of TSA, incubation of BxPC-3 cells with the drugs did not significantly affect BrdU incorporation. In the presence of TSA, BrdU incorporation of SB202190-treated BxPC-3 cells exceeded the one of cells exposed to SB202474 (Figure 4). Although the difference was small, it reached statistical significance. In AsPC-1 and CAPAN-1 cells, SB202190 had no effect on the incorporation of BrdU; independent of the presence or absence of TSA (data not shown). The data therefore suggest a (limited) contribution of p38 MAP kinase to the inhibition of DNA synthesis by TSA selectively in BxPC-3 cells; the cell line which had also shown the strongest TSA-dependent increase of p38 MAP kinase phosphorylation (Figure 3D).

Expression of TSA target genes in PC cell lines

Various HDACI target genes identified so far have been linked to the induction of cell cycle arrest or apoptosis. We chose two of the best-studied candidate genes, *BAX* and *p21^{Waf1}* (CDKN1A, Cip1), to compare the effects of TSA on gene expression in the three PC cell lines. *BAX*, a pro-apoptotic member of the *BCL* gene family^[25], and the cell-cycle inhibitor gene *p21^{Waf1}* were found to be ex-



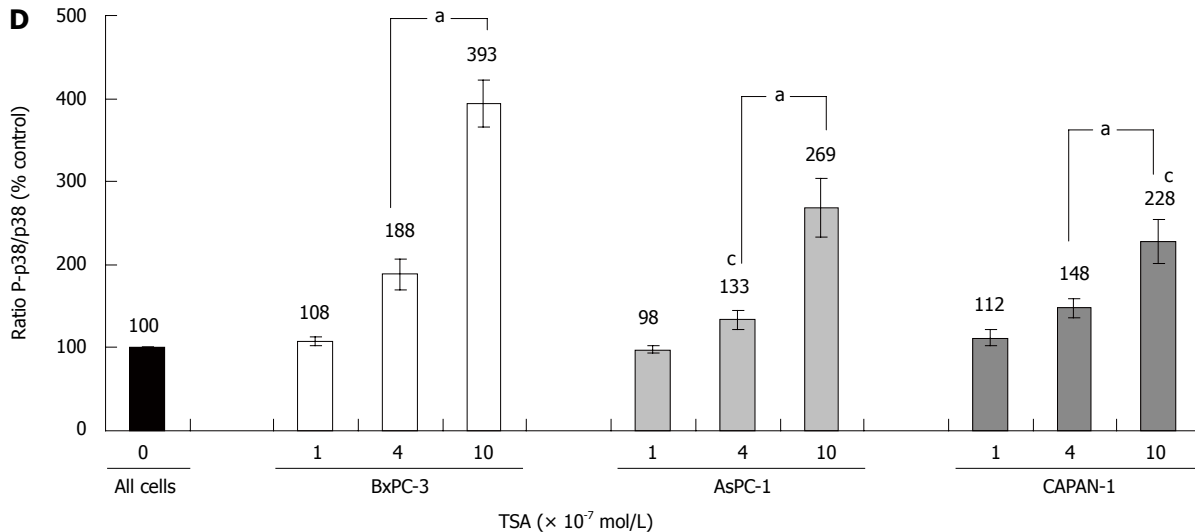


Figure 3 Expression and phosphorylation of signal transduction proteins in TSA-treated PC cell lines. BxPC-3, AsPC-1 and CAPAN-1 cells were treated with TSA at concentrations up to 10×10^{-7} mol/L for 24 h. A: Expression and phosphorylation of the indicated proteins was analyzed by immunoblotting. Phospho-proteins were detected first, followed by a reprobing of the blots with anti-protein specific antibodies. Actin was used as a housekeeping control protein; B-D: Fluorescence signal intensities of phospho (P) proteins and total proteins were quantified using Odyssey® software version 3.0. Subsequently, the ratios phospho-ERK/ERK protein (B), phospho-AKT/ERK protein (C) and phospho-p38/p38 protein (D) were determined. A ratio of 100% corresponds to control cells cultured without TSA. Data of 8 independent experiments were used to calculate mean values and SE; ^a $P < 0.05$ vs control cultures, ^c $P < 0.05$ vs BxPC-3 cells (Wilcoxon's rank sum test).

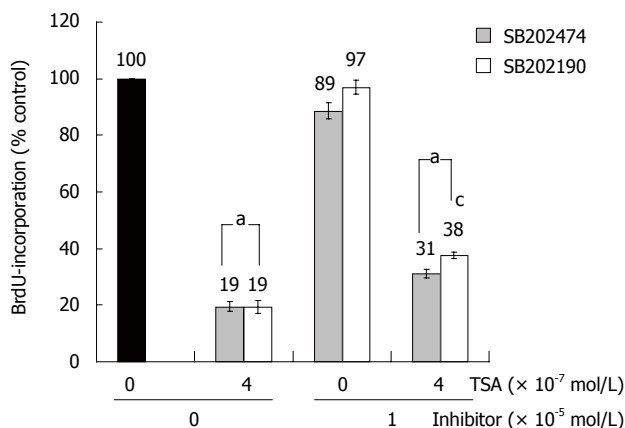


Figure 4 SB202190 diminished inhibition of BrdU incorporation by TSA in BxPC-3 cells. The cells were treated with TSA, SB202190 and SB202474 at the indicated concentrations for 24 h, before DNA synthesis was assessed with the BrdU incorporation assay. 100% BrdU incorporation corresponds to untreated cells. Data are presented as mean \pm SE ($n = 16$ separate cultures); ^a $P < 0.05$ vs control cultures, ^c $P < 0.05$ vs SB202474-treated cells (identical concentration of TSA) (Wilcoxon's rank sum test).

pressed in all cell lines (Figure 5A and B). In TSA-treated BxPC-3 cells, a significant increase of bax mRNA levels was observed, whereas AsPC-1 cells displayed a non-significant tendency towards higher bax mRNA levels, only. Surprisingly, CAPAN-1 cells responded to TSA application by a dose-dependent, significant decrease of bax mRNA expression (Figure 5A).

Treatment of BxPC-3 and AsPC-1 cells with TSA caused a strong increase of p21^{Waf1} mRNA expression, while in CAPAN-1 cells this effect was less pronounced (Figure 5B). Similar results were obtained at the level of p21^{Waf1} protein expression (Figure 5C). Here, in CAPAN-1 cells the TSA effect did not reach statistical significance.

DISCUSSION

The molecular targets of HDACI in PC cells have been studied by several groups in recent years. The results suggest that inhibitors of cell-cycle progression, such as p21^{Waf1}/CDKN1A, and regulators of cell survival/death are involved in the mediation of antiproliferative and proapoptotic effects of HDACI^[13-15,17]. The molecular basis of variations in the biological efficiency of HDACI in different PC cells, however, has not been systematically studied so far. It is also largely unknown how precisely the increase of histone acetylation is linked to the biological and molecular effects of HDACI described above. To address these questions, we chose an experimental model system of three PC cell lines that differ significantly in their biological responsiveness to the HDACI trichostatin A. BxPC-3 cells, which displayed the strongest increase of histone H3 acetylation and decrease of DNA synthesis, were the only wild-type *KRAS* cells in this study. Interestingly, only BxPC-3 cells also showed a significant inhibition of ERK 1/2 and AKT phosphorylation in response to TSA treatment. Both ERK 1/2 (through Raf-1 and MEK) and AKT (through PI 3-kinase) are downstream of *KRAS*^[26]. We therefore hypothesize that oncogenic *KRAS* mutations may reduce, but not abolish, TSA efficiency by preventing an inhibition of AKT and ERK 1/2 signalling.

Although BxPC-3 cells were most sensitive to TSA treatment, DNA synthesis of CAPAN-1 and AsPC-1 cells could also be reduced by the drug. In CAPAN-1 cells, however, a significant effect was achieved at the high TSA concentration of 1×10^{-6} mol/L only. Searching for further molecular explanations of these phenomena, we found that CAPAN-1 cells showed the weakest increase of acetyl-histone H3, p21^{Waf1} and phospho-p38 levels. Furthermore, an unexpected decrease of bax mRNA expression

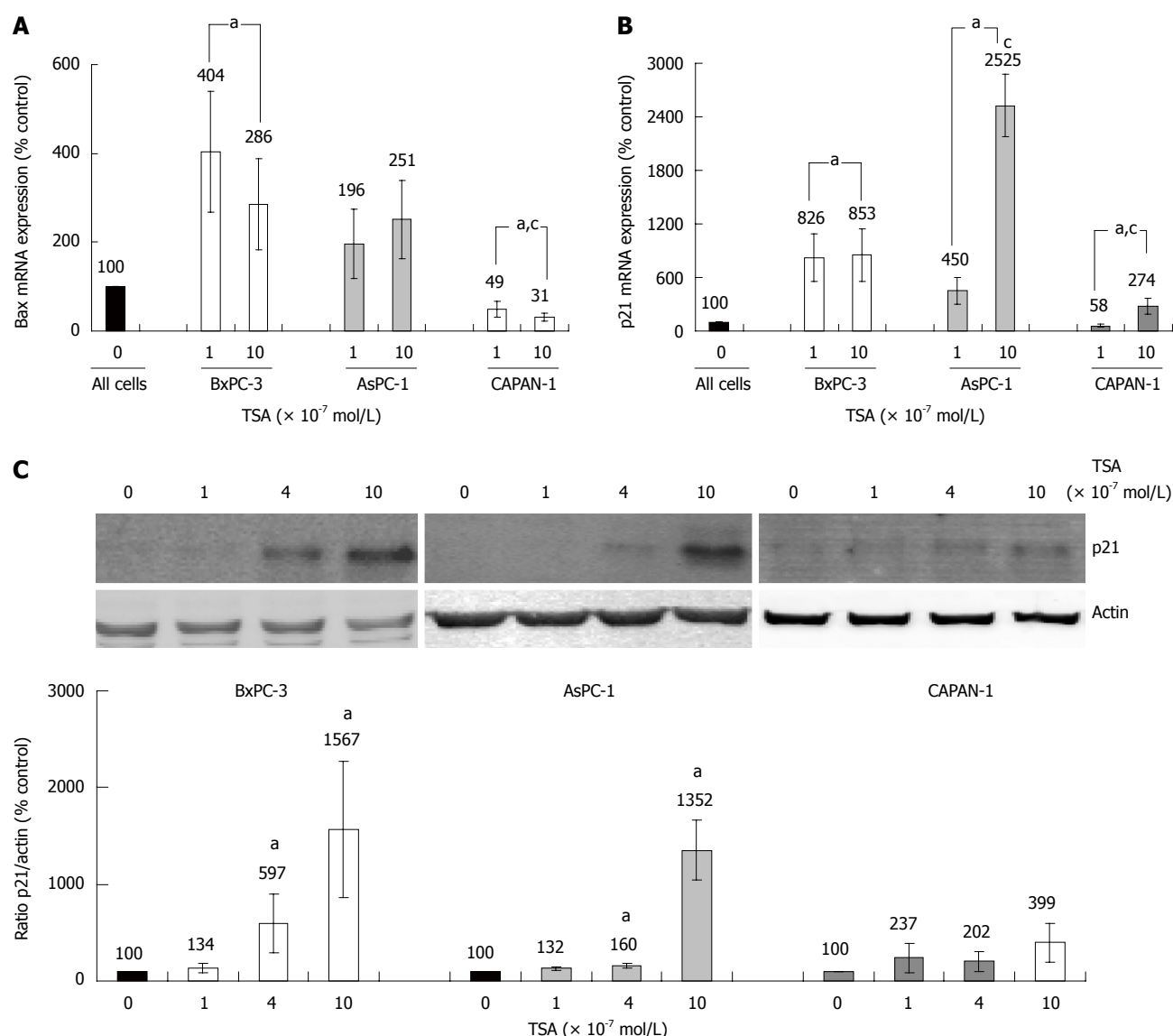


Figure 5 Effects of TSA on BAX and p21^{Waf1} expression in PC cell lines. A, B: The cell lines were incubated with TSA for 8 h as indicated. The mRNA expression of Bax (A), p21^{Waf1} (B) and the housekeeping gene GAPDH (A and B) was analyzed by real time PCR, and relative amounts of target mRNA were determined. 100% mRNA expression of each gene corresponds to cells cultured without TSA. Data of ≥ 6 independent experiments were used to calculate mean values and SE; ^a $P < 0.05$ vs control cultures, ^c $P < 0.05$ vs BxPC-3 cells (Mann-Whitney test); C: BxPC-3, AsPC-1 and CAPAN-1 cells were treated with TSA at concentrations up to 10×10^{-7} mol/L for 24 h. Expression of the p21^{Waf1} protein and actin (housekeeping control protein) was analyzed by immunoblotting. Fluorescence signal intensities of p21^{Waf1} and actin were quantified, and the ratio p21^{Waf1}/actin was determined. A ratio of 100% corresponds to control cells cultured without TSA. Data of 8 independent experiments were used to calculate mean values and SE; ^a $P < 0.05$ vs control cultures, ^c $P < 0.05$ vs BxPC-3 cells (Wilcoxon's rank sum test).

ssion was observed in these cells.

Taken together, our data suggest that the biological efficiency of TSA in the different PC cell lines correlates with the increase of histone H3 acetylation, p21^{Waf1} expression and p38 MAP kinase phosphorylation, and the decrease of ERK 1/2 and AKT phosphorylation. Using a specific inhibitor of p38, we also obtained evidence for a direct involvement of this kinase in the mediation of the antiproliferative effect of TSA in the cell line BxPC-3. Along these lines, the efficiency of TSA in PC cells might be restricted by antagonistic effects of other factors, such as oncogenic KR4S, on intracellular pathways that are critical for HDAC1 action.

Considering established crosstalks between growth-reg-

ulatory pathways, we hypothesize that the diverse molecular effects of TSA observed in this study are, at least in part, causally related. Specifically, we suggest direct links between modulation of MAP kinase (ERK, p38) signalling by the drug and induction of p21^{Waf1} expression, since activation of p38 and inhibition of ERK phosphorylation have previously been shown to enhance p21^{Waf1} promoter activation in other contexts^[27-29]. Furthermore, AKT has been implicated in functional inactivation of p21^{Waf1} through the phosphorylation of the protein, resulting in its cytoplasmic localization^[30]. While cellular localization of p21^{Waf1} was not investigated in this study, we observed an inverse correlation between p21^{Waf1} expression and AKT activity.

A detailed knowledge of the molecular mechanisms

of TSA action is essential to understand what determines the HDACi sensitivity and resistance of tumors. Our data encourage further studies in the field of pancreatic cancer, where novel therapeutic approaches are particularly needed.

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COMMENTS

Background

Pancreatic cancer has the worst prognosis of all common human tumors, indicating an urgent need for novel therapeutic approaches. Histone deacetylase inhibitors, such as trichostatin-A (TSA) or the clinically available drug SAHA, display anti-cancer activities *in vitro* and *in vivo*. They are therefore under experimental investigation for the treatment of different human malignancies. Histone deacetylase inhibitor (HDACi) act as epigenetic regulators by increasing histone acetylation, which is associated with a repressed chromatin state.

Research frontiers

While the immediate targets of HDACi action are clear, the precise molecular links between histone protein acetylation and suppression of cancer cell growth remain to be established. In most types of cancer cells, inhibitors of cell cycle progression and regulators of cell survival/apoptosis are important mediators of HDACi effects. However, a systematic overview of the underlying molecular mechanisms is missing. Thus, it is currently unknown which genes and proteins critically determine the efficacy or ineffectiveness of HDACi in pancreatic cancer (PC) cells.

Innovations and breakthroughs

The results of this study provide molecular insights into HDACi action in pancreatic cancer cells. The data suggest that the biological efficiency of TSA in different PC cell lines, indicated by the inhibition of DNA synthesis, correlates with the increase of histone H3 acetylation, but also both with genetic alterations and specific effects at the level of intracellular signal transfer as well as gene expression. In detail, expression of wild-type *KRAS*, a strong increase of bax, p21^{Waf1} and phospho-p38 levels, and a diminished phosphorylation of ERK 1/2 and AKT were found to be associated with a high biological efficiency of the drug. The authors hypothesize that PC cells differ in their biological sensitivity to TSA, since not all tumor cells exhibit the full range of molecular targets required for an effective HDACi action.

Applications

The results of this study open an avenue for the further investigation of the molecular determinants of HDACi action in PC cells. An improved understanding of HDACi effects at the molecular level may ultimately lead to the establishment of markers predicting the HDACi sensitivity or resistance of tumors.

Terminology

Pancreatic cancer is a malignancy of the pancreas with poor prognosis that typically displays the histological features of a ductal adenocarcinoma. HDACi, including trichostatin A, are substances that increase histone acetylation by inhibiting the activity of histone deacetylases. MAP kinases (such as ERK 1/2 and p38), PTEN and AKT are intracellular proteins that are involved in the intracellular transfer of receptor-derived signals regulating e.g. cell growth and survival. p21^{Waf1} belongs to a family of proteins that prevent cells from entering the S-phase of the cell cycle, while bax is an inducer of apoptotic cell death.

Peer review

It is an interesting and well written manuscript, and a scientifically sound modest extension of what is known regarding histone deacetylase inhibitors and cancer.

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Application of hepatitis B virus replication mouse model

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Abstract

AIM: To evaluate the value of the hepatitis B virus (HBV) replication mouse model with regard to several aspects of the study of HBV biology.

METHODS: To evaluate the HBV replication mouse model in detecting the efficacy of anti-HBV agents, the interferon inducer polyinosinic-polytidylin acid (polyIC) and nucleotide analogues adefovir and entecavir were administered to mice injected with wild type pHBV4.1, and the inhibiting effect of these agents on HBV DNA replication was evaluated. To identify the model's value in a replication ability study of HBV drug-resistant mutants and a HBx-minus mutant, telbivudine resistance mutants (rtM204I, ayw subtype), adefovir resistance mutants (rtA181V + rtN236T, ayw subtype) and HBx-minus mutants were injected respectively, and their corresponding HBV DNA replication intermediates in mouse liver were assessed.

RESULTS: Compared with the wild type HBV replication mouse model without antiviral agent treatment, the HBV DNA replication intermediates of the polyIC-treated group were decreased 1-fold; while in the entecavir- and adefovir-treated groups, the levels of HBV DNA replication intermediates were inhibited 13.6-fold and 1.4-fold, respectively. For the mouse models injected with telbivudine resistance mutant, adefovir resistance mutant and HBx-minus mutant, HBV DNA replication intermediates could still be detected, but the levels of HBV DNA replication intermediates of these mutants decreased 4.5-fold, 5.6-fold and 2.9-fold respectively, compared with the mouse model with wild type HBV plasmid.

CONCLUSION: The HBV replication mouse model we established was a useful and convenient tool to detect the efficacy of antiviral agents and to study the replication ability of HBV mutants *in vivo*.

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Key words: Hepatitis B virus; Antiviral agents; Drug resistance; Mutants; Mouse model

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INTRODUCTION

Hepatitis B virus (HBV) is an enveloped hepatotropic DNA virus associated with significant morbidity and mor-

tality. Globally, at least 2 billion people or one third of the world population have been infected with HBV, over 378 million people (or 6% of the world population) are chronic carriers, and approximately 620000 people die each year from acute and chronic sequelae secondary to HBV infection^[1]. As the pathogenic mechanisms of HBV remain to be elucidated, molecular biological studies for HBV are still required. Present options to treat chronic HBV infection are restricted to the use of interferon and nucleoside analogues; interferon was the first drug approved for the treatment of HBV although the response rates were not high and there were some side effects^[2,3]. The recent development of nucleoside analogues to treat HBV infection has accelerated with the characterization of the molecular mechanism of HBV replication. Nucleoside analogues interfere with the viral reverse transcriptase and thus inhibit viral replication^[4]. However, the development of drug resistance is a major limitation to long-term effectiveness of antiviral agents. Therefore, the current treatment is not ideal in clinical studies and it is necessary to develop new anti-HBV therapeutic options for chronic hepatitis B patients^[5]. Understanding the mechanisms of drug resistance mutants is also important for designing new agents and devising strategies to manage and prevent the development of antiviral drug resistance^[6-8].

As the host range of HBV is limited to humans and primates, most *in vivo* studies about the mechanism of HBV replication and HBV-associated liver diseases, and about the selection of anti-HBV drugs, have been performed by using HBV transgenic mice^[9-12]. However, the acquisition of transgenic mice is very time-consuming and laborious, needing expensive microinjection equipment and complicated technologies. It is costly and inconvenient to establish HBV transgenic mice with every mutant^[13]. Thus, the availability of a small model which can be easily established to study the *in vivo* efficacy of new antiviral drugs and mechanisms of HBV virulence would help to develop new options to control HBV replication. In our lab, a simple and convenient immunocompetent mouse model for HBV replication has been established by using a hydrodynamic-based procedure^[14].

As our mouse model is a transient HBV transfection model with viral replication and expression lasting for 10 d and peaking at day 3 and day 4 after transfection, whether this HBV replication mouse model can meet the applicable requirement and be used in studies about HBV is unclear. The present study was performed to identify these issues by utilizing the HBV replication mouse model to evaluate the effect of polyIC and nucleoside analogues on HBV replication and to investigate the replication ability of drug-resistant HBV mutants and an HBx-minus mutant^[15].

MATERIALS AND METHODS

Plasmids

Wild type pHBV4.1 is an HBV replication competent plasmid which contains 1.3 copies of the HBV genome (subtype ayw)^[16,17]; the telbivudine resistance mutant named as pLdT is a plasmid at which the residue rt204

ATG (M, Methionine) is changed into ATT (I, Isoleucine); the adefovir resistance mutant named as pADV2 is a plasmid at which the residue rt181 GCT (A, Alanine) is changed into GTT (V, Valine) and the residue rt236 AAC (N, Asparagine) is changed into ACC (T, Threonine). Both mutants were derived from wild type pHBV4.1 by site-directed mutagenesis (QuikChange mutagenesis kit, Stratagene) according to the manufacturer's instructions and were confirmed by sequencing. The plasmid payw1.2 is a replication competent construct that contains 1.2 copies of the wild type HBV genome (subtype ayw). The HBx-minus mutant payw*7, which contains an ochre termination signal (CAA to UAA) after codon 7 (at codon 8) in the HBx ORF and lacks the expression of HBx, was derived from payw1.2 by site-directed mutagenesis^[18,19].

Animals

Balb/c male mice (6-8 wk old) at specific pathogen-free (SPF) level, weighing 18-20 g, were purchased from the Huaxi Laboratory Animal Center of Sichuan University, China. All mice drank tap water and were fed rat chow *ad libitum* and were kept within a 12 h light-dark cycle at constant temperature and humidity. All mice were cared for in compliance with institutional guidelines. For establishment of the mouse model, the mice were injected with 10 µg HBV plasmid DNA in 2 mL phosphate-buffered saline (PBS) *via* the tail vein within 5-8 s^[20].

Study procedure

To evaluate the HBV replication mouse model in screening the efficacy of antiviral drugs *in vivo*, polyinosinic-polytidylin acid (polyIC) (Sigma USA) and nucleoside analogues adefovir (GlaxoSmithKline) and entecavir (Bristol-Myers Squibb) were administered by injection or oral gavage and their inhibiting efficacies on HBV replication (wild type HBV) were assessed. Twenty-four hours after the HBV replication mouse model was established, 200 µg polyIC in 200 µL PBS was given three times at 24 h intervals, and 200 µL PBS was used as a control. Twenty-four hours after hydrodynamic injection with pHBV4.1 plasmid, the nucleoside analogue-treated groups were administered adefovir by oral gavage at a dose of 1.5 mg/kg per day, and entecavir at a dose of 0.075 mg/kg per day in a medication course lasting 3 d, while the PBS group was used as a control. On the third day, the mice were sacrificed 4-6 h after the oral gavage. For each group there were at least 3 mice in parallel.

To identify the value of the HBV replication mouse model for assessing replication ability of HBV mutant strains, the drug-resistance mutants pLdT (rtM204I, ayw subtype) and pADV2 (rtA181V + rtN236T, ayw subtype) as well as HBx-minus mutant payw*7 were injected respectively. Three days after injection, all mice were sacrificed and samples of liver tissue and blood were collected.

Liver tissue was collected and frozen in liquid nitrogen. Blood samples were processed for collection of serum and stored at -20°C until assayed. The corresponding HBV DNA replication intermediates in liver were assessed as follows.

Detection of hepatitis B surface antigen and hepatitis B e antigen by enzyme-linked immunosorbent assay:

Hepatitis B e antigen (HBeAg) and hepatitis B surface antigen (HBsAg) qualitative analysis was performed with 50 μ L mouse serum by using enzyme-linked immunosorbent assay (ELISA). HBeAg and HBsAg detection kits (Shanghai Shiye Kehua Company, China) were used respectively, according to the manufacturer's instructions, and samples were considered positive at $A_{450} \geq 2.1$.

Detection of HBV DNA replication intermediates by Southern blotting:

The frozen liver tissue was mechanically pulverized in liquid nitrogen and HBV DNA replicative intermediates were isolated as described previously^[21]. One hundred and twenty micrograms of liver tissue powder was lysed in 0.6 mL of 100 mmol/L Tris hydrochloride (pH 8.0) and 2 mL/L NP40. The supernatant was adjusted to 6.75 mmol/L magnesium acetate plus 200 μ g DNaseI/mL, and incubated for 1 h at 37°C. The supernatant was readjusted to 10 mmol/L ethylenediaminetetraacetic acid (EDTA), 8 g/L sodium lauryl sulfate, 100 mmol/L NaCl and 1.6 mg pronase/mL, and was incubated for an additional 1 h at 37°C. The supernatant was extracted twice with phenol/chloroform, precipitated with 0.7 volume of isopropanol, and resuspended in 30 μ L of 10 mmol/L Tris hydrochloride (pH 8.0) and 1 mmol/L EDTA. DNA Southern blotting hybridization analysis was performed with 30 μ L viral replication intermediates, as previously described. Filters were probed with digoxigenin-labeled (Roche Applied Science) HBV aytw genomic DNA to detect HBV sequences. The level of HBV DNA replicative intermediates was calculated by the Quantity One software according to the manufacturer's instructions.

RESULTS

The effect of polyIC on HBV replication

PolyIC is a strong inducer of internal interferon and its efficacy on HBV replication *in vivo* was studied. As shown in Figure 1A and B, the level of HBV DNA replication intermediates in liver tissue of the polyIC-treated group was inhibited by about one-fold, as compared with that of the PBS-treated group. This result suggested that polyIC injection decreased the level of viral replication in the liver.

The effect of nucleoside analogues on HBV replication

To investigate whether the HBV replication mouse model can be used in the selection of anti-HBV agents, we used this model to test the effect of entecavir and adefovir, two of the approved nucleotide analogues for chronic hepatitis B, on HBV replication. Figure 2A and B show the levels of HBV DNA replication intermediates in liver tissue of the mouse model. Compared with the PBS-treated group, the HBV DNA replication intermediate levels in the entecavir-treated group and the adefovir-treated group were inhibited by around 13.6-fold and 1.4-fold, respectively. In contrast to these results, it appeared that both nucleo-

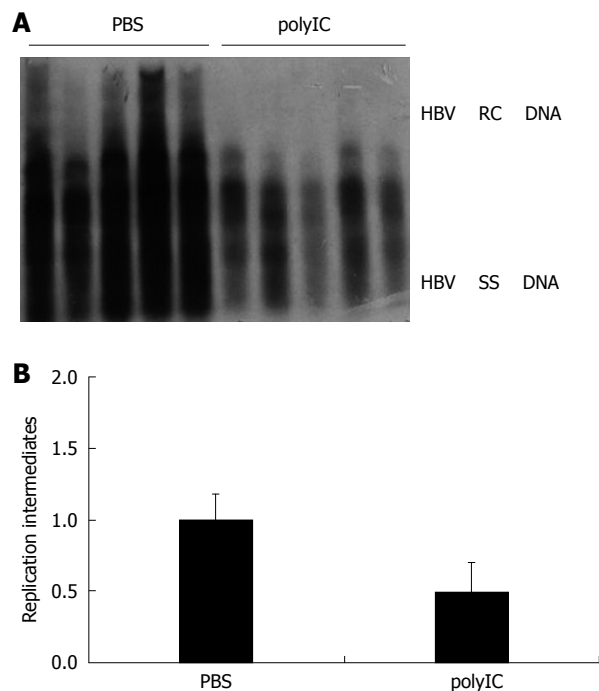


Figure 1 Evaluation of the effect of polyIC on hepatitis B virus (HBV) replication using the HBV replication mouse model. A: The effect of polyIC on HBV DNA replication intermediates in mouse model. After hydrodynamic transfection, mice were treated with phosphate-buffered saline (PBS) (control) or polyIC; B: The relative intensity of HBV DNA replication intermediates of the mouse model for PBS group and polyIC group which inhibited the HBV replication by about one fold. The levels of HBV DNA replication intermediates in PBS-treated group were set to 1, and the relative levels of polyIC-treated group were calculated accordingly.

side analogues did not influence the expression of HBV-associated antigens, as the HBeAg and HBsAg were both positive in the serum of the mouse model after nucleoside analogue treatment for 3 d.

Replication ability of drug-resistant HBV mutants

To investigate whether our HBV replication mouse model can be used in studies about replication ability of drug-resistant HBV mutants, the mouse model was used to study the replication ability of two drug-resistant HBV variants: an adefovir resistance mutant and a telbivudine resistance mutant. The HBV DNA replication ability of both drug-resistant HBV mutants decreased by a large amount compared with the wild type control mouse group. HBV DNA replication in the telbivudine resistance mutant pLdT (rtM204I) was decreased about 4.5-fold and in the adefovir resistance mutant pADV2 (rtA181V + N236T) was decreased about 5.6-fold, compared with the wild type mice (Figure 3A-D). This result suggested that these mutations in the drug-resistant HBV mutants could result in the decline of replication ability. However, the HBsAg and HBeAg were both positive in the serum of the mouse model as compared with the wild type control.

Replication ability of HBx-minus mutant

In order to evaluate whether the HBV replication mouse model can be used in the study of the mechanism by which HBV replicates, we utilized this model to identify

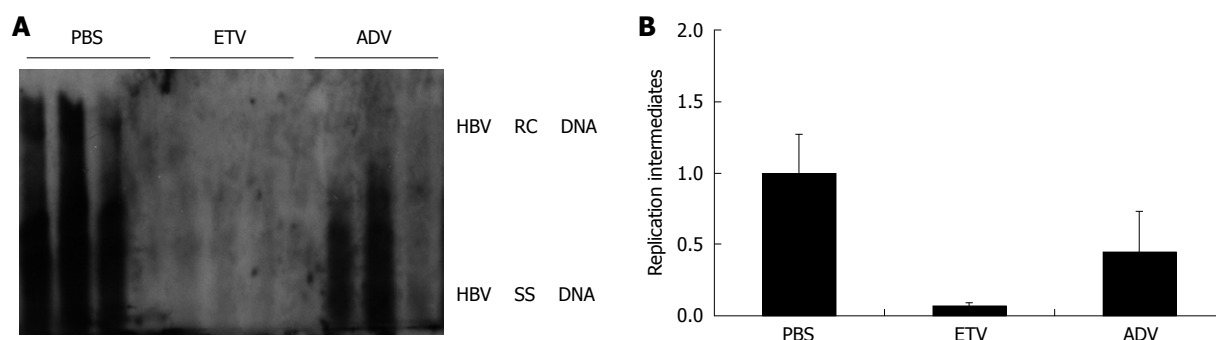


Figure 2 Evaluation of the effect of nucleoside analogues entecavir (ETV) and adefovir (ADV) on HBV replication using the HBV replication mouse model. After hydrodynamic transfection, mice were administered by oral gavage with two different nucleoside analogues: entecavir at a dose of 0.075 mg/kg per day and adefovir at a dose of 1.5 mg/kg per day for 3 d. A: HBV DNA replication intermediates in the mouse model treated with PBS (control), entecavir and adefovir, respectively; B: The relative levels of HBV DNA replication intermediates in the mouse model among different groups treated with PBS (control), entecavir and adefovir, respectively. The levels of HBV DNA replication intermediates in PBS treated group were set to 1, and the relative levels of treated groups were calculated accordingly.

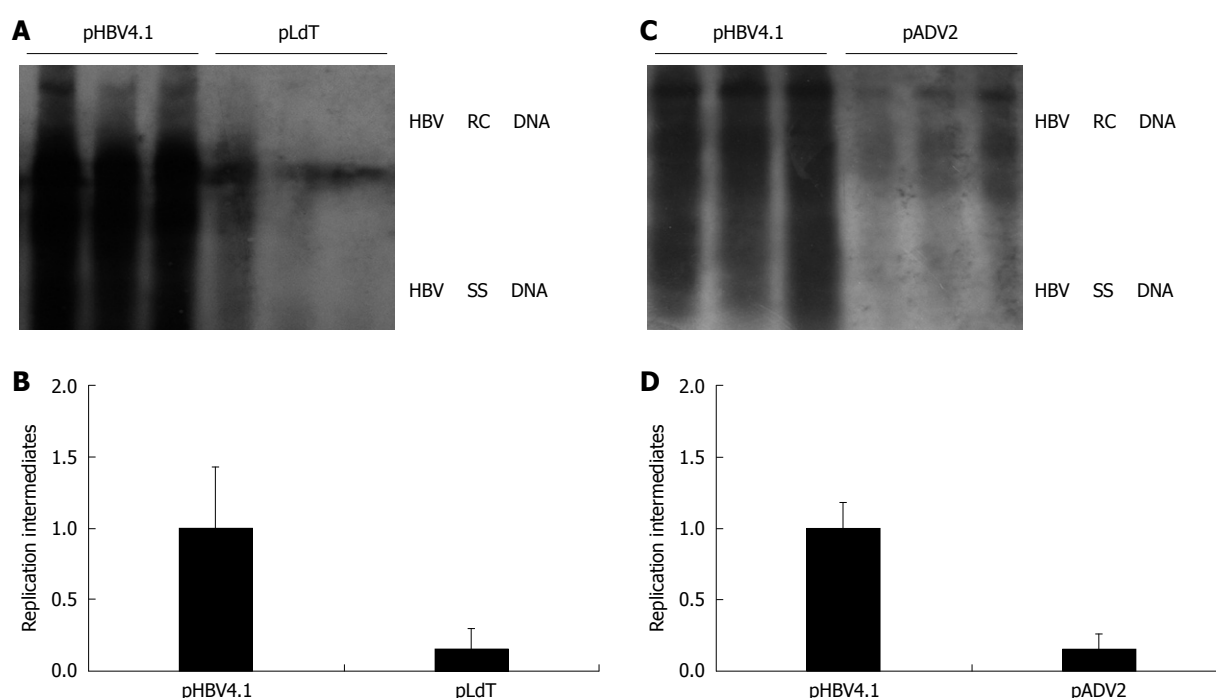


Figure 3 Assessment of HBV replication ability of drug-resistant HBV mutants using the HBV replication mouse model. The levels of HBV DNA replication intermediates in wild type group were set to 1, and the relative levels of mutant groups were calculated accordingly. A, B: The HBV DNA replication intermediates and relative intensity between wild type injected with pHBV4.1 and telbivudine resistance mutant injected with pLdT (rtM204I); C, D: The HBV DNA replication intermediates and relative intensity between wild type injected with pHBV4.1 and adefovir resistance mutant injected with pADV2 (rtA181V + N236T).

the role of the HBx-minus mutant in HBV replication. Plasmids of wild type and HBx-minus mutant were transferred into Balb/c mice, respectively. The levels of HBV DNA replication intermediates from these two constructs were compared (Figure 4A). In both wild type and HBx-minus mutant groups HBV DNA replicated in this mouse model, which suggested that both plasmids had ability to replicate *in vivo*. However, the levels of HBV DNA replication intermediates of the HBx-minus mutant were decreased by nearly 2.9-fold compared with the payw1.2 control in this mouse model (Figure 4B).

DISCUSSION

The development of animal models for HBV infection is

crucial for understanding viral replication, disease pathogenesis, and selecting candidate drugs for the treatment of HBV infection^[22]. However, the narrow range of hosts for the virus has limited the availability of animal models. Chimpanzees are susceptible to HBV infection, but experiments with chimpanzees are limited by cost, availability and ethical considerations. Hepadnaviruses such as ground squirrel hepatitis virus, woodchuck hepatitis virus and duck HBV have offered opportunities to investigate some mechanisms of viral pathogenesis, but these hepadnaviruses have some differences to HBV in terms of genome structure, transcription and replication regulation. Therefore, they cannot serve as a substitute for research into HBV^[23]. Recently, much attention has been paid to HBV transgenic mice, especially HBV

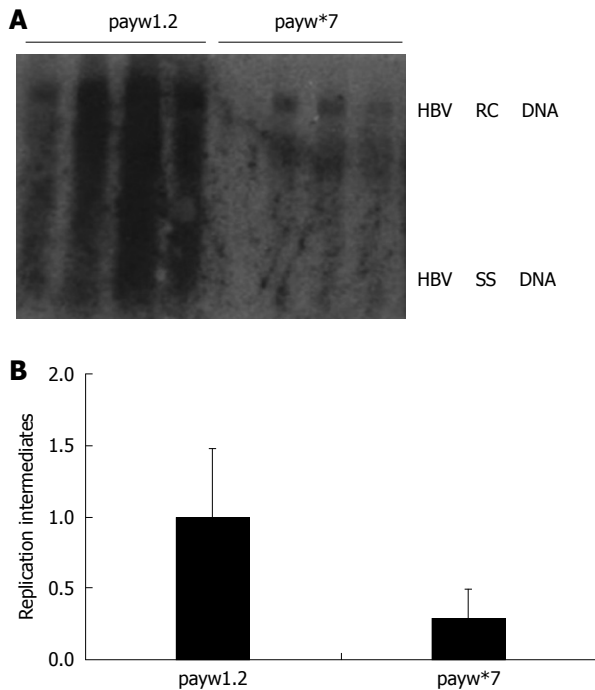


Figure 4 Assessment of HBV replication ability of HBx-minus mutant using the HBV replication mouse model. A: The levels of HBV DNA replication intermediates between wild group injected with payw1.2 and HBx-minus mutant group injected with payw*7; B: The relative intensity of HBV DNA replication intermediates between wild group injected with payw1.2 and HBx-minus mutant group injected with payw*7. The levels of HBV DNA replication intermediates were reported to the levels of the mice injected with payw1.2, which were set at 1.

replication-competent transgenic mice with 1.3 copies of HBV genome. These transgenic mice have provided information on pathogenesis and virus replication and are widely used in studies of HBV. However, a plasmid needs to be transferred into a male pronucleus, then injected embryos are implanted into pseudopregnant females and live births tested for transgene, before transgenic mice can be cultivated; this process requires a great deal of time, cost and labor^[24,25]. Furthermore, in HBV transgenic mice all viral RNA is synthesized from a chromosomally integrated copy of the virus, whereas no cccDNA, the natural template of viral transcription *in vivo*, is produced^[26,27]. By using a hydrodynamic-based procedure, our laboratory has established a rapid and convenient mouse model with a high level of HBV replication. In this study, this mouse model was used to evaluate the inhibitory effect of some anti-HBV agents on HBV replication, the replication ability of HBV-resistant mutants and the effect of HBx on HBV replication; the results shown above suggesting that this model can be used for studies of HBV biology.

The principal treatments for chronic hepatitis B include the use of interferon and nucleoside analogues. PolyIC is a strong inducer of internal α/β interferon which could inhibit the HBV replication. In our study, it was shown that after treating the animal models with polyIC, the level of HBV replication decreased; there were at least 3 mice in parallel for each group to ensure the reliability and accuracy of results. The effect of polyIC injections on HBV replication in these mice was less than that in HBV trans-

genic mice reported previously^[28] but supportive of the suggestion that an inhibitory effect of α/β interferon can be achieved in this mouse model. Similarly, we found that entecavir and adefovir, two of the approved nucleoside analogue treatments for chronic hepatitis B in the clinic, could also inhibit HBV replication in this mouse model. Furthermore, there are different inhibition levels according to the two different drugs as entecavir showed greater effects than adefovir in our study; this corresponds with the clinical report that entecavir has the greatest inhibitory effect on HBV^[29]. These results demonstrate that this animal model can be used to study existing and potential therapeutic approaches directed against HBV.

Long-term treatment of HBV with nucleoside analogues is associated with the emergence of resistant virus strains. The rate of development of lamivudine resistance increases progressively during treatment with rates of 20% to 25% reported annually and approaching more than 80% after 4 to 5 years of therapy^[30,31]. The resistance rate to adefovir is 2% at 2 years and 29% at 5 years. It was previously demonstrated that the HBV mutation resistant to adefovir is the rtA181V + N236T^[32-34], which was associated with a 5 to 10 fold decrease in sensitivity to adefovir and a moderately reduced replication capacity observed in *in vitro* assays^[35-37]. The viral mutant resistant to telbivudine is the rtM204I mutation. It has been reported that the presence of resistant variants is one of the factors that causes severe liver damage and even fulminant liver failure, while the mechanisms remain unclear^[38-40]. Therefore, the biological properties and mechanisms by which HBV-resistant variants cause deteriorated liver function need to be explored, and the effective agents against these variants need to be selected^[41]. If HBV transgenic mice are used, it is very impractical to construct transgenic mice with every mutant. In contrast, it was demonstrated that the properties of HBV-resistant variants could be tested in a convenient HBV replication mouse model. Our results suggest that the mutations in drug-resistant HBV mutants could affect the replication process and result in the decline of replication ability but that they do not affect the transcription process and the expression of HBsAg and HBeAg. This could be because mutations may cause allosteric changes in the catalytic region of polymerase and affect the enzyme function but the transcription of viral messenger RNA which is translated into viral proteins is not affected. Thus, this mouse model of HBV replication will greatly facilitate studies about HBV drug-resistant mutants.

To understand the mechanism of HBV transcription, replication and pathogenesis, it has been necessary to mutate the HBV genome and then survey the transcription, replication and expression levels of viral mutants. In this way, the function of different HBV genome sequences can be understood. However, this research would be very time-consuming, costly and laborious if HBV transgenic mice are used. In the present study, the results were consistent with previous observations in HBV transgenic mice and cell culture as mentioned above, suggesting that this mouse model could be used in functional studies about HBV and that this model of HBV replication will

also provide a useful tool for mechanism studies of HBV.

From these data we can draw the conclusion that this rapid and convenient model of HBV replication affords new opportunities and can be applied widely to many aspects of the study of HBV such as selection of antiviral candidates against HBV, the properties of drug-resistant variants, and the studies of HBV replication mechanisms. Furthermore, the model is reliable and simple for the study of HBV genomic function, and especially for investigating the mechanisms involved in HBV replication through the mutating HBV genome and for selection of new agents against HBV mutants.

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COMMENTS

Background

More than 378 million people are chronic carriers of hepatitis B virus (HBV), and approximately 620 000 people die each year related to HBV infection. The lack of convenient and reliable animal models has hindered progress in HBV research. Recently, the authors have established a simple HBV replication mouse model using hydrodynamic tail vein injection of HBV replication competent plasmid (pHBV4.1) into Balb/c mice.

Research frontiers

An HBV mouse model established by using a hydrodynamic *in vivo* transfection method is useful and convenient. Chronic HBV mouse models have been established by using immunodeficient mice. With the progress of tissue engineering and transgenic technology, some liver cell humanized transgenic mice for studying human HBV have also been constructed.

Innovations and breakthroughs

In this study using an HBV replication model, the authors found that the *in vivo* ability of HBV DNA replication of drug-resistant HBV mutants decreased greatly, while the HBsAg and HBeAg were both positive in the serum compared with the wild type. It was further discovered that the lack of HBx also decreased the ability of HBV DNA replication *in vivo*. The authors demonstrated that the properties of HBV mutant variants could be tested in this convenient HBV replication mouse model without constructing transgenic mice.

Applications

The HBV replication mouse model established in this study is a rapid and convenient tool to detect the efficacy of antiviral agents and to study the replication ability of HBV mutants *in vivo*.

