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AIMS AND SCOPE

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EDITORIAL

Field cancerisation in colorectal cancer: A new frontier or pastures past?

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Abstract

Despite considerable advances in our understanding of cancer biology, early diagnosis of colorectal cancer remains elusive. Based on the adenoma-carcinoma sequence, cancer develops through the progressive accumulation of mutations in key genes that regulate cell growth. However, recent mathematical modelling suggests that some of these genetic events occur prior to the development of any discernible histological abnormality. Cells acquire pro-tumourigenic mutations that are not able to produce morphological change but predispose to cancer formation. These cells can grow to form large patches of mucosa from which a cancer arises. This process has been termed "field cancerisation". It has received little attention in the scientific literature until recently. Several studies have now demonstrated cellular, genetic and epigenetic alterations in the macroscopically normal mucosa of colorectal cancer patients. In some reports, these changes were effectively utilised to identify patients with a neoplastic lesion suggesting potential application in the clinical setting. In this article, we present the scientific evidence to support field cancerisation in colorectal cancer and discuss important limitations that require further investigation. Characterisation of the field defect is necessary to enable early diagnosis of colorectal cancer and identify molecular targets for chemoprevention. Field cancerisation offers a promising prospect for experimental cancer research and has potential to improve patient outcomes in the clinical setting.

Key words: Colorectal cancer; Carcinogenesis; Biomarkers; Epigenetics; Synchronous

Core tip: There is a great deal of interest in developing non- invasive tests that are able to detect colorectal cancer in the asymptomatic population. Most current research activity is focussed on investigating the biological changes found in tumour tissue itself. This

review evaluates the biological alterations found in the normal mucosa around a neoplastic lesion and critically analyses the concept of field cancerisation. It highlights recent advances and identifies important molecular targets that could play a role in early colorectal carcinogenesis. In particular, the available evidence for field cancerisation is scrutinised and future avenues for further scientific enquiry are outlined.

INTRODUCTION

Colorectal cancer is the third most common cancer worldwide, affecting 1.36 million people[1] and is the largest killer amongst non-smokers in the United Kingdom[2]. The greatest chance of cure is with disease confined to the bowel wall, hence, early diagnosis and prompt treatment are important[3]. It is generally accepted that cancers develop through accumulation of mutations in key genes[4,5]. Traditionally, a three step process comprising initiation, promotion and progression was proposed[6,7]. Later, it became apparent that the colonic epithelium undergoes an ordered sequence of genetic events with corresponding histological abnormalities on its journey to cancer formation[8]. However, several recent analyses have revealed that the mutations found in colorectal cancer occur long before the onset of a clinically visible lesion[9,10]. In many cancers, cells have been shown to acquire pro tumorigenic mutations that are not able to produce morphological change but predispose to subsequent malignant transformation[11-14]. These cells can expand creating patches of mucosa which have an increased risk of developing into cancer. This process has been described as "field cancerisation"[15,16]. It is a concept that has previously received little attention in the scientific literature. Most studies investigating colorectal carcinogenesis have focused solely on the cancer tissue or assumed that the mucosa adjacent to the neoplastic lesion is normal[17,18]. However, based upon the field cancerisation theory, characterization of the biological events that occur in the "mucosa at risk" could enable identification of the earliest steps in colorectal cancer formation. This could aid the scientist in discovery of how neoplasia develops and enable the clinician to develop more reliable tests to risk stratify patients. This article discusses the evidence for field cancerisation, its limitations and its potential clinical application to improve patient outcome.

FIELD CANCERISATION THEORY - DEFINITION AND MECHANISM

Field cancerisation was first described by Slaughter in 1953 for head and neck squamous cell carcinoma[15]. It was based upon the observation that a statistically significant proportion of oral cancers developed in multifocal areas and often had histologically abnormal cells surrounding the cancer. Since its inception, field cancerisation has been applied to several other cancers including cancer of the oesophagus[19], stomach[20], lung[21], bladder[22], pancreas[23] and skin[24]. In the colon, it has been described as "the process whereby colonic epithelial cells acquire pro-tumorigenic mutations that are insufficient to cause morphological change but which predispose to tumour"[25]. Multiple mechanisms have been proposed to explain how a patch of altered mucosa forms around a cancer (Figure 1). Genetic analysis has revealed that the field defect consists of a clonal proliferation of a mutated cell. Based on this observation, it was proposed that a mutation or epigenetic alteration in stem cells gives it reproductive advantage so that it generates clonal descendents that outcompete neighbouring stem cells[26]. These stem cells replace other stem cells through the process of niche succession[27] and eventually, the entire crypt is occupied by the mutant or epigenetically altered cells[28,29]. Crypt fission of this mutated or epigenetically altered crypt results in a patch defect (Figure 1A). Other mechanisms include alteration in the adjacent mucosa by the presence of the tumour itself or as a result of chemicals released by the tumour[30,31] (Figure 1B). Whilst others have proposed that, like oral cancer[32,33] and bladder cancer, colonic epithelial cells shed in the lumen at one place could migrate to another site, seed and give rise to synchronous cancer[16] (Figure 1C). Field changes could be more widespread which has led some authors to suggest that dietary exposure, for example, vitamin B and folate, could alter the methylation state of the entire colonic mucosa predisposing it to cancer[25]. Further investigation is required to elucidate the precise biological mechanism and causal events that underly field cancerisation. Despite this, however, a large body of evidence exists that supports the presence of a field defect in colorectal cancer.