Peer review

The work done by Gao *et al* has a great value and the studied issue is applicable. It includes HBV gene expression and replication by HBV plasmids, which were delivered to the mouse liver by hydrodynamic injection. Development of methods that allow an efficient expression of exogenous genes in animals would provide tools for gene function studies, treatment of diseases and for obtaining gene products.

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Neoadjuvant chemoradiotherapy for esophageal cancer: Impact on extracapsular lymph node involvement

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METHODS: Two hundred and ninety-eight patients with advanced esophageal cancer underwent esophagectomy between 1997 and 2006. One hundred and ninety patients (63.8%) were treated with neoadjuvant CRT prior to resection. A total of 986 metastatic LNs were examined. Survival of the patients was analyzed according to intra- and extra-capsular LNI.

RESULTS: Five-year survival rate was 22.5% for the entire patient population. Patients with extracapsular LNI had a 5-year survival rate of 16.7%, which was comparable to the 15.8% in patients with infiltrated nodes of the celiac trunk (pM1lymph). In contrast to patients treated with surgery alone, neoadjuvant therapy resulted in significantly ($P = 0.001$) more patients with pN0/M0 (51.6% vs 25.0%). In 17.6% of the patients with surgery alone vs 16.8% with neoadjuvant CRT, extracapsular LNI was detected. Neoadjuvant therapy does not reduce the occurrence of extracapsular LNI.

CONCLUSION: Extracapsular LNI is an independent negative prognostic factor not influenced by neoadjuvant CRT. In a revised staging system for esophageal cancer, extracapsular LNI should be considered.

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Key words: Esophageal cancer; Neoadjuvant therapy; Chemotherapy; Radiotherapy; Adenocarcinoma; Squamous cell carcinoma; Lymph node metastasis; Extracapsular lymph node involvement; Prognosis

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Abstract

AIM: To assess the effects of neoadjuvant chemoradiotherapy (CRT) on the presence of extracapsular lymph node involvement (LNI) and its prognostic value in patients with resected esophageal cancer.

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INTRODUCTION

Surgery remains the treatment of choice for most localized esophageal cancers. However, despite complete tumor resection and extensive lymphadenectomy, systemic and local recurrence is common, and the 5-year survival rate is 15%-39%^[1]. There is increasing evidence to include combined neoadjuvant chemoradiotherapy (CRT) as an alternative to surgical resection alone, to improve survival for locoregional esophageal cancer. The aim of combining neoadjuvant chemotherapy and radiotherapy is to reduce the tumor size and maximize local control. A meta-analysis from GebSKI *et al*^[2] that included data from 10 neoadjuvant CRT trials of 1209 patients has shown an absolute overall survival benefit of 13% when compared with surgery alone. This result prompted some investigators to consider neoadjuvant treatment as the standard of care in esophageal cancer^[3].

Regarding prognostic factors for esophageal cancer treated with neoadjuvant CRT, histomorphological tumor regression and the extent of lymphatic dissemination are among the most important predictors for survival in gastrointestinal malignancies^[4,5]. More recently, attention has been paid to the prognostic value of extracapsular lymph node involvement (LNI) in patients with gastrointestinal malignancies. Extracapsular LNI is the extension of cancer cells through the nodal capsule into the perinodal fatty tissue: a common phenomenon in gastrointestinal cancer patients^[6]. Its presence identifies a subgroup of patients with a significantly worse long-term survival.

However, the effects of neoadjuvant therapy on the presence of extracapsular LNI and its prognostic relevance are unclear because to date, most studies have not included patients undergoing such therapy.

The aim of this study was to assess the prevalence as well as the prognostic impact of extracapsular LNI in patients with resected esophageal cancer who were treated with neoadjuvant CRT.

MATERIALS AND METHODS

Patients

Four hundred and thirty-six patients with esophageal cancer were treated in the Department of Surgery between January 1, 1997 and December 31, 2006. Two hundred and ninety-eight patients had locally advanced tumors with cT3-4. The histological distribution was 155 (52.0%) squamous cell carcinoma and 143 (48%) adenocarcinoma. One hundred and ninety of these patients were treated with neoadjuvant CRT prior to resection. The reasons for primary surgical resection

Table 1 Demographic data of 298 patients with advanced esophageal cancer *n* (%)

Variables	Surgery alone	Neoadjuvant CRT	Significance
Patients	108	190	
Sex			NS
Male	86 (80.0)	151 (79.4)	
Female	22 (20.0)	39 (20.6)	
Age (yr)	63.6	60.1	<i>P</i> < 0.01
< 50	11 (10.2)	38 (20.0)	
50-70	65 (60.2)	124 (65.3)	
> 70	32 (29.6)	18 (14.7)	
Histological subtype			0.871
Squamous cell carcinoma	55 (51.0)	100 (52.6)	
Adenocarcinoma	53 (49.0)	90 (47.4)	
No. of resected LNs (median, range)	30 (3-74)	27 (4-55)	<i>P</i> = 0.007
pN category			<i>P</i> < 0.001
pN0	27 (25.0)	98 (51.6)	
pN1	81 (75.0)	92 (48.5)	
LN ratio			<i>P</i> < 0.001
pN0	27 (25.0)	98 (51.6)	
LN ratio < 0.2	52 (48.1)	69 (36.3)	
LN ratio ≥ 0.2	29 (26.9)	23 (12.1)	
pM1 organ	11 (10.1)	8 (4.2)	NS
R category			NS
R0	98 (92.6)	179 (94.2)	
R1/R2	10 (7.4)	11 (5.8)	
Response after neoadjuvant therapy			
Minor response	-	112 (59.0)	
Major response	-	78 (41.0)	

LN: Lymph node; CRT: Chemoradiotherapy; NS: Not significant.

were exclusion criteria for chemoradiation, such as age, comorbidity, and lack of patient consent. Relevant patient characteristics are summarized in Table 1. Written informed consent was obtained from all patients in this study.

Staging

TNM staging was performed according to the criteria of the International Union Against Cancer^[7]. Clinical staging was based on results from barium swallow examination, endoscopic ultrasound, and computed tomography (CT) of the chest and abdomen (4-mm sections). Endoscopy and endoscopic ultrasound were performed by two experienced examiners for all patients.

Surgical resection and CRT regimen

Surgical treatment of choice was subtotal *en bloc* esophagectomy using a right transthoracic approach including two-field lymphadenectomy of mediastinal and abdominal LNs. The specimens were removed *en bloc* including the LNs. To ensure primary tumor integrity, the LNs were dissected partially in the operating theater and partially by pathologists according to a standardized protocol. The examined LNs were documented according to the sixth edition of the TNM classification^[7]. The median number of examined LNs was 28 (4-74).

Standard reconstruction for patients receiving trans-

thoracic esophagectomy was done by stomach interposition with high intrathoracic esophagogastronomy^[8,9].

One hundred and ninety patients received preoperative CRT according to a standardized protocol, which is described in detail elsewhere^[5]. Locally advanced tumors (cT3-4) were included unless documented systemic metastases or bronchoscopically proven invasion of the tracheobronchial tree was present. Patients with cT2 tumors were offered this treatment protocol when CT showed a tumor mass compatible with a T3 category, while endoscopic ultrasound showed complete invasion of the muscularis propria without clear invasion of the adventitia (so-called near T3 categories). Cisplatin (20 mg/m² per day) was administered as a short-term infusion on days 1-5 and 5-fluorouracil (1000 mg/m² per day) as a continuous infusion over 24 h on days 1-5. Radiotherapy was administered by linear accelerators with 10-15-MV photons. Radiotherapy was simulated to encompass the tumor volume with 5-cm cephalo-caudal margins and 2-cm radial margins, and treatment ports were designed to include enlarged regional nodes based on CT evaluation and endoscopic ultrasound. Radiation was delivered in daily fractions of 1.8 Gy with a total dose of 36 Gy using a multiple field technique. Surgical resection was performed 4-5 wk following completion of CRT, after clinical restaging. If there was evidence for tumor progression, patients underwent definitive CRT without surgery.

Histopathology

Histopathological examination of all resected specimens consisted of thorough evaluations of tumor stage, residual tumor (R) category, grading, and number of examined and involved LNs. The specimens were fixed in 5% formaldehyde and embedded in paraffin. The LNs were counted and a series of sections from each node was selected and stained with hematoxylin and eosin, as well as periodic acid-Schiff. All dissected LNs were microscopically analyzed for metastatic disease.

Extracapsular LNI was defined as metastatic cancer extending through the nodal capsule into the perinodal fatty tissue. Examination of the LNs was performed by two experienced pathologists (S.E.B. and U.D.). Deposits of metastatic cancer cells without a recognizable LN were considered as extracapsular LNI, unless these deposits were associated with perineural and/or had vessel involvement. In cases of desmoplastic reactions resulting in difficulty in identifying the preexisting LN capsule, an imaginary line representing the original capsule was drawn to facilitate this interpretation (see also Lagarde *et al*^[10]).

The ratio of the number of involved to examined regional LNs was termed the lymph node ratio. According to the sixth edition of the TNM classification for tumors of the lower esophagus, metastasis in the celiac lymph node group (LNG 9) is classified as M1a, and in other non-regional locations, as M1b^[7]. After neoadjuvant therapy, the pathological assessment is difficult because of potential tumor regression. For this reason, such clas-

sification is identified with the prefix “y” to indicate that it does not have the same reliability as the pTNM classification after surgery alone^[11].

Statistical analysis

Beginning in 1997, data were collected prospectively according to a standardized protocol. Analysis of the data was performed retrospectively. The median, with the lower quartile and upper quartile, was used for descriptive statistics. χ^2 statistics were calculated for factor frequencies, with a significance level of $P < 0.05$.

The median follow-up time for the study patients was calculated using the time between surgical procedure and the end of follow-up for censored data; for deceased patients until December 31, 2007^[4]. The median follow-up time was 4.1 years (range: 0.5-10.5 years). All living patients had follow-up of > 1 year.

Kaplan-Meier plots were used to describe survival distribution^[12]. The log-rank test was used to evaluate survival differences^[13]. For multiple comparisons, the Holm-Sidak method was used. In addition, 95% CI for the different survival curves was calculated. Postoperative mortality was included in the calculation of prognosis. The 30-d postoperative mortality was 3.4%. The multivariate analysis of survival used Cox regression analysis to identify independent prognostic variables. The level of significance was set at $P < 0.05$.

All statistical analyses were performed using SPSS for Windows version 15.0. For graphic presentation of the results, Sigma-Plot version 8.0 was used.

RESULTS

Two hundred and ninety-eight patients with locally advanced esophageal cancer were included in this study. The median age of the patients was 61.0 years (range: 22-82 years). Neoadjuvant CRT was administered to 190 of the 298 patients (63.8%). The demographics and the established prognostic factors for the patients are shown in Tables 1 and 2.

A total of 8376 LNs (median 28; range: 4-74) were resected. All positive LNs ($n = 986$) were reexamined for the presence of extracapsular LNI. Tumor growth beyond the LN capsule was detected in a total of 351 lymph nodes.

Age, sex and differentiation grade were comparable between the patients with and without extracapsular LNI. The number of LNs with extracapsular involvement was significantly ($P < 0.001$) correlated with the number of positive nodes (data not shown). Extracapsular LNI was seen more often when the numbers of resected and identified nodes, the numbers of positive nodes, and the lymph node ratios were higher.

To analyze the effect of neoadjuvant CRT on the prevalence of extracapsular LNI, patients with and without neoadjuvant therapy were compared. 190 patients received neoadjuvant therapy, and 108 underwent surgery alone (Tables 2 and 3).

Table 2 Univariate survival analysis of 298 patients with advanced esophageal cancer

Variables	P	Surgery alone		Neoadjuvant CRT	
		n	5-yr survival (%)	n	5-yr survival (%)
Patients	NS	108	20.4	190	25.6
Sex					
Male	NS	86	22.0	151	24.4
Female	NS	22	13.8	39	32.6
Age (yr)					
< 50	NS	11	58.3	38	33.7
50-70	0.015	65	15.8	124	24.7
> 70	NS	32	15.1	18	0.0
Histological subtype					
Squamous cell carcinoma	NS	55	31.3	100	19.8
Adenocarcinoma	0.030	53	16.9	90	35.4
pN category					
pN0	NS	27	36.5	98	41.6
pN1	NS	81	15.4	92	12.3
LN ratio					
pN0	NS	27	36.5	98	41.6
LN ratio < 0.2	NS	52	23.7	69	10.3
LN ratio ≥ 0.2	NS	29	0.0	23	0.0
pM1 organ	NS	11	0.0	8	0.0
R category					
R0	NS	98	22.1	179	26.4
R1/R2	NS	10	0.0	11	0.0
Response after neoadjuvant therapy					
Minor response		-		112	11.4
Major response		-		78	42.4

Table 3 Influence of neoadjuvant CRT on extent of LN metastases and extracapsular LNI n (%)

T3/4 Category only (n = 298)	Surgery alone		Neoadjuvant CRT		Surgery vs neoadjuvant CRT	
	Total	pN1 caps (+)	Total	pN1 caps (+)	Total	pN1 caps (+)
Total	108 (100.0)	45 (41.7)	190 (100.0)	43 (22.6)		P < 0.001
pN0M0	27 (25.0)	-	98 (51.6)	-	P < 0.001	
pN1M0 caps (-)	28 (27.8)	-	38 (20.0)	-	NS	
pN1M0 caps (+)	19 (17.6)	19 (100)	32 (16.8)	32 (100.0)	NS	
pM1 lymph	23 (21.6)	18 (78.3)	14 (7.4)	8 (57.1)	P < 0.001	NS
pM1 organ	11 (10.1)	8 (72.7)	8 (4.2)	3 (37.5)	NS	NS

LNI: Lymph node involvement; Caps (-): Without extracapsular LNI; Caps (+): With extracapsular LNI.

The 5-year survival rate was 22.5% for the entire patient population. LN-negative patients had a 5-year survival rate of 34%. Intracapsular LNI decreased the 5-year survival rate to 20%. Detection of extracapsular LNI resulted in a 5-year survival rate of 7% ($P < 0.001$). The resection plane (R0 resection vs R1/2 resection) also proved to be an independent prognostic parameter ($P < 0.001$) in univariate analysis.

In the group with the neoadjuvant regimen, we observed a decrease in distant metastases (pM1 lymph + pM1 organ). Neoadjuvant therapy also resulted in a significant ($P = 0.001$) increase of patients with pN0/M0 (51.6%), compared with surgery alone (25%) (Table 3). After neoadjuvant therapy, there was also a survival benefit for patients with negative LNs. However, this was not significant compared to surgery alone (Table 2).

Neoadjuvant therapy did not reduce the occurrence of extracapsular LNI. In 19/108 patients (17.6%) with surgery alone vs 32/190 patients (16.8%) with neoad-

juvant CRT, extracapsular LNI was detected (Table 3, Figure 1).

All LNs with extracapsular involvement were within the radiation field. We did not detect a difference between patients treated with neoadjuvant CRT and those who were resected primarily.

Univariate and multivariate analysis (Table 4) revealed that pN, pM category, extracapsular LNI, and radicality of the resection plane were all significant prognostic parameters for esophageal cancer.

DISCUSSION

The presence and extent of lymphatic dissemination are among the most important predictors for survival in gastrointestinal malignancies^[14,15]. More recently, attention has been paid to the presence of extracapsular LNI, which identifies a subgroup of patients with significantly worse long-term survival^[16,10,16,17]. For breast cancer,

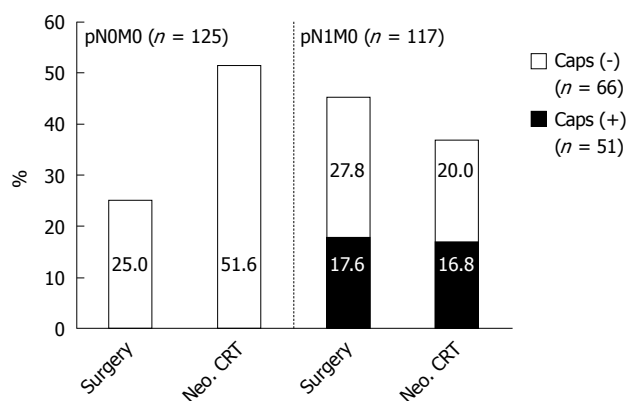


Figure 1 Extent of lymph node (LN) metastases and extracapsular LN involvement (LNI), comparing surgery alone with neoadjuvant chemoradiotherapy (CRT) and surgery. pN0M0: Neoadjuvant CRT resulted in an increase of patients with pN0/M0 compared to surgery alone ($P = 0.001$); pN1M0: Neoadjuvant CRT did not reduce extracapsular LNI. Caps (-): Without extracapsular LNI; Caps (+): With extracapsular LNI; Neo.: Neoadjuvant.

extracapsular spread is such an important factor that it has been appended to the TNM classification as a specific subcategory^[18].

In a recently published systematic review on the significance of extracapsular LNI in gastrointestinal malignancies, Wind and coauthors have identified seven papers that discuss the impact of extracapsular LNI in esophageal cancer^[6,10,17,19-23]. In general, these studies applied variable inclusion and exclusion criteria with respect to the type and stage of tumors and the use of (neo)adjuvant therapies. Only three studies from Tachikawa *et al.*^[23], Lerut *et al.*^[17], and Lagarde *et al.*^[10] have provided some detailed information on the prognostic value of the number of LNs with extracapsular involvement.

Due to the heterogeneity of the design of the existing studies, the effect of preoperative CRT on the presence and extent of extracapsular LNI is unclear.

In the present study, we demonstrated that extracapsular LNI was an independent prognostic parameter for esophageal cancer. Lerut *et al.*^[17], and Lagarde *et al.*^[10] have analyzed relatively uniform groups of patients with adenocarcinoma of the esophagus and have found a similar result. Also, for patients with squamous cell carcinoma, Tachikawa *et al.*^[23] have concluded that prognosis is significantly worse when extracapsular disease is confirmed. However, Tachikawa *et al.*^[23] analyzed a non-homogeneous cohort of patients with squamous cell carcinoma, treated with surgery and different chemotherapeutic regimens or no adjuvant therapy.

The present study comprised a consecutive series of 298 patients with adenocarcinoma and squamous cell carcinoma of the esophagus. There was a significant correlation between the number of positive nodes resected and the number with extracapsular involvement. The greater the number of positive LNs, the higher the number with extracapsular involvement. These results underline the findings of Lerut *et al.*^[17] and Lagarde *et al.*^[10], that extracapsular LNI is an independent prognostic parameter for esophageal cancer.

Table 4 Multivariate analysis of prognostic factors for patients with neoadjuvant CRT

Variable	HR	95% CI	P
Age	1.00	0.99-1.03	0.376
Sex			0.353
Male	1 (ref)		
Female	0.77	0.44-1.34	
pN/pM category			0.000
pN0/pM0	1 (ref)		
pN1 (-)/pM0	1.89	1.10-3.22	0.021
pN1 (+)/pM0	2.70	1.57-4.64	0.000
pM1 lymph	2.92	1.49-5.74	0.002
pM1 organ	3.92	1.76-8.72	0.001
Histology			0.145
Squamous cell carcinoma	1 (ref)		
Adenocarcinoma	0.73	0.48-1.11	
Response			0.010
Minor response	1 (ref)		
Major response	0.56	0.36-0.87	

pN1 (-): pN1 category without extracapsular LNI; pN1 (+): pN1 category with extracapsular LNI.

To explain the aggressiveness of tumors with extracapsular LNI, investigators have discussed the ability of cancer cells not only to spread into an LN, but also to invade through the node capsule in an immunologically hostile environment^[24,25]. The prognostic relevance of this extracapsular spread is heightened by the fact that patients with only one positive node have significantly worse survival rates if tumor involvement extends beyond the LN capsule^[10]. As a result of this, several authors have proposed designing a new or revised staging system for esophageal cancer that includes the presence and extent of extracapsular LNI as an additional parameter^[15,26]. For breast cancer, the occurrence of extracapsular LNI is such an important factor that it has been added to the TNM classification as a specified subcategory^[18].

There is increasing evidence to support neoadjuvant CRT for treatment of esophageal cancer. A meta-analysis published by Gebiski *et al.*^[2] has shown significant survival benefits from preoperative CRT for patients with esophageal cancer. However, until now, the effects of such a neoadjuvant regimen on the presence of extracapsular LNI in esophageal cancer have not been analyzed.

The present study is believed to be the first, in which a relatively uniform group of patients with esophageal carcinoma was evaluated to determine the extent to which neoadjuvant CRT influences the presence of extracapsular LNI. We compared the data from patients receiving neoadjuvant therapy with those from patients treated with primary esophagectomy. This comparison showed some selection bias. However, although patients who were excluded from neoadjuvant CRT might have been older or have had greater comorbidity, the preoperative staging for both groups was the same.

In accordance with earlier reports from our group, neoadjuvant therapy resulted in a significant increase of patients with pN0/M0 compared to those having surgery alone^[5]. Mariette *et al.*^[27] also recently have reported a

significant decrease of LN metastases after neoadjuvant CRT. Approximately 13% of patients develop tumor progression under neoadjuvant CRT^[5]. To ease comparison between patients treated with surgery alone *vs* those receiving preoperative CRT, patients with progressive disease (i.e. pM1 lymph + pM1 organ) were excluded from our calculations of extracapsular LNI.

Regarding extracapsular LNI, neoadjuvant therapy did not reduce its occurrence. Extracapsular LNI was detected in 19 patients (17.6%) with surgery alone *vs* 32 (16.8%) with neoadjuvant CRT.

This observation demonstrates that extracapsular LNI reflects highly aggressive biological behavior of the primary tumor, which is not influenced by neoadjuvant CRT in a multimodal treatment setting. An issue that remains unresolved is the optimal radiation dosage for neoadjuvant CRT. In a review by Geh *et al.*^[28], a dose-response relationship between increasing radiotherapy, 5-FU and cisplatin doses, and pathological complete response (pCR) was reported. In contrast, increasing radiotherapy treatment time and increasing median age reduced the probability of pCR. However, low dosage of radiation reduced the risk of postoperative morbidity and mortality. Our study protocol, with 36 Gy, 5-FU and cisplatin is in the median range of published protocols^[2,28]. The advantage of our current study is that all patients were treated with the same protocol regardless of histological tumor type, so that varying therapies could not affect the results.

Due to lack of evidence, the accuracy of the 6th UICC/TNM classification is suboptimal, especially when the extent of lymphadenectomy and the effects of neoadjuvant therapy are ignored^[7,29]. There is no doubt that the ratio of positive LNs and the extent of extracapsular LNI have a significant impact on prognosis^[10,17,27,30]. Nothing is known about the effects of neoadjuvant therapy on the extent and prognostic role of extracapsular LNI. By clarifying this issue, whether neoadjuvant treatment influences the spread of extracapsular LNI, the request for a revised staging system for esophageal cancer must be supported^[29,31].

Extracapsular LNI identifies a subgroup of esophageal cancer patients with significantly worse long-term survival rates. The present study provides evidence to suggest that neoadjuvant CRT does not influence the occurrence of extracapsular LNI. This hypothesis has to be proven in a real prospective study.

Extracapsular LNI is an independent negative prognostic factor that reflects particularly aggressive biological behavior of tumors and has valuable prognostic potential. Therefore, in a revised staging system for esophageal cancer, extracapsular LNI should be taken into account as a negative prognostic factor.

about the effects of neoadjuvant chemoradiotherapy (CRT) on the presence of extracapsular lymph node involvement (LNI) and its prognostic value in patients with resected esophageal cancer. To clarify this issue might help to set up novel treatment strategies and lead to more individualized therapeutic strategies with better survival.

Research frontiers

In the present study, the authors describe, perhaps for the first time, the impact of neoadjuvant CRT on the presence of LNs with extracapsular tumor spread in esophageal cancer. The study demonstrates that extracapsular LNI is not influenced by neoadjuvant CRT.

Innovations and breakthroughs

The present study is believed to be the first in which a relatively uniform group of patients with esophageal cancer was evaluated to determine the extent to which neoadjuvant CRT influences the presence of extracapsular LNI.

Applications

Detection of extracapsular LNI might identify a subgroup of esophageal cancer patients with significantly worse long-term survival rates, who do not benefit from neoadjuvant CRT.

Terminology

Extracapsular LNI is defined as extension of cancer cells through the nodal capsule into the perinodal fatty tissue.

Peer review

This was an interesting study in a reasonably large population, with a novel aspect of the importance of extracapsular LNI in patients receiving neoadjuvant CRT for esophageal cancer.

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COMMENTS

Background

The presence and extent of lymphatic dissemination are among the most important predictors for survival in gastrointestinal malignancies. Little is known

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MRI-determined fat content of human liver, pancreas and kidney

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Abstract

AIM: To assess and correlate the lipid content of various organs in obese subjects and in persons with a normal body weight.

METHODS: Magnetic resonance spectroscopy and a previously validated gradient echo magnetic resonance imaging method with Dixon's two point technique were used in this study to quantify fat in liver, pancreas as well as kidney.

RESULTS: In 36 volunteers with body mass index (BMI) ranging from 20.0 to 42.9 kg/m², the median fat content of liver, pancreas and kidney was 2.3% (interquartile range: 0.2%-7.8%), 2.7% (1.0%-6.5%) and 0.7% (0.1%-1.4%), respectively. BMI and subcutaneous fat correlated significantly with liver and pancreas fat content. We show for the first time the significant correlation of liver and pancreas fat content in healthy

controls ($r = 0.43$, $P < 0.01$). These observations are related to body weight as measured by BMI and the amount of subcutaneous fat. Kidney fat content is very low and correlates with none of the other fat depots.

CONCLUSION: Renal lipid accumulation, unlike the coupled accumulations of fat in liver and pancreas, is not observed in obese subjects. Unlike suggestions made in previous studies, renal lipid accumulation appears not to be involved in the pathogenesis of renal disease in humans.

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Key words: Lipids; Magnetic resonance imaging; Magnetic resonance spectroscopy; Kidney; Liver; Pancreas

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Sijens PE, Edens MA, Bakker SJL, Stolk RP. MRI-determined fat content of human liver, pancreas and kidney. *World J Gastroenterol* 2010; 16(16): 1993-1998 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v16/i16/1993.htm> DOI: <http://dx.doi.org/10.3748/wjg.v16.i16.1993>

INTRODUCTION

Storage of fat within the peritoneal cavity (visceral fat) and within other tissues (ectopic fat) rather than in subcutaneous adipocytes, is accompanied by adverse metabolic and lipotoxic effects^[1,2]. Both visceral fat^[1] and liver fat^[3] are known for secreting numerous atherogenic factors into the blood system, e.g. lipids and inflammatory cytokines, predisposing to cardiovascular diseases, one of which is atherosclerotic renovascular disease (ARVD)^[4,5]. It has been hypothesized that in obesity and metabolic syndrome, ectopic fat causes lipotoxic damage to organs^[4,5], e.g. liver (steatohepatitis and cirrhosis)^[6], pancreas (β-cell

dysfunction)^[7] and lipotoxicity of the kidney^[4].

Whereas liver steatosis has been quantified in dozens of magnetic resonance imaging (MRI) studies, and pancreatic lipomatosis^[8-10] and muscle fattening^[8-11] in some, quantitative documentation of the content of fat in human kidneys *in situ* appears to be lacking entirely. In rats, however, steatosis of the kidney was recently associated with an alteration in renal acidification^[12]. In that study, the fat accumulated in the renal cortex, as shown quantitatively by enzymatic triglyceride measurement and qualitatively by oil red O staining^[12]. This fits the notion that, within the cortex, the proximal tubule is vulnerable to lipid accumulation due to its role in the reabsorption of free fatty acids bearing albumin^[13,14].

The most accurate, and therefore preferable, method for quantifying hepatic fat content is metabolic MRI, i.e. multivoxel magnetic resonance spectroscopy (MRS)^[15-17]. Other MRI methods are less reproducible or have systematic errors. In a previously published comparison of subjects examined by MRS and by a Dixon-based dual-echo breath hold gradient echo method, we found strong correlation between the liver fat content according to both methods and concluded that MRI can be used for quantifying fat content, provided that the small systematic overestimation of fat content at the lower end range is corrected for^[17]. The comparison is now extended to 36 volunteers, varying from lean to obese, with application of the Dixon-based MRI fat quantification method to pancreas and kidney as well as liver. Only the liver was additionally examined by MRS. Our approach was to apply the equation converting the liver MRI fat values to the MRS values, to the pancreas and kidney MRI values. Additional MRS examinations of pancreas and kidney would have made the total MRI examination times too lengthy. Furthermore, reliable MRS of the renal cortex, a thin layer of tissue sandwiched between the peripheral fat and the fatty core of the kidney, is technically not feasible.

High hepatic lipid content is common in obese subjects who are otherwise healthy, i.e. overweight persons not suffering from metabolic disease^[15]. In this study we examined 36 volunteers with a wide range of body mass index (BMI) values, using previously validated MRI methods to quantify the fat content of liver, pancreas as well as kidney. The purpose was to quantify the lipid content of these organs in obese subjects and in persons with a normal body weight. Our hypothesis was that in obese persons the lipid content of liver, pancreas and kidney would be higher than in thin persons. Our comparatively large study was also intended to assess possible relationships between the fat content in different organs.

MATERIALS AND METHODS

Thirty-six adult volunteers were recruited by advertisement to undergo abdominal MRI and liver MRS. These volunteers had a BMI ranging from 20.0 to 42.9 kg/m² with a mean value of 27.5 kg/m² and were average in that they were neither profound exercisers nor sedentary. Eight

out of 36 subjects were obese, i.e. they had BMI values exceeding a value of 30. Two-thirds of the participants were men; the median age was 39 years (range: 22-64 years). Everyone was interviewed to assess the status of his health and cases of any disease, including diabetes, were excluded. All volunteers gave informed consent. The studies were approved by the medical ethics committee.

Magnetic resonance studies

MRI and MRS studies took place in one measurement session, using a 1.5 Tesla whole-body scanner (MAGNETOM Avanto; Siemens Medical Solutions, Erlangen, Germany) equipped with gradients of up to 40 mTm⁻¹ (maximal slew rate = 200 mT m⁻¹/ms) and a six-channel spine array coil. Subjects were in the supine position. In both MRI and MRS a large flex coil placed over the liver was used simultaneously with the spine array coil as receiver. T₁-weighted gradient echo images were recorded to assess the anatomy of liver, kidneys and pancreas.

Dixon's two point MRI technique

Breath-hold T₁-weighted gradient echo MR images, a dual flip angle adaptation of the wide spread gradient-recalled echo MRI method based on Dixon's two point technique^[18], were acquired with 6 mm slice thickness, section gap 0 mm, matrix 256 × 160 and a repetition time (TR) of 155 ms, and TEs of 2.4 ms (OP) and 4.8 ms (IP). Flip angles of 20° and 70° were used to generate intermediate-weighted and T₁-weighted images, respectively. Images were corrected for T₂* decay: $S_{corrected} = S e^{(\tau/T_2^*)}$, where τ is the echo time difference between IP and OP images, and S represents the signal intensity in a defined region of interest (ROI)^[19]. Under these conditions $\tau = 2.4$ ms combined with $T_2^* = 19.44$, calculated from the mean spectral line width of the water peak in human liver measured by MRS in the 36 volunteers (details are given in a next paragraph), gave a correction factor of 1.13 for S_{IP} relative to S_{OP}.

The algorithm used for estimating fat content, modified to prevent occasional mix-ups of water and fat signals^[17], consists of: (a) adjustment for T₂* relaxation as described above, (b) calculation of the apparent fat content using the following equation %fat = (S_{IP} - S_{OP})/2S_{IP} × 100% (equation 1) for both intermediate- or hydrogen density-weighted, (%fat_{Hwt} at 20° flip angle) and T₁-weighted (%fat_{T1wt} at 70° flip angle) image pairs, (c) if fat_{Hwt} AND %fat_{T1wt} ≤ 20%, then %fat = MIN [%fat_{Hwt}, %fat_{T1wt}], if fat_{Hwt} AND %fat_{T1wt} > 20% and %fat_{Hwt} ≤ %fat_{T1wt}, then %fat = %fat_{Hwt}, otherwise, %fat = 100% - %fat_{Hwt}, where AND is a logical operator, and MIN (*a*, *b*) is a mathematical operator computing the minimum value between *a* and *b*. A T₂* of 19.4 ms for liver tissue was adopted from the results of a previous study^[17] for correcting S_{IP} in the above mentioned MRI algorithm. This value was used for correcting all MRI fat content figures in Table 1.

Liver MRS for correcting abdominal MRIs

Hybrid 2D-spectroscopic imaging (chemical shift imaging,

Table 1 Spearman's correlation coefficients (r_s) between measures of obesity, and MRI fat measurements according to Dixon's two point technique in kidney, liver and pancreas after correction by MRS

$n = 36$	Correlation				
	BMI	Subc.fat	Kidney fat	Liver fat	Pancreas fat
BMI	-	0.765 ^b	0.264	0.517 ^b	0.349 ^a
Subcutaneous Fat (cm)	0.765 ^b	-	0.256	0.447 ^b	0.442 ^b
Liver fat (%)	0.517 ^b	0.447 ^b	0.231	-	0.428 ^b
Pancreas fat (%)	0.349 ^a	0.442 ^b	0.081	0.428 ^b	-
Kidney fat (%)	0.264	0.256	-	0.231	0.081

^a $P < 0.05$, ^b $P < 0.01$. MRI: Magnetic resonance imaging; MRS: Magnetic resonance spectroscopy; BMI: Body mass index.

CSI), point resolved spectroscopy (PRESS) with a repetition time (TR) of 5000 ms and an echo time (TE) of 30 ms, was performed using a field of view of $16 \times 16 \text{ cm}^2$ and a volume of interest of $5 \times 8 \times 4 \text{ cm}^3$ positioned inside the liver (Figure 1C)^[20]. The CSI measurement lasted $16 \times 16 \times 5 = 1280 \text{ s}$ or approximately 21 min. Shimming was automated and water suppression was not applied in order to be able to calculate the fat-water ratio distributions in the liver directly^[17]. At the used TR of 5 s, T_1 saturation of the water and fat signals is negligible, that is $TR > 5T_1$ ^[21], and at the used TE of 30 ms the correction applied to our data to compensate for the fact that the fat signal has a longer T_2 (78 ms) than water (60 ms) was 12.2%^[22]. In our MRS method respiratory compensation was not applied as this was previously shown not to affect the quality and composition of our liver spectra^[17].

The liver MRS data were co-registered to the MR images in the liver by using the coordinates of the anterior right (AR) and posterior left (PL) at the same height position as regional checkpoints. The kidney and pancreas MRI fat contents were derived from a single slice centered on the organ involved. Figure 1 shows the regions of interest for kidney (a), pancreas and liver tissue (b) and the matching liver MRS spectral map (c) for one of the volunteers examined. In the analysis of kidney data the results for left and right kidney volumes of interest containing medulla and renal column tissue were averaged. MRS measurements of the renal cortex and pancreas were not performed because, apart from measurement time considerations, accurate quantification of small tissue volumes adjacent to the fatty tissue surrounding these organs is impossible.

The thickness of the layer of subcutaneous fat was measured at the two most left and right points on the same MRI slice to obtain a measure of the amount of subcutaneous fat tissue (Figure 1A). A more precise method^[23] was not feasible in our study, as the MRI field of view was often too small to depict the entire abdominal body cross section.

The pancreas measurements were confined to the tail (cauda) because that tended to be easier to depict than caput and corpus (Figure 1B). The difference be-

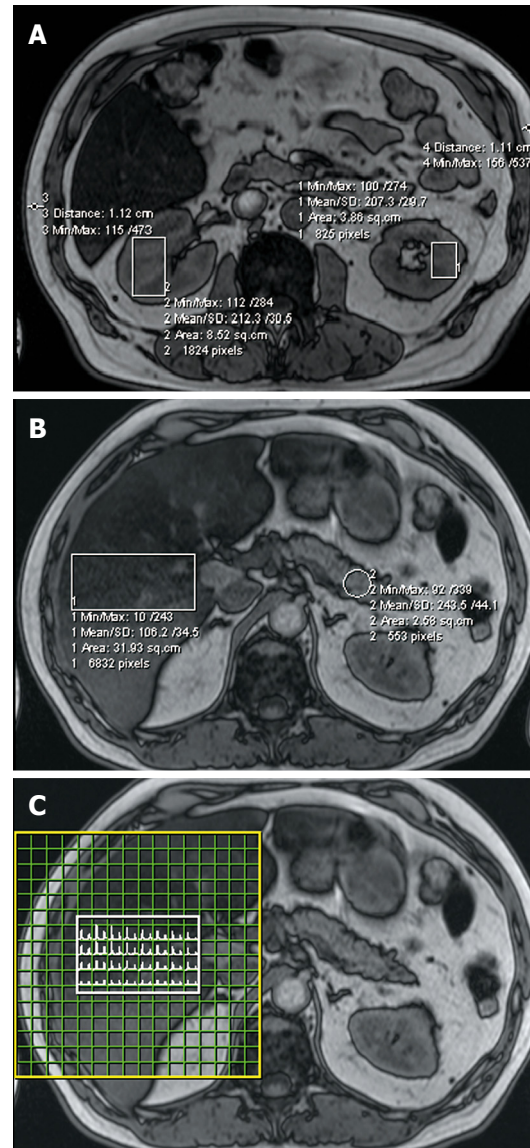


Figure 1 Regions of interest on magnetic resonance imaging (MRI) (OP series with 70° pulse angle and TE = 2.4 ms) and the corresponding magnetic resonance spectroscopy (MRS). A: Kidney and subcutaneous tissue measurement; B: Liver and pancreas; C: Liver MRS spectral map, showing an array of water and fat peaks.

tween the liver fat content according to MRS, the gold standard^[17], and liver fat content according to MRI was used to correct all MRI content figures determined in this study (kidney, liver, pancreas). This approach is reasonable because with the combined use of the flex and spine array as receiver MRI coils, the B1 field in the positions corresponding with the locations of these organs did not show significant differences. In other words, the outcome of phantom experiments gave us reason to expect that equivalent Dixon's two point technique MRI signal intensities in liver, pancreas and kidney should correspond to the same fat content.

Statistical analysis

The distributions of the obtained fat contents were not

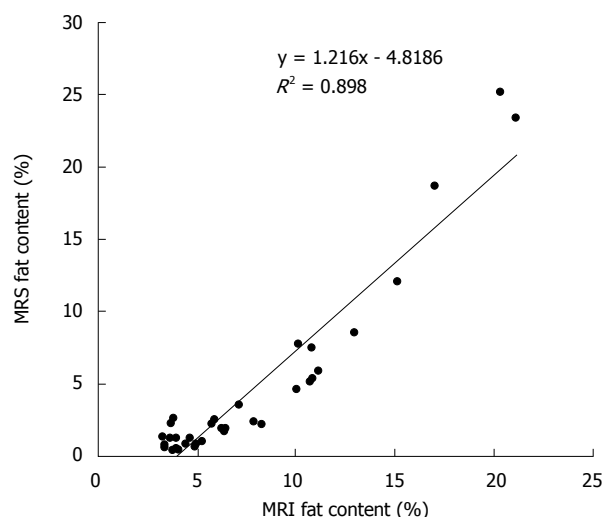


Figure 2 Liver fat content according to MRS plotted against the fat content of the same volume of interest according to MRI.

normal, thus non-parametric correlations were calculated using Spearman's correlation coefficients (with 2-tailed testing of significance).

RESULTS

MRI correction

Mean liver fat content according to MRS was 3.2% lower than that according to MRI (4.4% *vs* 7.6%). The linear correlation between liver fat content according to MRI and MRS is shown in Figure 2 ($r = 0.95$, $P < 0.0001$). The obtained slope ($y = 1.216x - 4.82$) was used to correct all MRI-determined tissue fat contents.

MRI fat measurements

After correction for this systematic overestimation, which is inherent to the MRI method^[13], calculated fat content of liver, pancreas and kidney was 2.3% (interquartile range: 0.2%-7.8%), 2.7% (1.0%-6.5%) and 0.7% (0.1%-1.4%), respectively. For the kidney fat measurements the variance between subjects was lower (interquartile range: 1.8%) than in liver (7.6%) and pancreas (5.5%). Illustrated by Figure 3 is the observation that, compared with the normal body weight subjects, the subgroup of obese subjects tended to have comparatively high fat in the liver (median 4.57% *vs* 1.11%), slight elevation in the pancreas (3.60% *vs* 2.26%) and equally low fat contents in the kidney (1.35 *vs* 0.64). The interquartile ranges for these organs in obese and normal weight persons showed great overlap and were of comparable magnitude, i.e. 2.93-7.70 (0.18-7.76), 2.32-8.88 (0.62-5.45) and 0.61-1.49 (0.10-1.17), respectively. Table 1 shows that BMI, a measure of overall fat deposits in the human body, correlated significantly with the amount of subcutaneous fat ($r = 0.77$, $P < 0.01$), liver fat content ($r = 0.52$, $P < 0.01$) and pancreas fat content ($r = 0.35$, $P < 0.05$). The amount of subcutaneous fat also correlated significantly with liver fat content and pancreas fat content ($r = 0.45$ and $r = 0.44$, respectively;

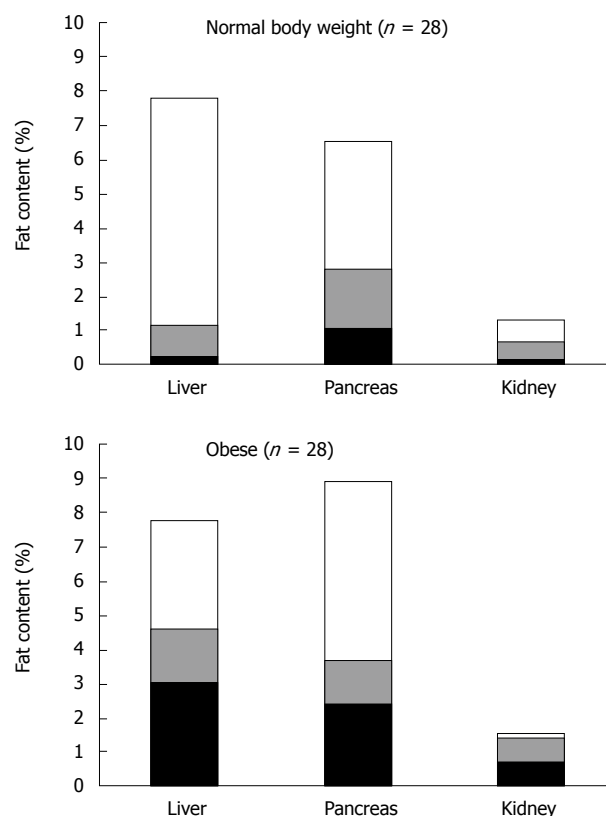


Figure 3 Tissue fat content, median values and interquartile ranges, according to MRI (i.e. after correction by MRS) for subjects with normal body weight and for obese subjects.

$P < 0.01$). Liver and pancreas fat contents were significantly correlated ($r = 0.43$, $P < 0.01$). Kidney fat content was comparatively low, showed little intersubject variation and correlated with none of the other fat depots.

DISCUSSION

These healthy volunteers, who varied in body weight from lean to obese, had kidney fat contents that were comparatively low and showed little variation between subjects. The kidney fat content correlated with none of the other fat depots, indicating that in otherwise healthy subjects obesity does not affect kidney fat content. In other words, renal lipid accumulation, unlike the accumulation of fat in liver and pancreas, is not observed in obese healthy subjects.