EARLY EVIDENCE BASED ON THE TRANSITIONAL MUCOSA

The term "transitional mucosa" was used to describe the patch of mucosa around a cancer that was abnormal compared to the rest of the mucosa. Although field cancerisation had not been formally proposed at the time, these were some of the early studies supporting the concept.
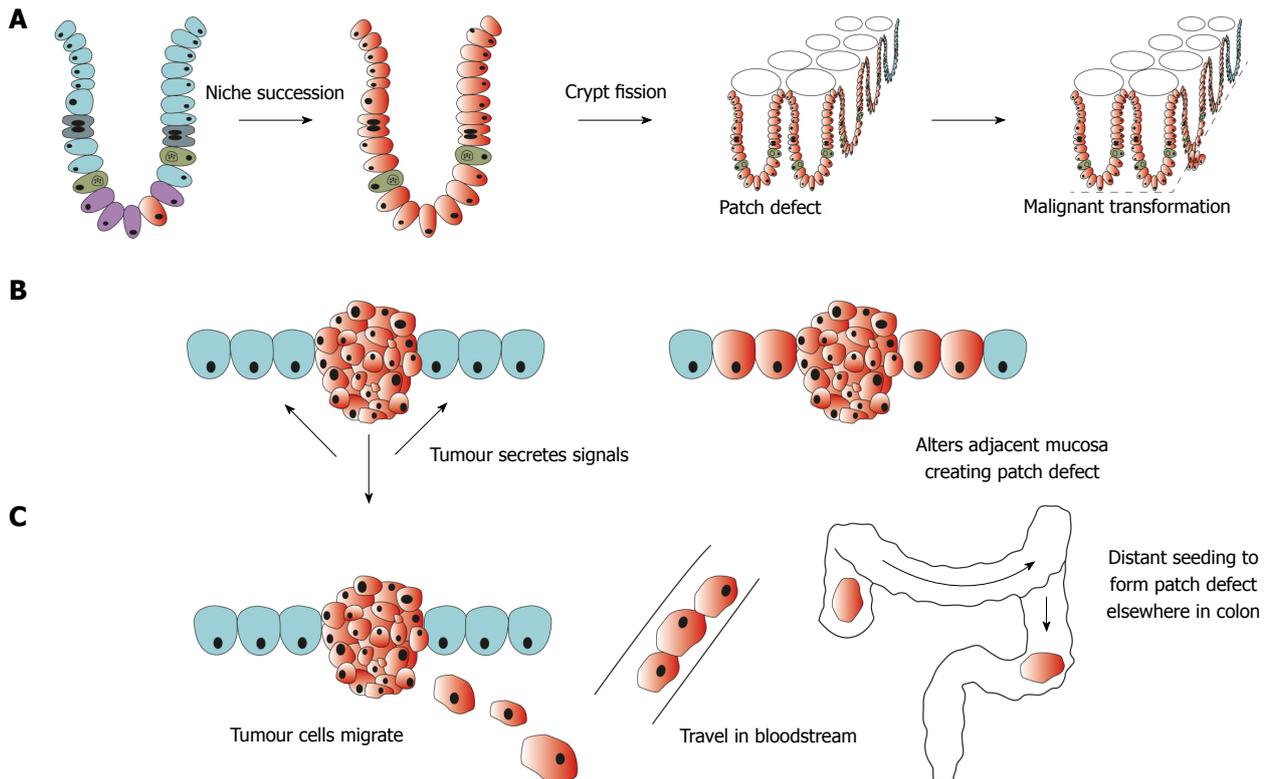
Figure 1 Schematic representation of proposed mechanisms for formation of field defect. A: A mutation or epigenetic alteration in a stem cell (depicted in red) is inherited by all cells within the crypt through niche succession. Crypt fission results in several crypts becoming biologically altered creating a patch defect. Further mutation within this field of altered mucosa leads to malignant transformation; B: Tumour secretes chemical signals that alter the adjacent mucosa resulting in a field defect; C: Malignant cells shed from a tumour travel in the bloodstream and seed in a distant site rendering the mucosa susceptible to malignant transformation.

In 1969, Filipe described abnormal histochemical properties in the transitional mucosa[34] with a decrease in sulfomucins, usually found in normal colorectal tissue and concurrent increase in sialomucin content. Sulfomucins protect against luminal insults by increasing mucus viscosity which increases the resistance against bacterial degradation and microbe adhesion[35]. Replacement by sialomucin has been previously observed in colorectal cancer tissue. Based on the finding of increased sialomucin content in the transitional mucosa, Filipe proposed that these changes could represent an early stage of carcinogenesis[36]. Further exploration using light microscopy revealed that there were alterations in crypt morphology and cell type within this mucosa. Saffos and Rhatigan[37] found an increase in the length of the crypts, increased distension and branching of the crypts and an increase in the number of goblet cells in the crypt. They were unable to demonstrate similar changes in tissue samples taken along the rest of the colon in these patients. They concluded that these changes were confined to the rim around the tumour.

In the late 1970's, several investigators characterised the ultrastructural properties of the transitional mucosa[38,39]. They found that the crypts were larger in diameter and composed of larger cells with larger nuclei compared to those found in the normal colon. There was also a change in cell distribution with an increase in mature goblet cells in the lower half of the crypt and an increase in immature goblet cells and "intermediate" cells in the upper half of the crypt.

Subsequent studies have highlighted alterations in the nuclear morphology of cells in the transitional mucosa, often as far as 50 mm from the tumour[40,41]. Many nuclear features were found to differ in the transitional mucosa compared to that of healthy controls including total optical density, nuclear area, chromatin texture, chromatin coarseness, average optical density and increased tendency of peripherally placed chromatin[42]. However, because of the considerable inter-patient and inter gland variation in these parameters, the authors cautioned against the use of any single feature to identify those at risk. Instead, it was suggested that a combination of parameters be used to develop a tool for risk stratifying patients. More recent studies, using computer based karyometric analysis[43] or electron microscopy[44] have confirmed these earlier findings. Although there are differences in nuclear appearance of cells in the transitional mucosa, the variability seen between patients and between samples taken from the same patient preclude the use of nuclear analysis as a discriminant factor to risk stratify patients. Investigators have therefore sought to identify other biological changes that could be indicative of a field defect.
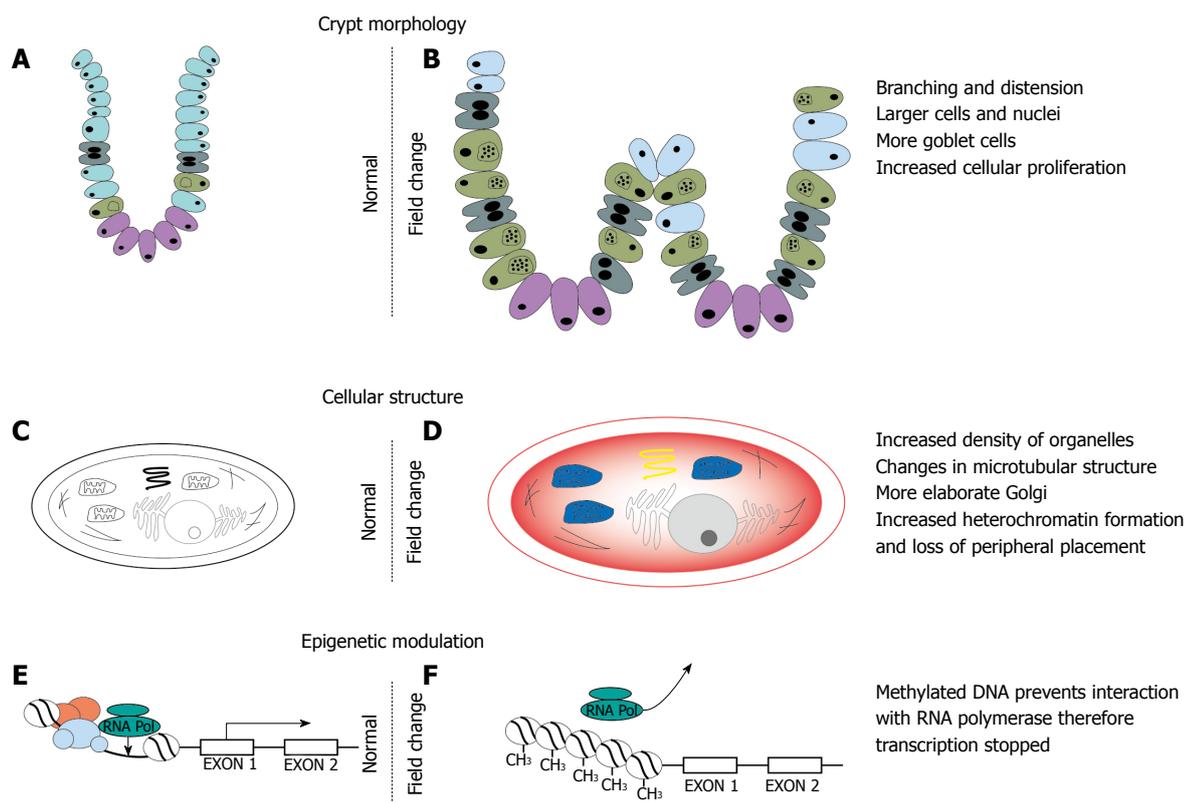
Figure 2 Changes in crypt morphology, cellular ultrastructure and epigenetic modulation in the field defect. A, B: Changes in crypt morphology characterised by increased branching and distension of crypts, increased cell division and a change in proportion of cells with increase in goblet cells; C, D: Changes in the cell cytoskeleton, organelles and nuclear composition; E, F: Epigenetic modulation of DNA leading to transcriptional silencing of certain genes involved in regulation of cell division, apoptosis and DNA repair.

SUPPORTING EVIDENCE BASED ON CANCER BIOLOGY

Colorectal cancer usually arises from multiple dysfunctional cellular processes which enable the cell to evade homeostatic signals and grow in an autonomous manner. Similarly, alterations at the genetic, epigenetic and protein level in a number of cellular processes and function have been described in the colonic field (Figure 2).

Cellular proliferation and apoptosis

Many studies have demonstrated that there is an increase in cellular proliferation and concurrent reduction in apoptosis in the macroscopically normal appearing mucosa around a malignant lesion[45-48]. Using 3H thymidine autoradiography, the rate of proliferation in normal mucosa was found to be significantly higher in patients harbouring a colorectal cancer or large adenoma compared to those with small adenomas or healthy individuals[45]. These changes were most prominent in the upper third of the mucosal crypt[47,48] and could potentially be utilised as a predictive marker to identify patients with a neoplastic lesion[49,50]. Not only have these changes in proliferation been linked to the presence of neoplastic lesions but they have also been shown to be predictive for the risk of polyp recurrence[49]. Epithelial cell proliferation in

the macroscopically normal rectal mucosa of patients with and without polyps was assessed. The crypt was divided into five compartments along the longitudinal axis and the labelling index was calculated for the entire crypt and each of the five compartments. In the 22 patients whose polyps recurred, the upper compartments 3, 4 and 5 demonstrated a significantly higher labelling index compared to the 33 patients without recurrence. There was an upward shift in the proliferative zone of the crypt that was associated with polyp recurrence. Interestingly, there was no difference in the labelling index between the first and second biopsy suggesting that an underlying genetic defect or persisting environmental insult may have been responsible for the field defect detected in this study.

Genetic and epigenetic modulation

The genetic and epigenetic abnormalities found in colorectal cancer have also been shown to extend into the macroscopically normal mucosa supporting the field cancerisation theory. Early studies using flow cytometry confirmed that mucosa adjacent to diploid cancers is diploid in nature and in patients with aneuploid tumours, is often aneuploid[51,52]. Similarly, epigenetic modulation of genes has been found to differ in patients with classical adenomas compared to

those with serrated polyps[53]. These lesions represent the two different pathways to colorectal cancer. Classical adenomas are linked to carcinogenesis that occurs along the CIN (chromosomal instability) pathway compared to serrated polyps which are the precursor for the CpG island methylator phenotype (CIMP) pathway. The authors demonstrated that age-related methylation was inversely associated with the presence of classical adenomas compared to methylation of cancer specific genes that was more likely in patients with serrated polyps. This suggests that the background mucosa of these patients had detectable epigenetic differences that conferred a predisposition to a specific pathway of cancer prior to the development of any discernible histological abnormality.

Differences in expression of genes across a wide number of cellular processes have been implicated in the field defect and may potentially play a role in early tumourigenesis. Based on a 15-gene signature encompassing genes that play a role in the APC/Beta catenin, NFκB, cell cycle and inflammation pathways, significant alterations in gene expression were found in the normal mucosa of human cancer resection specimens, often extending into the margins[54]. In a further study, based on analysis of a macroscopically normal appearing rectosigmoid biopsy, this 15-gene signature could discriminate between individuals with and without polyps[55].

Epigenetic silencing of genes has been implicated in both mismatch repair deficient and CIMP cancer. Several investigators have reported epigenetic changes in the normal colonic mucosa in patients with cancer. Reduced protein expression of the DNA repair proteins, mismatch repair endonuclease (Pms2), DNA excision repair protein (ERCC1) and DNA excision repair protein XPF (ERCC4) was found up to 10 cm longitudinally from the tumour edge supporting the field theory[56]. In a study by Shen *et al*[57], O-6-methylguanine-DNA methyltransferase (MGMT) methylation was found in the normal adjacent mucosa of 50% patients whose tumours also had methylated MGMT compared to only 6% when MGMT was not methylated in the tumour. In 10 out of 13 patients, methylation changes were seen as far as 10 cm away from the tumour and hypermethylation was more pronounced at 1 cm compared to 10 cm. These findings raise the possibility that MGMT methylation may play a role in the field defect representing an early step in the carcinogenesis pathway of tumours with hypermethylated MGMT. Similarly, others have also reported that MGMT hypermethylation is more likely to be found in the surrounding mucosa of microsatellite unstable tumours compared to microsatellite stable cancers[58,59]. Other studies have investigated the methylation profile of combinations of multiple genes confirming that the apparently normal mucosal field has undergone significant epigenetic change that could represent the earliest stages of colorectal cancer development[60,61].

Epigenetic modulation through methylation of micro-RNAs (miR) may also contribute to a field defect. Grady *et al*[62], found expression of hsa-miR-342, a microRNA encoded in an intron of the gene EVL, is commonly suppressed in human colorectal cancer. They found methylation at the EVL/has-miR-342 locus in 56% of histologically normal mucosa from patients with colorectal cancer compared to only 12% of patients without colorectal cancer. Similarly, methylation of miR-124a and miR-34b/c in the histologically normal mucosa, was observed in 59% and 26% of patients with cancer but was not found in patients without cancer or Ulcerative Colitis[63]. In another study, the level of methylation of miR-137 was found to be higher in the macroscopically normal mucosa in cancer patients compared to healthy controls[64] (10.3% *vs* 7.7%, P = 0.035). These findings suggest that changes at the micro-RNA level could also play an important role in field defect around a tumour.

Although, there are considerable genetic and epigenetic alterations in the "normal" mucosa surrounding a cancer, it is not yet clear which of these changes are most important. Epigenetic changes are particularly interesting as they can be modified by changes in diet or pharmacological agents unlike the germline mutations often linked with cancer. Elucidation of the specific epigenetic marker that underlies the field defect could enable specific chemopreventative agents to be designed to target these early changes prior to the development of any precancerous lesions such as adenomas.

FIELD CANCERISATION - POTENTIAL PITFALLS

Although there is sufficient evidence to support the field cancerisation theory in colorectal cancer, a number of pertinent questions remain.

Pre malignant change or a secondary phenomenon?

Similar changes in crypt and cellular morphology to those observed in the transitional mucosa have also been described in mucosa adjacent to squamous cell carcinoma of the anus[65,66], sarcoma of the colon[66,67] and in non-neoplastic lesions such as endometriosis[67]. This has led to the conclusion that these alterations do not represent premalignant change but rather, a reactive phenomenon in response to tumour or non-neoplastic injury such as that induced by inflammation or necrosis. Early studies showed that the width of the transitional mucosa was related to the size of the tumour[36] where it became larger with increasing stage of the tumour. However, if these changes precede cancer formation, it would be expected that as a tumour grows, it replaces the transitional mucosa from which it arose resulting in a smaller area containing the initial cellular changes. Therefore, one would expect that

the area of the transitional mucosa would be inversely related to the size of the tumour and the changes seen in the transitional mucosa would be demonstrated throughout the entire colonic mucosa. Investigators were unable to provide evidence to support this hypothesis which led to the proposal that the transitional mucosa represents the neoplastic phenotype, however, is likely to be a secondary phenomenon as a result of factors released by the tumour. This may also explain how it was found adjacent to non-neoplastic lesions which would be expected to release similar growth factors, in response to the inflammatory process, to those secreted by the tumour. However, subsequent studies have successfully correlated genetic mutations found in the tumour with those demonstrated in the surrounding mucosa confirming that these tumour cells share a common clonal origin. Also, the reports of these changes persisting despite removal of the offending lesion[49,68] suggest that this is a primary phenomenon rather than reactive change in response to the presence of a neoplastic lesion. Hence, these field changes are most likely to be pre-malignant events that represent some of the very early steps along the path to colorectal cancer.