In this study, we used a previously validated gradient echo MRI method^[17] for determining the fat content in liver pancreas and kidney. In our liver measurements we compared the MR spectroscopy results in the thirty-six volunteers with the MRI liver content results to refine the correction equation needed to adapt the MRI method for quantitative use. Specifically, we determined the coefficient of correlation between the two-point Dixon-based MRI method and the MRS results in liver and used the result to correct all MRI data for systematic overestimation at the lower end range, a phenomenon also observed by others^[24] and most probably related to Rician noise

distribution-related overestimation of the magnitude images of weak fat signals. Thus we have used our liver data for correcting all MRI data based on the assumption that the MRI method works out the same for liver and other organs (pancreas, kidney). This is the best one can do, considering that (1) MRS examinations of small fat embedded organs are inaccurate; and that (2) it is not an option to collect tissue biopsies from healthy volunteers. Furthermore, as stated in Materials and Methods, control experiments had made sure that with the radiofrequency coils used, the MRI signal intensities in positions corresponding with those of liver, pancreas and kidney corresponded to the same fat contents.

Renal lipotoxicity and its role in the pathogenesis of renal disease are not fully understood^[4,5]. It has been assumed that renal disease progression is promoted by the accumulation of lipids in the kidneys, a phenomenon in which triglyceride-rich lipoproteins, free fatty acids and their metabolites, and albumin-loaded free fatty acids appear to be involved. Quantitative documentation of the content of fat in human kidneys *in situ* appears not to exist, so we cannot compare our MRI-determined kidney fat content figures to literature data. In a bovine growth hormone transgenic mouse line, kidney triglycerides, while lower than those found in liver, recently showed a similar trend of reduced levels as compared with non-transgenic littermate controls showing overall fat mass increases^[25]. Furthermore, in rats, steatosis of the kidney was recently associated with an alteration in renal acidification^[12]. The fat accumulated in the renal cortex, as shown quantitatively by enzymatic triglyceride measurement and qualitatively by oil red O staining^[12]. This fits the notion that, within the cortex, the proximal tubule is vulnerable to lipid accumulation due to its role in the reabsorption of free fatty acids bearing albumin^[13,14]. Our result in humans, with regard to the finding of very low fat content (up to 2%) in the cortex of kidneys of both lean and obese individuals, is not in line with the findings of the above experimental studies.

Our observation of significant correlation between liver and pancreas fat content according to MRI ($r = 0.43$, $P < 0.01$) is also in disagreement with a recent study involving a smaller number of volunteers than we included here (17 *vs* 36)^[10], and with another small study ($n = 15$) in which the existence of significant correlation between liver and pancreas fat content is not mentioned^[8]. The small scale of both earlier studies in humans probably explains their failure to demonstrate the correlation between the fat content in liver and pancreas. The presence of such a correlation does fit a previous demonstration that obese nondiabetic subjects have increased fat in the pancreas^[8]. It is also in line with a notion that lipomatosis of the pancreas reflects early events in the pathogenesis of diabetes^[9], confirming what has been shown in numerous publications dealing with the fattening of the liver.

The significant correlations of BMI with both liver and pancreas fat content in this study are in agreement with a previous report^[8]. Why would obese subjects tend to accumulate fat in liver and pancreas and not

in the kidney, despite the observation that obesity and the metabolic syndrome are involved with initiation of chronic kidney disease^[4]? It seems that due to specific requirements, such as albumin loading needed to facilitate the uptake of fat into kidney tissue, obese but otherwise healthy subjects do not accumulate fat in the kidneys.

In conclusion, this is the first demonstration of the use of an MRI method for determining kidney fat content. Observed for the first time are significantly correlated liver and pancreas fat contents in (otherwise) healthy persons varying in body weight from lean to obese. These observations are related to body weight as measured by BMI and the amount of subcutaneous fat. The amount of fat in the kidney in obese persons is small and not related to the amount of body fat or the fat content of liver and pancreas. We have thus shown that in obesity, the first step in the pathogenesis of renal disease, lipid is not accumulated in the kidney. Therefore, the role of lipid accumulation in the pathogenesis of renal disease, diabetes and metabolic disease in humans should be reconsidered. That is not to say that fat metabolites, rather than the triglyceride levels detected here by MRI, may have profound effects. In future studies we propose to examine patients suffering from the above illnesses in order to validate our current hypothesis that the lipid content is always low in the kidneys.

COMMENTS

Background

Worldwide obesity is on the rise and so are diseases associated with accumulation of fat in various organs.

Research frontiers

This is the first study providing quantitative fat content data for human liver, pancreas and kidney *in situ*.

Innovations and breakthroughs

Shown here for the first time is significant correlation of the liver and pancreas fat contents in persons with body weights varying between thin and obese.

Applications

This study shows that magnetic resonance imaging can be used to study metabolic disease noninvasively. The fat content of multiple organs can thus be quantified and correlated.

Terminology

The fat content of human tissues can be quantified by using magnetic resonance imaging methods to quantify and compare the signals of water and fat. The fat content of human tissues is typically expressed as fat/(water+fat).

Peer review

The study assessed renal lipid accumulation, as well as fat accumulation in the liver and pancreas of a series of volunteers varying in body weight from thin to obese. Results confirmed the correlation between liver and subcutaneous fat content as well as showing the absence of fat storage in the kidney. The latter is the real novel finding of the study.

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Comparison of two nutritional assessment methods in gastroenterology patients

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Abstract

AIM: To investigate and compare efficacy and differences in the nutritional status evaluation of gastroenterology patients by application of two methods: subjective global assessment (SGA) and nutritional risk index (NRI).

METHODS: The investigation was performed on 299 hospitalized patients, aged 18-84 years (average life span 55.57 ± 12.84), with different gastrointe-

stinal pathology, admitted to the Department of Gastroenterohepatology, Clinical and Hospital Center "Bezanijska Kosa" during a period of 180 d. All the patients, after being informed in detail about the study and signing a written consent, underwent nutritional status analysis, which included two different nutritional indices: SGA and NRI, anthropometric parameters, bioelectrical impedance analysis, and biochemical markers, within 24 h of admission.

RESULTS: In our sample of 299 hospitalized patients, global malnutrition prevalence upon admission varied from 45.7% as assessed by the SGA to 63.9% by NRI. Two applied methods required different parameters for an adequate approach: glucose level (5.68 ± 1.06 mmol/L vs 4.83 ± 1.14 mmol/L, $F = 10.63$, $P = 0.001$); body mass index (26.03 ± 4.53 kg/m² vs 18.17 ± 1.52 kg/m², $F = 58.36$, $P < 0.001$); total body water (42.62 ± 7.98 kg vs 36.22 ± 9.32 kg, $F = 7.95$, $P = 0.005$); basal metabolic rate (1625.14 ± 304.91 kcal vs 1344.62 ± 219.08 kcal, $F = 9.06$, $P = 0.003$) were very important for SGA, and lymphocyte count was relevant for NRI: $25.56\% \pm 8.94\%$ vs $21.77\% \pm 10.08\%$, $F = 11.55$, $P = 0.001$. The number of malnourished patients rose with the length of hospital stay according to both nutritional indices. The discriminative function analysis (DFA) delineated the following parameters as important for prediction of nutritional status according to SGA assessment: concentration of albumins, level of proteins, SGA score and body weight. The DFA extracted MAMC, glucose level and NRI scores were variables of importance for the prediction of whether admitted patients would be classified as well or malnourished.

CONCLUSION: SGA showed higher sensitivity to predictor factors. Assessment of nutritional status requires a multidimensional approach, which includes different clinical indices and various nutritional parameters.

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Key words: Nutritional status; Subjective global assessment; Nutritional risk index; Comparison

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INTRODUCTION

Many methods of assessment of nutritional status have been developed to identify malnourished patients or the risk for malnutrition. Most of them, such as subjective global assessment (SGA), Malnutrition Universal Screening Tool, Mini Nutritional Assessment, Nutritional Risk screening-NRS 2002, dealt with multiple components: medical history, dietary intake, amount of weight loss, biochemical variables, and anthropometric measurements^[1-3]. SGA is a clinical assessment index which appeared to be the most widely used and applied in a variety of patient populations, especially in surgical, oncology patients and subjects with chronic renal disease^[1,4-6]. The nutritional risk index (NRI), developed by Veterans Affairs Total Potential Nutrition Cooperative Study Group, is an objective screening nutritional tool and has predictive potential to identify patients who become nutritionally depleted during hospitalization or are at increased risk for disease complications^[7]. There is divided opinion about “gold standard” techniques for determining a patient’s nutritional status: some authors are of the opinion that SGA is more sensitive to any biochemical markers or anthropometric measurements alone and is the best predictor for hospital-related outcome than other nutritional indices^[8-11], while others considers that NRI must reflect real risk for malnutrition independent of severity of disease^[12,13].

In the present study we attempted to investigate differences in baseline nutritional parameters in patients with different digestive diseases and disorders and intended to estimate degrees of concordance between two applied nutritional assessment methods, SGA and NRI. Also, we aimed to evaluate parameters which significantly influence the nutritional state.

MATERIALS AND METHODS

The investigation was performed on 299 hospitalized patients, aged 18-84 years (average life span 55.57 ± 12.84), with different gastrointestinal pathologies, admitted to the Department of Gastroenterohepatology, Clinical and

Hospital Center “Bezanijska Kosa” during a period of 180 d. Patients with the following diagnosis: gastritis/oesophagitis, inflammatory bowel disease, peptic ulcer disease, functional bowel disorders, chronic pancreatitis, decompensate chronic liver disease, compensate chronic liver disease, autoimmune hepatitis, were classified as the benign group, while individuals with gastric, colorectal and pancreatic cancer were classified as the malignant group. The patients included in the study met the following criteria: subjects older than 18 years, metabolic and clinical stability, stable state of consciousness, absence of any kind of morphological or abnormalities of extremities. Exclusion criteria for all subjects were edema, major cardio-respiratory resuscitation, severe hyperhydration in patients with liver cirrhosis, estimated by clinical and ultrasound examination.

Nutritional measurements

All the patients, after being informed in detail about the study and signing a written consent, underwent nutritional status analysis upon admission, using 2 different nutritional indices: SGA and NRI, anthropometric parameters, bioelectrical impedance analysis (BIA), and biochemical markers. All measurements were performed within 24 h of admission. The SGA^[14] is a clinical nutritional index, which involved a standardized questionnaire consisting of dietary intake change, recent body weight changes, gastrointestinal symptoms, functional capacity, and physical signs of malnutrition (loss of subcutaneous fat or muscle mass, oedema, ascites). The standardized questionnaire was performed by a previously trained investigator, gastroenterologist (Filipović BF). Patients were classified into to three different groups: well-nourished (SGA-A), moderately malnourished (SGA-B) and severely malnourished (SGA-C). The NRI^[15] was computed by equation, which included concentration of albumin and the ratio of actual to usual weight: $NRI = (1.519 \times \text{serum albumin (g/L)} + 0.417 \times (\text{present weight/usual weight} \times 100))$. NRI score higher than 100 indicates that the patient is at no risk, a score of 97.5 to 100 indicates low risk, a score of 83.5 to 97.5 indicates medium risk, and a score lower than 83.5 indicates high risk. In order to perform appropriate statistical analysis we merged all malnourished individuals into one group. The usual weight was defined as a stable weight over the last six months before admission. The standard laboratory tests were analyzed: complete blood count, concentration of albumin, total protein, cholesterol, C-reactive protein (CRP) and glucose level. The lymphocyte count was calculated from the total blood cell count, and the differential white blood cell count was obtained by an automated analyzer. Anthropometric variables were determined: body weight and height, triceps skin fold thickness, mid-arm and waist circumferences. Body height was measured to the nearest 0.5 cm with a stadiometer and body weight was measured with mechanical scales. Triceps skin fold thickness was measured with a skin caliper on the posterior upper arm, midway between the acromion and olecranon process. A skin fold

Table 1 Distribution of nutritional status upon admittance according to the gastrointestinal diagnosis observed by SGA and NRI

Nourishment status/diagnosis	SGA assessment ¹		NRI assessment ²	
	Well	Malnourished	Well	Malnourished
Benign	140	76	90	126
Malignant	23	60	18	65
Total	163	136	108	191

¹ $\chi^2 = 46.24$, DF = 1, $P < 0.001$; ²Cochran's κ index = 0.367; $P < 0.001$. SGA: Subjective global assessment; NRI: Nutritional risk index.

thickness of 5 to 8 mm was determined as borderline fat stores, and of 3 mm or less as severe depletion. Mid-arm circumference was measured with non-stretch measuring tape, midway between the acromion and olecranon of the non-dominant arm, and 15 cm or less was an indicator of severe depletion of muscle mass. Both parameters were used to compute mid-arm muscle circumference (cm) according to the formula, as reported by Frisanchio^[16]: mid-arm circumference (cm)-[triceps skin fold thickness (mm) \times 0.3412], as an estimate of muscle mass or lean tissue stores. Bioelectrical impedance was performed using a single frequency (50 kHz) Bioelectrical Impedance Analyzer, standard platform-based electrode system, model TANI-TA BC-418MA (TANITA CORPORATION, Tokyo, Japan). This analyzer has a Goal Setter function which calculates the amount of fat mass to be lost in order to achieve a selected target. All measurements were performed in the morning within 24 h of admission. Patients had fasted overnight. Patients were told to stand barefoot on the platform-based electrode system with both feet and to grip two electrodes with both hands. The following parameters were by default revealed by the built-in software: body mass index (BMI), basal metabolic rate (BMR), body fat mass (FM), fat free mass (FFM), total muscle mass (MM), total body water (TBW), impedance of whole body (IWH). Resistance was directly measured in Ohms at 50 kHz, 550 mA using BIA. "Impedance/index", which is defined as body height squared divided by resistance, was determined. The length of hospital stay was actual number of hospitalization days and was recorded retrospectively from the Hospital Administration. The study was approved by the Ethical Committee, Clinical and Hospital Center Bezanijska Kosa.

Statistical analysis

Statistical analysis was performed with a commercially available statistical software program (SPSS 13.0, Inc, Chicago IL, US). As well as the usual methods of descriptive statistics (mean, standard deviation- SD), we tested and obtained differences by Student *t* test for independent samples. Entire testing was performed at a 95% probability level. Concordance between the two assessment methods was analyzed by Cochran's κ (k) index. The value of κ index varies from 0 to 1: a value < 0.00

Table 2 Differences in laboratory values of obtained parameters according to the applied nutritional scores (mean \pm SD)

Parameters (serum)	Well	Malnourished	Significance <i>t</i> test
SGA assessment	<i>n</i> = 163	<i>n</i> = 136	
Glucose (mmol/L)	5.68 \pm 1.06	4.83 \pm 1.14	$F = 10.63$, $P = 0.001$
Cholesterol (mmol/L)	5.52 \pm 0.96	3.54 \pm 0.79	$F = 10.61$, $P = 0.001$
C reactive protein (mg/L)	12.42 \pm 16.48	22.08 \pm 18.46	$F = 11.44$, $P = 0.001$
NRI assessment	<i>n</i> = 108	<i>n</i> = 191	
Lymphocyte count (%)	25.56 \pm 8.94	21.77 \pm 10.08	$F = 11.55$, $P = 0.001$
Cholesterol (mmol/L)	5.25 \pm 1.04	4.27 \pm 1.35	$F = 8.27$, $P < 0.05$
C reactive protein (mg/L)	11.25 \pm 8.86	19.97 \pm 20.93	$F = 6.48$, $P < 0.05$

Degrees of freedom = 297; NS: Not significant.

indicates less than chance, 0.00-0.20 poor, 0.21-0.40 fair, 0.41-0.60 moderate, 0.61-0.80 substantial and 0.81-1.00 almost perfect concordance. The predictive potential of analyzed parameters was evaluated by the analysis of discriminant function.

RESULTS

Prevalence

In our sample of 299 hospitalized patients in the gastroenterohepatology department the frequency of any degree of malnutrition at admission varied from 45.7% as assessed by the SGA to 63.9% by NRI (Table 1). No significant differences were obtained in gender distribution of malnutrition (χ^2 for SGA = 1.05, DF = 1, $P > 0.05$; χ^2 for NRI = 2.63, DF = 1, $P > 0.05$). Age did not significantly differ between well-nourished and malnourished patients ($F = 0.53$, DF = 1, $P > 0.05$). A degree of corroboration between the two screening methods for assessment nutritive status, SGA and NRI, was revealed as fair concordance (Table 1).

Nutritional parameters

The mean values and SD of the laboratory, anthropometric characteristics and parameters of bioelectrical impedance analysis according to both applied nutritional methods were shown on Tables 2, 3 and 4. Moreover, the number of malnourished patients rose with the length of hospital stay according to both nutritional indices (Table 5). The discriminative function analysis (DFA) delineated the following parameters as important for prediction of nutritional status according to SGA assessment: concentration of albumins, level of proteins, SGA score and body weight. The DFA extracted MAMC, glucose level and NRI scores as the variables of importance for the prediction to whether admitted patients will be classified as well or malnourished (Table 6). The correctness of the equations was emphasized by the accuracy test computed from the examined group (93.7% and 100%).

Table 3 Distribution of anthropometric measures and parameters of bioelectrical impedance analysis obtained by SGA (mean \pm SD)

Parameters	SGA assessment		Significance <i>t</i> test
	Well <i>n</i> = 163	Malnourished <i>n</i> = 136	
MAC (cm)	28.28 \pm 2.70	23.21 \pm 2.06	<i>F</i> = 10.20, <i>P</i> = 0.002
MAMC (cm)	11.56 \pm 12.62	8.23 \pm 10.47	<i>F</i> = 73.22, <i>P</i> < 0.001
TSF (mm)	9.92 \pm 2.47	5.25 \pm 1.77	<i>F</i> = 5.92, <i>P</i> = 0.02
Waist circumferences (cm)	90.16 \pm 10.92	70.03 \pm 8.01	<i>F</i> = 24.56, <i>P</i> < 0.001
Body mass index (kg/m ²)	26.03 \pm 4.53	18.17 \pm 1.52	<i>F</i> = 58.36, <i>P</i> < 0.001
Total body water (kg)	42.62 \pm 7.98	36.22 \pm 9.32	<i>F</i> = 7.95, <i>P</i> = 0.005
Resistance of whole body (Ω)	545.86 \pm 89.97	661.50 \pm 98.89	<i>F</i> = 4.77, <i>P</i> = 0.03
Basal metabolic rate (kcal)	1625.14 \pm 304.91	1344.62 \pm 219.08	<i>F</i> = 9.06, <i>P</i> = 0.003
Impedance-index	55.3184 \pm 13.00	46.4059 \pm 9.33	<i>F</i> = 6.73, <i>P</i> = 0.01

Degrees of freedom = 297. MAC: Mid-arm circumferences; MAMC: Mid-arm muscle circumferences; TSF: Triceps skinfold thickness.

Table 4 Differences in anthropometric measures and parameters of bioelectrical impedance analysis obtained by NRI (mean \pm SD)

Parameters	NRI assessment		Significance <i>t</i> test
	Well <i>n</i> = 108	Malnourished <i>n</i> = 191	
MAC (cm)	27.51 \pm 2.73	25.11 \pm 3.60	<i>F</i> = 7.88, <i>P</i> = 0.005
MAMC (cm)	10.63 \pm 12.42	9.68 \pm 11.41	<i>F</i> = 11.55, <i>P</i> = 0.001
TSF (mm)	9.09 \pm 2.72	7.06 \pm 3.20	<i>F</i> = 11.55, <i>P</i> = 0.007
Waist circumferences (cm)	88.51 \pm 10.71	76.76 \pm 13.81	<i>F</i> = 7.14, <i>P</i> = 0.008
Resistance of whole body (Ω)	554.40 \pm 91.07	623.37 \pm 112.57	<i>F</i> = 5.98, <i>P</i> = 0.02
Impedance-index	54.36 \pm 12.20	45.51 \pm 12.03	<i>F</i> = 6.82, <i>P</i> = 0.01

Table 5 SGA and NRI assessments of nourished and malnourished patients according to the length of hospitalization

Length of hospital stay	SGA assessment		NRI assessment	
	Well <i>n</i> = 163	Malnourished <i>n</i> = 136	Well <i>n</i> = 108	Malnourished <i>n</i> = 191
< 10 d	90	26	58	58
> 10 d	73	110 ¹	50	133 ²
Total	163	136	108	191

¹ χ^2 = 40.69, DF = 1, *P* < 0.001; ² χ^2 = 15.82, DF = 1, *P* < 0.001.

Table 6 Discriminant function analysis for particular groups of analyzed variables

Status	Centroids	Section points	Selecting equation	Percentage of accuracy
SGA				
Well	-2.466	0.420	-2.58 + 0.04 \times [albumins] - 0.038 \times [proteins] + 2.83 \times SGA score - 0.03 \times body weight	100%
Malnourished	2.884			
NRI				
Well	1.72	0.365	-11.75 + 0.17 \times MAMC - 0.20 \times [glucose] + 1.04 \times NRI score	93.7%
Malnourished	-0.99			

DISCUSSION

In this paper, the authors intended to estimate the adequacy of nutritional assessments comparing two of the most often used methods: SGA and NRI. The prevalence of malnutrition in hospitalized patients was reported to vary between 20% and 60%. Higher prevalence has been revealed in the elderly and in patients with malignant diseases^[17-20]. In our investigation the overall prevalence of malnutrition was significantly higher by NRI assessment methods (63.9%) than SGA nutritional score (45.7%). Observed differences in prevalence of malnutrition between indices could be the result of different scoring systems. The problem appears with the classification of the mildly malnourished, who, according to SGA, are adequately classified, while NRI assigns them to the group of moderately malnourished^[21]. Schneider and Hebutterne^[22] claimed that nutritional clinical indices are more sensitive and more accurate compared with a single nutritive parameter. The nutritional parameters used to determine malnutrition varied in different studies. Most authors revealed that levels of serum albumin and cholesterol decreased in malnourished individuals and this result indicated that hypoalbuminemia and low levels of cholesterol could be

a predictor of risk for malnutrition, rather than a parameter for identifying and quantifying nutritional status^[23-26]. However, some authors suggested that serum albumin and body mass index are overestimated factors in the malnutrition assessment^[27]. Our results sustained this opinion, at least considering albumins. In our clinical study cholesterol in lower concentrations correlated with poor nutritional status, according to both applied nutritional indices. Scalfi *et al.*^[28] have claimed that impedance-index was decreased in malnourished patients and several studies have demonstrated that BIA is strictly associated with fat free mass and total body water in healthy subjects and

in patients. Nevertheless, it is still debated whether and for what purpose BIA can be used in the evaluation of body composition changes^[29-33]. Furthermore, our investigation showed that the impedance-index is significantly lower in malnourished patients when compared with other examinees.

Apparently, two applied methods requiring different parameters are needed for an adequate approach: glucose level, body mass index, total body water, basal metabolic rate are very important for SGA, and lymphocyte count is relevant for NRI. Results of several studies have suggested moderate to perfect concordance between the SGA and the NRI or between SGA and the mini nutritional assessment^[13,34]. Some authors reported poor overlapping levels between the same assessment methods^[21]. SGA has some limitations in evaluating nutritional status. First, the SGA is a clinical index which consists of subjective parameters to determine malnutrition. Second, the SGA failed to recognize the group of patients with mild degrees of malnutrition and some cases of malnutrition, particularly early and acute malnutrition. In prospective studies, SGA was demonstrated to be a good predictor of complications related with poor nutritional state^[22,35,36]. On the other hand, the combination of serum albumin and weight loss, as presented in the NRI, would reflect nutritional risk and indicate severity of illness and adverse outcome^[2,12]. According to our results, malnourished patients had a longer hospital stay than well-nourished patients, applying results from both nutritive techniques. Discriminant function analysis has outlined some nutritive variables such as concentrations of serum albumins, level of total protein, SGA score and body weight according to the SGA nutritional assessment method, while different nutritional parameters (MAMC, glucose level and NRI score) by the NRI assessment method have been extracted as predictors of whether individuals will be classified as well or malnourished.

In conclusion, SGA showed higher sensitivity to predictor factors, although the sensitivity of NRI methods was also very high. Assessment of the nutritional status requires a multidimensional approach, which includes different clinical indices and various nutritional parameters.

COMMENTS

Background

Many methods of assessment of nutritional status have been developed to identify malnourished patients or risk for malnutrition. Most of them such as subjective global assessment (SGA), Malnutrition Universal Screening Tool, Mini Nutritional Assessment, Nutritional Risk screening-NRS 2002-dealt with multiple components: medical history, dietary intake, amount of weight loss, biochemical variables, and anthropometric measurements.

Research frontiers

In the authors' investigation overall prevalence of malnutrition was significantly higher by nutritional risk index (NRI) assessment methods (63.9%) than SGA nutritional score (45.7%). The corroboration between the two methods, was, according to Cochran's κ index = 0.367, which could be interpreted as fair concordance.

Innovations and breakthroughs

The discriminative function analysis extracted MAMC, glucose level and NRI scores as the variables of importance for the prediction to whether admitted patients will be classified as well or malnourished. The correctness of the equa-

tions was emphasized by the accuracy test computed from the examined group (93.7% and 100%).

Applications

SGA showed higher sensitivity to predictor factors, although the sensitivity of NRI was also very high. Assessment of the nutritional status requires a multidimensional approach, which includes different clinical indices and various nutritional parameters.

Terminology

The SGA is a clinical nutritional index, which involved a standardized questionnaire consisting of dietary intake change, recent body weight changes, gastrointestinal symptoms, functional capacity, and physical signs of malnutrition (loss of subcutaneous fat or muscle mass, oedema, ascites). The NRI was computed by equation, which included concentration of albumin and the ratio of actual to usual weight: $NRI = [1.519 \times \text{serum albumin (g/L)} + 0.417 \times (\text{present weight/usual weight} \times 100)]$. The bioelectrical impedance analysis is a software based electrode platform variant system for the evaluation of the parameters of nutritional status.

Peer review

The authors have compared the two nutritional indices currently in use-SGA and NRI- for their efficacies in delineating malnutritional cases. The study has been conducted with a large number of patients. The data generated for comparison seems appropriate and suggests the corroboration between two screening methods for assessment of nutritional status.

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Distribution of different hepatitis C virus genotypes in patients with hepatitis C virus infection

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patients. Multiple HCV genotypes were found in 3 (2.3%) of 133 plasma samples, 9 (6.8%) of 133 PBMC samples, and 8 (18.2%) of 44 liver biopsy specimens. It is notable that the different genotypes found in PBMCs were not the same as those found in plasma and liver biopsy specimens.

CONCLUSION: Our study shows that a significant proportion of patients with chronic hepatitis C are affected by multiple HCV genotypes which may not be detectable only in serum of patients.

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Key words: Chronic hepatitis C virus infection; Mixed hepatitis C virus infection; Peripheral blood mononuclear cells; Hepatocyte

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Abstract

AIM: To investigate the presence of mixed infection and discrepancy between hepatitis C virus (HCV) genotypes in plasma, peripheral blood mononuclear cells (PBMCs), and liver biopsy specimens.

METHODS: From September 2008 up to April 2009, 133 patients with chronic hepatitis C referred to Firouzgar Hospital for initiation of an antiviral therapy were recruited in the study. Five milliliters of peripheral blood was collected from each patient and liver biopsy was performed in those who gave consent or had indications. HCV genotyping was done using INNO-LiPA™ HCV II in serum, PBMCs, and liver biopsy specimens and then confirmed by sequencing of 5'-UTR fragments.

RESULTS: The mean age of patients was 30.3 ± 17.1 years. Multiple transfusion was seen in 124 (93.2%) of

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INTRODUCTION

Hepatitis C virus (HCV) is an enveloped positive-stranded RNA virus of the family Flaviviridae and genus Hepacivirus that causes both acute and chronic hepatitis^[1-3]. HCV is a major health problem affecting 170 million people worldwide^[4]. It is estimated that chronic HCV infection is responsible for approximately 250 000 to 350 000 deaths per year, mainly related to decompensation of cirrhosis, end-stage liver disease, and hepatocellular carcinoma^[5].

Phylogenetic analysis of HCV sequences resulted in a nomenclature that recognizes distinct virus types and subtypes^[6]. Six large groups of viral genotypes^[7-12], in addition to over 70 different subtypes (termed a, b, c, *etc.*) are distributed worldwide^[6,13]. The HCV genotypes should be systematically determined before treatment, as it determines the indication, the duration of treatment, the dose of ribavirin and the virological monitoring procedure^[14].

The HCV is considered as essentially hepatotropic^[15], but virus sequences have also been found in other important extrahepatic sites, including peripheral blood mononuclear cells (PBMCs), the central nervous system, and bone marrow from chronically-infected patients^[16-19].

HCV replicates through a negative-strand intermediary. Although hepatocytes are the primary sites for HCV replication, there is evidence of negative-strand HCV RNA in PBMCs. The HCV genomic sequences present in PBMCs have been found to differ from those found in serum and the liver biopsy specimens^[20-24]. Detection of HCV RNA in extrahepatic reservoirs has important implications for transmission, disease progression, and effective treatment^[25]. Furthermore, the PBMC compartment may be a privileged site for HCV, which is able to reinitiate viral replication after termination of HCV treatment when conditions again become more favorable. Re-infection of HCV after orthotopic liver transplantation has postulated that extrahepatic sites are suitable for HCV replication^[26]. Thus, even if clearance of HCV from hepatocytes is achieved by treatment, re-infection from extrahepatic sites, such as the PBMC compartment, may occur^[27].

The purpose of the present study is to determine the presence and frequency of different HCV genotypes in plasma, PBMC, and liver biopsy specimens of chronically infected patients.

MATERIALS AND METHODS

Patients

In this cross-sectional study, 133 consecutive patients with established chronic hepatitis C referred to Firouzgar Hospital from September 2008 to July 2009 were enrolled. Informed consents were obtained from the patients, and the study was approved by the local ethics committee of GI and Liver Disease Research centre (GILDRC) of Iran University of Medical Sciences.

Inclusion criteria were positive anti-HCV antibodies along with positive plasma HCV RNA. Also none of the included patients had been treated for HCV previously.

Collection and preparation of samples

About 5 mL of peripheral blood were collected from each patient into EDTA-containing vacutainer tubes. Plasma was stored at -70°C until analysis. PBMCs were isolated from EDTA-treated blood by centrifugation over density gradient (Lymphoprep, Oslo, Norway). PBMCs were then washed three times with phosphate-buffered saline (pH = 7.4), counted and stored at -70°C for later detection. Some of the patients who gave consent underwent a liver biopsy for diagnostic purpose. Liver biopsy specimens were di-

vided into 2 portions: one used for histological diagnosis, and the second was submerged into RNALater (Ambion Inc., Austin, TX) and stored at -70°C for HCV genotyping. Samples of serum and PBMCs were collected from all patients on the same day the liver biopsy was performed.

Isolation of RNA from serum, PBMCs, and liver

RNA was extracted from 140 µL of plasma by using the QIAamp Viral RNA Extraction kit (Qiagen GmbH, Hilden, Germany), from a pellet of approximately $3-5 \times 10^6$ PBMCs using the RNA Virus Mini Extraction Kit (Invitex GmbH, Germany), and from approximately 2 mm³ of liver biopsy specimens using the DNA/RNA Virus Mini Extraction Kit (Invitex GmbH, Germany), according to the manufacturer's instructions.

HCV genotyping

RNA was isolated from plasma, PBMC, and liver biopsy specimens as described above. 5'-untranslated region (5'-UTR) genotyping was done using a standard methodology with the INNO-LiPATM HCV II kit (Innogenetics, Ghent, Belgium) according to the manufacturer's instructions. Briefly, the 5'-UTR is amplified with biotinylated primers. Biotin-labeled PCR products are reverse hybridized to specific probes attached to nitrocellulose strips and then incubated with a chromogen. Developing results in a purple precipitate that forms a line on the strip. The HCV type is deduced on the basis of the patterns of hybridizing bands, using the line probe assay interpretation chart^[28].

INNO-LiPATM HCV II genotyping has also been confirmed by sequencing of 5'-UTR fragments. The HCV 5'-UTR was amplified from total RNA isolated from plasma, PBMC, and liver biopsy specimens of 10 randomly selected patients by reverse transcriptase-PCR as described previously^[29]. The 211-bp 5'-UTR PCR products were sequenced by dye termination method using the ABI 3130 XL sequencer. The sequencing results were compared with other sequences posited in gene bank by Blast software.

Statistical analysis

Data analysis was done by SPSS software version 16.0, using descriptive statistical indexes such as mean and standard deviation (SD), 95% confidence interval (CI) and *t* test. *P* value less than 0.05 was considered statistically significant.

RESULTS

One hundred and thirty three patients with established chronic hepatitis C were recruited in this study. Multiple blood transfusion was the most important related risk factor found in 124 (93.2%) of patients, 87 (65.4%) with thalassemia and 37 (27.8%) with hemophilia. The source of infection was unknown in 9 (6.8%) patients. The mean age of patients was 30.3 ± 17.1 years. Out of 133 patients, 100 (75.2%) were male. The mean viral load of all of the patients was $6.3 \times 10^5 \pm 7.8 \times 10^5$ (range from 45000 to 5.59×10^6). Out of 133 patients, 44 patients gave consent to undergo liver biopsy. Genotype 1a was the most fre-

Table 1 Distribution of different HCV genotypes in plasma, PBMCs, and liver biopsy specimens

No.	Gender/age (yr)	Viral load	Disease	HCV genotypes in plasma	HCV genotypes in PBMCs	HCV genotypes in liver biopsy specimen
1	F/15	76500	Thalassemia	3	3/1a	3
2	F/36	138000	Thalassemia	1a	3	1a
3	M/17	597000	Hemophilia	1a/3	1a	
4	M/23	989000	Thalassemia	1a/1b	1b	1a/1b/3
5	M/23	226000	Thalassemia	1a/1b	1b	1a/1b
6	M/33	450	Thalassemia	1a	3	
7	M/26	250000	Thalassemia	3	1a	1a/3
8	M/17	106000	Thalassemia	1a	1a/3	
9	M/25	780000	Thalassemia	1a	1a	1a/3
10	F/30	10800	Thalassemia	1b	1a/1b	1b/3
11	F/27	750000	Thalassemia	3	3	3/1b
12	M/14	5590000	Thalassemia	3	3	1a/3
13	M/28	1190000	Thalassemia	1a	1a/3	1a
14	M/19	1150000	Hemophilia	1a	1a/3	
15	M/20	890000	Thalassemia	1a	1a/1b	
16	F/22	618000	Thalassemia	3	3	3/2a
17	M/27	167000	Thalassemia	3	3/2a	
18	M/46	230000	Hemophilia	3	3/2a	
19	M/27	273000	Hemophilia	3	3/2a	
20	M/57	480000	Unknown	4	3	

HCV: Hepatitis C virus; PBMCs: Peripheral blood mononuclear cells.

Table 2 Frequency of HCV genotypes in plasma, PBMCs, and liver biopsy specimens *n* (%)

HCV genotypes	HCV genotypes in plasma	HCV genotypes in PBMCs	HCV genotypes in liver biopsy specimen
1a	85 (63.9)	81 (61.0)	24 (54.5)
1b	8 (6.0)	10 (7.5)	3 (6.8)
2	0 (0.0)	0 (0.0)	0 (0.0)
3	35 (26.2)	33 (24.8)	9 (20.0)
4	2 (2.3)	1 (0.8)	0 (0.0)
Mixed infection	3 (2.3)	9 (6.8)	8 (18.2)
Total	133	133	44

quent genotype in serum (63.9%), PBMCs (61.0%), and in liver biopsy specimens (54.5%). HCV genotypes in serum, PBMCs, and liver biopsy specimens of all patients were similar except for 20 patients (15%).

The complete data of these patients have been summarized in Table 1. As shown in Table 2, more than one HCV genotype has been found in some patients, as 3 (2.3%) patients had different HCV genotypes in their serum samples. In 9 (6.8%) patients, different HCV genotypes were found in PBMCs. In 8 (18.2%) patients (who were not exactly same as the 9 patients with different HCV genotypes in PBMCs) multiple HCV genotypes were found in liver biopsy specimens. The INNO-LiPA™ HCV II genotyping was confirmed with sequencing of the 5'-UTR. A 100% correlation was demonstrated between INNO-LiPA™ HCV II genotyping and sequencing of the 5'-UTR.

DISCUSSION

The present study was performed on 133 chronically

HCV infected patients to evaluate the prevalence of potential mixed HCV infection in their plasma, PBMCs, and liver biopsy specimens. In 15% of these patients different HCV genotypes were found in these compartments. Multiple HCV genotypes were detected in 3 (2.3%) of 133 plasma, 9 (6.8%) of 133 PBMC, and 8 (18.2%) of 44 liver biopsy specimens.

Mixed infection is infection of an individual with two or more distinct HCV genotypes. Mixed viral infection is of great clinical importance as it may result in more severe disease, unresponsiveness to antiviral therapy or recurrence after the completion of antiviral therapy course^[30].

In our study, various HCV genotypes presented in PBMCs were different from those found in plasma or liver biopsy specimens (Table 1). It means that PBMCs may present some divergent types, which are not detectable in liver biopsy specimens. It reinforces the previous proposed theories in which PBMCs have been known as an extrahepatic replication site for HCV^[17,25,26,31-34].

It is suggested that infection with one HCV type doesn't make a barrier to acquisition of other genotypes, therefore multiple exposures to HCV especially in potential risk groups, might lead to several episodes of re-infection and to the establishment of mixed infection in some patients. It is also well known that super infection with a new HCV strain leads to suppression of one virus under the detection limit of PCR while the other one prevails as under antiviral therapy, the displaced strain may become viremic again and may alter the outcome of therapy^[30,35-39].

In our study the prevalence of mixed HCV infection was estimated about 2.3% in plasma, 6.8% in PBMC, and 18.2% in liver biopsy specimens of chronically infected patients with HCV. Mixed infection with two HCV genotypes have been detected in 1% of HCV-positive patients,

using type-specific primers^[40,41]. Also figures of 1.6% to 31% have been reported in multi-transfused hemophiliacs^[42,43].

Our study demonstrated that a significant proportion of HCV infected patients have divergent HCV genotypes in their PBMCs and liver biopsy specimens which were not detectable in their plasma.

In the present study we used INNO-LiPA™ HCV II genotyping which is currently the most applied method and has more sensitivity than RFLP. One of the problems of INNO-LiPA™ HCV II is that it may underestimate the actual rate of mixed infection^[9,30,44]. According to this, the true prevalence of mixed infection may be higher than estimated in this study.

The second shortcoming is that only 44 of our patients had indication or gave consent to undergo liver biopsy, however, 18.2% of them had multiple HCV genotypes which is a significant proportion of the total. It indicates that hepatocytes are the main reservoirs for HCV. On the other hand since performance of liver biopsy is not possible for all patients, we suggest the assessment of PBMCs as another HCV reservoir for detection of HCV mixed infection^[26,34,45].

In conclusion, our study shows that patients with hemophilia and thalassemia are from the most high risk groups in whom mixed infection is relatively common. On the other hand failure to treatment and relapse of infection is also frequent in these groups. So it seems that considering the plasma genotype as the target genotype for scheduling of an anti HCV therapy may be one of the factors that leads to the failure of treatment. HCV genotyping in PBMCs or liver biopsy specimens might be beneficial.

COMMENTS

Background

Relapse and failure of antiviral therapy have been frequently seen in patients with chronic hepatitis C especially in those who are on multiple blood transfusions.

Research frontiers

It has been suggested that infection with multiple hepatitis C virus (HCV) genotypes in patients affected by chronic HCV infection might result in frequent relapse after antiviral therapy.

Innovations and breakthroughs

The authors found that a significant proportion of patients with chronic HCV infection, especially who are on chronic blood transfusion, might present more than one HCV genotype in their serum, hepatocytes and peripheral blood mononuclear cells (PBMCs). It is notable that detected HCV genotypes in serum may be different from those found in PBMCs or hepatocytes.

Applications

According to the above findings the authors suggest HCV genotyping in hepatocytes and PBMCs along with serum of patients. This may result in choosing a more appropriate antiviral therapy in these patients.

Peer review

This is a well written manuscript and it addresses a relevant topic of multiple HCV infections/superinfection/humoral protection and others. Moreover, the material itself is of some value.

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Change in renal function after sodium phosphate preparation for screening colonoscopy

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Abstract

AIM: To investigate the changes in renal function at 12-24 mo in patients following sodium phosphate (NaP) preparation for screening colonoscopy.

METHODS: We carried out a retrospective study on the results from patients who received health check-up services as part of an employer-provided wellness program performed between August 2006 and May 2008 and who were followed up for 12-24 mo. Prior to screening colonoscopy, 224 patients underwent bowel cleansing

with NaP (NaP group) and 113 patients with polyethylene glycol (PEG group). The control group comprised 672 age-matched patients. We compared the changes in the creatinine levels and the glomerular filtration rates (GFRs) from baseline to 12-24 mo between the NaP, PEG, and control groups using two-way repeated measured analysis of variance. In addition, multivariate linear regression analysis was performed to assess the risk factors for a decreased GFR.

RESULTS: The baseline mean serum creatinine level in the NaP, PEG, and control groups was 1.12 ± 0.15 , 1.12 ± 0.16 , and 1.12 ± 0.15 mg/dL, which increased to 1.15 ± 0.15 , 1.15 ± 0.18 , and 1.15 ± 0.15 mg/dL, respectively, after 12-24 mo. The baseline mean GFR in the NaP, PEG, and control groups was 69.0 ± 7.7 , 68.9 ± 8.0 , and 69.6 ± 6.7 mL/min per 1.73 m^2 , which decreased to 66.5 ± 7.8 , 66.5 ± 8.3 , and 67.4 ± 6.4 mL/min per 1.73 m^2 , respectively, after 12-24 mo. The changes in serum creatinine levels and GFRs were not significantly between the NaP, PEG, and control groups ($P = 0.992$ and $P = 0.233$, respectively). Using multivariate linear regression analysis, only the baseline GFR was associated with the change in GFR ($P < 0.001$). Indeed, the bowel preparations were not associated with the change in GFR ($P = 0.297$).

CONCLUSION: NaP bowel preparation in subjects with normal renal function was not associated with renal injury, and NaP can thus be used safely for screening colonoscopy.

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Key words: Sodium phosphate; Polyethylene glycol; Purgatives; Colonoscopy; Creatinine; Glomerular filtration rate

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INTRODUCTION

A bowel cleansing preparation is an essential prerequisite for safe, efficient, and accurate colonoscopy^[1]. Oral sodium phosphate (NaP) and polyethylene glycol (PEG) are the most widely used bowel cleansing agents before colonoscopy. Because small-volume NaP is better tolerated than 4-L PEG, NaP is more effective in bowel cleansing than PEG for bowel preparation^[2,3].

However, with the widespread use of NaP for bowel preparation, case reports and case series have suggested a possible association between the use of NaP and acute kidney injury, particularly in those patients with potentially compromised renal phosphate handling capacity^[4-8]. Several studies have reported an association between NaP exposure and incident kidney injury^[9-14]. Indeed, the findings have been mixed, with some studies reporting a strong association^[11,12,14] and other studies reporting a non-significant trend toward better kidney outcomes after NaP^[9,10,13] compared with other purgatives.

However, the populations evaluated in previous studies had considerable heterogeneity with a variety of indications for colonoscopy and differing health status. The comparisons for renal functional changes have been performed between 2 groups (i.e. NaP *vs* PEG or NaP *vs* healthy control). Furthermore, analyses of Asian populations have been limited.

Given the large number of patients exposed to NaP annually, clarification of this association is essential from clinical and public health perspectives. We analyzed the results of health screening services as part of an employer-provided wellness program. The primary end points were the changes in creatinine levels and glomerular filtration rates (GFRs) between 12 and 24 mo after NaP bowel preparation for screening colonoscopy, and the results were compared with those of patients who received PEG or no bowel preparation agents during the same study period.

MATERIALS AND METHODS

The Healthcare Center of Konkuk University Medical Center provides medical screening programs to individuals employed at corporations that offer medical screening services annually or biannually as part of their corporate wellness plans. According to the policy of the company, colonoscopy screening may or may not be provided as part of the screening program. Examination data have been recorded electronically in a centralized digital medical record system. The Konkuk University Medical Center Institutional Review Board approved the study protocol.