How far along the colon does a field defect extend?

If the macroscopically normal mucosa is biologically altered in response to the tumour, it would be limited to the area immediately in the vicinity of the tumour. In an early study investigating the field defect based on histochemical analysis, transitional mucosa was found in 90/100 cases, extending as far as 17 cm from the tumour[69]. The change in sialomucin content that was identified in the transitional mucosa was found at the resection margins and in a subset of patients, it was a direct extension of the zone of altered mucosa surrounding the tumour. Several other studies have described biological changes in mucosa as far as 10 cm from the tumour[43,70,71] whilst others have reported that the field defect extends as far as the rectum in these patients[49,72]. Some authors have shown that the hypermethylation changes observed in the field defect are more pronounced 1 cm away from the tumour compared to 10 cm[57] whereas others were unable to corroborate their findings with distance from the lesion[54] Some investigators have proposed that the field of altered mucosa does not occur in a contiguous manner but occurs in discrete patches. Bernstein *et al*[46] measured the bile salt induced apoptosis rate in 68 patients (17 colorectal cancer, 37 adenoma and 14 with neoplasia). Biopsies were taken 20 cm from the anal verge, caecum and descending colon. Site to site variability, both between regions of the colon and adjacent biopsies was greater than the inter-patient variability for individuals with a history of colorectal cancer suggesting that there was ''patchiness'' of the susceptibility of regions of the colon to bile acid

induced apoptosis. In other words, the field defect was not continuous along the entire colon; there were areas which showed greater changes in rate of apoptosis, however, these areas did not correspond to site of previous neoplasia. If these changes occur as a consequence of the interplay between an underlying genetic predisposition and environmental insult, patchy mucosal alterations could be explained by differences in luminal factors along the colon. Hence, there would be areas that are more susceptible to carcinogens found in the lumen or areas where cells are defective at protecting against the harmful effects of carcinogens. Further study is required to characterise the nature of the field defect and examine the causative agents responsible.

Are these alterations passengers or drivers in carcinogenesis?

Colon cancers have been found to contain a median of 76 non-silent sequence mutations of which, only 15 represent driver mutations[73]. These are mutations in key oncogenes or tumour suppressor genes that confer a selective advantage to the cell enabling it to divide uncontrollably and survive in unfavourable conditions. In comparison, passenger mutations occur during normal cell division that takes place to replenish the colonic epithelium and have no role in driving carcinogenesis. They can be over-represented in cancer tissue due to aberrant DNA repair mechanisms and defective anti-apoptotic machinery. Similarly, it is difficult to discriminate which of the molecular changes found in the field defect are integral in driving cancer formation from those that are innocent bystanders. Roy *et al*[74], used 4-dimensional elastic light scattering fingerprinting (4D-ELF) to probe the nanoarchitecture of colonocytes in the Azoxymethane treated rat model *vs* the saline treated rat. They measured 4D-ELF at different time points and correlated the changes observed with the emergence of the aberrant crypt focus. Their finding that changes in 4D-ELF were apparent 2 wk prior to development of aberrant crypt foci (ACF) and that they correlated both spatially and temporally with subsequent development of ACF suggests that these changes were integral in early colorectal cancer formation.

Mathematical modelling suggests that it is not the rate of mutations which is important but rather the selection of clones of cells with specific advantages in autonomous growth that drives malignant transformation[75]. It has also become apparent that this selective advantage is not conferred by mutations in one or few genes but is the accumulated benefit of several genes that have low individual selective advantage[76]. Therefore, it is crucial that mechanistic studies are conducted based upon the gene targets found in the mucosal field to discern the driver mutations from those that are innocent bystanders.

CLINICAL APPLICATION

Despite some of these shortcomings, field cancerisation in colorectal cancer is a promising prospect upon which to develop potentially diagnostic and therapeutic modalities. Elucidation of the underlying molecular mechanism could enable more accurate screening tests to be designed that are able to identify individuals with a malignant lesion. Current research is focused on developing tools that are capable of identifying patients with colorectal cancer based on analysis of a "normal" biopsy from a distant site. Using light scattering technology, three manifestations of tissue alteration in the colonic field have been shown[77]: changes in microcirculation [early increase in blood supply (EIBS)], changes in the extracellular matrix from abnormal cross linking and alignment of collagen fibres [as assessed by low coherence backscattering (LEBS)] and differences in the internal structure of colonocytes [as assessed using partial wave spectroscopy (PWS)]. EIBS can be detected within 30 cm of a polyp using a spectroscopic probe on 222 patients undergoing colonoscopy. The magnitude of EIBS correlated with the size and proximity of the adenoma. Based on a rectal biopsy, EIBS was found to be increased in 50% patients with an adenoma. A logistic regression model using EIBS, mucosal oxyhaemoglobin and patient age gave a sensitivity of 83% and specificity of 82% with an AUC of 0.88 for the detection of advanced adenomas[72]. A progressive change from control patients to those with advanced adenomas was demonstrated using LEBS parameters[78]. LEBS was able to discriminate between patients with and without advanced adenomas with 100% sensitivity, 80% specificity and an AUC of 0.90. An *in vivo* study was subsequently performed where a fibre optic probe was used to measure LEBS parameters in the rectum of 574 subjects[79] and was shown to reliably identify patients with an advanced adenoma. Similarly, PWS has been shown to correlate with risk of developing colorectal cancer[80]. The differences in EIBS, LEBS and PWS parameters detected in these studies were not confounded by demographics, presence of non-neoplastic lesions or site of adenoma suggesting true potential for development into a screening tool.

The presence of a field defect may indicate a higher risk of metachronous neoplastic lesions and could help to identify which patients require more radical surgery. Field cancerisation could also be utilized to ascertain risk of disease progression, hence, could enable risk stratification of patients with inflammatory bowel disease or a family history of colorectal cancer. However, the most exciting use of field cancerisation theory is its potential application in chemoprevention. Individuals at risk of malignancy could be identified based on field defects in their mucosa. Pharmacological therapy could be developed, targeted at the underlying signaling pathway, to modify the field change and reduce the risk of subsequent malignant transformation.

FUTURE DIRECTIONS

There is considerable evidence in the literature to support the field cancerisation theory in colorectal cancer. However, important questions about the underlying mechanism and extent of the field defect require further investigation before it can be applied in a clinical setting.

In other conditions that results in an increased risk of colorectal cancer such as Ulcerative Colitis, mutations in KRAS, CDNK2A (p16) and TP53 have been detected in non-tumour, non-dysplastic and dysplastic epithelium. In two patients, these changes were detected 4 years before the development of tumour suggesting that they represent some of the very early genetic events that led to colorectal carcinogenesis[81].