We retrospectively analyzed cohort data of the health

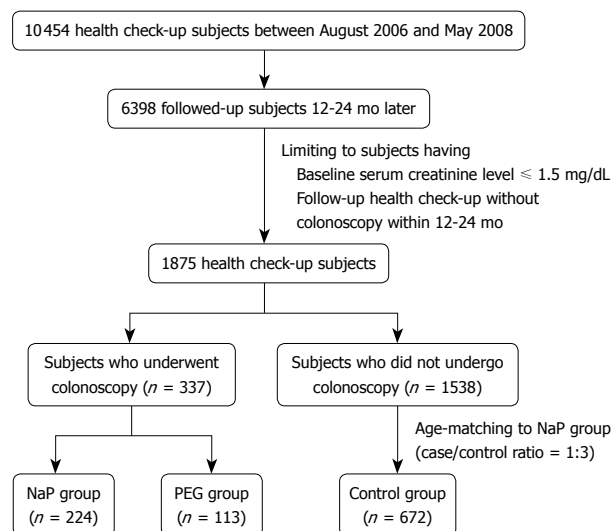


Figure 1 Flow diagram for selection of case and control subjects. NaP: Sodium phosphate; PEG: Polyethylene glycol.

screening program in subjects with normal creatinine levels who had undergone a health check-up between August 2005 and May 2008 and follow-up evaluations 12-24 mo later. During this period, 10,454 patients had health check-ups in the Healthcare Center of Konkuk University Medical Center. Of these, 6,398 were followed up 12-24 mo later. Two hundred and twenty-four subjects underwent bowel cleansing with NaP for colonoscopy during the initial health check-up and had a follow-up health check-up without a colonoscopy (NaP group). One hundred and thirteen subjects took PEG for colonoscopy preparation and had a follow-up health check-up without a colonoscopy (PEG group). Of 1,538 patients who had undergone health check-ups without colonoscopy and follow-up health check-ups without colonoscopy during the same period, 672 subjects, age-matched with the NaP group, were randomly selected as the control group. Figure 1 shows the flow diagram for selection of NaP, PEG, and control subjects.

The patients received written information about the medical screening program and a standard questionnaire, which included questions regarding personal medical history, such as hypertension and diabetes mellitus. Telephone interviews were conducted to ensure that the examinees who called to make an appointment were asymptomatic (i.e. no recent changes in bowel habits, lower abdominal pain, or visible rectal bleeding). Persons with symptoms were urged to seek medical care.

An oral NaP solution (Fleet®; Unimed Pharm. Inc., Seoul, Korea) or PEG (Colyte 4L®; Taejun Pharm. Co. Ltd., Seoul, Korea) was used for bowel preparation. Our center used PEG as a purgative for all subjects who underwent colonoscopy until July 2006 and then changed to NaP, except for those patients who had previous renal problems, heart failure, liver cirrhosis, or were > 65 years of age, because of better patient tolerance and cost-effectiveness. The dosing of NaP was as follows. Only liquid foods were consumed on the day of bowel preparation. Two doses of oral NaP solution (45 mL) were

Table 1 Baseline characteristics of subjects in the NaP, PEG and control groups *n* (%)

Characteristics	NaP group (<i>n</i> = 224)	PEG group (<i>n</i> = 113)	Control group (<i>n</i> = 672)	<i>P</i>
Age (yr)	46.9 ± 8.6	51.1 ± 10.5	46.9 ± 8.6	< 0.001
Gender				0.453
Male	149 (67)	81 (72)	475 (71)	
Female	75 (33)	32 (28)	197 (29)	
Body mass index (kg/m ²)	23.4 ± 3.1	23.8 ± 2.7	23.6 ± 2.9	0.496
Hypertension	50 (22)	34 (30)	188 (28)	0.186
Diabetes mellitus	29 (13)	23 (20)	134 (20)	0.056
Serum phosphate ¹ (mg/dL)	88.9 ± 14.5	95.3 ± 17.4	91.6 ± 17.4	0.004

¹Measured in 94 subjects in the sodium phosphate (NaP) group, 87 subjects in the polyethylene glycol (PEG) group, and 219 subjects in the control group.

Table 2 Comparison of the creatinine level and glomerular filtration rate (GFR) among NaP, PEG and control groups (mean ± SD)

Evaluation time	Creatinine level (mg/dL)				GFR (mL/min per 1.73 m ²)			
	NaP group	PEG group	Control group	<i>P</i>	NaP group	PEG group	Control group	<i>P</i>
Baseline	1.12 ± 0.15	1.12 ± 0.16	1.12 ± 0.15	0.985	69.03 ± 7.74	68.91 ± 7.95	69.57 ± 6.67	0.467
Follow-up 12–24 mo later	1.15 ± 0.15	1.15 ± 0.18	1.15 ± 0.15	0.928	66.46 ± 7.78	66.47 ± 8.26	67.42 ± 6.39	0.118

given at least 10–12 h apart. Each dose was taken with at least 250 mL of liquid, followed by an additional fluid intake of at least 1 L. The second dose was taken in the same manner and at least 3 h before the procedure. The dosing of PEG was as follows: No solid food was consumed for at least 2 h prior to ingestion of the solution. PEG (240 mL every 10 min) was consumed until the rectal output was clear or 4 L had been consumed. The colonoscopy was performed 4–6 h after bowel cleansing.

Age, race, gender, and clinical data sufficient to calculate an abbreviated Modification of Diet in Renal Disease Study Group (MDRD) glomerular filtration rate (GFR) were collected. The creatinine level on the day of the health check-up was recorded as the patient's baseline renal function. The creatinine concentration at the follow-up health check-up 12–24 mo later was recorded. The GFR was calculated using the abbreviated MDRD formula: $GFR (mL/min \text{ per } 1.73 m^2) = 186 \times (\text{serum creatinine})^{-1.154} \times (\text{age})^{-0.203} \times 0.742$ (if female).

Serum creatinine and GFR at the initial health check-up were considered the baseline levels and at the follow-up health check-up at 12–24 mo were considered the follow-up level. In the current study, we excluded subjects with a baseline creatinine level > 1.5 mg/dL or chronic renal disease.

Statistical analysis

Continuous variables are expressed as the mean ± SD, while categorical variables are presented as absolute values and percentages. A one-way analysis of variance (ANOVA) was used to examine the differences among the characteristics at baseline of the NaP, PEG, and control groups. The changes in the creatinine levels and GFRs between the NaP, PEG, and control groups were compared using the Student *t*-test. The changes in the creatinine levels and GFRs between baseline and follow-up among the PEG, NaP and control groups were compared using two-way repeated measures ANOVA. The data for the NaP, PEG

and control groups were included in a multivariate linear regression analysis to assess the relationship of the baseline creatinine level, group, age, gender, medication for hypertension, medication for diabetes mellitus, body mass index (BMI), and baseline phosphate level to the decline in renal function. A categorical “group effect” variable was defined to specify whether the patient was in the NaP, PEG, and control groups and was used in the multivariate regression model. For each variable, the odds ratio (OR) and 95% confidence interval (CI) were reported. A two-tailed *P*-value < 0.05 was considered statistically significant, and all analyses were performed with SPSS (version 12.0K; SPSS Inc., Chicago, IL, USA).

RESULTS

The baseline characteristics of subjects in each group are outlined in Table 1. The mean age of the PEG group, 51.1 ± 10.5 years, was significantly older than in the NaP and control groups (46.9 ± 8.6 years). Although there was a smaller proportion of subjects with diabetes mellitus in the NaP group compared with the PEG or control groups, there was no statistically significant difference among the groups. The phosphate level was significantly higher in the NaP group compared with the PEG and control groups on the day of the baseline health check-up.

Table 2 illustrates the changes in renal function between the groups. There was no significant difference between the baseline and follow-up levels of mean creatinine levels and the calculated MDRD GFR between groups. The baseline creatinine level for the NaP group ranged from 0.5–1.5 mg/dL (mean, 1.12 ± 0.15 mg/dL), with a calculated MDRD GFR ranging from 35 to 143 mL/min per 1.73 m² (mean, 69.03 ± 7.74 mL/min per 1.73 m²). Serum creatinine at 12–24 mo follow-up ranged from 0.4–3.0 mg/dL (mean, 1.01 ± 0.15 mg/dL), and GFR was 22–175 mL/min per 1.73 m² (mean, 66.46 ± 7.78 mL/min per 1.73 m²). There was no significant difference in the

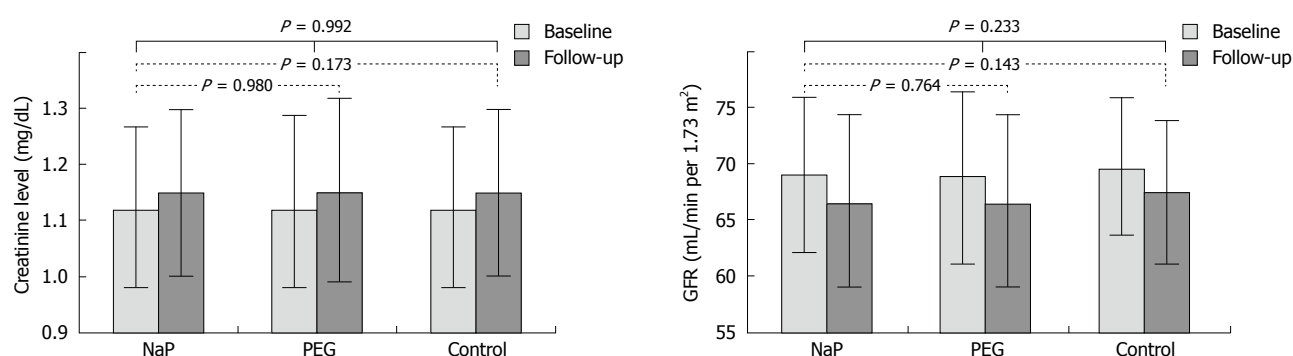


Figure 2 Comparison of changes of creatinine level and glomerular filtration rates (GFR) among NaP, PEG and control groups.

Table 3 Multivariate linear regression model on change in creatinine and GFR from baseline to 12-24 mo

Variable	Creatinine ($R^2 = 0.016$)		GFR ($R^2 = 0.053$)	
	Correlation coefficient (95% CI)	P	Correlation coefficient (95% CI)	P
Baseline	-0.018 (-0.057 to 0.021)	0.366	-0.135 (-0.187 to -0.083)	< 0.001
Group effect (NaP, PEG and control groups)	0.002 (-0.004 to 0.009)	0.489	-0.247 (-0.685 to 0.191)	0.269
Age at baseline creatinine and GFR measurement	0.000 (0.000 to 0.001)	0.433	-0.036 (-0.077 to 0.006)	0.094
Hypertension	0.007 (-0.009 to 0.023)	0.365	-0.748 (-1.774 to 0.277)	0.152
Diabetes mellitus	-0.016 (-0.034 to 0.002)	0.079	1.082 (-0.076 to 2.240)	0.067
Body mass index	0.001 (-0.001 to 0.003)	0.208	-0.038 (-0.162 to 0.086)	0.545
Serum phosphate	-0.001 (-0.004 to 0.003)	0.733	0.020 (-0.205 to 0.246)	0.859

changes in serum creatinine between the NaP and PEG group ($P = 0.980$) or the NaP and control group ($P = 0.173$). No significant difference was not found in the changes in GFR between the NaP and PEG group ($P = 0.764$) or the NaP and control group ($P = 0.143$). In addition, although creatinine level and GFR deterioration occurred at the 12-24 mo interval, there was no significant difference in the changes in creatinine levels and GFRs between the NaP, PEG, and control groups ($P = 0.992$ and $P = 0.233$, respectively; Figure 2).

To further identify the variables involved in the deterioration of renal function in patients who were exposed to the NaP preparation, we applied a linear regression model with the dependent variables (change in creatinine and GFR from baseline to 12-24 mo) and the following independent variables: age, creatinine or GFR at baseline, hypertension, diabetes, BMI, and serum phosphate. No significant risk factor for changes in serum creatinine (Table 3) was found. The NaP bowel preparation also was not associated with the creatinine change ($P = 0.366$). On the other hand, results of multivariate linear regression analysis indicated that the GFR change was only associated with the baseline GFR ($P < 0.001$). Based on the multiple linear regression model in Table 3, when all other variables remained constant, the change in GFR decreased by 0.14 units for every 1-unit increase in GFR at baseline. The NaP bowel preparation was not associated with the GFR change ($P = 0.269$). However, the model was used to select the significant variables for the change in the GFR rather than predicting the change in the GFR level since the R^2 value was low.

DISCUSSION

The demand for colonoscopy has steadily increased, with improved colorectal cancer screening uptake^[15]. Most patients undergoing colonoscopy receive NaP- or PEG-based purgatives. Multiple studies have shown that NaP is better tolerated and more cost-effective, and at least as efficacious as PEG^[2,3]. In a meta-analysis that compared NaP with PEG for bowel preparation in 25 randomized studies, the occurrence of serious adverse events with oral NaP was zero^[16,17]. Thus, there may be a reason to preferentially use NaP.

However, recent case reports and case series have suggested a possible association between NaP and acute kidney injury^[4-8,18], termed acute phosphate nephropathy. These cases presented to their medical care providers with impaired renal function and elevated serum creatinine levels from a few days up to a few weeks after ingestion of NaP. All of these patients had histopathologic evidence of phosphate nephropathy. Although the mechanism by which NaP may cause renal injury is unknown, acute phosphate nephropathy is characterized histologically by deposition of calcium phosphate crystals in tubular epithelial cells and lumens, interstitial inflammation and fibrosis, and evidence of tubular cell apoptosis^[6]. In many reported cases, the serum creatinine levels have remained increased long after disease recognition, leading some investigators to conclude that NaP may be an under-recognized cause of chronic kidney disease^[18].

The present study examined changes in renal functions in patients who had received oral NaP as a colon cleansing

Table 4 Description of the design, demographic characteristics, and effect estimate in previous studies

	Design	Population	Race (%)	Control	Timing of Follow-up	Association between NaP exposure and incident kidney injury
Abaskharoun <i>et al</i> ^[9]	RC	Any patients who had outpatient CFS	Not reported	PEG	3 mo-9 yr	No
Brunelli <i>et al</i> ^[10]	CC	Any patients who had outpatient CFS	White 46%, Non-white 52%, Unknown 2.3%	Any non-NaP preparation	Within 6 mo	No
Hurst <i>et al</i> ^[11]	RC	Any patients who had screening CFS	White 54%, Black 19%, Unknown 27%	PEG	Within 1 yr	Yes
Khurana <i>et al</i> ^[12]	RC	Any patients ¹	White 84%, Black 8%, Hispanic 7%, Asian 0.5%	No bowel preparation	6-9 mo/ 12-18 mo	Yes
Russmann <i>et al</i> ^[13]	RC	Any patients who had CFS	Not reported	PEG	Within 6 mo	No
Singal <i>et al</i> ^[14]	RC	Any patients who had CFS	White 27%, Black 53%, Hispanic 20%, Asian 0.3%	PEG	Within 1 yr	Yes

¹Excluded patients who had acute colonoscopic indication or systemic conditions which affect the renal functional deterioration. RC: Retrospective cohort; CFS: Colonofiberoscopy; CC: Case-control.

agent before colonoscopy and compared the changes with a group who received PEG or did not receive any purgatives. If a potent association existed, use of NaP would be inadvisable. If a weak, but significant, association existed, the risk of NaP must be weighed against such potential benefits as improved visualization of bowel mucosa and perhaps increased willingness of patients to undergo colonoscopy. If no association exists, NaP may be used without reservation. In this study, during the 12 to 24 mo follow-up, the changes in serum creatinine and MDRD GFRs were similar between the NaP, PEG, and control groups.

A number of previous controlled human studies have reported an association between NaP exposure and incident kidney injury^[9-14]. Table 4 summarizes the design, demographic characteristics, and effect estimates across previous studies^[9-14]. Unfortunately, it is impossible to determine whether an association between NaP and deterioration of renal function exists because of interstudy heterogeneity^[19].

Previous studies, except one study by Hurst *et al*^[11], enrolled any colonoscopy recipients^[9,10,12-14]. Inpatient colonoscopy recipients are more likely to have an underlying disease and more likely to develop kidney injury compared with asymptomatic healthy persons. In addition, because serum assays were not routinely used in previous studies, the authors had to derive this information by retrospectively identifying those patients whose conditions required the measurement of serum creatinine levels before and after bowel preparation. Therefore, the previous study population was not representative of all patients who received a bowel preparation for colonoscopy, especially for the purpose of screening colonoscopy. On the other hand, our participants had scheduled serum assays and colonoscopies as a part of health check-up services for an employer-provided wellness program, and this examination served the employees annually or biannually. Thus, all of our participants were an asymptomatic and relatively healthy homogenous group. In addition, studies in Asian subjects have been very rare, and our analysis could be relevant for this population.

Another noteworthy point is the role of the control group. Some studies considered PEG-treated controls^[9,11,13,14], whereas other studies also included any non-NaP bowel purgative^[10,12]. Because there is negligible systemic absorption of PEG^[20] and experimental data have not shown kidney lesions, even after prolonged daily exposure^[21], the PEG-treated group is a reasonable control for the NaP-treated group. However, because there was no recognizable possibility of purgative-induced renal impairment, such as volume depletion or patient condition necessitating PEG instead of NaP (renal impairment, heart failure, hepatic dysfunction, electrolyte imbalance, acute colitis, or inflammatory bowel disease), the subjects who were not exposed to PEG or NaP were also an appropriate control. In our study, both control groups were used and we found no significant differences in renal functional deterioration in the NaP group compared with either control group.

Lastly, the follow-up timing was important to compare the renal functional deterioration because purgative-induced volume depletion (expected to be greater with NaP, which is hyperosmolar, than with PEG, which is iso-osmolar) may have impaired renal function early after exposure without causing irreparable kidney damage. However, previous studies, apart from that of Abaskharoun *et al*^[9], followed the serum creatinine level up to 12 mo^[10-14]. Although the choice of a 12-24 mo follow-up interval was not based on firm evidence, we believe that it was a reasonable choice because the resultant kidney injury may take longer than 12 mo to become clinically manifest, and NaP exposure might result in a transient rise in serum creatinine^[18].

Our multivariate analysis identified that baseline renal function was an independent predictor of deterioration in renal function. This is a well-established risk factor for renal disease^[22]. On the other hand, in our multivariate analysis, the deterioration of GFR was not associated with NaP use or the serum phosphate level. A typical bowel preparation regimen for colonoscopy using NaP contains 5-10 times the usual daily intake of inorganic phosphate, and studies have shown a significant increase in serum phosphate levels^[23-25].

Hyperphosphatemia has been shown to predispose to acute kidney injury in rat models^[26], and an analogy to tumor lysis syndrome suggests a similar effect in humans. In addition, data from rat models suggest that hyperphosphatemia causes progressive deterioration in kidney function^[27,28]. An experimental model suggested that hyperphosphatemia resulted in calcium phosphate deposition in tubular cells and lumens, with subsequent apoptosis of tubular epithelial cells and interstitial fibrosis, consistent with histologic findings from case reports of acute phosphate nephropathy^[6,18]. Differences in predisposition to NaP-related kidney injury may result from allelic variations in sodium phosphate transporters or factors influencing their expression and cellular trafficking^[28].

With respect to exposure misclassification, our patients lacked data on nephrotoxic medications, including renin angiotensin system inhibitors, diuretics, non-steroidal anti-inflammatories, and radio-contrast material. There was unavoidable selection bias involving patients who received PEG because of our purgative selection. Thus, the presumably high-risk population was a lower proportion of the NaP group compared with the PEG group.

In conclusion, the changes in renal function in patients who used NaP was similar to healthy controls or PEG-treated patients. Further studies are warranted to validate and generalize our findings. Nonetheless, careful attention must be taken with patient selection, especially in patients with impaired renal function, and appropriate dosing of oral NaP (45 mL dose taken twice daily, 6-12 h apart) to prevent renal toxicity. Adequate hydration (at least 2-3 L of clear fluid throughout the cleansing period) is important during colon cleansing with oral NaP.

COMMENTS

Background

Case reports and case series have suggested a potential association between use of oral sodium phosphate (NaP) bowel preparations and kidney injury. Given the large numbers of patients exposed to NaP and the large and increasing incidence of chronic kidney disease, this association is of great public health importance.

Research frontiers

In the past year, a number of controlled human studies have evaluated the association between NaP exposure and incident kidney injury. However, results of these studies have been mixed, because considerable heterogeneity among population, control, and timing of follow-up.

Innovations and breakthroughs

As a potential risk of NaP is suggested, it is unethical to evaluate the association between NaP exposure and incident kidney injury using a prospective, randomized, controlled study. Thus, the selection of the study population is most important to reduce bias. Unlike previous studies, the present study evaluated the results from routine health check-ups for an employer-provided wellness program. Thus, although the analyses were retrospective, the selection bias was small and follow-up timing was consistent compared with previous studies. In addition, the changes in creatinine level and glomerular filtration rates (GFRs) in the NaP-treated group were compared not only to the polyethylene glycol-treated group, but also the healthy control group who did not use any purgatives.

Applications

Bowel preparation with oral NaP in patients who have normal renal function seems safe and effective; however, adequate hydration must be maintained before and after colonoscopy. To optimize safety, other agents should be considered in patients with increased serum creatinine or decreased GFR at baseline and in those predisposed to nephropathy.

Peer review

The study by Seol *et al* has been designed to answer a relevant clinical question: is the bowel cleaning NaP-prep safe in general population? Although retrospective, due to the large sample size and adequate control population, the study clearly reassures on this issue.

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Risk factors and therapeutic response in Chinese patients with peptic ulcer disease

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Abstract

AIM: To assess the risk factors and the efficacy of medications of patients with gastric and duodenal ulcers among Chinese patients in Taiwan.

METHODS: Patients with peptic ulcers, diagnosed by upper endoscopy, were retrospectively collected between January 2008 and December 2008. The differences were compared.

RESULTS: Among all 448 cases, 254 (56.6%) and 194 (43.4%) patients had gastric ulcers and duodenal ulcers respectively. Patients with gastric ulcers were younger than those with duodenal ulcers. Although more men existed, there was a female predominance in middle-aged cases. Patients with duodenal ulcers had a higher rate of *Helicobacter pylori* (*H. pylori*) infection (62.4% vs 43.3%, $P = 0.001$), and those with gastric ulcers owned a significantly higher amount of aspirin and nonsteroidal anti-inflammatory drug (NSAID) use (7.5% vs 1.5%, 6.7% vs 2.1%, $P = 0.001$). Tobacco smoking and alcohol drinking had no different impact between these two groups. Proton-pump inhibitors and H₂-receptor antagonists (H₂RA) were

effective, but significantly less so in cases with duodenal ulcers receiving H₂RAs, or in those with *H. pylori* infection and a history of NSAID use.

CONCLUSION: Patients with gastric ulcers had lower *H. pylori* infection but more aspirin or NSAID use. Antisecretory therapy was ineffective in gastric ulcers underwent H₂RA treatment, and cases combined *H. pylori* infection and NSAID use.

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Key words: Epidemiology; Peptic ulcer disease; Therapy

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INTRODUCTION

Peptic ulcer disease develops when the protective mechanisms of the gastrointestinal mucosa, such as mucus and bicarbonate secretion, are overwhelmed by the damaging effects of gastric acid and pepsin^[1]. Peptic ulcers occur mainly in the stomach (gastric ulcer) or proximal duodenum (duodenal ulcer), and they continue to be a common disease that causes a substantial socioeconomic burden and negatively impacts on quality of life.

Helicobacter pylori (*H. pylori*) infection was originally identified as the main cause of peptic ulcers. As the prevalence of *H. pylori* infection has declined in western

countries, gastric ulcer has become more commonly associated with the use of nonsteroidal anti-inflammatory drugs (NSAID) and aspirin^[2]. Tobacco smoking and alcohol drinking were also meaningful risk factors for peptic ulcer disease^[3].

Effective pharmacologic suppression of gastric acid secretion began with the introduction of H₂-receptor antagonists (H₂RA) in the 1970s, which greatly improved clinical outcomes. The development of proton-pump inhibitors (PPI) further improved inhibition of gastric acid secretion, and ensured very high healing rates for duodenal and gastric ulcers^[4]. The aim of this study was to compare the association of *H. pylori* infection, NSAID and aspirin use, tobacco smoking and alcohol drinking with gastric and duodenal ulcers, and further assess the efficacy of therapeutic medications in the patients with peptic ulcer disease.

MATERIALS AND METHODS

Data from the medical records of consecutive patients with peptic ulcers, diagnosed by open-access transoral upper endoscopy in our hospital, a 1155-bed academic urban tertiary-care center, were retrospectively analyzed between January 2008 and December 2008. Exclusion criteria were as follows: (1) cirrhosis with varices or portal hypertensive gastropathy, (2) combined other structural gastrointestinal disorders, such as gastroesophageal reflux disease, esophageal or gastric malignancy, (3) prior gastric surgery, (4) use of chronic anti-acid medication, such as PPI or H₂RA, for more than 2 mo prior to enrollment, and (5) combined gastroduodenal ulcers so undistinguished clearly.

Written informed consent for upper endoscopy was obtained from all patients before the procedure. Medications taken longer than 3 mo prior to the enrollment, including aspirin or other NSAID, were recorded. Aspirin and NSAID were recorded separately due to discrepant doses of these drugs. All cases underwent rapid urease test (CLO test, Delta West, Bentley, Australia) for *H. pylori* status from antral biopsy.

Patients were assigned to the gastric ulcer group or the duodenal ulcer group according to the locations of peptic ulcers, which were confirmed by experienced gastroenterologists. The definition of peptic ulcers was mucosal break of stomach or duodenum over 3 mm in diameter, or more than 10 gastric or duodenal erosions. The patients with *H. pylori* infection underwent standard eradication therapy, including oral PPI 40 mg, amoxicillin 2 g and Klaricid 1 g a day for one week. All patients enrolled in the study received PPI (omeprazole 20 mg, lansoprazole 30 mg, pantoprazole 40 mg once per day) or H₂RA (ranitidine 150 mg, cimetidine 400 mg twice a day). The efficacy of therapeutic medications was defined as ulcer healing and negative rapid urease test evaluated by repeated endoscopy pulse antral biopsy at our outpatient clinic 3 mo following initial endoscopy. Patients who received aspirin were also given PPI or H₂RA, and those who received NSAID were shifted to Coxibs in addition to standard PPI or H₂RA use.

Table 1 Clinical features of the two groups of patients at examination *n* (%)

	Upper endoscopy finding		Total	<i>P</i> -value
	Gastric ulcers	Duodenal ulcers		
Case numbers (prevalence)	254 (56.6)	194 (43.4)	448 (100)	
Age (yr)	60.44 ± 15.27	51.99 ± 16.24		0.001 ¹
Gender				
Male	142 (55.8)	110 (56.7)	252 (56.2)	0.866 ²
Female	112 (44.1)	84 (43.3)	196 (43.8)	
<i>H. pylori</i>				
Positive	110 (43.3)	121 (62.4)	231 (56.7)	0.001 ²
Negative	144 (56.7)	73 (37.6)	217 (43.3)	
Smoking				
Yes	36 (14.2)	31 (16.0)	67 (15.8)	0.595 ²
No	218 (85.8)	163 (84.0)	381 (86.0)	
Alcohol				
Yes	21 (8.3)	22 (11.3)	43 (9.6)	0.274 ²
No	233 (91.7)	172 (88.7)	405 (90.4)	
Co-medications				
Nil	218 (85.8)	187 (96.4)	405 (90.4)	0.001 ²
Aspirin	19 (7.5)	3 (1.5)	22 (4.9)	
NSAIDs	17 (6.7)	4 (2.1)	21 (4.7)	

¹*t* test; ²Pearson's χ^2 test. NSAIDs: Nonsteroidal anti-inflammatory drugs.

Statistical analysis

Data are expressed as standard derivation of mean for each of the measured parameters. Gender, *H. pylori* infection, and combined medication are expressed as a percentage of the total patient number. Statistical comparisons were made using Pearson's χ^2 test to compare the effects of gender, *H. pylori* infection, tobacco smoking, alcohol drinking, combined medications, and risk factors. *t* test was used to compare the mean ages of patients, and Fisher's exact test was applied to evaluate the therapeutic efficacy of cases with aspirin or NSAID use. A *P* value below 0.05 was considered statistically significant.

RESULTS

Among all consecutive patients who received open-access upper endoscopy between January and December 2008, there were 448 cases enrolled in our study and the characteristic of them were displayed in Table 1. Among these patients, 254 patients (56.6%) had isolated gastric ulcers and 194 patients (43.4%) had isolated duodenal ulcers. Comparing the chronic medication use of patients with gastric ulcers and duodenal ulcers, more patients with gastric ulcers took aspirin and NSAID than did those with duodenal ulcers (7.5% *vs* 1.5%, 6.7% *vs* 2.1%, respectively), and the difference was significant (*P* = 0.001).

Most of the patients with gastric ulcers were in the fifth decade of life, whereas most patients with duodenal ulcers were in the sixth decade, as shown in Figure 1. There were more male patients than female patients in both groups, and a similar gender ratio existed between these two groups. The ratio of gender according to age distribution is shown in Figure 2. More female cases were noted in the 50-60-year-old patients with gastric ulcers and the 60-70-year-old patients with duodenal ulcers. Male predominance was noted

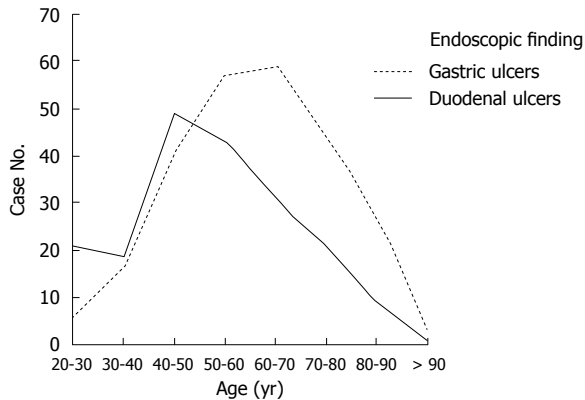


Figure 1 Age distribution of the two groups of patients.

Table 2 The impact of therapeutic efficacy according to *H. pylori* infection and ulcer location of patients *n* (%)

	Therapeutic efficacy		Total	P-value
	Ulcer healed	Ulcer unhealed		
<i>H. pylori</i>				
GU				
Positive infections	77 (70.6)	32 (29.4)	109	0.661
Negative infections	99 (68.3)	46 (31.7)	145	
DU				
Positive infections	87 (71.9)	34 (28.1)	121	0.505
Negative infections	55 (75.3)	18 (24.7)	73	
Medications				
GU				
Treat with PPI	131 (79.4)	34 (20.6)	165	0.007
Treat with H2RA	56 (62.9)	33 (37.1)	89	
DU				
Treat with PPI	115 (78.2)	32 (21.8)	147	0.430
Treat with H2RA	34 (72.3)	13 (27.7)	47	

All analyzed with Pearson's χ^2 test. GU: Gastric ulcer; DU: Duodenal ulcer; PPI: Proton-pump inhibitor; H2RA: H2-receptor antagonist.

in both the younger and older gastric ulcer and duodenal ulcer patients.

The rate of *H. pylori* infection, shown in Table 1, was significantly higher in the patients with duodenal ulcers than in those with gastric ulcers (62.4% *vs* 43.3%, $P = 0.001$). Similarly, more patients with duodenal ulcers smoked tobacco and drank alcohol than those with gastric ulcers (16% *vs* 14.2%, 11.3% *vs* 8.3%, respectively), but these differences in our study were insignificant ($P = 0.595$ and 0.274).

There was a similar proportion of patients in each of the two groups with positive *H. pylori* infection according to age distribution, as shown in Figure 2. The lowest rate was noted in gastric ulcer patients in the second and ninth decades of life, and the highest rate was found in duodenal ulcer patients in the ninth decade of life. However, the numbers in these groups were very small.

In regard to the impact of therapeutic efficacy on *H. pylori* infection, all had effective eradication proven by negative secondary rapid urease test. As illustrated in Table 2, the results revealed there were similar rates of peptic ulcer healing among patients with and without *H. pylori* infection in both groups who underwent eradication

Table 3 The impact of therapeutic efficacy according to co-medication and ulcer location of patients *n* (%)

Co-medications	Therapeutic efficacy		Total	P-value
	Ulcer healed	Ulcer unhealed		
None				
Positive <i>H. pylori</i> infection	159 (75.7)	51 (24.3)	210	0.370 ^a
Negative <i>H. pylori</i> infection	140 (71.8)	55 (28.2)	195	
ASP				
Positive <i>H. pylori</i> infection	8 (80)	2 (20)	10	0.195 ^b
Negative <i>H. pylori</i> infection	12 (100)	0	12	
NSAID				
Positive <i>H. pylori</i> infection	6 (60)	4 (40)	10	0.035 ^b
Negative <i>H. pylori</i> infection	11 (100)	0	11	

^aPearson's χ^2 test; ^bFisher's exact test. ASP: Aspirin.

therapy. There was no significant difference between the efficacy of PPI and H2RA among patients with duodenal ulcers (78.2% *vs* 72.3%, $P = 0.430$). On the contrary, PPI was significantly more effective than H2RA in patients with gastric ulcers (79.4% *vs* 62.9%, $P = 0.007$).

As shown in Table 3, the therapeutic efficacy of PPI and H2RA was compared among patients who used aspirin or NSAIDs regularly or took no medicine. In regard to the combined influence of *H. pylori* infection and chronic aspirin or NSAID use in patients with peptic ulcer disease, there was a lower rate of response to therapeutic medicine, especially in the cases taking NSAID (60% *vs* 100%, $P = 0.035$).

DISCUSSION

Peptic ulcer disease is associated with gastric hypersecretion, a physiological imbalance between antagonistic gastric hormones, cholinergic hypersensitivity, psychological stress, tobacco smoking, alcohol consumption, or use of aspirin and NSAID. Duodenal ulcer is essentially an *H. pylori*-related disease and is caused mainly by an increase in acid and pepsin load^[5]. Gastric ulcer in Western countries is most commonly associated with NSAID ingestion or *H. pylori* infection. As the prevalence of *H. pylori* infection has declined in western countries, gastric ulcer has become more commonly associated with the use of NSAID and aspirin^[6].

The prevalence of peptic ulcers in developed countries has been declining for several decades owing to improvements in sanitation and a reduced rate of *H. pylori* acquisition^[7]. Recently, a study in Taiwan reported incidences of gastric and duodenal ulcer diseases decreased 42%-48% and 41%-71%, respectively, in a 10-year period^[8]. The birth cohorts with the highest risk of developing gastric ulcer were born 10-20 years before those with the highest risk for duodenal ulcer in all European countries, the USA, Australia and Japan^[9]. Our results were similar, although our study was not population-based.

Previous studies have shown that male gender is an independent risk factor for peptic ulcer disease in populations in the USA, Japan and Norway^[10-12]. Our results documented male predominance in both the patients

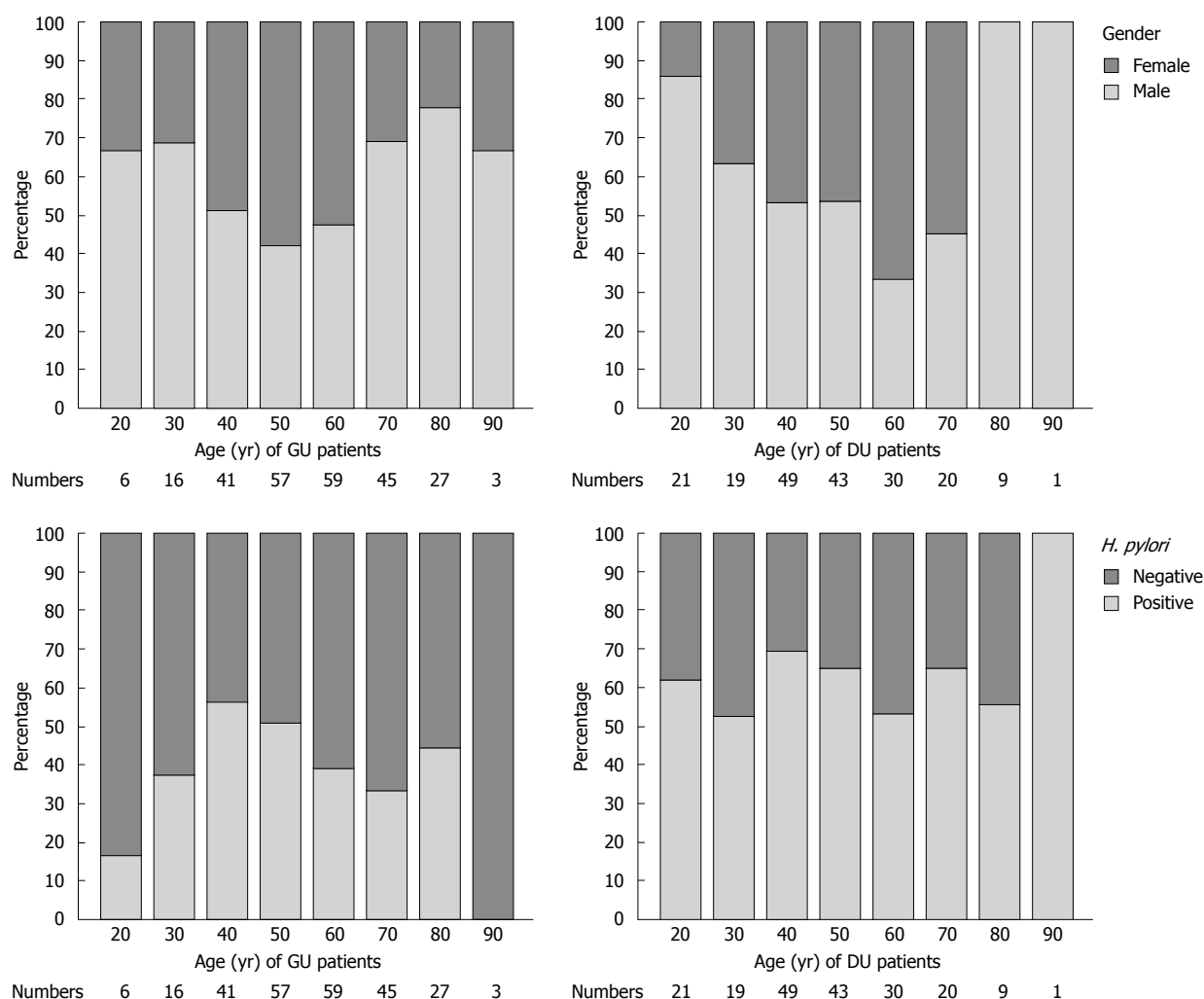


Figure 2 The ratio of gender and *Helicobacter pylori* (*H. pylori*) infection according to age distribution of the two groups of patients. GU: Gastric ulcer; DU: Duodenal ulcer.

with gastric ulcers and in those with duodenal ulcers. Interestingly, we also found there was relative female predominance in middle-aged patients, although no previous studies had similar findings. The reason may be that middle-aged women are more likely to seek medical treatment than men in the same age group.

Previous studies had documented tobacco smoking and alcohol drinking as firm risk factors for peptic ulcer disease^[3,5], however, no available data focused on the different impacts of these factors in achieving gastric ulcers and duodenal ulcers. Our study found similar influences of these two groups, implying the excellent benefit of quitting tobacco smoking and alcohol drinking in patients with peptic ulcer disease, regardless of the location of the ulcers.

During the 1980s, *H. pylori* infection was found in more than 90% of patients with duodenal ulcers, and around 70% of patients with gastric ulcers^[13]. An increased prevalence correlates with lower educational levels, income, and other socioeconomic factors^[14]. A recent study reported a seroprevalence of *H. pylori* infection in 56.4% of patients with duodenal ulcers and a lower rate in those with gastric ulcers^[3]. Similarly, our patients had

a lower prevalence of *H. pylori* infection than previously expected, with a rate of 42.9% in cases with gastric ulcers, and 62.4% in those with duodenal ulcers. The result may be due to improvements in hygiene, and it emphasizes the fact that eradication therapy should not be initiated without prior verification of the infection.

On the contrary, aspirin and NSAID significantly increase the risk of adverse gastric or duodenal ulcers and complications, especially bleeding^[3]. Gastric ulcers are strongly linked to NSAIDs, and this was confirmed by the positive relationship between aspirin or NSAID use and gastric ulcers in our study. *H. pylori* infection and NSAID have additive or synergistic effects on adverse gastrointestinal outcomes: *H. pylori* damages through direct pathogenic mechanisms, and NSAID causes disruption of mucosal defensive mechanisms indirectly^[2]. Our study did not include this conclusion because there were too few cases with *H. pylori* infection taking aspirin or NSAID to be analyzed sufficiently.

In regard to the patients with peptic ulcer disease owing to *H. pylori* infection, studies indicate that eradication of infection not only heals peptic ulcers, but also reduces the ulcer recurrence rate^[15]. Maintenance treatment should

be continued with PPI use following *H. pylori* eradication in all patients to prevent ulcer recurrence or complications^[16]. Standard dose PPI treatment should be prescribed for 4 wk in patients with duodenal ulcers and for 8 wk in patients with gastric ulcers^[17].

The results of our study revealed an adequate ulcer healing rate in patients with gastric or duodenal ulcers who received antisecretory therapy, PPI or H2RA, whether they had *H. pylori* infection or not, and whether they were taking aspirin and other NSAID or not. There were two subgroups of patients with a significantly poor therapeutic response: patients with gastric ulcers who underwent H2RA treatment (62.9%), and patients with both *H. pylori* infection and NSAID use who accepted antisecretory therapy (60%).

One previous study noted that pantoprazole was more effective than ranitidine in the treatment of duodenal ulcers, providing not only symptom remission but also faster ulcer healing^[18]. Another study reported *H. pylori* infection and NSAID use synergistically increase the risk of peptic ulcers and ulcer bleeding^[19]. These results implied that PPI, at least in duodenal ulcers, is more effective than H2RA, and prolonged antisecretory therapy may be necessary in the treatment of peptic ulcers caused by *H. pylori* infection and NSAID use. One recent study among a Japanese population also mentioned that it is desirable to administer antiulcer agents to patients with risk factors, such as *H. pylori* infection, when taking NSAID^[20].

There were some limitations in our study. Firstly, our study, a form of referral-based endoscopy, as opposed to a population-based survey, was less representative of the general population due to selection bias. Secondly, we only recorded the recent use of NSAID. There is a possibility that some patients were unaware that they were taking NSAIDs. Besides, the kinds of NSAID and PPI were heterogeneous. Thirdly, only an incomplete previous history of peptic ulcer disease or *H. pylori* infection was available to be analyzed. Furthermore, previous *H. pylori* eradication was not listed in the exclusion criteria of this study, and the *H. pylori* infection rate may be an underestimation. Lastly, major limitations of this study included the retrospective study design and the lack of randomization. Although the duration of this study was designed to be as long as one year, it is possible that there were unmeasured differences among these subgroups.

In the present study, Chinese patients in Taiwan with peptic ulcers had different characteristics. The patients with gastric ulcers were older, had a lower ratio of *H. pylori* infection and a greater association with aspirin or NSAID use, compared with those with duodenal ulcers. Antisecretory therapy was very effective in patients with peptic ulcer disease, except cases with gastric ulcers who underwent H2RA treatment, and those who had combined *H. pylori* infection and NSAID use and accepted antisecretory therapy.

and alcohol drinking. Effective pharmacological therapy includes H2-receptor antagonist (H2RA) and proton-pump inhibitor (PPI).

Research frontiers

Although risk factors and therapeutic efficacy are well known in Western countries, the information in the Chinese population is relatively rare. The aim of this study was to assess the risk factors and the efficacy of medications of patients with gastric and duodenal ulcers among Chinese patients in Taiwan.

Innovations and breakthroughs

The authors' result found that Chinese patients in Taiwan with gastric ulcers were older, had a lower ratio of *H. pylori* infection and a greater association with aspirin or NSAID use compared with those with duodenal ulcers. Antisecretory therapy was very effective against peptic ulcers, except in cases with gastric ulcers who underwent H2RA treatment, and those with combined *H. pylori* infection and NSAID use who accepted antisecretory therapy.

Applications

Maintenance treatment should be continued with PPI use, following *H. pylori* eradication, in all patients to prevent ulcer recurrence or complications, especially in cases who used NSAID.

Peer review

The study compares a group of gastric ulcer patients with a group of duodenal ulcer patients in terms of demographic characteristics, certain risk factors, and response to treatment. It is of moderate importance with minimal novelty.

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COMMENTS

Background

Peptic ulcer disease is associated with *Helicobacter pylori* (*H. pylori*) infection, aspirin and nonsteroidal anti-inflammatory drug (NSAID) use, tobacco smoking

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Application of acellular dermal matrix for intestinal elongation in animal models

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hesions were found between the graft and surrounding intestine. The grafts were completely absorbed within postoperative two or three months except one. Histological observation showed inflammation in the grafts with fibrinoid necroses, infiltration of a large amount of neutrophils and leukomonocytes, and the degree varied in different stages. The neointestine with well-formed structures was not observed in the study.

CONCLUSION: It is not suitable to use acellular dermal matrix alone as a scaffold for the intestinal elongation in animal models.

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Key words: Acellular dermal matrix; Intestine; Elongation

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Xu HM, Wang ZJ, Han JG, Ma HC, Zhao B, Zhao BC. Application of acellular dermal matrix for intestinal elongation in animal models. *World J Gastroenterol* 2010; 16(16): 2023-2027 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v16/i16/2023.htm> DOI: <http://dx.doi.org/10.3748/wjg.v16.i16.2023>

Abstract

AIM: To investigate the efficacy of acellular dermal matrix (ADM) for intestinal elongation in animal models.

METHODS: Japanese white big-ear rabbits ($n = 9$) and Wuzhishan miniature pigs ($n = 5$) were used in the study. Home-made and commercial ADM materials were used as grafts, respectively. A 3-cm long graft was interposed in continuity with the small bowel and a side-to-side anastomosis, distal to the graft about 3-4 cm, was performed. The animals were sacrificed at 2 wk, 4 wk, 8 wk and 3 mo after surgery and the histological changes were evaluated under light microscope and electron microscope.

RESULTS: The animals survived after the operation with no evidence of peritonitis and sepsis. Severe ad-

INTRODUCTION

Short bowel syndrome (SBS) is defined as a spectrum of small intestinal dysfunction for a major portion of small intestines being resected and the patients need chronic total parenteral nutrition (TPN) in lifetime. However, a long-term use of TPN is associated with significant

morbidity and mortality^[1-3], such as system infection and progressive cholestatic liver disease and many patients could not afford the high cost of the nutrition support. Several bowel elongation procedures were successful in increasing the absorptive surface area, but these methods were not widely applied for serious complications. Small intestinal transplantation may be a valid option, but the result is unsatisfactory for immunologic problems.

Recently, acellular biomaterials have received extensive attentions, including acellular dermal matrix (ADM). ADM provides a biologic scaffold that allows native cellular ingrowth and tissue remodeling. In this study, we applied the ADM as scaffold for the mucosal growth and intestinal elongation in animal models.

MATERIALS AND METHODS

Animal

Japanese white big-ear rabbits ($n = 9$, body weight 2.0-2.2 kg) and Wuzhishan miniature pigs ($n = 5$, body weight 41-44 kg) were used in the study. Animal care and use complied with the institutional regulations established and approved by the Animal Care and Use Committee of the Capital Medical University for Animal Research Program. All the animals were housed in individual cages under standard laboratory conditions and fed animal chow and water *ad libitum*.

Preparation of ADM

A Japanese white big-ear rabbit and a Wuzhishan miniature pig were used as the donors, respectively. Segments of the small intestine were harvested and cut into 5-cm lengths. The grafts were sterilized in 75% ethanol, rinsed in saline solution, and stored at 4°C in a Penicillin-Streptomycin solution until use. The dermis was acellularized in accordance with the method reported previously by Takami *et al.*^[4]. In brief, the segment of the small intestine was treated with 0.25% Trypsin (Roche corp.) at 4°C for 24 h to remove the epidermis and other cellular components from the dermal matrix. Subsequently, the dermal matrix was incubated in buffered 0.5% Triton X-100 (Sigma-Aldrich Corp.) for 24 h at 37°C. An oscillator was used during these procedures. Dispase-Triton ADM was then extensively washed with phosphate buffered solution (PBS) and stored in PBS at 4°C until use (Figure 1). All solutions were treated with filter sterilization.

Surgical procedures

Surgical procedures were performed under general anesthesia with 3% Pentobarbital (25 mg/kg). All the Japanese white big-ear rabbits (group A) and two Wuzhishan miniature pigs (group B) were transplanted with the allograft materials respectively, and the other two pigs (group C) with the human acellular dermal matrix (HADM, Ruinuo®, Qingyuanweiye Bio-Tissue Engineering Ltd, Beijing, China).

A laparotomy in the right lower abdominal quadrant

was performed, and the small bowel was transected in the middle portion. A graft (3 cm long) was interposed in continuity with the small bowel using interrupted end-to-end two layers anastomosis (muscularis-serosa layer and full-thickness layer) with 1-0 nonabsorbable sutures. Then a side-to-side anastomosis, distal to the graft about 3-4 cm (Figure 2), was performed. The defect of the mesentery was repaired to avoid entocoele. All the viscera were laid back to the abdominal cavity. The abdominal wall was closed with running suture, while the skin with interrupted suture. In the group of HADM, the graft was sewn at the edges with sutures to construct a tube, and then the same operation was repeated as mentioned above.

All the animals were maintained on a liquid diet and water for 48 h and then on a full diet. Two rabbits were sacrificed at 2, 4 and 8 wk, respectively after surgery. The study endpoint was 3 mo after the operation. Pathologic specimens including the anastomotic site were fixed in formalin, embedded in paraffin, and processed routinely for light microscopy. Histological changes were evaluated under light microscope and transmission electron microscope.

RESULTS

The animals survived after the operation with no evidence of peritonitis or sepsis. All the rabbits had gradual weight loss from median 2.1 to 1.8 kg and the survival after the operation was no longer than 8 wk, while the body weight of pigs increased from median 42 to 51 kg which were executed at 3 mo after the surgery according to the scheme.

In group A, severe adhesions were found between the graft and surrounding intestine. There was almost no blood supply in the grafts and the lumens were full of dry stool or pus at 2 and 4 wk after surgery. At the 8th postoperative week, the grafts totally disappeared, similar to group B and one pig in group C and there were only two blind-ended pouches. The other one in group C seemed to be the most successful one considering the small intestinal continuity was restorative. But the graft shrank from 3 to 0.2 cm and became a narrow-ring without flexibility (Figure 3).

Histological observation showed acute inflammation in the tissue during the first two weeks. In the first week, there were focal fibrinoid necroses and infiltration of a large amount of neutrophils and leukomonocytes (Figure 4A and B). In the second week, there was a further development of the inflammation. The fibrinoid necroses and cell infiltration became dispersed and inflammatory exudates of neutrophils and pus cells could be seen (Figure 4C and D). At the same time, a lot of micrangiums and fibroblasts appeared in the scaffold. By the 4th postoperative week, the infiltration of leukocytes and leukomonocytes mitigated. No well-formed structure could be seen and all the layers were contorted in the narrow-ring of group C (Figure 5). The thickening serosa and infiltration of a small amount of leukocytes and leukomonocytes could be discovered. Immunocytochemical stain with smooth muscle actin showed smooth muscle cells in the narrow-ring of ADM with disordered structure.

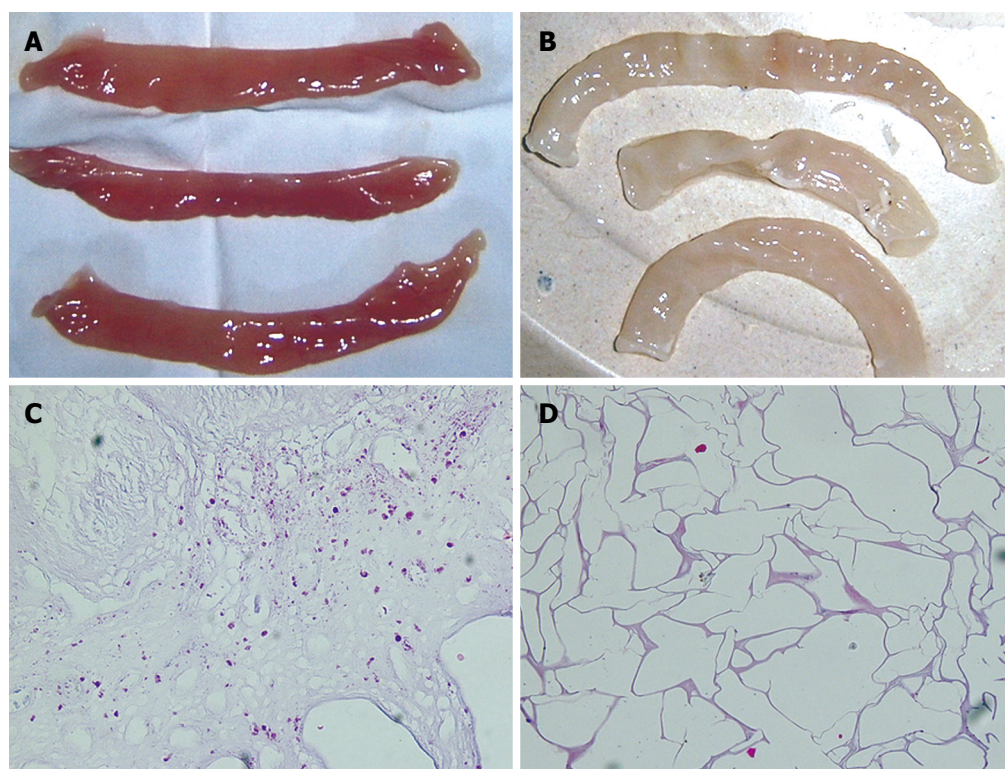


Figure 1 Acellular dermal matrix. A: Segments of the small intestine; B: Segments of acellular dermal matrix; C, D: Hematoxylin and eosin-stained histological photomicrography showed that all the cellular elements of the small intestine have been removed (C: × 100, D: × 400)

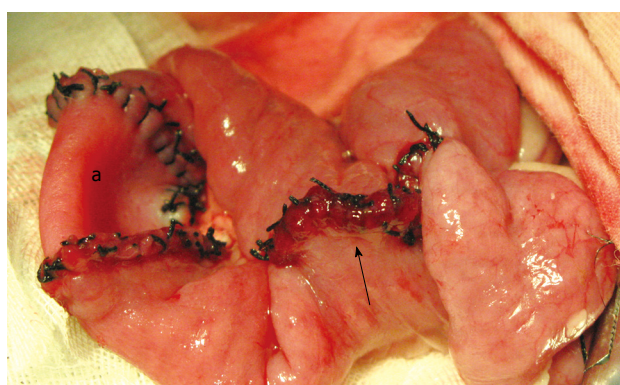


Figure 2 Surgical procedures. a: ADM; arrow: Side-to-side anastomosis.

DISCUSSION

Several surgical procedures have been used to improve the SBS^[5,6]: (1) to decrease transit time of the bowel content by creation of intestinal valves or interposition of bowel segments; (2) to increase mucosal surface area; and (3) to increase the bowel length or to transplant the bowel. Objective assessment of these surgical techniques was deficient due to a relatively small number of patients. The small intestine transplantation is becoming a less morbid therapy in recent years, but it remains complicated and is limited due to the donor availability.

ADM is a dermal biomaterial in which all of the cellular elements have been removed. The biologic properties of ADM, including its ability to support tissue regeneration repopulation with fibroblasts, revascularization, new collagen deposition and eventual absorption and replacement with native tissue permit its use in tissue reconstruc-

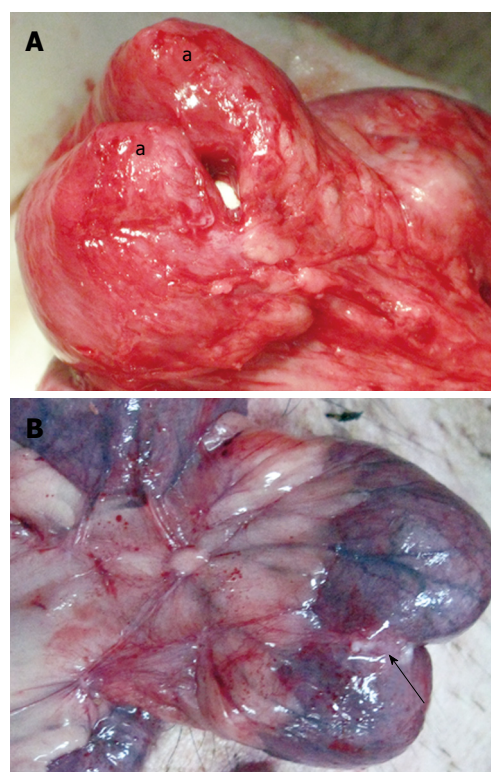


Figure 3 Postoperative gross specimen. A: ADM has been absorbed completely (a: Blind-ended pouch); B: The graft was shrinking (arrow: The ADM material).

tion. Now ADM has been used successfully in repairing the defect or damaged tissues and organs both in preclinical animal studies and in clinical applications^[7-11], such as burn surgery, herniorrhaphy, urethroplasty, pelvic floor

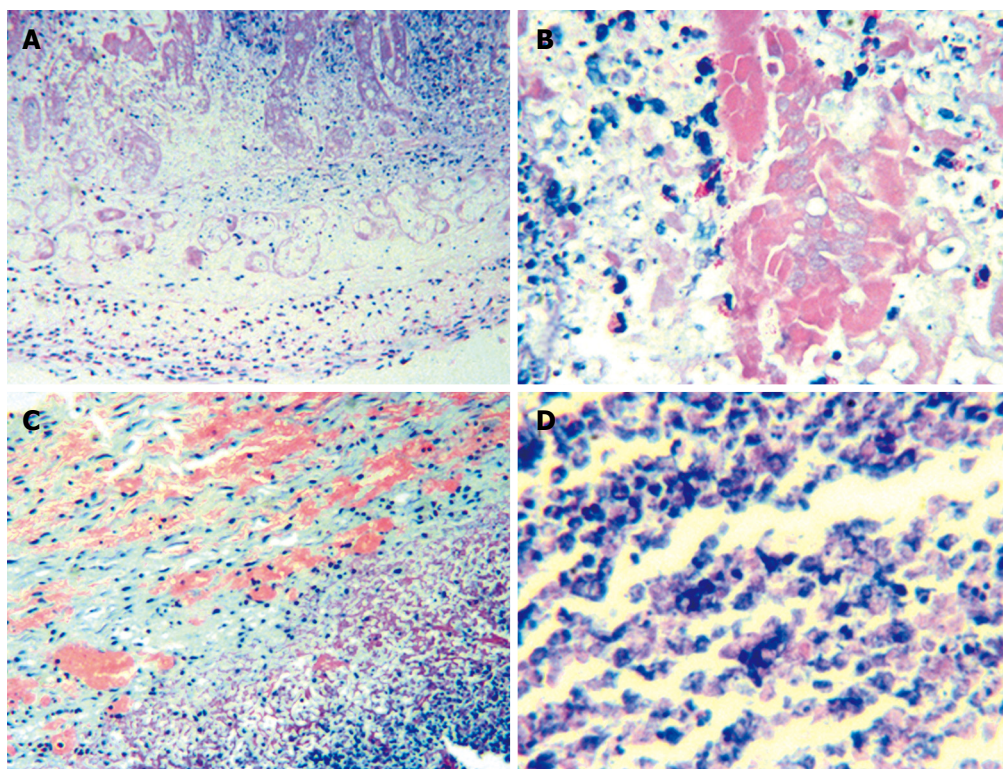


Figure 4 Histological photomicrography of postoperative ADM. Focal fibrinoid necroses and infiltration of a large amount of neutrophils and leukomonocytes in the first week (HE stain, A: $\times 100$, B: $\times 400$); a further development of the inflammation in the second week (HE stain, C: $\times 100$, D: $\times 400$).

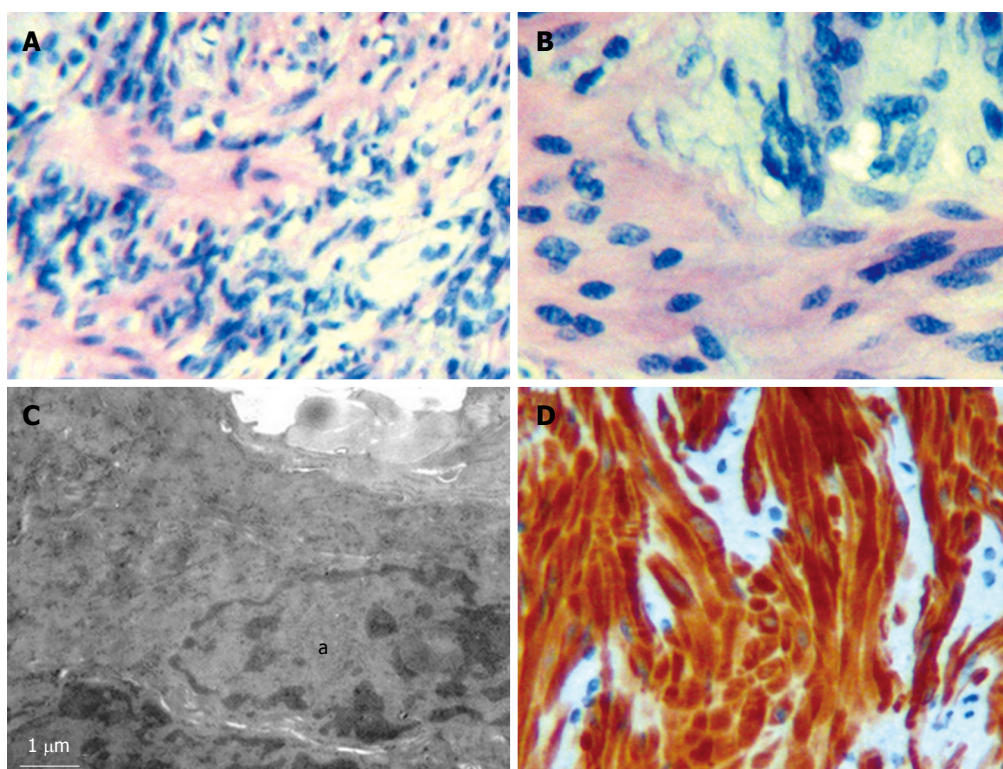


Figure 5 Structures of the ADM were contorted in the narrow-ring. A: $\times 100$; B: $\times 200$; C: Transmission electron microscopy, $\times 20\,000$; a: Fibroblast; D: Immunocytochemical stain with smooth muscle actin showed smooth muscle cells in the narrow-ring.

reconstruction, contaminated abdominal wall defects and fistula.

We supposed that the ADM can be used as a scaffold for tissue regeneration with the normal morphology of the mucosa, submucosa, muscle and serosa layers. An *in vivo* study in dog models showed that an extracellular matrix (ECM) plus autologous muscle could facilitate the

reconstruction of the esophagus with near normal biomechanical properties and structure^[12]. Pahari *et al*^[13] used the acellular human dermis in the form of tubular scaffolds for intestinal elongation. Intact mucosa with well-formed crypts was evident at 6 mo after operation^[13].

But it did not develop like what we had designed in the study. The grafts were almost completely absorbed

in two or three months. Pus could be seen in the lumens of ADM and histological examination showed the inflammatory exudates of neutrophils and pus cells. Thus, the ADM might be a source of infection in this study, though we have tested that the ADM can be used in contaminated wound for its resistance to infection^[14]. Inadequate blood supply and the rapid absorption were the most likely reasons. All the ADM depends on the blood infiltrating from the two anastomotic ends and the supply was very limited comparing with the granulation tissue of the wound surface. Immunocytochemical stain with smooth muscle actin showed smooth muscle cells in the narrow-ring at 3 mo after surgery, which is an exciting event and consistent with our previous study^[14].

The biggest challenge of bowel tissue engineering is the recovery of peristaltic activity of the regenerated bowel, which needs both smooth muscle and reinnervation of the regenerated bowel. It seemed that there was no evidence of innervations of the neointestine to date. Even the neointestine had well-formed structures, the dysmotile segment may become dilated, resulting in fecal stasis, bacterial overgrowth and malabsorption.

Our conclusion is different from some of the previous studies. We have made several reviews of the whole procedure, but no obvious errors could be found. Even if there were some problems in the process of preparation of the ADM, which was more elaborate, group C (HADM, Ruinuo[®]) had the same result. So we believe that the result is reliable. Though ADM has been successfully used to repair and reconstruct the tissue defects in many fields, it seemed that the single use of ADM is not suitable to the intestinal elongation in the present study and there is a long way to go before the ADM can be used for intestinal elongation.

COMMENTS

Background

Short bowel syndrome (SBS) has puzzled surgeons for a long time. Though some treatments have been reported, the effects are unsatisfactory. In recent years, acellular dermal matrix (ADM) has been used in many fields to provide a biologic scaffold for tissue regeneration or scarless healing, and few studies for SBS have been performed, and the results are uncertain.

Research frontiers

There are only few studies about ADM used for intestinal elongation. This study was designed to use both the home-made and commercial ADM materials. At the same time, different species were transplanted with the allograft materials. All of these can improve the credibility of this research.

Innovations and breakthroughs

This study was designed according to the previous work, but it showed different results. The authors gave some possible reasons, which were worthy of in-depth studies.

Applications

ADM may be used for intestinal elongation, but some reforms are necessary in its future application.

Terminology

ADM is a new material that all of the cellular elements have been removed. The kind of material has special biologic properties, including support of tissue regeneration with concomitant revascularization and repopulation with fibroblasts, new collagen deposition, absorption and replacement with native tissues.

Peer review

The treatment of short bowel syndrome has always been a challenge for the surgeons and any serious work regarding this issue will be very important for surgeons and patients. The aim of this work was to evaluate the efficacy of acellular dermal matrix for intestinal elongation, which has been well designed and performed.

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Effect of oxymatrine on the replication cycle of hepatitis B virus *in vitro*

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Abstract

AIM: To determine the antiviral mechanism or target of oxymatrine against hepatitis B virus (HBV).

METHODS: HepG2.2.15 cells were incubated with culture medium containing 500 µg/mL of oxymatrine for 2 and 5 d. The surface antigen of HBV (HBsAg) and e antigen of HBV (HBeAg) in supernatant were determined by ELISA. HBV DNA in supernatant, and intracellular covalently closed circular DNA (cccDNA), relaxed circular DNA (rcDNA) and pregenomic RNA (pgRNA) were quantified by specific real-time polymerase chain reaction (PCR) or reverse transcription (RT)-PCR.

RESULTS: Treatment with oxymatrine for 2 d and 5 d reduced the production of HBV by the cell line, as

indicated by the decline of HBsAg (22.67%, $t = 5.439$, $P = 0.0322$ and 22.39%, $t = 5.376$, $P = 0.0329$, respectively), HBeAg (55.34%, $t = 9.859$, $P = 0.0101$ and 43.97%, $t = 14.080$, $P = 0.0050$) and HBV DNA (40.75%, $t = 4.570$, $P = 0.0447$ and 75.32%, $t = 14.460$, $P = 0.0047$) in the supernatant. Intracellular cccDNA was also markedly reduced by 63.98% ($t = 6.152$, $P = 0.0254$) and 80.83% ($t = 10.270$, $P = 0.0093$), and intracellular rcDNA by 34.35% ($t = 4.776$, $P = 0.0413$) and 39.24% ($t = 10.050$, $P = 0.0097$). In contrast, intracellular pgRNA increased by 6.90-fold ($t = 8.941$, $P = 0.0123$) and 3.18-fold ($t = 7.432$, $P = 0.0176$) after 500 µg/mL of oxymatrine treatment for 2 d and 5 d, respectively.

CONCLUSION: Oxymatrine may inhibit the replication of HBV by interfering with the process of packaging pgRNA into the nucleocapsid, or inhibiting the activity of the viral DNA polymerase.

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Key words: Oxymatrine; Hepatitis B virus; Replication intermediates; Covalently closed circular DNA; Pregenomic RNA

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INTRODUCTION

Despite efficient vaccines, chronic hepatitis B virus (HBV)

infection remains a major public health problem, which involves more than 350 million people in the world^[1]. Antiviral agents play a key role in the treatment of chronic hepatitis B infection by inhibiting the replication of the virus, and delaying or preventing progression to cirrhosis, hepatocellular carcinoma and death. Interferon- α (IFN- α) has been shown to be effective in suppressing HBV replication and inducing remission of liver disease^[2]. Its principal antiviral mechanism has been thought to include both a direct antiviral effect and an immunomodulatory effect^[3-5]. However, its efficacy is limited to a small percentage of highly selected patients and is often associated with adverse effects such as flu-like symptoms, fatigue, leucopenia, depression, anorexia, hair loss, *etc*^[2]. Nucleoside analogues such as lamivudine, adefovir dipivoxil and entecavir could competitively inhibit the activity of HBV DNA polymerase or terminate the elongation of newly synthesized DNA chain by incorporation, and demonstrated potent anti-HBV efficacy *in vitro* and *in vivo*^[2]. However, nucleoside analogues are also limited to certain adult patients, and related adverse effects such as renal tubular dysfunction by adefovir have also been reported^[2]. In addition, viral mutation may induce drug resistance to nucleotide analogues and relapse of hepatitis B^[2].

In China, many herbs or their derivatives have also been widely used in the treatment of viral hepatitis and associated complications such as liver cirrhosis and liver failure^[6,7]. Oxymatrine, a type of alkaloid extracted from the herb *Sophora alopecuroides* L.^[8], had shown a promising anti-HBV effect in a HBV-transfected cell line, in a HBV transgenic mice model and in patients with chronic hepatitis B^[9-11]. Oxymatrine had also been found to be capable of relieving hepatic fibrosis or severe injury independently^[12-14]. It has been approved for the treatment of hepatitis B by the State Food and Drug Administration of China, and is listed as one of the recommended anti-HBV agents in the Guideline for Prevention and Treatment of Chronic Hepatitis B jointly proposed by the Chinese Society of Hepatology and the Chinese Society of Infectious Diseases^[15]. Unfortunately, unlike IFN- α or antiviral nucleoside analogues, little is known about the exact mechanism or target of oxymatrine against HBV.

The replication cycle of the hepadnavirus involves a pathway by reverse transcription of an RNA intermediate^[16], during which several important replicative intermediates are generated, including covalently closed circular DNA (cccDNA), pregenomic RNA (pgRNA) and progeny virus relaxed circular DNA (rcDNA), and the viral particle is secreted outside after maturation^[17]. The antiviral agents usually target one or more specific sites of the HBV replication cycle. It can be reasoned that any intervention or interruption in the replication cycle of HBV will result in the fluctuation or alteration of the product occurring in the specific site, which will in turn provide clues to illuminate the target of antiviral agents. In this paper, we investigated the effect of oxymatrine on the replication cycle in the HepG2.2.15 cell line, and explored the possible antiviral target of oxymatrine.

MATERIALS AND METHODS

Compounds

Oxymatrine was provided by Jiangsu Chia-tai Tianqing Pharmaceutical Co. Ltd, China, the purity of which had been determined to be greater than 99% by high-performance liquid chromatography. The compound powder was stored at room temperature in light-resistant containers, and stock solutions were prepared when required by dissolving reagents in dimethyl sulfoxide to a concentration of 200 mg/mL and stored at -4°C.

Cell line

The HepG2.2.15 cell line, which could support persistent replication of HBV and produce intact HBV particles^[18], was provided by the Molecular Viral Laboratory of Fudan University with permission from the Mount Sinai Medical Center, NY, USA. It was maintained with Dulbecco's Modified Eagle Medium (Gibco-BRL, Grand Island, NY, USA) supplemented with 10% (vol/vol) heat-inactivated fetal bovine serum (Gibco-BRL, Grand Island, NY, USA), 380 µg/mL G418 (Gibco-BRL, Grand Island, NY, USA) 125 µg/mL penicillin and 50 µg/mL streptomycin at 37°C in a humidified incubator containing 50 mL/L CO₂. Then cells were harvested from the flask by treating the monolayer with 0.25% trypsin (Gibco-BRL, Grand Island, NY, USA) and 1 mmol/L EDTA, and resuspended in culture medium for further use.

Cytotoxicity

HepG2.2.15 cells were inoculated on a 96-well culture microplate (Costar, Corning Inc., NY, USA) at a density of 2×10^4 /mL (200 µL for each well). After incubating for 48 h, supernatant was substituted by fresh culture medium containing serial 1:2 dilutions of oxymatrine, varying from 8000 µg/mL to 62.5 µg/mL (triplicates for each concentration), and was refreshed every other day. After treatment for 6 d, cytotoxicity of oxymatrine was determined by a MTT assay as previously described in detail^[19].

Treatment of HepG2.2.15 cells with oxymatrine

HepG2.2.15 cells were inoculated in 12 flasks (75-cm², NUNC, Roskilde, Denmark) at a density of 2×10^5 /mL (12 mL for each flask). Forty-eight hours after cell inoculation, HBV DNA could be easily detected in the culture medium. Then the culture medium was removed, and fresh culture media containing 500 µg/mL of oxymatrine were added, while the normal control group was refreshed with new culture media. At different times (2 d and 5 d) after treatment, supernatant from 3 flasks of each group were collected independently for determination of HBV surface antigen (HBsAg), HBV e antigen (HBeAg), and HBV DNA, and cells were harvested by trypsin digestion and washed 3 times with phosphate buffered solution (PBS, pH 7.3). Then cells were counted and different numbers of cells were used to determine different HBV replicative intermediates: 1×10^6 cells for cccDNA, 3×10^6 cells for

Table 1 Sequences of primers and probes for PCR or RT-PCR

Primer set			Taqman MGB probe			Target
Name	Sequence	Position ¹	Name	Sequence	Position ¹	
CCP1	5'TTCTCATCTGCCGACCG 3'	1562-1579	TCC	5'FAM-CCTAATCATCTCTTGTCAT-MGB 3'	1836-1855	cccDNA
CCP2	5'CACAGCTGGAGGCTTGAAC 3'	1883-1864				
RCP1	5'TCCTCTTCATCCTGCTGCTATG 3'	404-425	TRC	5'FAM-TGTTGGTTCTTCTGGACTA-MGB 3'	437-455	rcDNA
RCP2	5'CGTGCTGGTAGTTGATGTTCTT 3'	510-489				
PGP1	5'CTCAATCTCGGGAATCTCAATGT 3'	2429-2451	TPG	5'FAM-CCTTGGACTCATAAGG-MGB 3'	2459-2474	pgRNA
PGP2	5'TGGATAAAACCTAGCAGGCATAAT 3'	2659-2636				
BAP1	5'ACGGCCAGGTCATCACCAT 3'	2396-2415	TBA	5'FAM-CAATGAGCGGTTCCG-MGB 3'	2418-2432	β-actin
BAP2	5'AGGCTGGAAGAGTGCCTCAG 3'	2438-2457				

¹Nucleotide position in U95551 HBV sequence or M10277 β-actin gene sequence. HBV: Hepatitis B virus; cccDNA: Covalently closed circular DNA; rcDNA: Relaxed circular DNA; pgRNA: Pregenomic RNA; MGB: Minor grooving binder; PCR: Polymerase chain reaction; RT-PCR: Reverse transcription polymerase chain reaction.

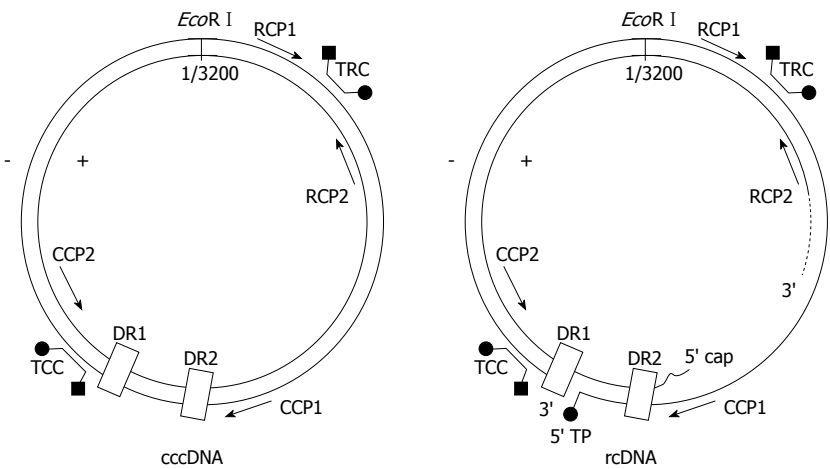


Figure 1 Schematic diagram of intracellular covalently closed circular DNA (cccDNA), relaxed circular DNA (rcDNA) and design of PCR primers and probes. When rcDNA is detected by primer set CCP1 and CCP2, new strand extension priming by CCP1 or CCP2 will cease at the site of the nick in the minus strand or gap in the plus strand, respectively, and no PCR product is generated. Therefore primer set CCP1 and CCP2 will preferentially amplify cccDNA and not (or to a lesser extent) rcDNA. Primer set RCP1 and RCP2 can amplify both cccDNA and rcDNA.

pgRNA and another 1×10^6 cells for rcDNA. At each interval, those unharvested flasks continued to be incubated with fresh culture media containing the same amount of antiviral agent as before.

Determination of HBsAg, HBeAg and HBV DNA in supernatant

Supernatant from each flask was collected at different times after oxymatrine treatment and stored at -20°C until measurement. HBsAg and HBeAg were simultaneously detected by ELISA kits (Sino-America Biotechnology Co. Ltd, Shanghai, China) according to the manufacturer's instruction. HBV DNA in the supernatant was purified with QIAamp DNA Mini Kit (QIAGEN Inc., Chatsworth, CA, USA) following the manufacturer's instruction, and then was determined by real-time fluorescent PCR with primer RCP1/RCP2 and Taqman[®] minor grooving binder (MGB) probe DRC, targeting the S open reading frame (ORF) of HBV genome (Figure 1 and Table 1).

Real-time fluorescent PCR was performed on the ABI prism 7000 Sequence Detection System (Applied Biosystems Inc., USA) with following PCR cycling parameters: 2 min at 50°C for uracil N-glycosylase incubation, 10 min at 95°C for Hotstart Taq DNA polymerase activation, followed by 40 cycles of denaturation for 30 s at 95°C and annealing for 60 s at 60°C . The reaction system consisted

of 0.5 μL of sense primer (25 $\mu\text{mol/L}$), 0.5 μL of anti-sense primer (25 $\mu\text{mol/L}$), 1.25 μL of Taqman MGB probe (10 $\mu\text{mol/L}$), 25 μL of Taqman[®] Universal PCR master mix ($2 \times$ concentration, P/N 4304437, Applied Biosystems Incorporation, USA), 5 μL of template, supplemented with double-distilled water to a final volume 50 μL . Uniform real-time PCR system and cycling mode were utilized in the determination of cccDNA, total HBV DNA and cDNA because different prime sets and probes were designed based on the same principle for Taqman[®] real-time PCR technology.

Purification and quantification of intracellular cccDNA

Since HBV cccDNA is structurally similar to plasmid, intracellular cccDNA was extracted from cells by an alkali lysis procedure with Plasmid Mini Kit (QIAGEN Inc., Chatsworth, CA, USA), which would remove most of cellular chromosomal DNA and non-supercoiled rcDNA. Purified cccDNA was dissolved in 50 μL TE buffer (10 mmol/L, pH 8.0), and 10 μL product was further treated with Plasmid-Safe[™] ATP-Dependent DNase (PSAD, Epicentre Technologies, Madison, WI, USA) to remove any remaining rcDNA, single-stranded virus DNA and cellular chromosomal DNA. Briefly, DNA was digested with 10 U PSAD for 1 h at 37°C in the presence of $1 \times$ buffer (33 mmol/L Tris-acetate pH 7.8, 66 mmol/L

potassium acetate, 10 mmol/L magnesium acetate and 0.5 mmol/L DTT) and 1 mmol/L ATP, followed by incubation in 70°C water for 30 min to inactivate PSAD. Intracellular cccDNA was quantified by selective fluorescent PCR with primer set spanning DR1 and DR2 region, which had been documented to be capable of amplifying cccDNA more efficiently than rcDNA ($10^4:1$)^[20,21]. Design of the primer set CCP1 and CCP2, and the Taqman® MGB probe DCC was demonstrated in Figure 1 and Table 1. The mean level of cccDNA pool of each cell at different times was calculated according to total cccDNA and the number of cells for cccDNA extraction (1×10^6).

Isolation of intracellular core particles and determination of viral DNA

Cytoplasmic core particles in HepG2.2.15 cells were isolated as described with modification^[22]. Briefly, cells were incubated at 37°C with 1 mL of lysis buffer (1 mmol/L EDTA, 0.1% Nonidet P-40, 50 mmol/L NaCl, 8% sucrose) for 10 min, then nuclei and other insoluble materials were removed by centrifugation at 15000 r/min for 2 min. Supernatants were treated with 10 U RQ1 DNase (Promega) and 0.1 µg of RNase A (Sigma) at 37°C for 15 min, then 1/4 volume of polyethylene glycol 8000 (PEG 8000, Amresco) solution (10% PEG 8000, 0.6 mol/L NaCl) were added, followed by incubation at 4°C for 30 min and centrifugation at 15000 r/min for 10 min. Pellets containing core particles were re-suspended in 500 µL of digestion buffer (50 mmol/L Tris, 10 mmol/L EDTA, 150 mmol/L NaCl, 1% sodium dodecyl sulfate, 0.5 mg/mL proteinase K, pH 8.0) and were incubated at 50°C for 2 h. Nucleic acids were extracted with phenol:chloroform (25:24) and then with chloroform:isoamylol (24:1), and were precipitated from the aqueous fraction with ethanol, dissolved in TE buffer (10 mmol/L Tris hydrochloride, 1 mmol/L EDTA, pH 8.5). Viral DNA in core particles was quantified by real-time fluorescent quantitative PCR with primer RCP1/RCP2 and Taqman MGB probe TRC (Figure 1 and Table 1). The mean viral DNA in core particles of each cell was calculated in the same way as cccDNA.

Extraction of total RNA and analysis of intracellular pgRNA

Total RNAs were extracted from cells with Total RNA Miniprep System (Viogene, Sunnyvale, CA, USA) and dissolved in 50 µL RNase-Free ddH₂O, and 10 µL product was treated with 8 U RQ1 DNase to eliminate DNA contamination from virus or cells. Then cDNAs were synthesized by reverse transcription with SuperScript™ III First-Strand Synthesis System for RT-PCR (Invitrogen Corp., San Diego, CA, USA) according to the manufacturer's instruction.

Because HBV mRNAs of different lengths (3.5, 2.4, 2.1 and 0.7 kb) are transcribed from the same cccDNA template, and ORFs overlap with each other in various regions (Figure 2), it is crucial to find a specific target region for RT-PCR in order to discriminate pgRNA from other mRNAs. In addition to its role in viral DNA replication, the pgRNA is also a bicistronic mRNA that encodes C

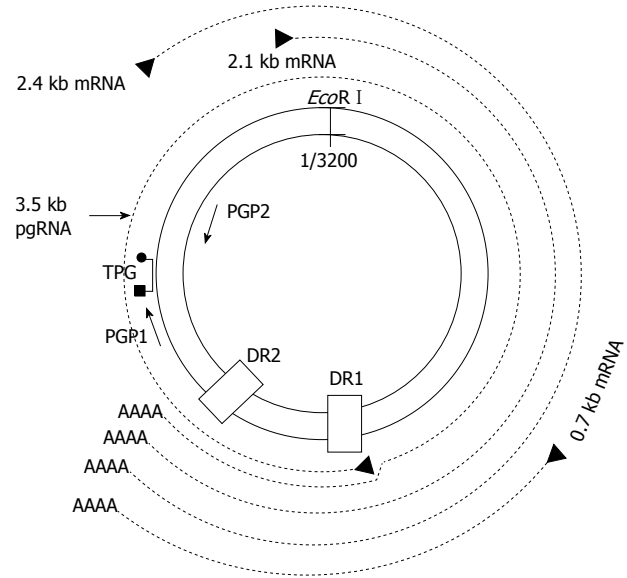


Figure 2 Schematic diagram of HBV mRNAs and design of PCR primer and probe for determining pregenomic RNA (pgRNA). Four kinds of HBV mRNAs are transcribed from the same cccDNA template and overlap with each other in various regions (dashed lines, dark triangle indicating the initiate site of transcription). The largest mRNA, 3.5 kb pgRNA, encodes both core protein and DNA polymerase of HBV. The promoter region of the DNA polymerase gene, which does not exist in the other 3 smaller mRNAs, was selected as the amplifying target for determining pgRNA by real-time RT-PCR with primer PGP1, PGP2 and Taqman MGB probe TPG.

and P proteins^[22,23], which contain an approximate 1010 bp nucleotide sequence (nt1838-2850, from the end of X ORF to the origin of S ORF) not existing in the other 3 smaller mRNAs (Figure 2). The primer set PGP1/PGP2 and Taqman MGB probe TPG targeting this region was designed to detect pgRNA (Table 1 and Figure 2). Results of RNA extraction and RT-PCR were normalized with the housekeeping gene β -actin as control^[24]. The β -actin cDNA of each sample was determined simultaneously by real-time PCR with primer BAP1/BAP2 and Taqman MGB probe TBA (designed on the basis of M10277 β -actin gene sequence). The mean expression level of β -actin in HepG2.2.15 was assumed to be similar to that in human tissues, namely, 5320 copies per cell^[25].

Statistical analysis

All results were expressed as mean \pm SD. Data from the treatment group and normal control group were analyzed by the Student unpaired *t* test using statistical software SPSS 10.0. Differences were considered statistically significant at $P < 0.05$.

RESULTS

Cytotoxicity of oxymatrine to HepG2.2.15 cells

Cytotoxicity to HepG2.2.15 cells was determined with fresh culture medium containing serial 1:2 dilutions of oxymatrine. The survival percentage of HepG2.2.15 cells under different concentrations of oxymatrine is shown in Figure 3. The mean half toxic concentration (TC₅₀) of oxymatrine was 1219.66 µg/mL, calculated by the Reed-

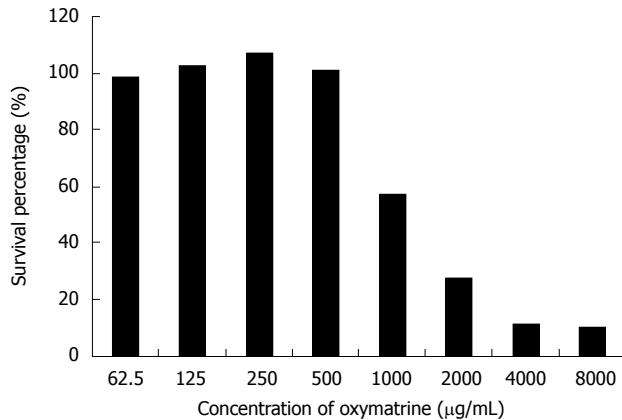


Figure 3 Survival of HepG2.2.15 cells after oxymatrine treatment. Two days after being inoculated, cells were incubated with different concentrations of oxymatrine for 6 d, and then the percent survival of cells was determined by MTT assay. The cytotoxicity experiment was repeated twice.

Muench formula^[26]. In further experiments, the effect of 500 µg/mL of oxymatrine (approximately equal to the largest nontoxic concentration) on the replication cycle of HBV was analyzed *in vitro*.