Furthermore, a recent study using a mouse colitis model showed persisting epigenetic alteration in the mucosa despite removal of the toxic insult that initiated it[68]. Lessons learnt from these studies could shed light upon the interactions that take place between the environment and the mucosa in the journey along the cancer pathway.

Cancer research has traditionally focused on characterization of the genetic/epigenetic events that occur in a malignant cell to understand the processes that contribute to malignancy. This approach seems somewhat backwards, especially in a disease where early intervention is important. Future research needs to identify early events that occur along the cancer pathway. Hence, a paradigm shift in scientific enquiry is required which focusses on the temporal sequence of mutational events to elucidate early molecular targets in colorectal cancer. The field cancerisation theory offers such an approach whereby, based on the changes occurring in the surrounding mucosa, the initial events leading to colorectal carcinogenesis can be discerned.

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EDITORIAL

Helicobacter pylori eradication in gastric diffuse large B cell lymphoma

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Abstract

Diffuse large B cell lymphoma (DLBCL) of the stomach is a heterogenous disease. There are tumors without histological evidence of mucosa-associated lymphoid tissue (MALT) lymphoma, which are classified as pure or *de novo* DLBCL and those with evidence of MALT, which are classified as DLBCL (MALT). The association between *Helicobacter pylori* (*H. pylori*) and gastric MALT lymphoma and remission with *H. pylori* eradication was shown in the 1990s. In recent years, scientists from Taiwan and others have shown that high-grade gastric lymphomas may be dependent on *H. pylori* and eradication of this microorganism is effective in these cases. This entity is biologically distinct from *H. pylori* (-) cases and has a better clinical outcome. There are sufficient data about the complete remission in some of these cases with brief treatment with antibiotics. With this strategy, it is possible to save some of these cases from the harmful effects of standard chemotherapy. It is time to treat these cases with *H. pylori* eradication. However, strict histopathological follow-up is crucial and histopathological response must be evaluated according to the scoring system proposed by Groupe d'Etude des Lymphomes de l'Adulte. If there is no sufficient response, chemotherapy must be given immediately. These results suggest that *H. pylori* dependency and high-grade transformation in gastric MALT lymphomas are distinct events.

Key words: *Helicobacter pylori*; Eradication; Gastric diffuse large B cell lymphoma

Core tip: The association between *Helicobacter pylori* (*H. pylori*) and gastric mucosa-associated lymphoid tissue lymphoma has a long history and *H. pylori* eradication is the standard of care in these cases. In recent years, it has been shown that high-grade, early-stage gastric lymphoma may be dependent on *H. pylori* and eradication of this microorganism may be curative in some of the cases with gastric diffuse large B cell lymphoma. However, chemotherapy is a standard approach in cases unresponsive to *H. pylori* eradication.

Diffuse large B cell lymphoma (DLBCL) of the stomach is a heterogenous disease entity and includes lymphomas with or without mucosa-associated lymphoid tissue (MALT) features in WHO classification[1]. Tumors without histological evidence of MALT lymphoma, dense infiltration of centrocyte like cells in the lamina propria, and typical lymphoepithelial lesions are classified as pure or *de novo* DLBCL and those with evidence of MALT are classified as DLBCL (MALT)[2,3]. The association between *Helicobacter pylori* (*H. pylori*) and gastric MALT lymphoma and remission with *H. pylori* eradication (HPE) is an old story. In recent years, this association has been shown in *de novo* gastric DLBCL and HPE is effective in this disease. There are sufficient data about HPE in these cases and it is time to treat these cases with 2 wk antibiotics and to save these cases from the harmful effects of standard chemotherapy. For this aim we can use large-scale data about HPE for MALT and we must be ready to draw an analogy from gastric MALT lymphoma for gastric DLBCL.

In the early 1990s, a high incidence of *H. pylori*-related gastritis was described by Wotherspoon in patients with gastric MALT lymphoma. Complete histological remission of gastric MALT lymphoma by HPE was detected in five of six cases in 1993[4,5]. In the past 20 years, complete pathological response (pCR) in *H. pylori (+)* gastric MALT lymphoma has been reported in 47%-100% of these patients[6-9]. The most important factors in response to HPE are tumor stage, depth of invasion, and localization, as well as geographic region[8-10]. Lymphoma regression has been reported in > 80% of cases confined to the submucosa, and in about 50% of cases with deeper invasion. Distal lesions are more responsive to HPE than proximal lesions (92% *vs* 76%). A higher response rate has been found in Asian than western populations (84% *vs* 74%). These factors may also determine the response to HPE in gastric DLBCL. Another important point is the use of highly heterogenous pathological response criteria after HPE in MALT lymphoma studies. Scoring for histopathological response proposed by Groupe d'Etude des Lymphomes de l'Adulte, which is European Gastro-Intestinal Lymphoma Study consensus report, is the recommended system for MALT lymphomas and this scoring system must be used also for gastric DLBCL[9,11].

In previous years, it was believed that MALT is dependent on *H. pylori* and this dependency is lost with high-grade transformation. However, it has been shown that low-grade MALT lymphomas and high-grade gastric MALT lymphomas remain *H. pylori* dependent and potentially can be cured by HPE only[12-14]. In an early German study, pCR by HPE was reported in seven of eight cases with high-grade gastric MALT in 2001[12]. Long-term results of HPE in early-stage gastric high-grade transformed MALT lymphoma were reported by Chen *et al*[15] in 2005. pCR was reported in 64% of cases. After these successful results about the regression of HG lymphomas with

HPE, the first prospective study was reported again by Chen *et al*[14]. The study of Chen *et al*[14] reported treatment with HPE in 16 cases with stage IE, high-grade MALT lymphomas between 1995 and 2000. *H. pylori* was eradicated in 15 of the 16 cases and rapid tumor regression with disappearence of large cells was seen in 10 of these 15 cases. The response was found to be adversely affected by the depth of tumor invasion, as reported in low-grade MALT lymphomas with an attractive pCR rate (66.6%) with long duration (31.2 mo)[3]. This study suggests that high-grade transformation is not associated with loss of *H. pylori* dependency and disappearance of large cells with HPE. This finding is similar to the results of a study from Japan in which pCR was found in four of six cases with high-grade MALT lymphoma restricted to the mucosa/submucosa, but in only one of four cases with invasion beyond the muscularis propria[13]. A retrospective but larger study was published by Kuo *et al*[16] 3 years ago. Fifty patients with stage IE (tumor limited from mucosa to subserosa) or stage II1E (tumor invasion detected in regional lymph nodes) *H. pylori* (+) gastric DLBCL between 2002 and 2009 were treated by HPE. pCR was detected in more than two-thirds of the cases with *de novo* DLBCL and more than half of those with DLBCL (MALT). Importantly, all the patients achieving pCR were alive and in remission at a median of 7.7 years[16]. These studies are the cornerstone for HPE in gastric DLBCL. Additionally, an important study published last year showed different biology of *H. pylori*-dependent gastric DLBCL. It was shown that *H. pylori* (+) cases had lower international prognostic score, earlier stage disease, fewer constitutional symptoms of lymphoma, higher sensitivity to standard chemotherapy, and better 5-year event-free survival (EFS) and overall survival than *H. pylori*-independent cases. *H. pylori* negativity was seen among the six detrimental prognostic factors for EFS, and CagA expression was related to better response to chemotherapy[17].