Effect of oxymatrine on secretion of HBsAg and HBeAg in HepG2.2.15 cell line

As shown in Figure 4, oxymatrine could inhibit secretion of HBsAg and HBeAg by HepG2.2.15 cells. After treatment with oxymatrine for 2 d and 5 d, the mean optical density at a wavelength of 450 nm (A_{450}) was 0.096 ± 0.011 and 0.175 ± 0.016 , respectively, when HBsAg in the supernatant was determined by microplate reader (Model 550, Bio-Rad), and decreased by 22.67% ($t = 5.439$, $P = 0.0322$) and 22.39% ($t = 5.376$, $P = 0.0329$) compared to the normal control ($A_{450} = 0.124 \pm 0.018$ and $A_{450} = 0.226 \pm 0.022$, respectively). The A_{450} of HBeAg in the supernatant after treatment for 2 d and 5 d was 0.259 ± 0.031 and 0.713 ± 0.031 , respectively, which indicated that secretion of HBeAg was reduced by 55.34% ($t = 9.859$, $P = 0.0101$) and 43.97% ($t = 14.080$, $P = 0.0050$) compared to the normal control ($A_{450} = 0.580 \pm 0.034$ and $A_{450} = 1.269 \pm 0.052$, respectively). Of note was that the reduction in HBeAg was at least twice that of HBsAg.

Effect of oxymatrine on production of virions in the supernatant

Since each virion contains only one copy of the genome, production of hepatitis B virions in the supernatant could be measured by quantification of supernatant HBV DNA. As demonstrated in Figure 5A, treating HepG2.2.15 cells with oxymatrine resulted in a significant reduction in hepatitis B virions in the supernatant. After treatment with 500 µg/mL oxymatrine for 2 d and 5 d, the level of HBV DNA in the supernatant was $(5.69 \pm 0.86) \times 10^4$ and $(7.86 \pm 0.99) \times 10^4$ copies/mL, respectively, while in the normal control the mean level was $(9.60 \pm 2.98) \times 10^4$ and $(31.85 \pm 4.74) \times 10^4$ copies/mL, respectively. In another words, production of virions was reduced by 40.75% ($t = 4.570$,

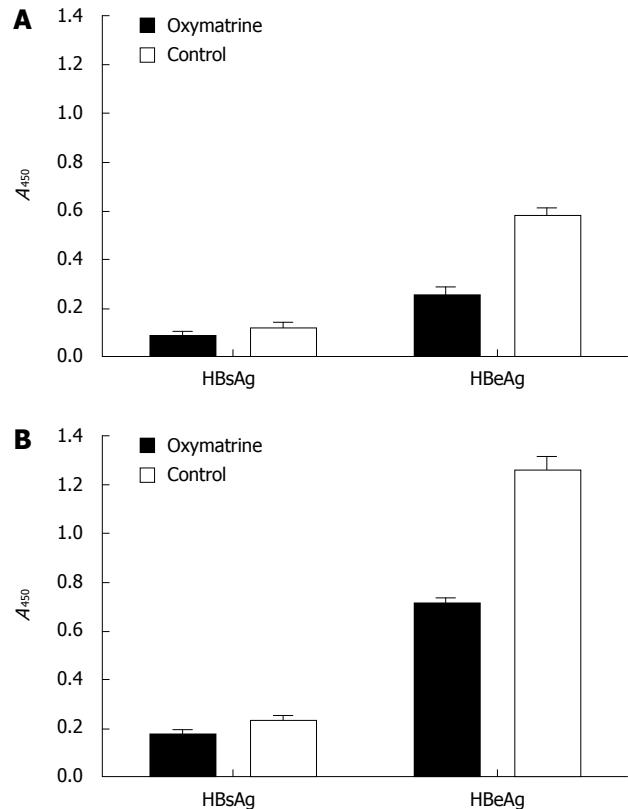


Figure 4 Effect of oxymatrine on the secretion of HBsAg and HBeAg in the HepG2.2.15 cell line. A: After treatment with oxymatrine for 2 d, HBsAg in the supernatant decreased by 22.67% compared to normal control, and HBeAg by 55.43%; B: After treatment with oxymatrine for 5 d, HBsAg in the supernatant decreased by 22.39% compared to normal control, and HBeAg by 43.97%.

$P = 0.0447$) and 75.32 % ($t = 14.460$, $P = 0.0047$), at 2 d and 5 d, respectively.

Effect of oxymatrine on the formation of intracellular HBV cccDNA pool

At different times after oxymatrine treatment, the HBV cccDNA pool in HepG2.2.15 cells diminished significantly compared with the normal control group (Figure 5B). After treatment for 2 d and 5 d, the size of the intracellular HBV cccDNA pool was downregulated to 0.75 ± 0.16 and 3.50 ± 0.26 copies per cell, respectively. The decrease was 63.98% ($t = 6.152$, $P = 0.0254$) and 80.83% ($t = 10.270$, $P = 0.0093$), respectively, after incubation with oxymatrine, compared with normal controls (2.09 ± 0.23 and 18.26 ± 3.43 copies per cell, respectively).

Effect of oxymatrine on viral DNA synthesis in the intracellular core particles

The effect of oxymatrine on the intracellular production of progeny viruses was indirectly measured by determination of HBV DNA in the intracellular core particles. As shown in Figure 5C, treatment with oxymatrine could lower the level of viral DNA in core particles isolated from HepG2.2.15 cells. After oxymatrine treatment for 2 d and 5 d, viral DNA in core particles was reduced to 144.95 ± 48.78 and 302.32 ± 36.36 copies per cell, respectively,

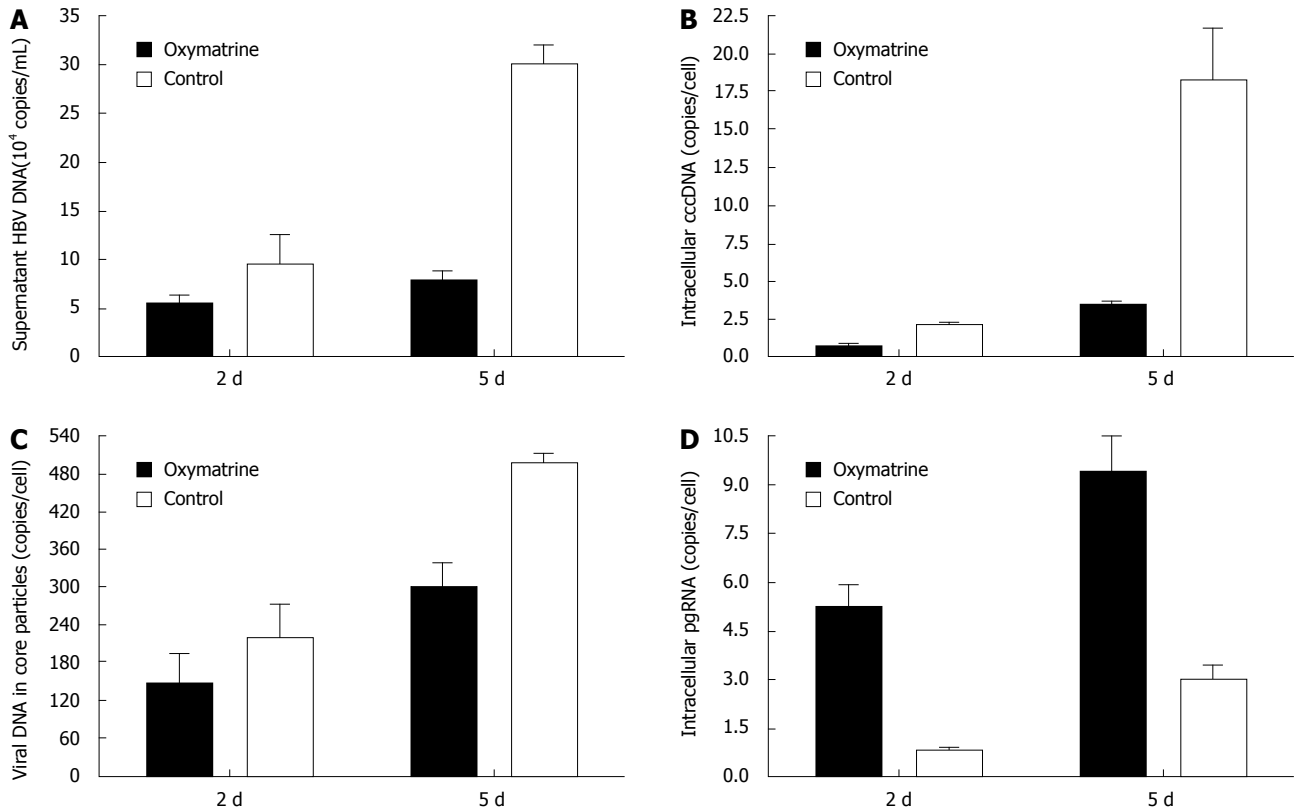


Figure 5 Effect of oxymatrine on the replication of HBV in HepG2.2.15 cell line after treatment for 2 d and 5 d. Compared to normal control, treatment with oxymatrine for 2 d and 5 d resulted in: (A) a decrease in HBV DNA in the supernatant by 40.75% and 75.32%, respectively; (B) a reduction in the intracellular HBV cccDNA pool by 63.98% and 80.83%; and (C) a reduction in viral DNA in the intracellular core particles by 34.35% and 39.24%. However, intracellular pgRNA increased by 6.90-fold and 3.18-fold (D).

which was 34.35% ($t = 4.776$, $P = 0.0413$) and 39.24% ($t = 10.050$, $P = 0.0097$) lower than that of normal controls (220.79 ± 51.73 and 497.57 ± 16.15 copies per cell, respectively). It could be noted that the reduction of viral DNA in core particles was not as great as that of the intracellular HBV cccDNA pool and HBV DNA in the supernatant after treatment with oxymatrine either for 2 or 5 d. In addition, the HBV cccDNA pool was significantly lower than that of viral DNA in core particles. After treatment with oxymatrine for 2 d and 5 d, the level of viral DNA in core particles was 193.27-fold and 86.38-fold the size of the cccDNA pool, respectively. For the normal control, it was 105.64-fold and 27.25-fold, respectively.

Effect of oxymatrine on intracellular HBV pgRNA

Unlike the other 2 replicative intermediates, the level of intracellular HBV pgRNA was upregulated after oxymatrine incubation, as shown in Figure 5D. With oxymatrine treatment for 2 d and 5 d, intracellular pgRNA accumulated to 5.25 ± 0.69 and 9.43 ± 1.13 copies per cell, respectively, which was 6.90-fold ($t = 8.941$, $P = 0.0123$) and 3.18-fold ($t = 7.432$, $P = 0.0176$) to that of normal control (0.76 ± 0.16 and 2.97 ± 0.48 copies per cell, respectively).

DISCUSSION

Because of the lack of a convenient and economic ani-

mal model with persistent HBV infection, the effect of oxymatrine on the replication cycle of HBV was investigated in the HepG2.2.15 cell line, which was established by transfecting a hepatoblastoma cell line (HepG2) with plasmids containing four 5'→3' tandem copies of the HBV genome, and could produce 42 nm Dane particles and more 22 nm spherical or filamentous particles^[18]. The cell line could support the full replication cycle HBV, as evidenced by identification of replicative intermediates or products including cccDNA, HBV-specific polyadenylated RNAs (pgRNA, 2.5 kb RNA and 2.1 kb RNA), and incomplete double- and single-stranded forms of the HBV genome, none of which were necessarily dependent on the chromosomally-integrated HBV DNA^[27]. In addition, HBV virions produced by the cell line were rich in endogenous polymerase activity^[27] and could induce hepatitis in chimpanzees^[28]. Therefore, the cell line is an appropriate model for identifying the molecular events in intracellular viral replication cycle as well as secretion of HBV particles.

As indicated by our results, secretion of HBsAg and HBeAg from HepG2.2.15 cells could be inhibited after incubation with oxymatrine for 2 d and 5 d. Of note was that secretion of HBeAg was reduced more than that of HBsAg after oxymatrine treatment either for 2 d (22.67% vs 55.43%) or 5 d (22.39% vs 43.97%). A discrepancy between the reductions in HBsAg and HBeAg has also

been observed by other researchers^[9,29]. HBsAg is encoded by S ORF of the viral genome, and is organized into spherical or filamentous HBsAg particles outside infected cells without a viral genome, which typically outnumber virions by 1000:1 to 10 000:1^[30]. Thus, expression of HBsAg can be independent of the replication of HBV. HBeAg is translated from the same template as core protein and polymerase, pgRNA, which is also the template for synthesis of progeny virus. In contrast to HBsAg, although HBeAg plays no role in viral assembly and its function is not clear^[30], expression and secretion of HBeAg were found to be associated with active replication of HBV^[31]. Therefore, inhibition of HBV replication would inevitably reduce the secretion of HBeAg while it may not affect or have a smaller effect on the secretion of HBsAg, as evident by the difference between the reductions in HBsAg and HBeAg by oxymatrine.

Consistent with previous research *in vitro*^[9,19,29], definite inhibition by oxymatrine of the replication of HBV was observed in our investigation. As far as we know, this was the first time that the effect of oxymatrine on production of virions in the supernatant and intracellular synthesis of the viral genome was simultaneously investigated. After treatment with oxymatrine for 2 d and 5 d, production of virions in the supernatant was reduced by 40.75% and 75.32%, while viral DNA synthesis in intracellular core particles was 34.35% and 39.24% lower than that of normal control. It appeared that extracellular viral DNA was more likely to be reduced by oxymatrine than intracellular viral synthesis. A similar effect has also been observed in cell lines treated with lamivudine or clevudine^[32], and in patients with chronic hepatitis B who were under antiviral therapy with adefovir or entecavir^[33,34]. A possible explanation for this may be that competitive inhibition of activity of HBV DNA polymerase or premature chain termination by these antiviral agents may result in predominant immature viral particles containing only intact or non-intact single-stranded viral DNA, which could not be discriminated from mature virions containing relaxed circular double-stranded DNA by routine PCR or fluorescent PCR. Therefore, the reduction in mature virions may not be intrinsically reflected in the determination of viral DNA in core particles by PCR. In contrast, because only mature virions could be secreted outside infected cells^[16], reduced intracellular production of mature virions could be more sensitively identified by detection of supernatant HBV DNA in cell lines or serum HBV DNA *in vivo*.

The effect of oxymatrine on intracellular HBV cccDNA was also first reported in this research. HBV cccDNA is the first replicative intermediate generated after HBV entering into hepatocytes, which indicates the origination of intracellular HBV replication and successful establishment of HBV infection^[17]. Reductions varying from 0.8 log₁₀ to 2.8 log₁₀ in the intrahepatic cccDNA have been reported in patients receiving mono- or combined antiviral therapy with IFN- α or nucleos(t)ide analogues for 48 wk^[33-35], and persistence of cccDNA in the nuclei of hepatocytes after withdrawal of antiviral agents was be-

lieved to be primarily responsible for the reoccurrence of hepatitis B^[36]. Therefore, the effect on nuclear cccDNA was an indispensable index when evaluating an anti-HBV agent, and a predictor of a sustained antiviral response to therapy^[37]. In our research, a marked decline in the intracellular cccDNA pool was observed after oxymatrine treatment for 2 d and 5 d (63.98% and 80.83%, respectively). The conversion of viral genome into cccDNA did not depend on viral polymerase activity^[20], and formation of the nuclear cccDNA pool in the nuclei of hepatocytes had been shown to be mainly dependent on recycling of cytoplasmic mature progeny virions into nuclei of hepatocytes^[38,39]. Therefore, the reduction in the cccDNA pool induced by oxymatrine may be attributed to reduction in cytoplasmic mature virions rather than direct inhibition of the conversion of rcDNA into cccDNA. It should be noted that, in contrast to hepatocytes in the liver, HepG2.2.15 cells are actively dividing, and whether cccDNA can survive cell division or not is still unknown^[40]. The reduction in the cccDNA pool should not be attributed to cell proliferation, because the survival of HepG2.2.15 cells incubated with 500 μ g/mL oxymatrine was found to be approximately 100% in the cytotoxicity experiment (Figure 2), and the cell proliferation rate after oxymatrine treatment was not significantly different to that of normal control when assessed by the total number of cells acquired from flasks at each time (data not shown).

Interestingly, although significantly lower than that of the normal control group ($P < 0.05$), the cccDNA pool after oxymatrine continued to expand slowly (from 0.75 copies per cell to 3.50 copies per cell). This was probably because production of virions in the cytoplasm was not completely inhibited, and more cytoplasmic rcDNAs were recycled into the nucleus instead of being secreted out so as to compensate for the loss of the cccDNA pool and maintain intracellular infection. The “wisdom” of HBV to maintain an intracellular existence by conserving cccDNA could be corroborated by other facts. It has been reported that even in chimpanzees or patients with sustained clearance of serum HBsAg and negative serum HBV DNA, residual cccDNA could still be detected in their hepatocytes^[41,42].

In our research, a special strategy was taken to determine intracellular cccDNA, which integrated extraction by alkali lysis, purification with Plasmid-Safe™ ATP-dependent DNase (PSAD) and quantitative real-time PCR with selective primer set. Because HBV cccDNA is similar to plasmid in spatial structure and physicochemical characteristics, a procedure for purification of plasmid DNA with a kit based on alkali lysis was adopted, which would irreversibly denature double-stranded DNA species (including HBV DNA integrated into the cellular chromosome and viral rcDNA) that were not covalently closed^[43,44]. PSAD can selectively hydrolyze linear double-stranded (ds) DNA to deoxynucleotides at slightly alkaline pH, and with a lower efficiency, hydrolyzes linear and closed circular single-stranded DNA. We had observed that overnight incubation of total chromosomal DNA from 20 mg of

liver tissues with 10 U PSAD could digest it to an invisible level in agarose electrophoresis, and approximately 1 log₁₀ reduction was observed by real-time PCR when HBV genome with 10⁸ to 10⁶ hepatitis B virions was treated with 10 U PSAD for 1 h (data not shown). PSAD was also frequently used by other researchers^[21,32,33,35] to minimize the background rcDNA before detection of cccDNA with selective PCR, which would preferentially amplify cccDNA rather than rcDNA (10⁴:1)^[20,21].

After oxymatrine incubation for 2 d and 5 d, intracellular pgRNA increased by 6.90-fold and 3.18-fold, respectively. It seemed a little incomprehensible why pgRNA accumulated distinctively in spite of a decline in the cccDNA pool and reduction of DNA synthesis in core particles. Two possible targets in the intracellular replication cycle of HBV interference by oxymatrine might account for such result. First, the packaging process of pgRNA into the protein nucleocapsid was interrupted by oxymatrine, and pgRNA accumulated in the nucleus or cytoplasm of hepatocytes, which resulted in further reduction of DNA synthesis in core particles and then nuclear cccDNA. Second, pgRNA was packaged into nucleocapsid as normal, but the activity of virus DNA polymerase was suppressed by oxymatrine. It is well known that accompanying extension of viral minus-strand DNA by reverse transcription with pgRNA as template (catalyzed by reverse transcriptase activity of HBV polymerase), pgRNA is degraded synchronously from pgRNA/minus-strand DNA hybrids by the RNase H activity of viral polymerase^[17,45]. Therefore, suppression of viral DNA polymerase would lead to reduction of rcDNA and cccDNA and less pgRNA degradation compared to the normal control group. This hypothesis could be evidenced by the phenomena discovered by other investigators, who reported that duck HBV reverse transcriptase expressed by a recombinant P vector would suppress viral pregenomic RNA accumulation by 3-4-fold in an LMH cell line^[24]. Whether the packaging process of pgRNA or viral DNA polymerase was the exact antiviral target of oxymatrine remains to be further investigated.

Very few investigations had studied the effect of antiviral agents on intracellular HBV RNAs as far as we know. In HBV-transgenic mice, animals treated with adefovir resulted in a significant reduction in serum and intrahepatic HBV DNA, but mean relative intrahepatic HBV RNA (log₁₀ pg/μg ± SD), seemed to be a little higher than that of saline-control animals (5.8 ± 2.5 *vs* 4.7 ± 2.4) after treatment, although the difference was not statistically significant^[46]. In the same research, animals treated with lamivudine had a significant reduction in serum HBV DNA, and slight but insignificant reduction in intracellular HBV DNA, and HBV RNA seemed to be even higher than that of saline-control animals (7.0 ± 1.4 *vs* 4.7 ± 2.4) although the difference was also statistically significant. It should be noted that the author did not clarify which fragment of HBV DNA had been chosen as a probe for detecting HBV RNA by Northern blot, so it was hard to know which of the 4 HBV mRNAs (3.5 kb,

2.4 kb, 2.1 kb and 0.07 kb) may contribute to the alteration of HBV RNA in the result. Other nucleoside analogues, such as 5-fluoro-2',3'-dideoxy-3'-thiacytidine (5-FSddC), failed to affect intrahepatic HBV-specific RNAs (3.5 kb, 2.5 kb and 2.1 kb) when detected by Northern blot, although they reduced intracellular viral DNAs (rcDNA and single-stranded DNA) in a dose-dependent manner^[47].

In conclusion, our research demonstrated an inhibitory effect of oxymatrine on the replication cycle of HBV, which may be attributed to interference with the process of packaging pgRNA into the nucleocapsid, or inhibiting the activity of the viral DNA polymerase. The independent effect of oxymatrine on these 2 targets remains to be studied further. In addition, comparative analysis of the chemical or spatial structure between oxymatrine and nucleoside analogues may help illuminate the antiviral mechanism of oxymatrine.

COMMENTS

Background

It is the consensus that anti-hepatitis B virus (HBV) therapy plays a decisive role in slowing disease progress, prolonging the survival, and improving the prognosis of patients with chronic hepatitis B. Anti-HBV agents now available include interferon-α and several nucleos(t)ide analogues. Although many patients have benefited from them, the adverse effect of interferon-α, virus-resistance to nucleos(t)ide analogues as well as the high cost of these agents have limited their utilization. Therefore, it is very necessary to screen for more cost-effective antiviral agents.

Research frontiers

China has unique resources for screening for candidate anti-HBV drugs in traditional medicine. In this area, most researchers work hard to find new derivatives or mono-components from various Chinese herbs to test their anti-HBV effect *in vitro* or *in vivo*. However, not enough importance has been attached to identification of the antiviral mechanism of the agents that seem to have promising anti-HBV activity.

Innovations and breakthroughs

Oxymatrine is a type of alkaloid extracted from the herb *Sophora alopecuroides* L. Prior research concerning oxymatrine involved determining its anti-HBV effect in cell lines, in animals and in double-blind, randomized, multicenter clinical trials. Some researchers had observed its effect in relieving hepatic fibrosis or severe liver injury. Unfortunately, no research had tried to explore the exact antiviral target of oxymatrine, and little was known about the mechanism of oxymatrine against HBV until now, which is a major obstacle to its marketing. In this paper, the authors reported the effect of oxymatrine on the main steps of the HBV replication cycle. The change in different replicative intermediates after oxymatrine incubation was documented and analyzed for the first time. Based on the results, the authors proposed that oxymatrine may inhibit the replication of HBV by interfering with the process of packaging pregenomic RNA (pgRNA) into the nucleocapsid, or inhibiting the activity of the viral DNA polymerase.

Applications

Although the authors have not confirmed the exact target of oxymatrine in inhibiting the replication of HBV in this article, the preliminary results proposed 2 candidate targets for further research.

Peer review

In this paper, the effect of oxymatrine on the replication cycle in the HepG2.2.15 cell line was investigated, and the possible antiviral targets of oxymatrine were explored. The inhibitory effect of oxymatrine on the replication cycle of HBV, interfering with the process of packaging pgRNA into the nucleocapsid, or inhibiting the activity of the viral DNA polymerase were demonstrated.

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CT volume measurement for prognostic evaluation of unresectable hepatocellular carcinoma after TACE

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Abstract

AIM: To analyze the value of computed tomography (CT) volume measurements for evaluation of the survival rate of unresectable hepatocellular carcinoma (HCC) patients after transcatheter arterial chemoembolization (TACE).

METHODS: One hundred and sixty-six unresectable HCC patients after TACE were involved in this retrospective study. Hepatic CT scan was performed for all patients before and 4 wk to 2 mo after TACE to define the morphologic features of HCC including its largest diameter, volume, product of the greatest axial dimension, tumor to liver volume ratio (TTLVR), and tumor shrinkage ratio. Clinical variables used in the study included gender, age, pattern of tumor growth, number of lesions, Child-Pugh classification of liver function, repeated TACE times, pre- or post-treatment α -fetoprotein (AFP) level, portal vein cancerous thrombus, tumor me-

tastasis, degree of lipiodol retention within the tumor, and percutaneous ethanol injection. The correlation between survival time and clinical variables of patients or lesions was analyzed by combining morphologic features with the corresponding clinical and general data as input. A Cox proportional hazard model was used to analyze prognostic factors. The Kaplan-Meier method was used to calculate the cumulative survival time. Influence of the parameters on prognosis was analyzed by the log-rank test.

RESULTS: The overall 6, 12, 24, 36 and 60 mo cumulative survival rates were 78.92%, 49.85%, 23.82%, 15.60% and 8.92%, respectively. The median survival time was 12 mo. Univariate and multivariate analysis showed that only 4 parameters were the independent prognostic factors including TTLVR ($\chi^2 = 14.328$, $P < 0.001$), portal vein cancerous thrombus ($\chi^2 = 5.643$, $P = 0.018$), repeated TACE times ($\chi^2 = 8.746$, $P = 0.003$), and post-treatment serum AFP level ($\chi^2 = 5.416$, $P = 0.020$). When the TTLVR value was less than 70%, the survival time was inversely correlated with the TTLVR value.

CONCLUSION: CT volume measurement technique can predict the prognosis of unresectable HCC patients after TACE.

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Key words: Hepatocellular carcinoma; Chemoembolization; Tumor volume; Computed tomography; Prognosis

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INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common malignancies in Africa and Asia, especially in China and Japan. The tumor is often already large in size when it is diagnosed, and the prognosis of HCC patients, especially those with unresectable lesions tends to be poor. The median survival time of untreated HCC patients is less than 6 mo from its diagnosis^[1,2]. The treatment of HCC is very challenging. The outcome of resection and liver transplantation is good. However, they are only suitable for less than 15% of patients. Although many treatment modalities for HCC are available, transcatheter arterial chemoembolization (TACE) has been shown to be the most effective procedure for unresectable HCC^[3]. Patients receiving TACE often demonstrate a remarkable embolization effect and consequently have a higher cumulative survival rate^[4].

Advances in computed tomography (CT) technology can quantify volumetric tumor burden due to its volumetric data acquisition and image processing^[5-8]. Compared with uni- and bi-dimensional measurement techniques, volumetric quantification can result in a different assessment result^[9-11].

The largest tumor diameter, product of the greatest axial dimension, and several volume measurements show a congruence with the actual volume when the measured lesion is a sphere, an ellipsoid, or a cube. However, these measurements may have significant differences if the tumor has an irregular shape^[12,13]. Three-dimensional (3D) measurements have advantages for the assessment of irregular masses, because they compensate for actual tumor shape rather than assume it to be a sphere, an ellipsoid, or a cube^[6,14]. Spiral CT theoretically offers advantages in terms of accuracy and reproducibility over 3D measurements^[14,15]. In follow-up studies, 3D measurements enable the evaluation of changes in the diameter of a neoplasm, and offer a more complete evaluation of changes in tumor size over time.

It has been reported that TACE in combination with cytostatic drugs (doxorubicin, cisplatin, mitomycin C) for treatment of HCC patients can reduce vital tumor tissue and prolong survival time^[1,3,4,16]. When an indication for palliative TACE is established, strict guidelines based on the condition of patients and tumor stage must be followed. In this study, we retrospectively reviewed the morphologic CT data about lesions in 166 unresectable HCC patients after TACE. The correlation between morphological variables and survival rate was also intensively investigated.

MATERIALS AND METHODS

Clinical data and variables

Segmental lipiodol-TACE was performed for 166 HCC patients (143 men, 23 women) at the age of 51 ± 12 years (range: 21-78 years) in February 1999 to August 2006. HCC was diagnosed based either on percutaneous liver needle biopsy (112 patients) or on imaging and clinical examination (54 patients). This retrospective study was approved by our institution's research ethics board. Informed consent was obtained from all patients. Cirrhosis was classified according to the Child-Pugh system. Child-Pugh grades A and B were found in 148 and 18 patients, respectively.

The morphologic features of HCC, including its largest diameter, product of the greatest axial dimensions, tumor volume (TV), tumor to liver volume ratio (TTLVR) and tumor shrinkage ratio (TSR) post-treatment, were evaluated for their correlation with treatment outcome. In addition, other study variables influencing treatment outcome were analyzed, including gender, age, pattern of tumor growth, number of lesions, Child-Pugh classification of liver function, repeated TACE times, pre-treatment or post-treatment serum α -fetoprotein (AFP) levels, portal vein cancerous thrombus, tumor metastasis, degree of lipiodol retention within the tumor, and percutaneous ethanol injection (PEI). A total of 17 variables were analyzed in the study.

Criteria for inclusion or exclusion of TACE

TACE was performed for HCC patients to control their local tumor growth and prolong their survival time. All patients undergoing TACE had contraindications for liver resection judged by a multidisciplinary team composed of hepatic surgeons, radiologists, hepatologists, and oncologists. The inclusion criteria for unresectable HCC include disease multifocality, early vascular invasion, decompensated liver disease, or poor performance status. To simplify the study, those whose main trunk of the portal vein was completely obstructed by a tumor thrombus or those who underwent surgical resection after TACE, were excluded from the study.

TACE procedures

Transcatheter hepatic segmental arterial chemoembolization using lipiodol mixed with anticancer drugs, including 40-60 mg adriamycin, 50-100 mg cisplatin (CDDP) or 10-20 mg mitomycin C (MMC) and a mixture of 2-30 mL iodized oil (lipiodol, André Guerbet, Aulnay-sous-bois, France), was carried out followed by injection of gel-foam particles. The mixture of lipiodol and anticancer drugs was super selectively infused into hepatic arteries supplying the tumor-bearing segment or subsegment until the lipiodol was densely accumulated in the tumor and tumor-bearing area. The arteries were then embolized with gelatin sponge particles. The volume of injected lipiodol in each patient was 2-30 mL depending on the

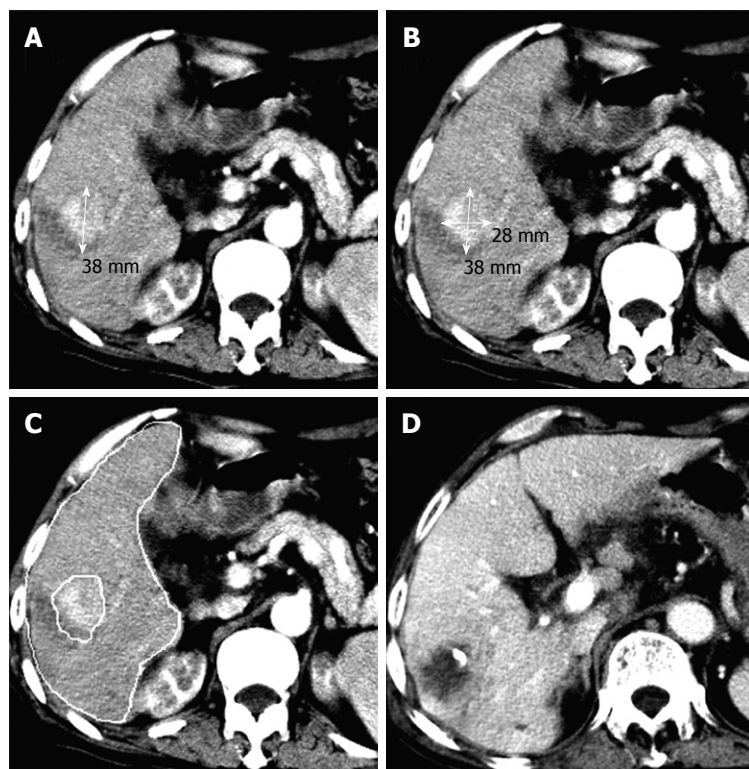


Figure 1 Transverse computed tomography (CT) scans showing a mononodular pattern of hepatocellular carcinoma (HCC) in the right posterior lobe of liver and an observed survival time of 33 mo in a 76-year-old man with a tumor volume of 55.64 cm³ and a tumor to liver volume ratio (TTLVR) of 5.16%. A and B: Arterial phase contrast-enhanced CT scan showing a high density nodule in the right lobe of liver before the first transcatheter arterial chemoembolization (TACE) procedure, while one-dimensional measurement showing the largest tumor diameter (3.8 cm), and two-dimensional measurement showing the tumor product of the greatest axial dimension (10.64 cm²); C: Contrast-enhanced CT scan showing hepatic and lesion volumetric measurements before the TACE procedure; D: Follow-up CT scan displaying complete tumor necrosis 44 d after the first TACE procedure.

tumor size, vascularity, and fluoroscopic findings^[4].

Tumor markers and CT/MR images were detected during the follow-up after TACE. TACE procedure was repeated if any residual tumor was detected after 4 wk. TACE was performed 1-7 times (1 time for 60, 2 times for 57, and ≥ 3 times for 49 patients) in the study, and the mean repetition frequency was 2.12 times. Of the 166 patients, 9 were treated with PEI after TACE.

CT scanning and measurement techniques

CT scanning was performed for patients using a helix-spiral CT scanner (Somatom Plus 4, Siemens, Germany) before treatment. Pre- and post-contrast enhanced images of the liver of all patients were obtained. Contrast medium (Omnipaque, 300 mgI/mL, Schering, Germany) was administered by intravenous bolus injection into a cubital vein during the arterial phase (volume = 100 mL, flow rate = 2-4 mL/s, scan delay time = 25 s), portal venous phase (scan delay time = 60 s) or venous phase (scan delay time = 90 s)^[16,17]. Biphasic and triphasic CT scans were performed for 76 and 90 patients, respectively.

CT scan and tumor measurements were performed using three measurement techniques during follow-up. Uni-dimensional measurement (Figures 1A and 2A) and product of the greatest axial dimension (Figures 1B and 2B) were obtained as previously described^[2]. TV was measured by manually delineating (with a computer mouse) its contours on all sections, followed by calculating the lesion area with computer software (Figures 1C and 2C) as previously described^[7,8,16,18]. Nonenhanced (Figure 2D) and contrast-enhanced CT (Figure 1D) were performed again within 4 wk to 2 mo (44 ± 8 d) after TACE during the

follow-up. Volumetric CT evaluation included analysis of TV, liver volume, TTLVR, and TSR. The TTLVR value was less than 25% ($n = 74$), less than 50% ($n = 48$), less than 70% ($n = 38$), and 70% or over ($n = 8$)^[13], respectively. The TSR value was greater than 50% reduction in TV ($n = 33$), greater than 25% reduction in TV ($n = 64$), 0%-25% reduction in TV ($n = 27$), and disease progression ($n = 42$), respectively, after treatment. The retention of lipiodol within tumor tissue was classified into four types according to the lipiodol accumulation pattern observed after lipiodol-TACE^[19]: type I = homogeneous ($n = 38$), type II = defective (defect is found in lipiodol accumulation in the main tumor, $n = 44$), type III = inhomogeneous (inhomogeneous lipiodol accumulation was observed in the main tumor, $n = 53$), and type IV = only slight accumulation ($n = 31$).

Statistical analysis

Statistical analysis was performed using SPSS11.5. Survival time from the commencement of treatment was analyzed using the Kaplan-Meier method. The influence of parameters on prognosis was evaluated by log-rank test. The influence of clinical variables on prognosis was evaluated by univariate analysis. When the result was statistically significant, multivariate regression analysis according to the Cox proportional hazard model was used to analyze the factors influencing the prognosis to avoid any confounding interaction between them. The largest tumor diameter, product of the greatest axial dimensions, and TV/TTLVR represented the 1D, 2D, and 3D measurements, respectively. Correlations between tumor size, each of the three different measurements, and survival time were analyzed

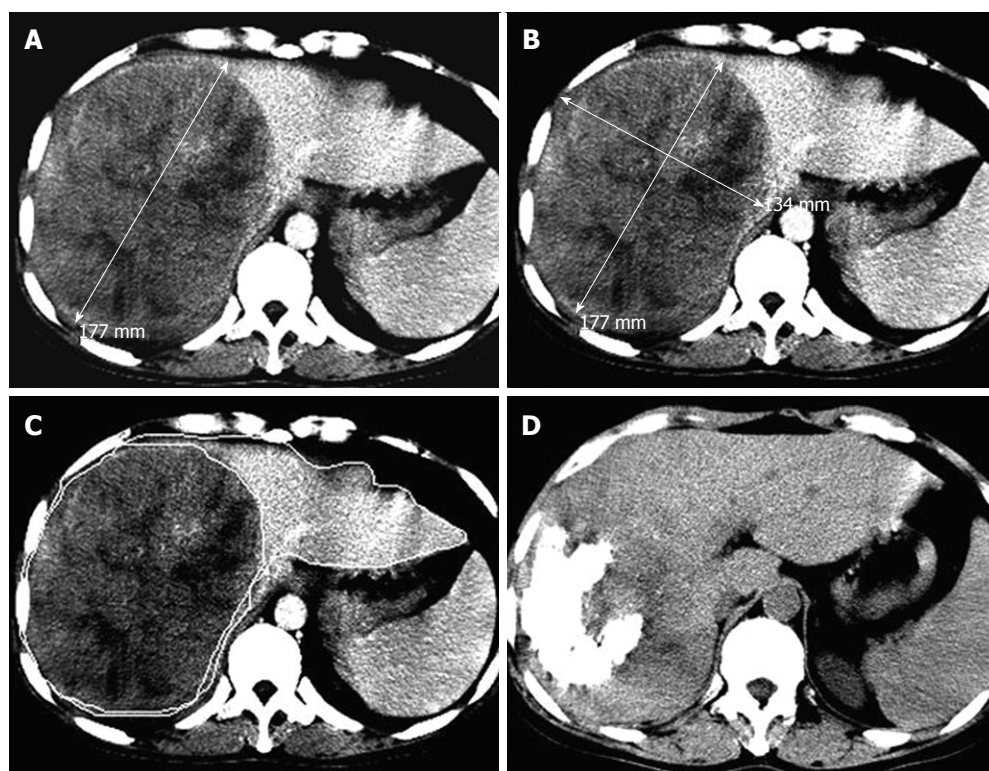


Figure 2 Transverse CT scans showing a massive pattern of HCC in the right lobe of a 46-year-old man with a tumor volume of 1936.70 cm³, a TTLVR of 67.52%, and a survival time of 6 mo. A and B: Arterial phase contrast-enhanced CT scan showing a high density tumor in the right lobe of liver before the first TACE procedure, while one-dimensional measurement showing the largest tumor diameter (17.7 cm) and two-dimensional measurement showing the tumor product of the greatest axial dimension (237.18 cm²); C: Contrast-enhanced CT scan showing hepatic lesion volumetric measurements before TACE; D: Nonenhanced CT follow-up scan showing lipiodol retention of grade III and a reduced tumor size 60 d after the first TACE procedure.

using the Spearman coefficient of correlation. $P < 0.05$ was considered statistically significant.

RESULTS

General features

The formed mass was divided into nodular type ($n = 39$), and massive type ($n = 127$) on the basis of imaging examination^[20]. Multiple lesions were identified in 31 patients and a mono-nodular pattern was detected in 135 patients. Portal vein cancerous thrombus was classified into four subgroups according to location: bilateral or trunk ($n = 20$), right branch ($n = 20$), left branch ($n = 6$), and no cancerous thrombus ($n = 119$). The serum AFP levels (< 20 ng/mL) were normal in 100 patients and elevated in 66 patients (≥ 20 ng/mL) before TACE. The patients with elevated AFP levels were monitored in 4 wk to 2 mo after TACE. The AFP level was decreased in 38 patients and increased in 28 patients after TACE. Tumor metastasis was divided into intrahepatic metastasis ($n = 24$), extrahepatic metastasis ($n = 6$), lymphoid node metastasis ($n = 13$), and no metastasis ($n = 123$).

Measurement data

The mean liver volume was 1621.39 ± 637.83 cm³ (range: 637.38–3514.73 cm³). The TV was 6 to 2432 cm³ with a mean of 590.13 ± 608.08 cm³ and a median of

384.26 cm³. The TTLVR was $30.54\% \pm 21.69\%$ (range: 0.44%–85%) with a median of 26.62%. The largest tumor diameter was 2.0–22 cm with a mean diameter of 9.47 ± 4.00 cm and a median diameter of 9.1 cm. The tumor product of the greatest axial dimension was 2.64–264 cm² with a mean of 79.76 ± 56.46 cm² and a median of 65.85 cm².

Cox proportional hazard model analysis

The survival time was analyzed using the Kaplan-Meier method. The overall results obtained by log-rank test are shown in Figure 3. The 6 mo-, 1-, 2-, 3-, and 5-year cumulative survival rates of patients after TACE were 78.92%, 49.85%, 23.82%, 15.60% and 8.92%, respectively. The median and mean survival time was 12 ± 1 mo and 20 ± 2 mo, respectively.

Univariate analysis showed that 13 parameters were the significant prognostic factors, including pattern of tumor growth, Child-Pugh's classification of liver function, repeated TACE times, post-treatment AFP level, portal vein cancerous thrombus, tumor metastasis, largest tumor diameter, tumor product of the greatest axial dimension, TV, TTLVR, TSR, degree of lipiodol retention within the tumor, and PEI (Table 1). Since these variables are related to each other, multivariate Cox regression analysis was performed using the significant variables identified in the univariate analysis model. Finally, 4 variables including

Table 1 Results of univariate Cox regression analysis

Parameter	B	χ^2	SE	P	Hazard ratio
Sex	0.319	1.652	0.248	0.199	1.375
Age	-0.006	0.706	0.007	0.401	0.994
Child-Pugh classification	0.546	5.189	0.240	0.023	1.727
Pre-treatment level of AFP	0.204	0.921	0.212	0.337	1.226
Post-treatment level of AFP	-0.931	11.238	0.278	0.001	0.394
Times of repeated TACE	-0.507	20.734	0.111	< 0.001	0.602
Number of lesions	-0.218	0.954	0.223	0.329	0.804
Pattern of tumor growth	0.743	16.507	0.183	< 0.001	2.102
Tumor largest diameter	0.132	31.708	0.023	< 0.001	1.141
Tumor product of diameters	0.009	30.972	0.002	< 0.001	1.009
TV	0.001	38.448	0.000	< 0.001	1.001
TTLVR	0.662	44.113	0.100	< 0.001	1.938
Portal vein cancerous thrombus	0.304	16.869	0.074	< 0.001	1.355
Tumor metastasis	0.287	10.160	0.090	0.001	1.333
The degree of lipiodol retention	0.336	15.208	0.086	< 0.001	1.399
TSR	0.286	13.014	0.079	< 0.001	1.331
PEI	0.739	3.120	0.418	0.077	2.094

B: Regression coefficient; AFP: α -fetoprotein; TACE: Transcatheter arterial chemoembolization; TV: Tumor volume; TTLVR: Tumor to liver volume ratio; TSR: Tumor shrinkage ratio; PEI: Percutaneous ethanol injection.

Table 2 Enter factors of multivariable Cox regression analysis

Parameter	B	χ^2	SE	P	Hazard ratio
Post-treatment level of AFP	-0.731	5.416	0.314	0.020	0.481
Times of repeated TACE	-0.597	8.746	0.202	0.003	0.550
TTLVR	0.765	14.328	0.202	< 0.001	2.150
Portal vein cancerous thrombosis	0.340	5.643	0.143	0.018	1.405

Method: Forward stepwise (likelihood ratio).

TTLVR, portal vein cancerous thrombus, repeated TACE times and post-treatment AFP level that entered the model could not be removed (Table 2).

In this study, the survival time was inversely correlated with the TTLVR values. The higher the TTLVR value was, the shorter the survival rate would be. The mean and median survival time was 28 and 18 mo, respectively, in 74 patients with their TTLVR less than 25%. Those whose TTLVR was 25%-50% and 50%-70% had a shorter mean and median survival time. Patients whose TTLVR was $\geq 70\%$ usually had a poor prognosis even after TACE. The survival time of 8 patients whose TTLVR was $\geq 70\%$ was no more than 6 mo after TACE, indicating that TTLVR is a significant factor influencing

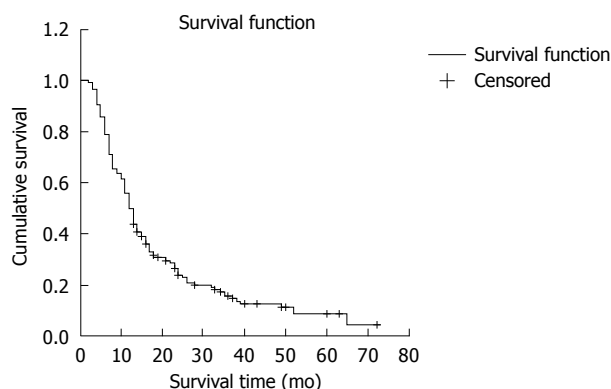


Figure 3 Cumulative survival time of 166 HCC patients after TACE. The 6 mo- 1-, 2-, 3-, and 5-year cumulative survival rates were 78.92%, 49.85%, 23.82%, 15.60% and 8.92%, respectively, in HCC patients after TACE.

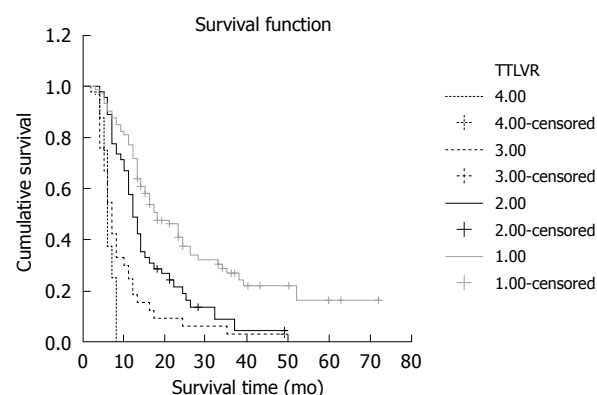


Figure 4 Reverse correlation between survival time and TTLVR values. The TTLVR value was 0%-25%, 25%-50%, 50%-70%, and 70%, respectively.

the cumulative survival rate of HCC patients after TACE ($\chi^2 = 14.328$, $P < 0.001$). Furthermore, TTLVR is a risk factor affecting the outcome of treatment because of a positive B value. The mean, median and cumulative survival rates of patients are shown in Figure 4 and Table 3.

Univariate analysis showed that the influence of tumor size measured with different methods including TV, TTLVR, largest tumor diameter, and tumor product of the greatest axial dimensions was significant on prognosis of HCC patients and their correlations are listed in Table 4.

DISCUSSION

It was reported that the median survival time of untreated HCC patients is less than 6 mo from its diagnosis^[2]. However, it was 12 mo after TACE in our study, indicating that TACE is an effective treatment modality for HCC, a finding in agreement with the reported data^[4].

Selection of optimal CT tumor measurement methods is still a challenge in treatment of HCC patients. Tumor size is widely considered a good indication for choosing an appropriate treatment modality for HCC patients, although it is difficult to accurately determine the lesion volume. In 1979, the World Health Organiza-

Table 3 Cumulative survival ratios of different TTLVR subgroups

Subgroup	Cumulative survival rate (%)					Median survival (mo)	Mean survival (mo)
	6 mo	12 mo	24 mo	36 mo	60 mo		
0%-25%	90.54	77.03	37.68	26.56	16.30	18	28
25%-50%	88.89	48.89	18.72	8.92	4.04	12	16
50%-70%	54.55	18.18	6.06	3.03	0.00	7	10
≥ 70%	37.50	0.00	0.00	0.00	0.00	6	6

Significant differences in survival were recognized for cumulative survival ratios of different TTLVR subgroups by log rank test: 0%-25% *vs* 25%-50% ($\chi^2 = 9.1$, $P = 0.003$), 0%-25% *vs* 50%-70% ($\chi^2 = 33.71$, $P < 0.001$), 0%-25% *vs* ≥ 70% ($\chi^2 = 38.98$, $P < 0.001$), 25%-50% *vs* 50%-70% ($\chi^2 = 9.08$, $P = 0.003$), 25%-50% *vs* ≥ 70% ($\chi^2 = 22.91$, $P < 0.001$), 50%-70% *vs* ≥ 70% ($\chi^2 = 1.71$, $P = 0.19$).

Table 4 Correlations between largest tumor diameter, tumor product of the greatest axial dimension, TV, and TTLVR

Parameter	Tumor largest diameter	Tumor product of diameters	TV
TTLVR	0.836	0.842	0.897
Tumor largest diameter		0.976	0.916
Tumor product of diameters			0.928

Largest tumor diameter *vs* TTLVR ($r = 0.836$, $P < 0.001$), tumor product of the greatest axial dimension *vs* TTLVR ($r = 0.842$, $P < 0.001$), TV *vs* TTLVR ($r = 0.897$, $P < 0.001$), largest tumor diameter *vs* tumor product of the greatest axial dimension ($r = 0.976$, $P < 0.001$), largest tumor diameter *vs* TV ($r = 0.916$, $P < 0.001$), tumor product of the greatest axial dimension *vs* TV ($r = 0.928$, $P < 0.001$).

tion (WHO) established the guidelines for tumor response to treatment. According to the WHO guidelines, the maximum diameter in the transverse plane and the largest perpendicular diameter of a tumor, measured on the same image, are multiplied to yield a cross product^[9]. In 1994, the European Organization for Research and Treatment in Oncology, the National Cancer Institute of the United States, and the National Cancer Institute of Canada Clinical Trials Group reviewed the tumor measurement techniques in view of the advances in imaging technologies. The revised response evaluation criteria in solid tumors guidelines advocate that uni-dimensional measurement (largest diameter in the transverse plane) can be used alone to quantify tumor burden^[6].

Tumor diameter, cross-sectional area or TV can be used to measure the size of tumors with a perfect sphere shape for the classification of their responses to TACE or other treatments. Unfortunately, since most tumors have an irregular geometry the size of tumors estimated with each parameter is usually significant different^[21]. Prasad *et al.*^[9] showed that 37 patients with secondary liver tumors responded to treatment with uni-dimensional and bi-dimensional techniques. However, tumor burden measured with TV gave a different response to treatment compared with uni-dimensional or bi-dimensional technique in a considerable proportion of patients, suggesting that methods to obtain directly TV are very important in treatment of HCC with an irregular geometry.

Advances in medical imaging have provided new means to assess tumor with volumetric imaging technologies such as helical CT and magnetic resonance imag-

ing, both of which allow physicians to accurately quantify tumor burden and to efficiently evaluate the outcomes of treatment^[5,9,16,22,23]. In our study, 3D measurements of HCC were made in patients with HCC using helical CT images. It has been shown that CT measurements of TV can provide a more sensitive assessment of tumor change than qualitative evaluation using linear methods^[24,25]. Moreover, changes in TV may be correlated with the prognosis of HCC patients^[25,26].

To our knowledge, only one study^[16] is available on the survival time of HCC patients after TACE. However, no studies are available on the correlation between tumor size measurements of unresectable HCC and survival time of HCC patients after TACE. In this study, we investigated not only the correlation between any variable and survival time, but also the correlation between multiple variables and survival data with confounding interaction effects taken into account.

In this study, univariate Cox regression analysis showed that there was an almost complete agreement between treatment response and TTLVR, TV, largest tumor diameter and tumor product of the greatest axial dimensions in 166 HCC patients after TACE ($P < 0.001$). However, multivariable Cox regression analysis demonstrated that TTLVR was the only significant factor for the prognosis of HCC patients after TACE, suggesting that TTLVR may be more important for evaluating the prognosis of HCC patients than either a simple volumetric measurement or a linear measurement after TACE. Moreover, a volumetric measurement would not only overcome the difficulties in estimating the size of lesions that are confluent and irregular in shape, but also permit the measurement of overall tumor burden in an organ. Kim *et al.*^[27] have shown the significance of TTLVR for prognosis of HCC patients compared with other treatment modalities. Using a multivariate Cox hazard model, they investigated the prognostic factors for 119 HCC patients after liver transplantation, and showed that the TV ratio greater than 10% can be used as an independent prognostic factor.

It is worth noting that some studies have shown that the survival time is not always associated with the size of tumors detected at the time of treatment with TACE^[2,28]. In some cases, volumetric tumor measurement for evaluating therapeutic response can not show any advantages over uni-dimensional and bi-dimensional measurements^[10,12]. To some extent, the difference is

thought to be due to the fact that the patients in these studies had different clinical characteristics. For instance, Sohaib *et al.*^[10] studied only nodal masses, which tend to have regular ovoid structures. Furthermore, TTLVR was not investigated in such studies.

Our study also showed a strong correlation between survival time and frequency of repeated TACE, which is consistent with the findings in other studies^[3,29,30]. The mean survival time of patients undergoing ≥ 2 times of TACE was significantly longer than that of those undergoing TACE once. The reason why repeated TACE can prolong the survival time of these patients is that additional embolization can make up for the incomplete effect of previous treatment, indicating that repeated TACE in treatment of HCC is very important for prolonging the survival time of patients^[30].

It has been shown that portal vein cancerous thrombosis is associated with the survival time of HCC patients after TACE^[26,30,31], which is consistent with the findings in our study. Portal vein cancerous thrombosis is an important factor for predicting survival time, as univariate or multivariable Cox regression analysis in our study has shown that it is statistically significant. Independent of initial values, a lower serum AFP level after TACE is another useful post-procedural predictor^[29,32], which usually implies that a favorable tumor response occurs.

In conclusion, volumetric CT technique can be reasonably used to measure tumor burden in an organ. TTLVR is a significant prognostic factor influencing the survival time of HCC patients after TACE. Compared with uni-dimensional and bi-dimensional criteria for evaluating the response of unresectable HCC to TACE, the volumetric measurement technique gives favorable results.

However, there are some limitations in our study. First, some bias may have occurred in this retrospective study due to the lack of complete clinical data. Second, the TV measurement technique relies on a mostly manual operation. Tracing individual tumor margins with mouse is time consuming and the results may have some variations if performed by different observers^[33]. Moreover, the software for accurate TV estimation must be installed and used^[9]. In the future, advances in post-processing of images may allow for automatic lesion contouring and TV calculation, leaving the radiologist the simple task of refining the outlined tumor contours^[12]. Using these techniques can further improve the reproducibility of volume measurements and allow precise measurement of tumor burden in an organ.

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COMMENTS

Background

Hepatocellular carcinoma (HCC) is one of the most common malignancies

in Asia and Africa. Transcatheter arterial chemoembolization (TACE) has been shown to be one of the most effective procedures for the treatment of unresectable HCC. Since quantification of tumor size with computed tomography (CT) is usually used to assess the prognosis of HCC patients after TACE, it is very important to measure the accurate tumor size. Many studies have shown that uni-dimensional or bi-dimensional measurements can be employed to evaluate the prognosis of HCC patients after TACE. However, it can not always achieve a satisfactory prognosis.

Research frontiers

Three-dimensional measurement has advantages over uni- and bi-dimensional measurements for the assessment of irregular masses. Spiral CT offers theoretical advantages in terms of the accuracy and reproducibility of three-dimensional measurements. Previous studies have shown that CT-based three-dimensional measurement provides a more sensitive assessment of tumor change than qualitative evaluation with linear methods. However, CT-based three-dimensional volumetric measurement for prognostic evaluation of unresectable HCC after TACE has not been systematically addressed. In this study, the authors demonstrate that a volumetric CT technique can predict the prognosis of unresectable HCC patients after TACE.

Innovations and breakthroughs

Previous studies evaluated the survival time of HCC patients after TACE on the basis of tumor to liver volume ratio (TTLVR) values, but multivariable Cox regression analysis was not done. In this study, the authors investigated not only the correlation between multiple variables and survival data, but also took the confounding interaction between these factors. Furthermore, this study showed that volumetric quantification might result in a more favorable assessment than uni-dimensional and bi-dimensional techniques.

Applications

The results of this study suggest that a volumetric CT technique can reasonably predict the prognosis of unresectable HCC patients after TACE.

Terminology

TTLVR: A measurement value for tumor burden in the liver. It may be a significant prognostic factor influencing the survival time of HCC patients after TACE.

Peer review

The authors studied the prognostic significance of CT volumetric measurements in HCC patients after TACE. This work states that simple response evaluation criteria in solid tumors and WHO criteria are insufficient for the adequate evaluation of HCC lesions after treatment. Furthermore, this study revealed that TTLVR might result in a more favorable assessment than uni-dimensional and bi-dimensional measurement techniques. The results are interesting and useful for readers.

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LOH analysis of genes around D4S2964 identifies ARD1B as a prognostic predictor of hepatocellular carcinoma

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affected by loss of heterozygosity (LOH) and their clinical implications.

METHODS: Four hundred and forty single nucleotide polymorphisms (SNPs) located at 49 genes around D4S2964 were selected from the National Center for Biotechnology Information website for the SNPs microarray fabrication. LOH of SNPs markers in 112 cases of hepatocellular carcinoma (HCC) tissues and paired adjacent liver tissues were investigated by the SNPs microarray. The correlation between allelic losses with clinicopathological features and overall survival was analyzed.

RESULTS: A fine map of LOH of SNPs in genes around D4S2964 was plotted. The average frequency of LOH in genes was 0.39. A correlation between cirrhosis and the FAL index (fractional allelic loss) was found ($P = 0.0202$). Larger tumor size was found to be significantly associated with LOH in genes ADP-ribosyltransferase 3 (ART3), nucleoporin 54 kDa (NUP54), scavenger receptor class B, member 2 (SCARB2) and coiled-coil domain containing 158 (CCDC158) ($P = 0.043$, $P = 0.019$, $P = 0.001$, $P = 0.037$, respectively). Kaplan-Meier analysis showed that patients with LOH in ARD1 homolog B (ARD1B) and septin 11 (SEPT11) had a significantly lower survival rate than those with retention ($P = 0.021$ and $P = 0.004$, respectively). A Cox regression model suggested that LOH in ARD1B and SEPT11, respectively, were predictors of the overall survival in HCC ($P = 0.006$ and $P = 0.026$, respectively).

CONCLUSION: LOH in genes around D4S2964 may play an important role in HCC development and progression. LOH in ARD1B and SEPT11 could serve as novel prognostic predictors in HCC patients.

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Key words: ARD1 homolog B; Hepatocellular carcinoma;

Abstract

AIM: To investigate genes around the locus D4S2964

Loss of heterozygosity; Septin 11; Single nucleotide polymorphism

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INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most prevalent cancers and the third leading cause of cancer mortality worldwide^[1]. It has been ranked the second leading cause of cancer death in China since the 1990s^[2]. HCC incidence and mortality rates continue to increase, particularly among middle-aged black, Hispanic, and white men in the United States^[3]. The main causes of HCC are hepatitis B and C virus (HBV and HCV) infection, dietary exposure to aflatoxin B1 and high-level alcohol consumption^[4]. Although many risk factors are known, the molecular mechanisms of carcinogenesis and progression of HCC have not been well elucidated.

Loss of heterozygosity (LOH) at microsatellite markers has been reported in nearly all types of human cancers. LOH regions found in a significant portion of tumors are thought to embody tumor suppressor genes (TSG). However, hundreds of LOH regions remain to be explored for the presence of TSGs. LOH in HCC is frequently detected in many chromosome regions, including 1p, 4q, 8p, 9p, 10q, 11p, 13q, 16q, 17p and 22q^[5-10]. Allelic losses in some of the regions were correlated to clinicopathologic features and HCC progression^[11]. An association between LOH and poor prognosis was also implicated in some studies^[9,12].

In our previous study, we performed a genome-wide LOH analysis in HCC with 382 microsatellite (MS) markers, and found that LOH frequency of D4S2964 located at 4q21 in HCC was as high as 50% and correlated with HBV infection^[5]. A high frequency of LOH in HCC at the D4S2964 locus was also observed by Nishimura *et al.*^[13]. The high frequency of LOH at D4S2964 was only found in HCC. Those results strongly suggest the presence of TSGs specifically for human HCC in this region. Because of the low density of the microsatellite markers, it is very difficult to use these markers for TSG identification. In the present study, we took advantage of the National Center for Biotechnology Information (NCBI) single nucleotide polymorphism (SNP) database and designed a novel strategy to gain deeper insight into the D4S2964 locus and to determine genes that may be involved in hepatocarcinogenesis. We

included all 49 genes located within 4 Mb upstream and downstream of the D4S2964 locus, and selected 440 SNPs from the 49 genes with an average of 9 SNPs in each gene from the NCBI website. With the microarray consisting of probes for all the 440 SNPs, we detected LOH in 112 HCC cases paired with non-cancerous liver tissues and analyzed the correlation between the LOH affected genes and clinical features. Our study provided important clues for the identification of candidate TSGs in the LOH affected region and their clinical implication.

MATERIALS AND METHODS

Patients and tissue specimens

All 112 HCC patients, 94 males and 18 females with an age range of 13 to 72 years (median, 45.5 years), underwent surgical resection at the Department of Hepatobiliary Oncology, Sun Yat-sen University Cancer Center between 2004 and 2007. All of the tumors removed from these patients were confirmed as HCC by pathological examination. None of the patients had received any other therapies such as chemoembolization or chemotherapy before surgery. The follow-up time for these patients ranged from 1 to 58 mo with a median follow-up time of 36 mo. Most (96 of 112, 85.7%) of the patients were HBsAg positive. This study was approved by the Committee for the Conduct of Human Research of the Sun Yat-Sen University Cancer Center. Informed consent was obtained from each patient.

Primary liver carcinoma and matched non-tumor liver tissues were obtained from 112 patients when they underwent hepatectomy. Both tumors and corresponding adjacent non-tumor tissues (not less than 2 cm away from the HCC) were sampled. The fresh tissues were immediately immersed in RNAlater (Ambion, Inc., USA) after surgical resection, stored at 4°C overnight to allow thorough penetration of the RNase inhibitor, and then frozen at -80°C until use. After total RNA extraction using TRIzol reagent (Invitrogen, USA), DNA in the interphase and phenol phase from the initial homogenate was isolated according to the manufacturer's instructions.

SNP genotyping by microarray

Forty-nine genes located within approximately 8 Mb (from 77 to 85 Mb on the long arm of chromosome 4) and flanking the D4S2964 locus were included in the study. A total of 440 SNP markers with a heterozygosity greater than 0.10 were selected from the NCBI SNP database (<http://www.ncbi.nlm.nih.gov/snp>). The SNPs were located either within or 5 kb upstream/downstream of the 49 corresponding genes. On average, 9 SNPs (2-14) were selected for each gene. The average distance between adjacent markers for each gene was 7.8 kb and ranged from 0.2 to 111 kb. The polymorphic sites of the SNPs were all transition variations (A/G-C/T) to facilitate microarray analysis by two fluorescent colors.

The high-throughput SNP microarray genotyping system described by Wang *et al.*^[14] was used with minor modifications. The microarray was printed using a SmartArray™.

136 printer and scanned by a LuxScan™-10K Scanner (both devices are products of CapitalBio Inc., Beijing, China). Genotype call for each SNP was determined by a computer program called “AccuTyping2b”^[15] based on two color signal intensities. If the log ratio of the two color (Cy5 and Cy3) intensities for a SNP was between -3.3 and 3.3, the SNP genotype was considered as heterozygous (C/T). If the log ratio was greater than 3.3 or less than -3.3, the genotype was assigned as homozygous, C/C and T/T, respectively.

LOH analysis and DNA sequencing validation

In the present study, LOH was defined as the SNP that was homozygous in tumor tissue and heterozygous in the corresponding non-tumor tissue. If any one of the SNPs in a gene had LOH, the gene was assigned as LOH. The FAL index (fractional allelic loss) for individual cases was defined as the ratio of the number of SNPs showing LOH to the total number of informative SNPs in a tumor patient; it reflects the degree of LOH of the tumor^[16].

In order to confirm the genotypes and LOH of SNPs, DNA sequences containing these SNPs were amplified separately using the same condition as described above for multiplex PCR, and the PCR products were sent to Invitrogen (Guangzhou, China) for DNA sequencing.

Statistical analysis

The χ^2 -test, Fisher's exact test and Mann-Whitney *U* test were used to analyze the correlations between clinicopathological features and the FAL index or LOH index. The overall survival curves were estimated using the Kaplan-Meier analysis. The log-rank test was used to evaluate the statistical significance of the differences. The prognostic significance of each clinicopathologic characteristic was determined using univariate Cox regression analysis. Parameters that were significantly related to survival rate in the univariate analysis were entered into the multivariate analysis. With a Cox proportional hazard model, multivariate analysis was carried out to identify the prognostic significance of clinicopathologic variables. A forward stepwise selection with the likelihood ratio criterion or an enter mode was used to identify the variables in the Cox model whenever appropriate. The SPSS version 15.0 software package and Graph-Pad Prism were used for statistical analysis.

RESULTS

SNP genotyping by microarray and validation by DNA sequencing

A total of 13 322 SNP genotypes were determined as informative (heterozygous) in non-tumor liver tissues. The heterozygosity rate was 0.27 [13322/(440*112)]. Typical microarray images for SNP genotyping of the tumor and corresponding non-tumor tissue in one patient are shown in Figure 1A and B. Twenty-five informative genotypes for six SNPs, rs17352824, rs2280101, rs3733329, rs6828114, rs6845080, and rs9307811 in 10 pairs of

Table 1 Clinicopathologic correlation of FAL index around D452964

Parameter	FAL index		P value
	Low	High	
Sex			
Female	9	9	1.000
Male	47	47	
Serum HBsAg			
Negative	9	7	0.589
Positive	47	49	
Cirrhosis			
Absent	10	2	0.021
Present	42	46	
Tumor size (cm)			
≤ 5	12	13	0.820
> 5	44	43	
Tumor stage			
I and II	44	41	0.608
III and IV	11	13	
Recurrence			
No	39	36	0.547
Yes	17	20	

FAL: Fractional allelic loss.

tumor/non-tumor tissues were validated by the sequence analysis. Typical results are shown in Figure 1C and D. The concordant rate of SNP genotypes from microarray and DNA sequencing was 96% (24/25). The inconsistent genotype was rs6845080 in a non-tumor tissue, in which the genotype by microarray was determined as C/T heterozygote, whereas that from DNA sequencing was C/C homozygote.

LOH detection in the 112 HCC cases

Among the 440 SNPs selected in the study, 22 were shown to be non-informative in all cases, and were excluded from the data analysis. Of the 13 322 informative genotypes, 2553 (19.2%) showed LOH in tumor tissues. The results from LOH analysis for the 418 SNPs in the 49 genes in the 112 HCC patients are summarized in Figure 2. The FAL values for the 112 cases ranged from 0 to 0.769. All genes had at least one SNP affected by LOH in at least two patients. The fraction of the 112 cases with at least one SNP affected by LOH for each gene ranged from 0.227 (the *FGF5* gene) to 0.643 (the *PPEF2* gene) with an average of 0.382. However, for some genes, such as *CXCL10* and *CXCL11*, the number of HCC patients with informative SNPs was very small, and therefore the fractions may have fluctuated in a rather wide range. The genes statistically shown to be associated with clinical features had > 30 informative cases.

Association between clinicopathological features and LOH

In order to elucidate the possible clinical significance of sample FAL indices, the 112 cases were divided into two groups according to the median FAL value and a χ^2 analysis was performed. Results showed a significant correlation between FAL and cirrhosis but not with gender, age, serum α -fetoprotein (AFP) concentration, HBsAg

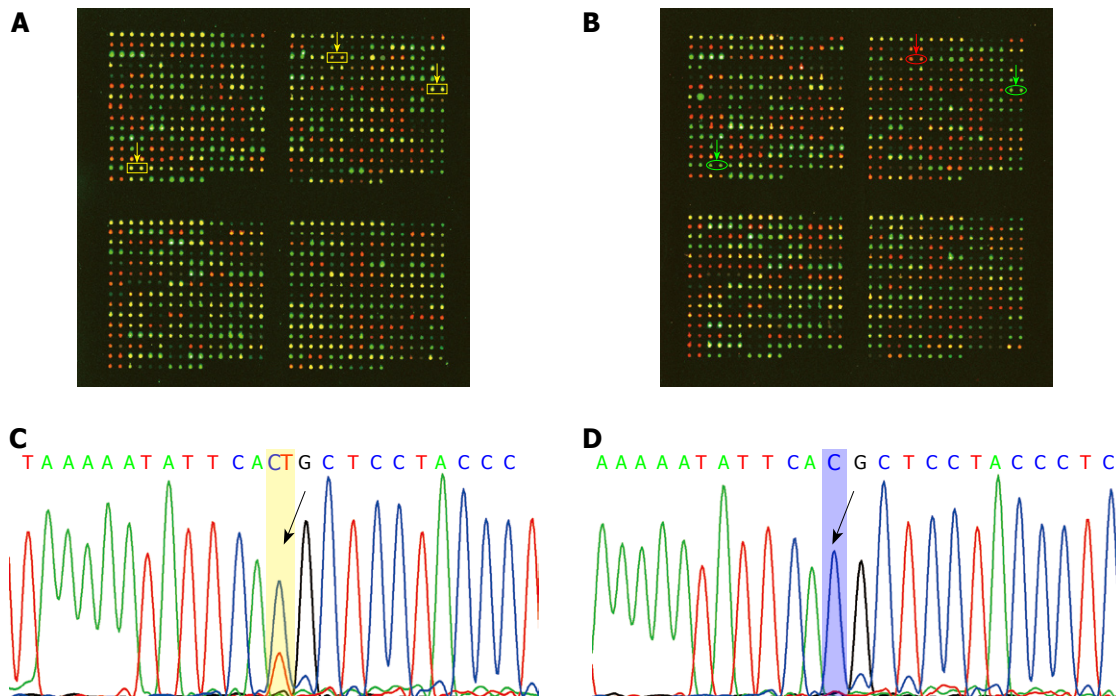


Figure 1 Representative case showing loss of heterozygosity. A: Informative normal adjacent tissue in SNP array. Yellow arrow shows the SNP loci with heterozygous genotype; B: Corresponding tumor tissue in SNP array. Red (or green) arrow shows the corresponding SNP loci with homozygous genotype; C: Informative normal adjacent tissue in DNA sequencing. Arrow shows the SNP loci with heterozygous genotype; D: Corresponding tumor tissue in DNA sequencing. Arrow shows the corresponding SNP loci with homozygous genotype.

status, tumor stage, tumor size, or recurrence (Table 1). The Mann-Whitney *U* test also revealed that HCCs with cirrhosis had significantly higher FAL indices than those without cirrhosis ($P = 0.020$, Figure 3).

Furthermore, we analyzed the correlation between LOH in genes and clinical features, including serum AFP concentration, HBsAg status, HBeAg status, tumor size, tumor stage, cirrhosis, recurrence and metastasis. The results are summarized in Table 2. Larger tumor size was found to be significantly associated with LOH in genes ADP-ribosyltransferase 3 (ART3), nucleoporin 54 kDa (NUP54), scavenger receptor class B, member 2 (SCARB2) and coiled-coil domain containing 158 (CCDC158) ($P = 0.043$, $P = 0.019$, $P = 0.001$, $P = 0.037$, respectively, Table 2), which are tandemly located. LOH in two genes, septin 11 (SEPT11) and chemokine (C-X-C motif) ligand 13 (CXCL13) was detected more frequently in HCC patients who were HBeAg-negative than in those who were HBeAg-positive ($P = 0.041$, $P = 0.024$, respectively, Table 2). However, LOH in cyclin I (CCNI) and SEC31 homolog A (SEC31A) were also more frequently detected in HBsAg-positive HCC patients than in HBsAg-negative patients ($P = 0.037$, $P = 0.013$ respectively, Table 2). The patients with LOH in ARD1 homolog B (ARD1B) showed a higher serum HBV-DNA level and a higher recurrence rate than those not affected by LOH in the gene ($P = 0.049$, $P = 0.046$, respectively, Table 2). LOH in genes helicase, POLQ-like (HELQ) and 1-acylglycerol-3-phosphate O-acyltransferase 9 (AGPAT9) was also found to be related with cirrhosis status ($P = 0.006$, $P = 0.033$, respectively, Table 2).

Correlation between LOH in genes ARD1B and SEPT11 and survival rate of HCC patients

Using the Kaplan-Meier curve, we showed that LOH in genes ARD1B and SEPT11 was a significant prognostic factor for poor overall survival rate in HCC patients. Patients with LOH in genes ARD1B and SEPT11 had a significantly lower survival rate than those without LOH in these genes ($P = 0.021$, $P = 0.004$, respectively, Figure 4).

Univariate and multivariate analyses of prognostic variables in HCC patients

Univariate and multivariate Cox regression models were performed to identify the variables of potential prognostic significance in all patients with HCC. The difference in predicting the prognosis was assessed by examining the ratio hazard and *P* value for each variable. Univariate Cox regression analysis showed that LOH in ARD1B and/or SEPT11 was significantly associated with overall survival rate (Table 3). Univariate Cox regression analysis also indicated that clinical variables including tumor size, recurrence, number of nodules and serum AFP were significantly associated with overall survival rate. Multivariate analysis with an enter mode was performed for LOH in ARD1B or in SEPT11, tumor size, recurrence, number of nodules, and serum AFP with overall survival rate. Results showed that LOH in ARD1B or in SEPT11 and number of nodules were predictors of the overall survival rate in HCC patients ($P = 0.006$, $P = 0.001$; and $P = 0.026$, $P = 0.005$, respectively, Table 4). LOH in ARD1B, LOH in SEPT11, tumor size, recurrence, number of nodules, and serum AFP altogether were analyzed with multivariate Cox regression model using

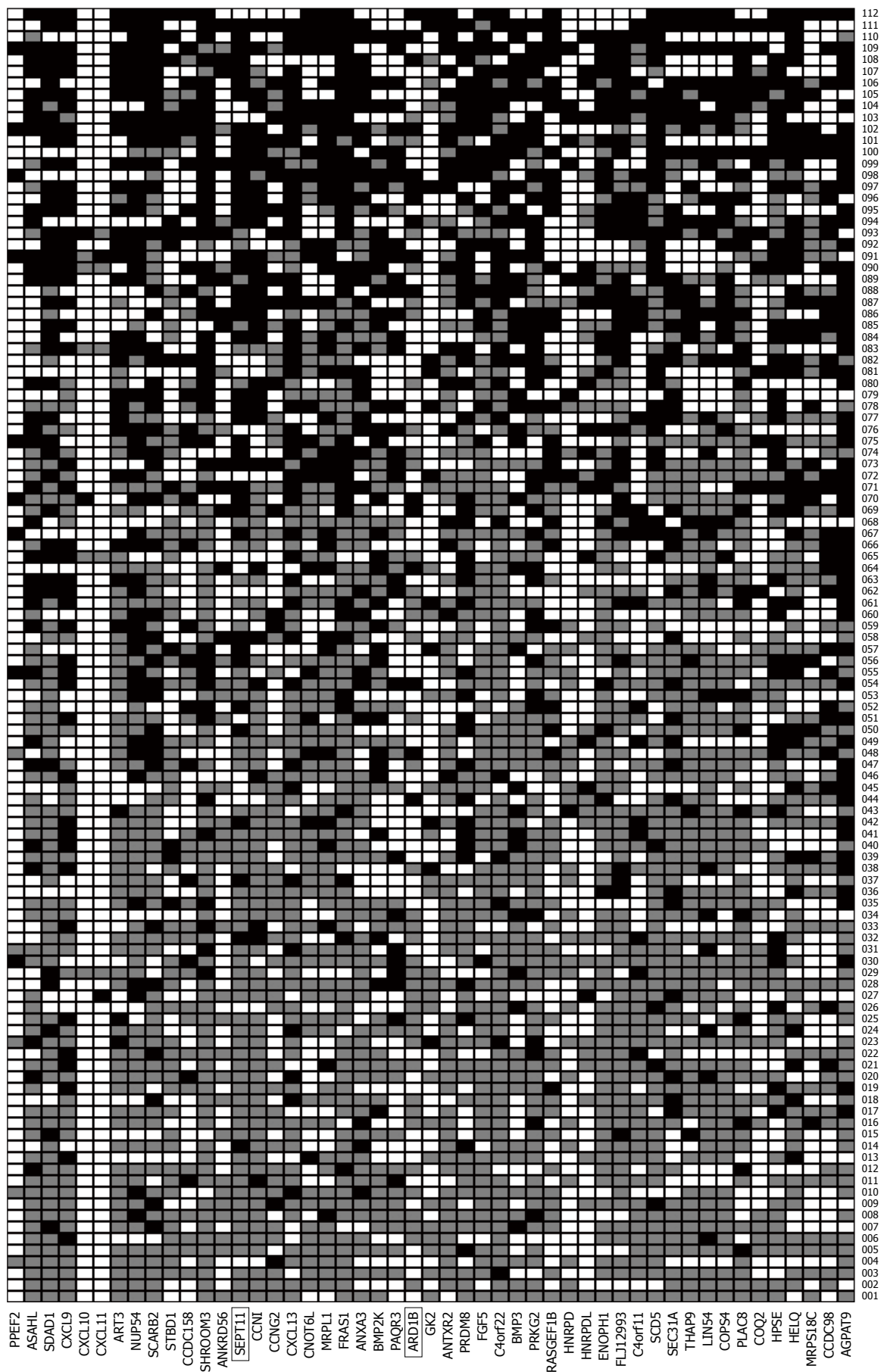


Figure 2 Summary of LOH analysis results. Case numbers are indicated in the right column. Gene names are listed in the bottom row, in the order of physical position from 77 Mb to 84 Mb (left to right). Gray boxes: Retention; White boxes: Noninformative; Black boxes: LOH.

Table 2 Representative clinicopathologic correlation of LOH in different genes around D4S2964

Parameter	LOH		P value
	Absent	Present	
Tumor size (cm)		ART3 ^a	
≤ 5	16	3	0.043
> 5	39	27	
Tumor size (cm)		NUP54	
≤ 5	15	6	0.019
> 5	30	41	
Tumor size (cm)		SCARB2	
≤ 5	18	3	0.001
> 5	33	39	
Tumor size (cm)		CCDC158	
≤ 5	11	3	0.037
> 5	19	22	
Serum HBeAg		SEPT11	
Negative	50	32	0.041
Positive	4	9	
Serum HBsAg		CCNI	
Negative	11	1	0.037
Positive	48	31	
Serum HBeAg		CXCL13	
Negative	50	37	0.024
Positive	2	8	
Recurrence			
No	40	26	0.044
Yes	12	19	
Tumor stage		ANXA3	
I and II	37	29	0.014
III and IV	15	2	
Serum HBV-DNA		ARD1B	
Negative	24	5	0.049
Positive	14	10	
Recurrence			
No	32	9	0.046
Yes	7	7	
Serum HBsAg		SEC31A	
Negative	4	10	0.013
Positive	47	26	
Metastasis			
No	40	34	0.039
Yes	11	2	
Cirrhosis		HELQ	
Absent	8	0	0.006
Present	32	34	
Cirrhosis		AGPAT9	
Absent	7	3	0.033
Present	27	50	

^aGenes are listed in order of the most centromeric (top) to the most telomeric (bottom). LOH: Loss of heterozygosity; HBV: Hepatitis B virus; ART3: ADP-ribosyltransferase 3; NUP54: Nucleoporin 54 kDa; SCARB2: Scavenger receptor class B, member 2; CCDC158: Coiled-coil domain containing 158; SEPT11: Septin 11; CCNI: Cyclin I; CXCL13: Chemokine (C-X-C motif) ligand 13; ANXA3: Annexin A3; SEC31A: SEC31 homolog A; HELQ: Helicase, POLQ-like; AGPAT9: 1-acylglycerol-3-phosphate O-acyltransferase 9.

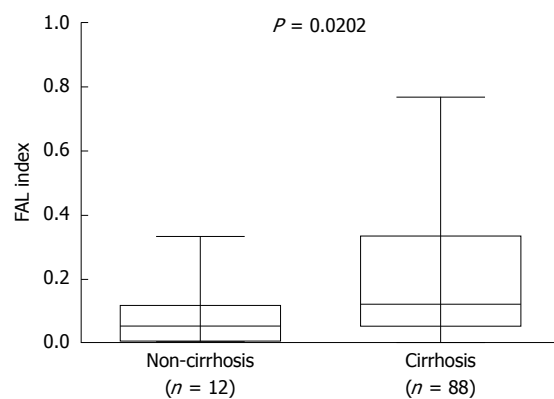
a forward stepwise selection method with the likelihood ratio criterion, only LOH in ARD1B and number of nodules were shown to be prognostic predictors of HCC ($P = 0.007$, $P = 0.004$, respectively, Table 4).

DISCUSSION

Allelic losses on chromosome 4q have been reported in

Table 3 Univariate analyses of individual parameters for correlations with overall survival rate: Cox proportional hazards model

Parameter	Hazard ratio	95% CI	P value
LOH in ARD1B	2.915	1.119-7.594	0.029
LOH in SEPT11	2.856	1.354-6.023	0.006
Sex	1.327	0.518-3.398	0.556
Tumor grade	1.693	0.948-3.023	0.075
Serum HBsAg	0.902	0.377-2.158	0.817
Serum AFP	3.319	1.295-8.504	0.012
Tumor size	4.266	1.31-13.888	0.016
No. of nodules	3.143	1.627-6.073	0.001
Cirrhosis	0.937	0.329-2.673	0.903
Metastasis	1.736	0.764-3.946	0.188
Recurrence	1.992	1.049-3.783	0.035

**Figure 3** Correlation between FAL index (fractional allelic loss) and tumor size. The box plot shows the median, quartile, and extreme values; the lines across the boxes indicate median FAL index.

several human cancers, including esophageal adenocarcinoma, cervical cancer, head and neck squamous cell carcinoma, oral squamous cell carcinomas, malignant mesothelioma, small-cell lung cancer and HCC^[17-27]. However, allelic losses on 4q occurred more frequently in HCC than in all other cancers and was found to be correlated with elevated α -fetoprotein production and $p53$ gene mutations^[17,26]. Many important genes encoding albumin, alcohol dehydrogenase (ADH3), fibrinogen and UDP-glucuronyltransferase, which are predominantly expressed in the liver, are located on chromosome 4q. Genes on the long arm of this chromosome including protein tyrosine phosphatase, non-receptor type 13 (PTPN13), nucleosome assembly protein 1-like 5 (NAP1L5), and PR domain containing 5 (PRDM5), were documented as potential TSGs^[28-30]. However, a large number of loci with high LOH frequency found in HCC have not been explored. Therefore, it is postulated that chromosome 4q should contain other unknown TSGs.

In the present study, based on our previous study and those by other researchers^[5,13], we detected LOH in 49 genes around the D4S2964 locus by a custom microarray consisting of 440 SNPs. The average distance between these SNPs in the genes was 7.8 kb. To our knowledge, this is the first time LOH has been detected in such a way. The high-density markers in genes would display

Table 4 Multivariate analyses of individual parameters for correlations with overall survival rate: Cox proportional hazards model

Parameter	ARD1B			SEPT11			ARD1B and SEPT11 ^a		
	HR	95% CI	P value	HR	95% CI	P value	HR	95% CI	P value
LOH in ARD1B	4.615	1.538-13.851	0.006				4.866	1.528-15.493	0.007
LOH in SEPT11				2.393	1.107-5.170	0.026			0.533
Serum AFP	1.781	0.468-6.774	0.397	2.166	0.748-6.274	0.154			0.208
Tumor size	2.146	0.459-10.033	0.332	3.316	1.001-10.984	0.050			0.393
No. of nodules	6.173	2.072-18.394	0.001	3.002	1.401-6.434	0.005	5.579	1.738-17.904	0.004
Recurrence	0.900	0.305-2.659	0.849	1.707	0.829-3.516	0.147			0.859

^aForward LR method was used. HR: Hazard ratio.

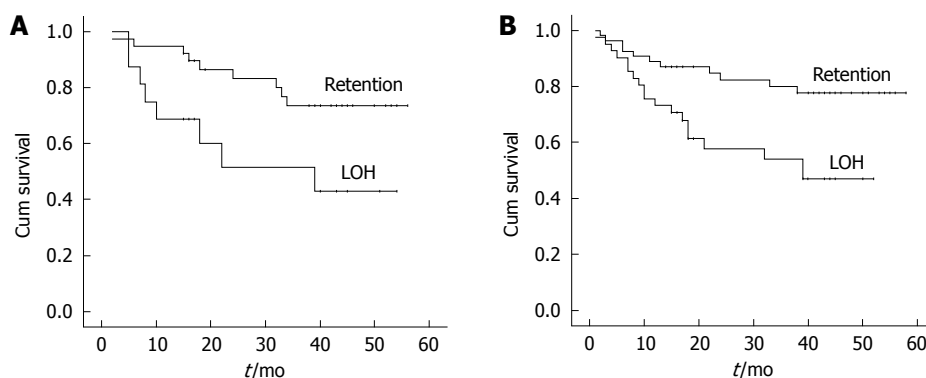


Figure 4 The association of loss of heterozygosity (LOH) in gene ARD1 homolog B (ARD1B) and septin 11 (SEPT11) with the postoperative survival of patients. A: Kaplan-Meier overall survival plot based on LOH in ARD1B (log-rank test, $P = 0.021$); B: Kaplan-Meier overall survival plot based on LOH in SEPT11 (log-rank test, $P = 0.004$).

more detailed and smaller LOH regions in single genes, and compensate for the weakness of SNP markers with much less informativeness than microsatellite markers.

Then we analyzed the correlations between allelic losses and clinicopathological features. The results showed that HCC patients with cirrhosis had significantly higher FAL indices (Table 1, Figure 3) and higher LOH fraction in HELQ and AGPAT9 (Table 2) than those without cirrhosis. These results showed that patients with or without cirrhosis can be discriminated by the FAL index and LOH in genes HELQ and AGPAT9. Cirrhosis is a definite, established risk factor for HCC. In patients with cirrhosis, cirrhotic livers with high liver cell proliferative activity have a higher risk of developing cancer^[31]. Higher LOH on several microsatellite markers on chromosome 16 and chromosome 17 was reported to be related to cirrhosis status in HCC patients^[32]. In our previous study, LOH was found in non-cancerous liver tissues with cirrhosis caused by chronic viral hepatitis B (data not published). Therefore, some LOH may be involved in hepatocarcinogenesis. Furthermore, the analysis revealed a correlation between larger tumor size and LOH in genes ART3, NUP54, SCARB2 and CCDC158 (Table 2). Interestingly, these genes are located in a cluster in the same chromosomal region. Therefore, further analysis of this region may lead to identification of new TSGs. The data also suggested that LOH in genes SEPT11, CXCL13, CCNI, SEC31A and ARD1B were correlated with HBV infectious status (Table 2). HBV infection in humans has been recognized as one of the main risk factors for liver cirrhosis and HCC. How-

ever, the molecular mechanism underlying HBV infection is still unclear. Our results may provide insight into the understanding of HBV-related HCC.

Most importantly, we found that LOH in genes ARD1B and SEPT11 were significantly correlated with poor overall survival in HCC patients (Figure 4). Univariate and multivariate Cox regression models further revealed that LOH in genes ARD1B and SEPT11 were significant prognostic factors for poor overall survival. However, when LOH in ARD1B, LOH in SEPT11, and clinical features altogether were analyzed by a multivariate Cox regression model, only LOH in ARD1B, not LOH in SEPT11, and the number of nodules were prognostic predictors of HCC (Table 4).