Although *H. pylori*-dependent DLBCL has a better biology and HPE may be curative in some of these cases, we must not forget that high-grade lesions may rapidly progress if they are unresponsive to HPE. For this reason, cellular and/or molecular markers predicting *H. pylori*-independent status of newly diagnosed high-grade gastric lymphoma are important. These markers are well known for low-grade MALT lymphoma and we can make an analogy for high-grade lesions. t(11:18) is a genetic aberration predictive for no response to HPE in low-grade lymphoma[18-21]. In contrast, t(11:18) activates the nuclear factor (NF)-κB pathway[22]. Aberrant nuclear BCL10 or NF-κB is predictive of *H. pylori*-independent status in low-grade gastric MALT lymphoma with or without t(11:18)[23]. However, t(11:18) is uncommon in gastric DLBCL with or without MALT properties and the absence of t(11:18) precludes its use in predicting the response to HPE in DLBCL[21]. Kuo *et al*[16] studied

BCL10 and NF-κB in 22 cases with stage IE high-grade gastric MALT. They found aberrant nuclear BCL10 expression in seven of eight *H. pylori*-independent cases and in none of 14 *H. pylori*-dependent high-grade MALT cases. Additionally, they found NF-κB expression in all seven cases with BCL10 expression and in only two of 15 cases without BCL10 expression. These results suggest that aberrant nuclear BCL10 or NF-κB expression is highly predictive of *H. pylori*-independent status[24]. In contrast, CD86 expression is associated with *H. pylori*- dependent status in high-grade gastric MALT lymphoma[25]. Another important biological feature is CagA expression, which is more frequent in *H. pylori*-dependent cases than *H. pylori*-independent cases, and response to HPE is more rapid in cases with Cag A expression[26]. These results identify the candidate patients with gastric DLBCL for HPE without chemotherapy.

In conclusion, HPE is not limited to *H. pylori*-dependent low-grade MALT lymphoma, and it may be used in patients with high-grade DLBCL. Essential points are as follows. (1) all the patients without pCR after HPE must be immediately treated by standard chemotherapy; (2) histological sections from a minimum of six endoscopic tumor biopsies should be evaluated according to the EGIL consensus. Endoscopic ultrasound is mandatory for initial staging and CR has to be confirmed in two subsequent follow-up biopsies[16]; (3) loss of *H. pylori* dependency and high-grade transformation are distinct events in the progression of gastric lymphoma and short-term antibiotics may be effective in some cases[6]; (4) there are many molecular and biological markers predicting *H. pylori* dependency. Markers associated with *H. pylori* dependency are CD86, CD4 CD56 Treg, p16[INK4A], serum/tissue CagA protein and antibodies. Markers showing no response to HPE are t(11:18), t (1:14), aberrant BCL10 nuclear expression, CXCR3, MAD2, miR203, miR 142-5p and miR 155[6]; (5) *H. pylori* (+) gastric DLBCL, particularly with Cag A expression, is *H. pylori* related and clinicopathologically distinct from *H. pylori*-unrelated gastric DLBCL[17]; and (6) CagA (+) cases with DLBCL tend to be localized and have a lower clinical stage[17]. CagA positivity is more frequent in East Asia compared with western countries and this may be related to higher response to HPE in eastern countries[6,27].

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EDITORIAL

Current pharmacological therapies for nonalcoholic fatty liver disease/nonalcoholic steatohepatitis

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Abstract

Nonalcoholic fatty liver disease (NAFLD)/nonalcoholic steatohepatitis (NASH) is considered to be a hepatic manifestation of metabolic syndrome, and its incidence is rapidly increasing worldwide. It is currently the most common chronic liver disease. NASH can progress to liver cirrhosis and hepatocellular carcinoma, and may result in liver-related death. Currently, the principal treatment for NAFLD/NASH is lifestyle modification by diet and exercise. However, pharmacological therapy is indispensable because obese patients with NAFLD often have difficulty maintaining improved lifestyles. The pathogenesis of NAFLD/NASH has not been completely elucidated. However, insulin resistance, inflammatory cytokines, and oxidative stress are thought to be important in the development and/or progression of the disease. Currently, insulin sensitizers (thiazolidinediones) and antioxidants (vitamin E) seem to be the most promising therapeutic agents for NAFLD/NASH, and lipid-lowering drugs, pentoxifylline, angiotensin receptor blockers, and n-3 polyunsaturated fatty acids also have promise. However, there is a lack of consensus regarding the most effective and appropriate pharmacotherapy for NAFLD/NASH. Animal experiments suggest that herbal medicines and natural products may be promising therapeutic agents for NAFLD/NASH, but their efficacy and safety are yet to be investigated in human studies. In this paper, we review the existing and potential pharmacological therapies for NAFLD/NASH.

Key words: Pharmacological therapy; Nonalcoholic fatty liver disease; Nonalcoholic steatohepatitis; Vitamin E; Thiazolidinedione

Core tip: Nonalcoholic fatty liver disease (NAFLD)/nonalcoholic steatohepatitis (NASH) is considered to be a hepatic manifestation of metabolic syndrome. Currently, the principal treatment for NAFLD/NASH is lifestyle modification by diet and exercise. However, establishment of pharmacological therapy is indispensable. Currently,

insulin sensitizers (thiazolidinediones) and antioxidants (vitamin E) seem to be the most promising agents for treating NAFLD/NASH, and lipid-lowering drugs, pentoxifylline, angiotensin receptor blockers, and n-3 polyunsaturated fatty acids also have promise. However, there is a lack of consensus regarding the most effective and appropriate pharmacotherapy for NAFLD/NASH. Here, we review the existing and potential pharmacological therapies for NAFLD/NASH.

INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) is characterized by accumulation of triglycerides in the liver of patients without a history of excessive alcohol consumption. NAFLD is classified into simple steatosis, in which only hepatic steatosis is observed, and nonalcoholic steatohepatitis (NASH), in which intralobular inflammation and ballooning degeneration of hepatocytes as well as hepatic steatosis are observed. NASH is a progressive disease and may lead to liver cirrhosis and hepatocellular carcinoma[1,2]. Twenty percent of NASH patients are reported to develop cirrhosis, and 30%-40% of patients with NASH cirrhosis experience liver-related death[3]. Recently, NASH has become the third most common indication for liver transplantation in the United States[4].

NAFLD/NASH is considered to be a hepatic manifestation of metabolic syndrome[5]. The incidence of NAFLD/NASH has been rapidly increasing globally in line with the increased prevalence of obesity, and is currently the most common chronic liver disease. Recently, the incidence of NAFLD and NASH was reported to be 46% and 12%, respectively, in a largely middle-aged population[6].

Currently, the principal treatment for NAFLD/NASH is lifestyle modification by diet and exercise. At present, there is a lack of consensus regarding the most useful and appropriate pharmacological therapy. However, establishment of pharmacological therapy is indispensable because obese patients with NAFLD often have difficulty maintaining improved lifestyles. In this paper, we review the existing and potential pharmacological therapies for NAFLD/NASH.