The ARD1B gene encodes a human protein with 81% sequence identity to ARD1 homolog A (ARD1A), which encodes the human protein N- α -acetyltransferase. Compared to lower organisms which only have one ARD, ARD1B potentially complements the functions of ARD1A, adding more flexibility and complexity to protein N- α -acetylation in human cells^[33]. Although the biological function of ARD1B in cancer remains unclear, it is recognized that the N-terminal acetylation of proteins influences multiple processes such as the cell cycle, heat-shock resistance, mating, sporulation, and telomeric silencing. However, RNAi-mediated knockdown of ARD1A and N (α)-acetyltransferase 15, NatA auxiliary subunit (NATH) induced apoptosis in human cell lines^[34]. Immunohistochemical analysis showed that ARD1A protein was expressed extensively in several cancer tissues

including glandular carcinoma and squamous cancer^[35]. It is proposed that ARD1A or the ARD1A-NATH complex could be drug targets in cancer treatment^[36]. Homologous to ARD1A, ARD1B is probably a functional protein in cancer development and/or progression. Although further study of ARD1B in cancer is necessary, our data suggest that LOH in ARD1B is a prognostic predictor of HCC and ARD1B is a potential TSG.

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COMMENTS

Background

High frequency of loss of heterozygosity (LOH) at locus D4S2964 was previously reported in hepatocellular carcinoma (HCC) by this research group and was confirmed by another group in Japan. The aim of this study was to investigate genes around the locus D4S2964 affected by LOH using a customized single nucleotide polymorphisms (SNP) microarray and their clinical implications.

Research frontiers

In HCC and nearly all other cancers, there are still a lot of important LOH regions which need to be explored with regard to what genes are involved in carcinogenesis and their biological and clinical implications. In general, LOH regions are thought to embody tumor suppressor genes (TSG), which are able to inhibit the development and progression of cancer.

Innovations and breakthroughs

This study reported LOH in genes at the D4S2964 locus that are proposed to be involved in the development and progression of HCC. With the SNP microarray, the authors showed that LOH in ARD1 homolog B (ARD1B) is an independent prognostic predictor of HCC and LOH in other genes correlated with tumor size, cirrhosis, hepatitis B virus infection, recurrence and metastasis.

Applications

These results provide novel and valuable information on LOH at the D4S2964 locus in HCC and provide clues for identifying TSGs related to HCC. In addition, this study proposes a new strategy to gain deeper insight into LOH regions existing in various cancers.

Terminology

Loss of heterozygosity (LOH): In a heterozygote, the loss of one of the two alleles at one or more loci in a cell lineage or cancer cell population due to chromosome loss, deletion, or mitotic cross-over.

Peer review

The study has been well conducted, including a reasonable series of paired samples from patients with HCC. Experimental procedures and statistics are correct. The results reported provide novel and valuable information that may contribute to our understanding and management of HCC.

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Prognostic impact of metastatic lymph node ratio on gastric cancer after curative distal gastrectomy

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Abstract

AIM: To investigate the prognostic impact of metastatic lymph node ratio (rN) on gastric cancer after curative distal gastrectomy.

METHODS: A total of 634 gastric cancer patients who underwent curative resection (R0) of lymph nodes at distal gastrectomy in 1995-2004. Correlations between positive nodes and retrieved nodes, between rN and retrieved nodes, and between rN and negative lymph node (LN) count were analyzed respectively. Prognostic factors were identified by univariate and multivariate analyses. Staging accuracy of the pN category (5th UICC/TNM system) and the rN category was compared according to the survival rates of patients. A linear regression model was used to identify the relation between rN and 5-year survival rate of the patients.

RESULTS: The number of dissected LNs was related with metastatic LNs but not related with rN. Cox regression analysis showed that depth of invasion, pN and rN category were the independent predictors of survival ($P < 0.05$). There was a significant difference in survival

between LN stages classified by the rN category or by the pN category ($P < 0.05$). However, no significant difference was found in survival rate between LN stages classified by the pN category or by the rN category ($P > 0.05$). Linear regression model showed a significant linear correlation between rN and the 5-year survival rate of gastric cancer patients ($\beta = 0.862$, $P < 0.001$). Pearson's correlation test revealed that negative LN count was negatively correlated with rN ($P < 0.001$).

CONCLUSION: rN category is a better prognostic tool than the 5th UICC pN category for gastric cancer patients after curative distal gastrectomy. Increased negative LN count can reduce rN and improve the survival rate of gastric cancer patients.

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Key words: Stomach neoplasm; Gastrectomy; Lymphadenectomy; Metastatic lymph node ratio; Prognosis

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INTRODUCTION

Gastric cancer, a common malignancy worldwide, is the

second most common cause of cancer-related death^[1]. Despite recent advances in multimodality treatment and targeted therapy, complete resection remains the only curative treatment modality. Lymph node metastasis is one of the most important prognostic factors for gastric cancer^[2-4], and accurate categorization of lymph node metastasis or optimization of pN category is critical for decision making of its subsequent therapies after surgery. Compared with total gastrectomy, distal gastrectomy is one of the most important operative procedures for gastric cancer, which can decrease postoperative morbidity and mortality and improve the postoperative life quality and nutrition condition of gastric cancer patients^[5-8]. Although there are some studies on the prognostic significance of rN in gastric cancer^[9-11], few relevant researches are available on gastric cancer after curative distal gastrectomy. Therefore, the aim of this retrospective study was to evaluate the prognostic value of metastatic lymph node ratio (rN) for gastric cancer after curative distal gastrectomy.

MATERIALS AND METHODS

Between January 1995 and November 2004, 1289 patients diagnosed as primary gastric cancer were treated with curative resection at Department of Oncology, Fujian Medical University Union Hospital (Fuzhou, China). Of the 1289 patients, 709 underwent distal gastrectomy, 75 were excluded from the study for T4 lesions in order to avoid data heterogeneity due to more extensive resections and higher positive margins. Finally, 634 patients were enrolled in this study. Curative surgery was defined as complete tumor removal with no macroscopic residual tumor, no invasion of carcinoma cells in the margin, and no evidence of distant metastasis. Clinical and histopathologic data about each patient were classified according to the Japanese Classification of Gastric Carcinoma (JCGC)^[12]. Lymph nodes were meticulously dissected from the *en bloc* specimens, and the dissected lymph nodes were classified by surgeons who reviewed the excised specimens after surgery based on the JCGC. Based on the 5th edition of UICC/TNM system^[13], pN category was defined as pN0 with no metastatic lymph nodes, pN1 with 1-6 metastatic lymph nodes, pN2 with 7-15 metastatic lymph nodes, and pN3 with > 15 metastatic lymph nodes, respectively. rN intervals were determined using the best cut-off approach and the survival rate of patients (log-rank statistic) was considered a dependent variable. The best-fit cut-off values of rN intervals were rN0: 0%, rN1: 1%-20%, rN2: 21%-50%, and rN3: > 50%, respectively. The patients were followed up by trained investigators through e-mail, telephone, visiting patients or recording the consultations of patients at the outpatient clinic. The survival time was the time when diagnosis was made to the last contact, or to the date of death, or to the date when the survival information was collected. All surviving patients were followed up for more than 5 years. The median follow-up time of

Table 1 Clinical parameters of patients included in this study
n (%)

Characteristics	Patients (<i>n</i> = 634)
Gender	
Male	467 (73.6)
Female	167 (26.4)
Age (yr)	56.1 ± 12.1
Tumor size (cm)	4.3 ± 1.7
Tumor location	
Lower	521 (80.8)
Middle	71 (11.2)
Lower and middle	42 (8.0)
Digestive tract construction	
Billroth I	469 (74.0)
Billroth II	148 (22.3)
Roux-en-Y	17 (3.7)
Pathology	
Differentiated	140 (22.1)
Undifferentiated	494 (77.9)
Depth of invasion	
pT1	118 (18.6)
pT2	174 (27.4)
pT3	342 (54.0)
pN category	
pN0	195 (30.8)
pN1	232 (36.6)
pN2	156 (24.6)
pN3	51 (8.0)
rN category	
rN0	195 (30.8)
rN1	172 (27.1)
rN2	156 (24.6)
rN3	111 (17.5)
Number of resected LNs	23.1 ± 8.6

the entire cohort was 62.0 mo (range 1-172 mo). A total of 591 patients were followed up, accounting for 93.2%.

Statistical analysis

Pearson's correlation coefficient (*r*) was used to study the relations between positive nodes and retrieved nodes, between rN and retrieved nodes, and between rN and negative LN count. Survival rate was calculated with the Kaplan-Meier method. Cox proportional hazard model was used in multivariate analysis, with a backward elimination model for all covariates. A linear regression model was established to correlate rN with survival based on Kaplan-Meier 5-year survival estimates at each rN interval, using 5% of rN interval as an independent variable. *P* < 0.05 was considered statistically significant. All statistical analyses were performed using the statistical package for social science (SPSS) version 13.0 for Windows.

RESULTS

Clinicopathological parameters of patients

The clinicopathological parameters of patients are summarized in Table 1. There were 467 males and 167 females at the age of 22-87 years (56.1 ± 12.1 years). The tumor diameter was 4.3 ± 1.7 cm. Gastric cancer was found at the lower third (L), lower and middle third (LM),

and middle third (M) of stomach in 521, 41, and 71 patients, respectively. Based on the Japanese Classification of Gastric Carcinoma^[12], 118 were at stage pT1, 174 at stage pT2, and 342 at pT3, respectively. As far as the histological grades were concerned, 140 were differentiated and 494 were undifferentiated. Lymph node metastasis was observed in 439 patients (69.2%). Based on the 5th UICC/TNM pN category^[13], 195 patients (30.8%) were classified as pN0, 232 (36.6%) as pN1, 156 (24.6%) as pN2 and 51 (8.0%) as pN3, respectively. Using the rN category, 195 patients (30.8%) were classified as rN0, 172 (27.1%) as rN1, 156 (24.6%) as rN2, and 111 (17.5%) as rN3, respectively. The median of total LN number was 23 (range 5-61, mean 23.1 ± 8.6) in each patient.

Correlation between LN metastasis and retrieved nodes

Pearson's correlation test showed that the number of metastatic lymph nodes was significantly related with that of retrieved nodes ($r = 0.252$, $P < 0.001$, Figure 1A), but the rN was not related with the number of retrieved nodes ($r = -0.075$, $P > 0.05$, Figure 1B).

Univariate and multivariate survival analysis

The 5-year survival rate of the entire cohort was 57.6%. The clinicopathological variables tested in univariate analysis are shown in Table 2. The factors influencing the 5-year survival rate were tumor diameter ($P = 0.036$), pathology types ($P = 0.003$), depth of invasion ($P < 0.001$), pN category ($P < 0.001$) and rN category ($P < 0.001$). The covariates of gender ($P = 0.991$), age ($P = 0.423$), tumor location ($P = 0.058$) and digestive tract construction ($P = 0.064$) had no significant influence on the survival rate. Multiple survival rates were calculated with the Cox's proportional hazard regression model. In order to confirm the influence of rN, the prognostic factors including tumor diameter, pathology types, depth of invasion and pN category except for rN category in univariate analysis were analyzed by stepwise regression. As a result, two independent prognostic parameters, namely depth of invasion ($P < 0.001$) and pN category ($P < 0.001$), were obtained. When rN category was included in the Cox's regression, the two independent prognostic parameters were also obtained. The risk ratios and their 95% confident interval are listed in Table 3.

Comparison of survival rates between subsets of patients in either LN classification

The subgroups of patients, defined by the pN category, were discriminated with rN category. A significant difference was observed in survival rates between LN stages classified by the rN category or by the pN category ($P < 0.05$, Table 4), whereas no significant difference was found in survival rates between LN stages classified by the pN category or by the rN category (Table 5).

Projected rN impact on overall survival rate

The linear regression model demonstrated that the regression equation of assumed linearity was $y = -0.814x + 74.4$ ($\beta = 0.862$, $P < 0.001$). The regression line of this

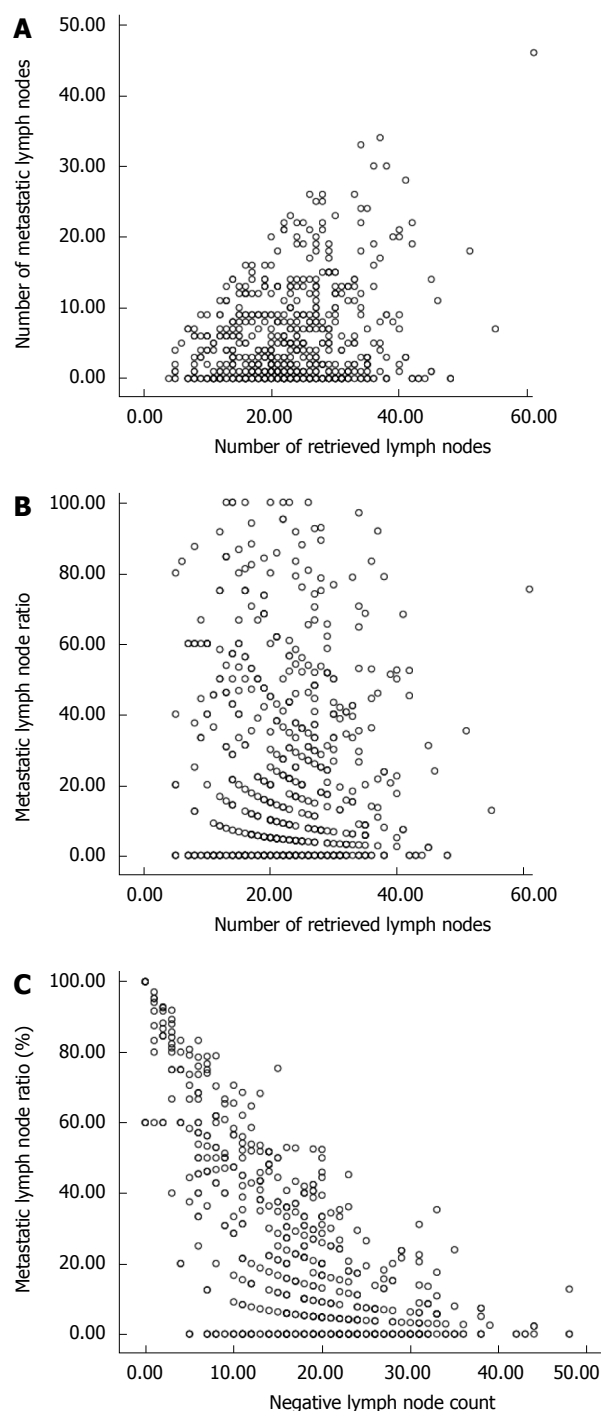


Figure 1 Pearson's correlation tests. A: A significant correlation between the number of lymph node metastases and retrieved lymph nodes; B: No significant correlation between the metastatic lymph node ratio and the number of retrieved lymph nodes; C: A significant correlation between rN and the negative lymph node count.

calculated rN effect on the 5-year survival rate is shown in Figure 2. The hypothetical baseline 5-year survival rate (based on the y-intercept, i.e. 0% rN) was 74.4%. When rN was increased 10%, the calculated 5-year survival rate was decreased by 8.14%.

Correlation analysis between rN and negative LN count

Pearson's correlation test showed that the negative LN

Table 2 Univariate analysis of variables for gastric cancer patients after curative distal gastrectomy

Parameters	n	5-yr survival (%)	χ^2	P
Gender			< 0.001	0.991
Male	467	58.1		
Female	167	56.3		
Age (yr)			0.641	0.423
< 60	370	58.9		
≥ 60	264	56.0		
Tumor size (cm)			4.409	0.036
≤ 4	343	61.0		
> 4	291	53.6		
Tumor location			5.693	0.058
Lower	521	57.7		
Middle	71	63.7		
Lower and Middle	42	45.4		
Digestive tract construction			5.508	0.064
Billroth I	469	60.7		
Billroth II	148	48.5		
Roux-en-Y	17	51.0		
Pathology			9.135	0.003
Differentiated	140	68.1		
Undifferentiated	494	54.6		
Depth of invasion			70.466	0.000
pT1	118	90.6		
pT2	174	64.4		
pT3	342	42.9		
pN category			142.279	0.000
pN0	195	83.3		
pN1	232	59.3		
pN2	156	33.9		
pN3	51	20.0		
rN category			217.494	0.000
rN0	195	83.3		
rN1	172	68.6		
rN2	156	40.7		
rN3	111	17.2		

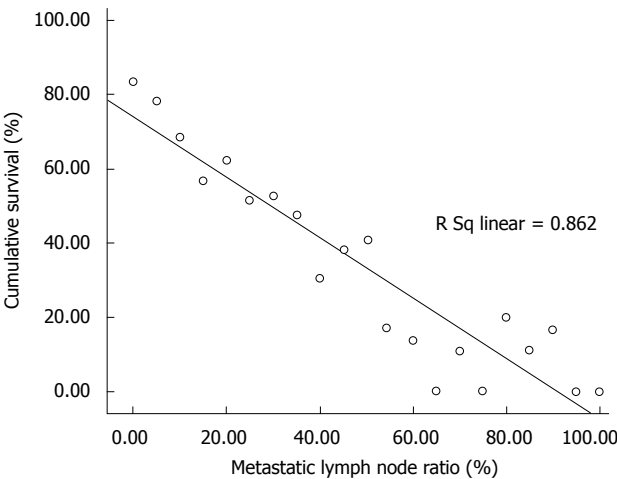


Figure 2 Regression line of rN impact on the 5-year survival rate.

count was negatively correlated with rN ($r = 0.684$, $P < 0.001$, Figure 1C).

DISCUSSION

Accurate staging of gastric cancer is of great importance

Table 3 Multiple stepwise regression analysis with the Cox proportional hazards model

Parameters	β	SE	Wald	P	RR	95% CI
rN excluded						
Tumor size	-0.025	0.117	0.045	0.832	0.976	0.776-1.227
Pathology	0.000	0.161	0.000	0.999	1.000	0.730-1.370
Depth of invasion			27.144	0.000		
pT2 vs pT1	0.956	0.259	13.59	0.000	2.601	1.565-4.325
pT3 vs pT1	1.253	0.247	25.668	0.000	3.500	2.156-5.683
pN category			20.572	0.000		
pN1 vs pN0	0.556	0.188	8.754	0.003	1.734	1.206-2.519
pN2 vs pN0	0.829	0.203	16.673	0.000	2.291	1.539-3.410
pN3 vs pN0	1.02	0.248	16.916	0.000	2.772	1.705-4.507
rN included						
Tumor size	-0.033	0.117	0.078	0.779	0.968	0.769-1.218
Pathology	0.011	0.161	0.005	0.945	1.011	0.738-1.385
Depth of invasion			24.388	0.000		
pT2 vs pT1	0.908	0.263	11.974	0.001	2.48	1.483-4.149
pT3 vs pT1	1.206	0.252	22.827	0.000	3.34	2.036-5.477
rN category			17.927	0.000		
rN1 vs rN0	0.502	0.199	6.349	0.012	1.651	1.118-2.440
rN2 vs rN0	0.842	0.205	16.928	0.000	2.322	1.554-3.468
rN3 vs rN0	0.867	0.234	13.758	0.000	2.380	1.505-3.763

β : Coefficient of regression.

Table 4 Comparison of survival rates for patients classified by rN category or by pN category

N stage	n	rN1		rN2		rN3		P
		n	OS (%)	n	OS (%)	n	OS (%)	
pN1	232	169	68.6	54	36.3	9	22.2	0.000
pN2	156	3	66.7	95	40.8	58	20.4	0.007
pN3	51	NA	NA	7	57.4	44	11.6	0.012

OS: Overall 5-year survival rate; NA: Not applicable.

Table 5 Comparison of survival rates for patients classified by pN category or by rN category

N stage	n	pN1		pN2		pN3		P
		n	OS (%)	n	OS (%)	n	OS (%)	
rN1	147	169	68.6	3	66.7	NA	NA	0.638
rN2	156	54	36.3	95	40.8	7	57.4	0.188
rN3	51	9	22.2	58	20.4	44	11.6	0.257

in clinical practice, especially when adjuvant treatment is considered. Metastasis to lymph nodes is considered the main prognostic factor for gastric cancer patients who undergo radical resection. In 1997, the International Union against Cancer (UICC) proposed that the category of lymph node involvement should be classified according to the number of metastatic lymph nodes. It has been reported that the category based on the number of metastatic lymph nodes is more sensitive than that based on the location of metastatic lymph nodes in prognostic eval-

uation of gastric cancer^[14-16]. However, the UICC/TNM classification (5th edition) only recommended that 15 or more lymph nodes should be examined for the accurate staging of gastric cancer. From the statistical viewpoint, the number of metastatic lymph nodes increases in proportion to the number of dissected nodes, suggesting that pN category can be influenced by the extent of lymphadenectomy. In addition, the pN category may be changed by adding or reducing one positive lymph node, suggesting that gastric cancer classified as N1 after limited lymph node dissection may be classified as N2 or N3 after extensive lymphadenectomy. This phenomenon is the so-called stage migration^[17,18]. Sun *et al.*^[19] reviewed 2159 gastric cancer patients who underwent gastrectomy with a curative intent, and found that there is a closed linear correlation between the number of retrieved LNs and the number of metastatic nodes, while there is no correlation between rN and the number of retrieved nodes, which is consistent with the results of our present study, suggesting that patients with identical rN, even with a different number of detected metastatic nodes, will have a similar outcome, and that rN category is superior to the 5th UICC/TNM classification in terms of minimizing stage migration.

Fukuda *et al.*^[20] compared the pN category of the UICC/TNM in 1997 with a classification based on the metastatic lymph node ratio in patients who underwent R0 resection using the regression analysis, and identified that the metastatic lymph node ratio is the most important prognostic factor for gastric cancer. The results of univariable and multivariable analyses of potential prognostic factors in this study showed that the rN category was one of the independent prognostic parameters. Kunisaki *et al.*^[21] analyzed 1472 early gastric cancer patients undergone curative gastrectomy, and found that rN can identify subsets of patients with a significantly different survival rate within UICC/TNM pN1, pN2 and pN3 categories, suggesting that the outcome of patients with the same number of metastatic nodes, or with a larger number of retrieved LNs, is unfavorable. In the present study, however, a significant difference was observed in survival rates between lymph-node stages classified by the rN category or by the UICC pN category, but no significant difference was found in survival rates between lymph-node stages classified by the UICC pN category or by the rN category, indicating that the UICC/TNM classification can demonstrate stage migration and heterogeneous stratification for disease-specific survival. However, the metastatic lymph node ratio showed less stage migration and homogenous stratification in this study. The N category is currently defined by the number of positive nodes, but routine H&E staining may not exactly reflect the prognosis of lymph node metastasis due to lymph node micro-metastasis^[22-25]. Therefore, the rN category may give more accurate prognostic information on nodal involvement than the current pN category of TNM, thus having a great impact on the selection of patients who may benefit from the adjuvant treatment.

A study from Italy^[11] showed that the number of metastatic lymph nodes is significantly related with rN, and that the more the number of metastatic lymph nodes is, the

higher the rN will be, indicating that gastric cancer patients with a higher rN have a greater risk of death after surgery and a shorter survival time. In this study, when the rN was increased 10%, the calculated 5-year survival rate was decreased by 8.14%, whereas when the rN was decreased 10%, the calculated 5-year survival rate was increased by 8.14%, suggesting that the long-term survival can be improved by reducing rN. Moreover, Person's correlation test showed that rN was negatively correlated with the negative LN count ($P < 0.001$), indicating that removal of more negative lymph nodes can reduce rN.

In conclusion, increased negative LN count can reduce rN, thus improving the long-term survival of patients with gastric cancer after curative distal gastrectomy.

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COMMENTS

Background

Distal gastrectomy is one of the most important operative procedures for gastric cancer. Although the staging system of gastric cancer has been refined step by step, staging techniques never stop updating. So far, few studies are available on the relative contribution of metastatic lymph node (LN) ratio to the prognostic evaluation of gastric cancer after curative distal gastrectomy.

Research frontiers

Some researches have shown that metastatic lymph node ratio (rN) is an excellent predictor for the survival rate of patients with colon cancer, pancreatic cancer, breast cancer and other carcinomas. Some related studies on gastric cancer have also found the potential effect of rN on prognostic evaluation, but no consensus on stratification cutoffs, especially lack of data on gastric cancer after curative distal gastrectomy.

Innovations and breakthroughs

The authors investigated the validity of metastatic LN ratio as a prognostic factor by retrospectively reviewing 634 gastric cancer patients who underwent curative resection (R0) with distal gastrectomy in 1995-2004. The relation between rN and survival was discovered.

Applications

Metastatic LN ratio can provide accurate information on LN metastasis and lymphadenectomy for gastric cancer after curative distal gastrectomy. Moreover, the rN category is an important prognostic factor and increment of negative LN count can reduce rN, thus improving the survival rate of gastric cancer patients.

Peer review

This is an interesting study about the prognostic value of metastatic lymph node ratio (MLR) for 634 gastric cancer patients who underwent curative subtotal gastrectomies at a single institute. The study was well designed.

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Active gastrointestinal bleeding: Use of hemostatic forceps beyond endoscopic submucosal dissection

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Abstract

To the best of our knowledge, this is the first report of the application of hemostatic forceps in active gastrointestinal (GI) bleeding that is not related to endoscopic submucosal dissection. An 86-year-old woman with chronic intake of low-dose aspirin had a Dieulafoy's lesion of the third duodenal portion. Bleeding control with epinephrine injection was unsuccessful. A 60-year-old man presented with a bleeding ulcer in the duodenal bulb. Ten days after combined endotherapy, he had recurrent bleeding from two minimal lesions in the same location. A 66-year-old woman under combined antithrombotic treatment was referred to us for chronic GI bleeding of unexplained origin. Endoscopy revealed active diverticular bleeding in the second duodenal portion. A 61-year-old woman underwent endoscopic mucosal resection of superficial gastric adenocarcinoma, which was complicated with immediate bleeding. In all cases, the blood was washed out using a water-jet-equipped, single-channel gastroscope with a large working channel. The bleeding points were pinched and retracted with hemostatic forceps. Monopolar electrocoagulation was performed using an electro-surgical current generator. Hemostasis was achieved. No complications occurred. In conclusion, hemostatic forceps may be an effective as well as safe alternative approach for active GI bleeding of various origins.

INTRODUCTION

Therapeutic endoscopy has greatly reduced the indications for urgent surgery in cases of gastrointestinal (GI) bleeding. Despite several advances in endoscopic technology, hemostasis can be technically challenging. In addition, the risk of rebleeding, which is associated with high mortality, cannot be eliminated^[1].

Hemostatic forceps is commonly included in the essential accessories for performing endoscopic submucosal dissection (ESD)^[2]. Therefore, we assumed that it could be also applicable in the treatment of active GI bleeding that is not related to ESD.

CASE REPORT

Case 1

An 86-year-old woman was admitted for melena. She had a medical history of ischemic heart disease and chronic intake of low-dose aspirin. She was intravenously administered omeprazole (8 mg/h). Her endoscopic findings were suggestive of a Dieulafoy's lesion that was located in the third duodenal portion at the level of the genu inferius. An initial attempt at bleeding control with injection of epinephrine solution at a dose of 25 mL proved

to be unsuccessful. At that point, the decision was made to use a hemostatic forceps (Coagrasper, FD-410LR; Olympus, Tokyo, Japan). The blood was washed out using a water-jet-equipped, single-channel gastroscope (GIF1T 140; Olympus), with a large working channel (diameter: 3.8 mm), and the hemostatic forceps was advanced through it. The bleeding point was gently grasped and retracted with the hemostatic forceps (Figure 1A). At that point, monopolar electrocoagulation was delivered using an electrosurgical current generator (ICC 200; ERBE, Tübingen, Germany) with forced mode at a setting of 60 W (Figure 1B). The coagulation effect was evaluated by washing out the blood again. The whole hemostatic procedure was carried out with success within 5 min. The bleeding point had to be grasped twice. The total duration of complete coagulation with this setting was about 1 min. The patient tolerated the procedure well. She had no perforation or rebleeding.

Case 2

A 60-year-old man presented with ulcer bleeding in the duodenal bulb. He was managed with injections of epinephrine solution in combination with the placement of two hemoclips (QuickClip II, standard size; Olympus) and intravenous administration of omeprazole (8 mg/h). However, during the next 10 d, he developed recurrent bleeding. A repeat endoscopy demonstrated two simultaneously oozing, bleeding, minimal lesions in the ulcer area. The endoclips remained attached to the site of application. Although high doses of epinephrine solution (60 mL) were injected again, they failed to achieve hemostasis. After that, a VIO 200 ERBE generator was set to soft coagulation mode (Effect 5, 80 W) to coagulate the bleeding lesions with hemostatic forceps. The same endoscope and technique were used (Figure 2). As it was difficult to keep the endoscope stable in the retro pyloric bulb, coagulation was also delivered by applying the tip of the unopened hemostatic forceps to the bleeding points. Prompt and effective hemostasis was achieved without any further episodes of bleeding. Following an uneventful recovery, the patient was discharged home a few days later.

Case 3

A 66-year-old woman was referred to our Endoscopy Unit for chronic GI bleeding of unexplained origin. She was receiving combined antithrombotic treatment with low-dose aspirin and clopidogrel for advanced cardiovascular disease. She was also receiving omeprazole for ulcer prevention. On upper endoscopy, she had signs of active diverticular bleeding of the second duodenal portion. She underwent endoscopic hemostasis by using an ERBE VIO 200 generator with either soft coagulation mode (Effect 5, 80 W) or forced mode (60 W), as well as hemostatic forceps, which grasped and retracted the bleeding point. The procedure was well tolerated and resulted in bleeding control. No late-onset complications were observed.

Case 4

A 61-year-old woman was diagnosed with depressed-

type IIc superficial adenocarcinoma in the stomach, with a diameter of approximately 1.5 cm. Cap-assisted endoscopic mucosal resection (EMR) of her neoplastic lesion was complicated with immediate bleeding. Coagulation of the spurting bleeding vessels using an ERBE VIO 200 generator (soft mode, Effect 5, 50 W) and hemostatic forceps allowed us to complete successfully the EMR procedure (Figure 3). The patient was treated with omeprazole 20 mg *bid*. She did not experience any further complications. She was discharged home within 48 h.

DISCUSSION

Several techniques or devices have been used for the endoscopic control of active GI bleeding. The widely available endoscopic hemostatic options include injection techniques such as epinephrine or sclerosant, ablative ones such as heater probe or argon plasma coagulation (APC), and mechanical methods such as endoclips or endoscopic banding^[1]. In several ulcer bleeding cases, combination endoscopic treatment with epinephrine injection and thermal coagulation or endoclippping has been recommended to achieve better outcomes^[3]. Bleeding control of a Dieulafoy's lesion may require hemostasis with multiple endoscopic treatment methods, whereas clinical experience with endotherapy of duodenal intradiverticular bleeding is limited to a small number of cases^[4,5].

GI bleeding is a major complication of endoscopic surgery. Minor bleeding during ESD can be managed with coagulation using an electrosurgical knife. When that fails, conventional endoscopic means such as hemoclips or APC can be applied^[6]. However, endoclippping may pose several difficulties in the completion of the dissection procedure^[7].

A hemostatic forceps is a valuable tool for performing safely ESD procedures, which are usually demanding^[8]. Its intraoperative use is extended from the coagulation of actively bleeding or oozing vessels to the pre-coagulation of visible, exposed vessels on the artificial ulcer. It does not only aim at facilitating an ESD procedure, so as to continue the endoscopic intervention safely, but also to prevent delayed bleeding as a late-onset complication^[9].

In our small study, a hemostatic forceps was used in the endoscopic management of GI bleeding that was unrelated to ESD. To the best of our knowledge, this is the first report of its application beyond ESD in the English-language literature. Our initial experience demonstrated that monopolar coagulation using a hemostatic forceps as: (1) a second-line therapeutic approach during the same hemostatic procedure; (2) second-line retreatment of two simultaneously bleeding sources after recent combined endotherapy with other modalities; and (3) first-line treatment of a non-iatrogenic bleeding lesion or an EMR-induced lesion, was effective as well as devoid of any post-procedural complications.

Although omeprazole was introduced prior to endotherapy in all our cases, the clinical efficacy of proton pump inhibitors (PPIs) in acute bleeding is a matter of debate. They can reduce the severity of endoscopic signs

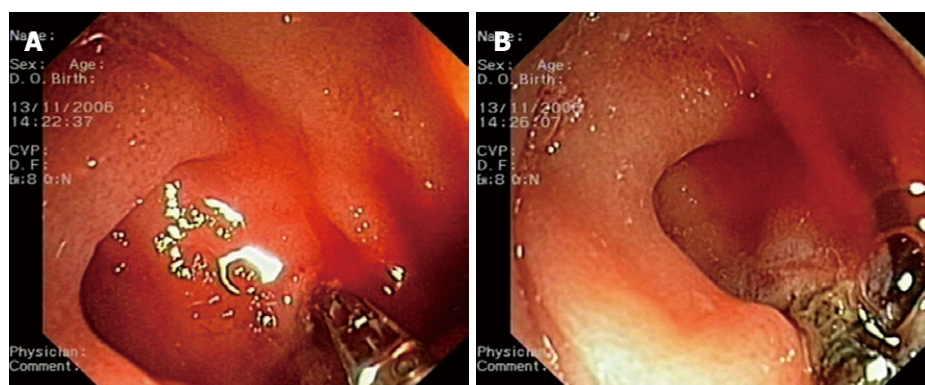


Figure 1 Endoscopic images. A: The bleeding point is pinched with a hemostatic forceps; B: Coagulation delivery at the retracted bleeding point.

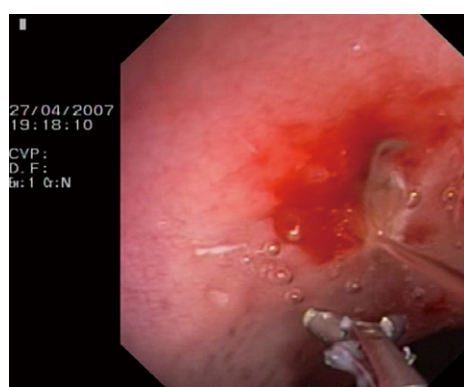


Figure 2 An opened hemostatic forceps while washing out the blood after coagulation.

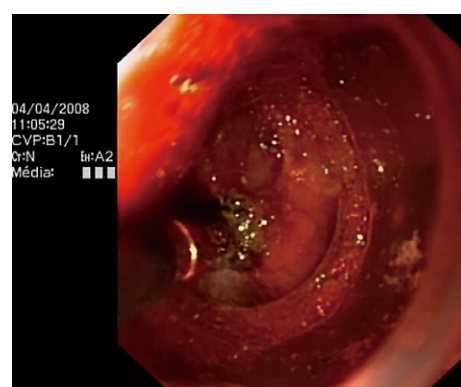


Figure 3 Coagulating the bleeding vessels during cap-assisted endoscopic mucosal resection (EMR).

of recent bleeding and the need for endotherapy, but they have not provided a clear benefit in terms of rebleeding, mortality or the need for surgery^[10]. In addition, the administration of PPIs prior to ESD does not seem necessary for the prevention of postoperative bleeding^[11]. However, they can prevent delayed bleeding more effectively than H₂-receptor antagonists^[12]. Moreover, epinephrine was initially injected in two cases. Epinephrine alone can provide initial hemostasis of 80%-100% in bleeding ulcers, but recurrent bleeding rates range from 6% to 36%. Similar to ulcers, it may only provide temporary hemostasis of bleeding of Dieulafoy's lesions^[13].

Although that hemostatic tools appears to be a reasonable alternative option for the endoscopic treatment of various bleeding lesions, a few practical and technical issues should be taken into consideration before encouraging endoscopists to use it for non-variceal, actively bleeding cases, to achieve definitive hemostasis. To date, the clinical experience of its hemostatic applications has not extended further than those interventionists that perform ESD procedures. However, it is conceivable that excessive coagulation with it might result in delayed perforation. Therefore, one should be aware of the mode of use of a hemostatic forceps, to avoid any complications. The bleeding point should be precisely pinched, retracted and coagulated with a minimal contact area for a minimum time^[7]. In difficult cases, it is also possible to achieve prompt tissue coagulation with the simple contact of an unopened hemostatic forceps, as shown in our second case. However, by pinch-

ing an artery, its use seems more advantageous than other endoscopic techniques, such as APC, for avoiding unnecessary maneuvers and providing accurate hemostasis. To facilitate capture, a hemostatic forceps can be also rotated.

Several experts in ESD procedures usually coagulate the blood vessels using a hemostatic forceps in soft mode^[7,9,14-20]. Others use the coagulation mode of the knife for hemostasis^[21,22]. In our endoscopy unit, we generally use an ERBE VIO 200 generator in soft mode (Effect 5, 50, 80 or 100 W) for a hemostatic forceps. However, an ICC 200 generator, which can be set to soft or forced mode, is the only available option for emergency cases. This is the reason why that was used for the coagulation of the Dieulafoy's lesion and the duodenal intradiverticular bleeding. In the case of the Dieulafoy's lesion, the field was blood-filled and large amounts of water were required to clear it. It took us a long time to grasp the spurting lesion twice and coagulate it with the forced mode to complete the hemostatic procedure. Despite the prolonged duration of coagulation, the bleeding was controlled with no early or delayed-onset complications. It seems doubtful if the use of soft coagulation would be effective in achieving hemostasis. In the case of the large bleeding diverticulum in the second duodenal portion, which was not easily accessible, we continued the coagulation using the forced mode (60 W), because the hemostatic effect of the soft mode with 80 W current was not rapid enough. Although there might be a higher risk of excess tissue injury from thermocoagulation

in thin-walled duodenal diverticula, an endoclip-induced perforation has been reported as the only complication of endotherapy of diverticular bleeding in a recent case series^[5]. Possible explanations include the sharp tip of the hemoclip and excessive air inflation during therapeutic endoscopy^[23].

In conclusion, our case series supports the potential value of the application of a hemostatic forceps in active GI bleeding of various origins. It suggests that it seems to be a reliable and safe therapeutic tool even for those cases of bleeding that are unrelated to ESD, which are difficult to treat with other endoscopic means, although more cases need to be studied before any firm conclusions can be made. Proper techniques and settings should be followed to avoid any procedure-related complications.

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Acute pancreatitis in pregnancy: An unresolved issue

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Abstract

Management of acute pancreatitis in pregnancy is based on expert opinion only, due to geographic and ethnic variations. Nonbiliary causes should be sought as they are associated with worse outcomes. Alcohol as a cause of acute pancreatitis is not rare. Hemoconcentration as a marker of fluid deficit and severity should be predicted with caution and fluid resuscitation should be done carefully by closely monitoring the central venous pressure, cardiac and respiratory system. Hypercalcemia of hyperparathyroidism may be falsely lowered due to hypoalbuminemia or suppressed by magnesium tocolysis.

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Key words: Acute pancreatitis; Pregnancy; Sedation; Nonbiliary cause

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Jain P. Acute pancreatitis in pregnancy: An unresolved issue. *World J Gastroenterol* 2010; 16(16): 2065-2066 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v16/i16/2065.htm> DOI: <http://dx.doi.org/10.3748/wjg.v16.i16.2065>

TO THE EDITOR

We read with interest the article "Acute pancreatitis in

pregnancy" by Pitchumoni *et al*^[1] in December 7, 2009 issue of *World Journal of Gastroenterology*. We agree that recommendation for management of acute pancreatitis is based on expert opinion only, due to geographic and ethnic variations. There are few points which need to be added regarding hyperlipidemic and alcoholic pancreatitis, as well as hemoconcentration as a marker of severity, analgesics and antibiotics dosage in pregnancy.

Nonbiliary causes of acute pancreatitis should be sought as they are associated with worse outcomes. Alcohol as a cause of acute pancreatitis is not rare. Eddly *et al*^[2] studied 89 cases of acute pancreatitis among 305 101 deliveries, and found that alcohol is an etiological factor associated with increased rates of recurrence, 12.3% of patients are in preterm delivery and pseudocyst, and 43% of patients are in the third trimester of pregnancy, 37% of women are nulliparous and 64% have one or more children. The average maternal age at presentation is 26.2 years and higher in patients with alcoholic pancreatitis.

Hyperlipidemic pancreatitis patients account for 4%-6% of all acute pancreatitis patients and have a poor outcome^[3]. Omega-3 fatty acids may prevent recurrent hypertriglyceridemia during pregnancy^[4].

During pregnancy, the maternal blood volume is increased progressively till the 30th wk of gestation, which is 50% greater than normal^[5]. This volume expansion is due to the effects of steroid hormones and elevated plasma levels of aldosterone and renin leading to dilution of red blood cells. So the hemoglobin level for hemoconcentration as a marker of fluid deficit and severity should be predicted with caution and fluid resuscitation should be done carefully by closely monitoring the central venous pressure, cardiac and respiratory system.

Meperidine and fentanyl are the preferred analgesic during pregnancy^[6]. In case of necrotizing pancreatitis, antimicrobial therapy with imipenem/cilastin is often started in view of its high morbidity. The pharmacokinetics of imipenem will change during pregnancy with a larger volume of distribution and faster total clearance from plasma^[7]. The dose adjustment during pregnancy should be considered.

The diagnosis of acute pancreatitis may be com-

plicated during pregnancy due to a mild physiological elevation of amylase, and magnetic resonance cholangio-pancreatography helps to detect acute pancreatitis and its complications^[8]. Very uncommonly, disseminated intravascular coagulation (DIC) can occur in acute pancreatitis. Tang *et al*^[9] studied 103 patients with acute pancreatitis during pregnancy, and found that 1 out of 3 patients with DIC would die in the third trimester of pregnancy.

A comprehensive search for causes should be taken even when the cause of pancreatitis is obscure. It has been reported that triglyceride level declines with bowel rest and hydration, and hypercalcemia of hyperparathyroidism may be falsely lowered due to hypoalbuminemia or suppressed by magnesium tocolysis^[10]. γ -glutamyl transpeptidase (GGTP) levels either are unchanged or fall slightly during gestation. An elevated GGTP level can help us to evaluate the history of alcohol use during pregnancy as patients might not be coming forth, due to stigmata associated with it^[11].

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Practice

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Conference

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26th Pakistan Society of
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14th Pan Arab Conference on
Diabetes PACD14

March 25-28
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12th World Congress of Endoscopic
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April 14-18
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- 6 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303 DOI:10.1136/bmj.325.7357.184]

Volume with supplement

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Statistical data

Write as mean \pm SD or mean \pm SE.

Statistical expression

Express *t* test as *t* (in italics), *F* test as *F* (in italics), chi square test as χ^2 (in Greek), related coefficient as *r* (in italics), degree of freedom as *v* (in Greek), sample number as *n* (in italics), and probability as *P* (in italics).

Units

Use SI units. For example: body mass, *m* (B) = 78 kg; blood pressure, *p* (B) = 16.2/12.3 kPa; incubation time, *t* (incubation) = 96 h, blood glucose concentration, *c* (glucose) 6.4 ± 2.1 mmol/L; blood CEA mass concentration, *p* (CEA) = 8.6 $24.5 \mu\text{g/L}$; CO₂ volume fraction, 50 mL/L CO₂, not 5% CO₂; likewise for 40 g/L formaldehyde, not 10% formalin; and mass fraction, 8 ng/g, etc. Arabic numerals such as 23, 243, 641 should be read 23 243 641.

The format for how to accurately write common units and quantum numbers can be found at: http://www.wjgnet.com/1007-9327/g_info_20100315223018.htm.

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Standard abbreviations should be defined in the abstract and on first mention in the text. In general, terms should not be abbreviated unless they are used repeatedly and the abbreviation is helpful to the reader. Permissible abbreviations are listed in Units, Symbols and Abbreviations: A Guide for Biological and Medical Editors and Authors (Ed. Baron DN, 1988) published by The Royal Society of Medicine, London. Certain commonly used abbreviations, such as DNA, RNA, HIV, LD50, PCR, HBV, ECG,

WBC, RBC, CT, ESR, CSF, IgG, ELISA, PBS, ATP, EDTA, mAb, can be used directly without further explanation.

Italics

Quantities: *t* time or temperature, *c* concentration, *A* area, *l* length, *m* mass, *V* volume.

Genotypes: *gyrA*, *arg 1*, *c myc*, *c fos*, etc.

Restriction enzymes: *EcoRI*, *HindI*, *BamHI*, *Kho I*, *Kpn I*, etc.

Biology: *H. pylori*, *E. coli*, etc.

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