PATHOGENESIS OF NAFLD/NASH

Understanding the pathogenesis of NAFLD/NASH is important for the development of suitable drugs. Although the pathogenesis of NAFLD/NASH has not been completely elucidated, the "two-hit"[7] and "multiple parallel hit"[8] hypotheses have been proposed. In the two-hit hypothesis, hepatic steatosis occurs first and progresses to NASH by subsequent second hits. Hepatic steatosis results from an imbalance between triglyceride accumulation and elimination in the liver. Insulin resistance (IR), which is frequently seen in obese individuals, is closely linked to this process, because it alters nutrient distribution among tissues and nutrient metabolism[9]. Peripheral IR leads to an influx of free fatty acids into the liver by decreased suppression of lipolysis and increased *de novo* lipogenesis[10]. The renin-angiotensin-aldosterone system plays a central role in IR and is associated with NAFLD/NASH[11]. Hepatic inflammation is caused by increased levels of inflammatory cytokines [*e.g.,* tumor necrosis factor (TNF)-α, interleukin-6], decreased levels of anti-inflammatory cytokines (*e.g.,* adiponectin), oxidative stress, and endotoxins originating from intestinal bacterial flora.

PHARMACOLOGICAL THERAPY FOR NAFLD/NASH

Insulin sensitizers

IR is a major mechanism in the development and progression of NAFLD, therefore, the potential therapeutic effect of insulin sensitizers on NAFLD/NASH has gathered much attention. Thiazolidinediones (TZDs) are peroxisome proliferator-activated receptor (PPAR)-γ agonists and increase insulin sensitivity. Rosiglitazone and pioglitazone are representative TZDs. Rosiglitazone was shown to improve steatosis and aminotransferase levels in patients with NASH in a randomized controlled trial[12]. However, usage of rosiglitazone is restricted because it can increase the risk of heart attack (ischemic heart disease). Usage of rosiglitazone is prohibited in Europe according to the recommendations of the European Medicines Agency, and it is highly restricted in the United States based on the recommendations of the Food and Drug Administration (FDA).

In randomized controlled trials, administration of 30 or 45 mg/d pioglitazone induced significant improvements in serum aminotransferase levels and liver histology (steatosis, inflammation, ballooning, and Mallory-Denk bodies) compared with placebo in NASH patients[13-15]. However, improvement in the extent of fibrosis was not significant. American guidelines for NAFLD have recommended the use of pioglitazone in patients with biopsy-proven NASH[16]. Pioglitazone has side effects including weight gain, edema, heart failure, and bone density reduction. In addition, it is reported that the risk of bladder cancer is increased if pioglitazone is used for > 2 years[17]. Therefore, the usage of pioglitazone for new patients is prohibited in France and Germany. In the United States, the FDA currently recommends avoidance of pioglitazone if

active bladder cancer is present, and caution if there is history of the disease[18].

Metformin is classified as a biguanide, and is used to treat type 2 diabetes mellitus. Metformin increases insulin sensitivity by decreasing hepatic gluconeogenesis and limiting triglyceride production[19]. In pilot studies, metformin was shown to improve fatty liver disease and reverse hepatomegaly, steatosis, and aminotransferase abnormalities in a mouse model of NAFLD[20], as well as improve serum aminotransferase levels and liver histology including steatosis, necroinflammation, and fibrosis in NAFLD/NASH patients[21,22]. However, in a subsequent randomized controlled trial, treatment with metformin for 6 mo showed no significant benefits compared with placebo in terms of improvement in liver histology in patients with NAFLD, although it was associated with a reduction in serum levels of lipids and glucose[23]. In a recent randomized controlled trial, metformin was not superior to placebo in attaining sustained reduction of alanine aminotransferase (ALT) levels in patients with pediatric NAFLD[24]. American guidelines for NAFLD do not recommend metformin for the treatment of adult NAFLD[25].

Antioxidants

Many studies have examined the therapeutic effects of antioxidants on NAFLD/NASH because oxidative stress is thought to be an important factor for the progression of NAFLD. Vitamin E (α-tocopherol) is a fat-soluble vitamin with antioxidant properties. In a pilot study, daily oral vitamin E administration was shown to normalize serum aminotransferase and alkaline phosphatase levels in children with NASH[26]. In a large randomized controlled trial, vitamin E administration (800 IU/d) for 96 wk significantly improved serum aminotransferase levels, hepatic steatosis, and lobular inflammation compared with placebo in adults with NASH and without diabetes. However, the extent of hepatic fibrosis was not significantly improved[15]. In another randomized controlled trial, administration of vitamin E and C (1000 IU/d and 1000 mg/d, respectively) for 6 mo resulted in significant improvement in hepatic fibrosis in patients with NASH[27]. In a recent randomized controlled trial, vitamin E (800 IU/d) administration for 96 wk significantly improved hepatocellular ballooning, but it was not superior to placebo in attaining sustained reduction in ALT level in patients with pediatric NAFLD[24]. Based on the results of earlier trials in non-diabetic patients with biopsy-proven NASH, the American guidelines for NAFLD recommend the use of vitamin E for non-diabetic patients with biopsy-proven NASH[16]. It is important to examine the effects of vitamin E in NASH patients with diabetes.

It is noteworthy that the long-term safety of vitamin E is questionable. It was reported that high-dosage ($\geq$ 400 IU/d) vitamin E supplements may increase all-cause mortality[28]. In addition, it is

reported that dietary supplementation with vitamin E significantly increases the risk of prostate cancer among healthy men[29]. It is necessary to investigate the long-term prognoses of NASH patients who take vitamin E supplements.

Lipid-lowering drugs

NAFLD is strongly associated with obesity and dyslipidemia. Statins prevent cholesterol synthesis by inhibiting 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase, and are used to treat dyslipidemia. In addition, owing to their possible anti-inflammatory effects, statins are an option for treating NAFLD[30]. Atorvastatin and simvastatin are representative statins. In a pilot study, serum aminotransferase and lipid levels were reduced significantly in all patients with NAFLD by atorvastatin treatment; however, histological assessment was not performed[31]. In a subsequent open-label study, liver steatosis and NAFLD activity score (NAS) significantly improved, whereas four of 17 (24%) patients with biopsy-proven NASH with hyperlipidemia had increased fibrosis stage after atorvastatin administration[32]. In a randomized controlled trial, atorvastatin (20 mg/d) combined with antioxidants (vitamin C and E) was effective in reducing the risk of hepatic steatosis by 71% after 4 years of active therapy in individuals with NAFLD at baseline[33]. In a randomized controlled trial, simvastatin treatment did not induce significant improvement in serum aminotransferase levels, hepatic steatosis, necroinflammatory activity, or stage of fibrosis in NASH patients[34]. Thus, the efficacy of statins for NAFLD/NASH has not been fully validated. Statins decrease lipid levels both peripherally and viscerally, specifically in the liver[9]. Serum aminotransferase levels may increase transiently when fat is removed from the liver. This transient elevation of serum aminotransferase levels does not progress to liver injury, and decreased fat deposition in the liver eventually decreases serum aminotransferase levels. Therefore, even if serum aminotransferase levels increase shortly after administration of statins, it is not necessary to discontinue treatment. American guidelines recommend against the use of statins for treatment of NASH until randomized controlled trials have confirmed their histological efficacy[16]. However, administration of statins may be beneficial in improving metabolic status and reducing the risk of cardiovascular disease.

Ezetimibe is a newer agent that decreases serum lipid levels by inhibiting cholesterol absorption. It was reported that combination therapy with ezetimibe and acarbose (an α-glucosidase inhibitor) for 24 wk improved histopathological findings (steatosis, inflammation, and fibrosis) in a mouse model of NAFLD[35]. In an open-label pilot study, serum aspartate aminotransferase (AST), ALT, and low-density lipoprotein (LDL) cholesterol levels were significantly improved in NASH patients by treatment

with ezetimibe (10 mg/d) for 6 mo. Follow-up liver biopsies revealed that steatosis grade and NAS also significantly improved; however, the fibrosis stage did not change significantly[36]. In a subsequent trial, long-term (10 mg/d for 24 mo) ezetimibe therapy significantly improved serum triglyceride, total cholesterol, LDL cholesterol, and ALT levels in NAFLD patients. In that study, histological features of steatosis, necroinflammation, and ballooning significantly improved from baseline, but fibrosis stage did not significantly improve[37]. In a recent randomized controlled trial, ezetimibe administration (10 mg/d for 6 mo) improved hepatic fibrosis but increased hepatic long-chain fatty acid and hemoglobin A1c levels in patients with NAFLD[38].

Pentoxifylline

Pentoxifylline is a methylxanthine derivative and nonselective phosphodiesterase inhibitor that inhibits synthesis of TNF-α. TNF-α is thought to be important in the progression of NAFLD, thus, pentoxifylline has been investigated as a treatment option for NAFLD/NASH. In addition, pentoxifylline has recently been shown to decrease oxidized lipid product levels in NASH patients[39]. In pilot trials, administration of pentoxifylline for 12 mo significantly decreased serum AST and ALT levels compared to baseline, and this correlated well with histological resolution in NASH patients[40,41]. However, randomized controlled trials led to mixed results. Van Wagner *et al*[42] reported that administration of pentoxifylline (1200 mg/d) for 12 mo failed to reduce aminotransferase levels compared to placebo in NASH patients. However, Zein *et al*[43] reported that administration of pentoxifylline (1200 mg/d) for 12 mo improved histological features of NASH (steatosis, lobular inflammation, NAS, and fibrosis) compared to placebo. Larger randomized controlled trials are needed in the future to validate the effects of pentoxifylline on NAFLD/NASH. Administration of pentoxifylline requires caution because it causes adverse effects such as nausea and vomiting.

Ursodeoxycholic acid

Ursodeoxycholic acid (UDCA) is a hydrophilic bile acid with antiapoptotic and cytoprotective properties. Its effects on NAFLD/NASH have therefore been examined. In a randomized controlled trial, 2 years of therapy with UDCA at a dose of 13-15 mg/kg per day did not have any significant benefit compared with placebo for patients with NASH[44]. In a subsequent randomized controlled trial, 23-28 mg/kg per day UDCA treatment for 18 mo failed to improve overall histology in patients with NASH compared with placebo[45]. A recent randomized controlled trial showed that treatment with high-dose (28-35 mg/kg per day) UDCA for 12 mo improved aminotransferase levels, serum fibrosis markers, and selected metabolic parameters in NASH patients, but histological assessment was not performed

in this study[46]. In a randomized controlled trial, 2 years treatment with UDCA in combination with vitamin E improved serum AST and ALT levels and hepatic steatosis in patients with NASH[47]; however, this may be primarily due to the effects of vitamin E. American guidelines do not recommend UDCA for the treatment of NAFLD or NASH[25].

Angiotensin receptor blockers

The renin-angiotensin-aldosterone system modulates insulin sensitivity and is associated with pathogenesis of NAFLD/NASH. Thus, the effects of angiotensin Ⅱ type 1 blockers (*e.g.,* telmisartan, valsartan and losartan) on NAFLD have been investigated. Telmisartan attenuated steatohepatitis progression by suppressing the infiltration of macrophages into the liver in a mouse model of NASH[48]. In a clinical trial, telmisartan and valsartan decreased serum ALT levels, homeostasis model assessment as an index of insulin resistance (HOMA-IR), and NAS in NASH patients with metabolic syndrome, with telmisartan showing a higher efficacy than valsartan for HOMA-IR and NAS[49]. Combined treatment with losartan and deferasirox (an oral iron chelator) attenuated progression of NASH in rats[50]. In a clinical trial, losartan improved serum aminotransferase levels and liver histology (necroinflammation and fibrosis)[51]. However, in an open-label trial, combination therapy with rosiglitazone and losartan conferred no greater benefit than rosiglitazone alone with respect to histopathology[52]. Well-designed randomized controlled trials are needed to confirm the effects of angiotensin receptor blockers on NAFLD/NASH. In addition, the use of angiotensin receptor blockers for normotensive patients requires caution because of their hypotensive effects.

N-3 polyunsaturated fatty acids

N-3 polyunsaturated fatty acids (n-3 PUFAs) are PPARα ligands, and are suggested to play a role in improving NAFLD. Supplementation with n-3 PUFAs ameliorated hepatic steatosis and the degree of liver injury in a rat model of NASH[53]. In a pilot human study, n-3 PUFA supplementation significantly decreased serum AST, ALT, triglyceride, and fasting glucose levels in patients with NAFLD compared with those in controls. Moreover, ultrasonography demonstrated improvement of liver echotexture after n-3 PUFA supplementation[54]. Eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) are major n-3 PUFAs. In a pilot trial, after administration of highly purified EPA (2700 mg/d) for 12 mo, serum ALT levels and liver histology, including steatosis, lobular inflammation, ballooning, and fibrosis improved in most NASH patients[55]. However, in a recent phase 2 trial, ethyl-eicosapentaenoic acid (EPA-E), a synthetic n-3 PUFA, had no significant effect on the histological features of NASH[56]. Although many studies have suggested positive effects of n-3 PUFAs on NAFLD/NASH, conclusions and recommendations
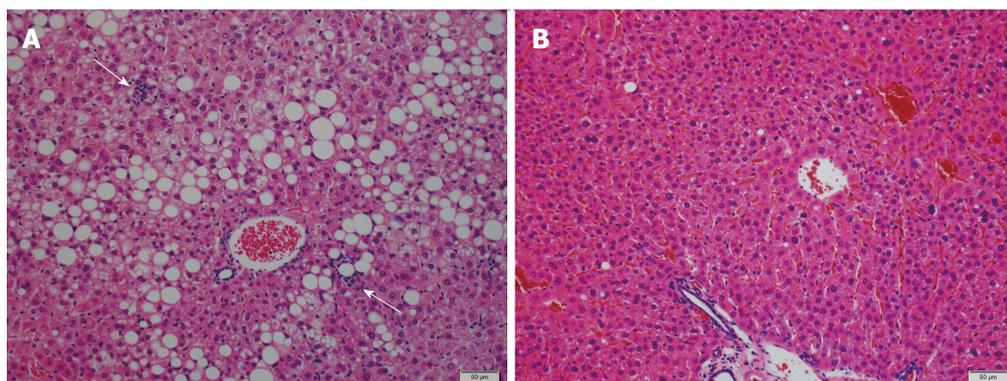
Figure 1 Effects of a Japanese herbal medicines on liver histology in an animal model of nonalcoholic steatohepatitis. A: When db/db mice were fed a methionine- and choline-deficient (MCD) diet, marked hepatic steatosis and scattered foci of lobular inflammation (arrows) were induced; B: When juzentaihoto was added to the MCD diet, liver histology markedly improved (hematoxylin and eosin staining).

for n-3 PUFA supplementation are difficult to establish because the specific quantities and ratios of EPA and DHA were unclear in most published trials.

Probiotics and synbiotics

Probiotics are microorganisms that provide health benefits when consumed. Prebiotics are chemicals that induce the growth and/or activity of microorganisms that contribute to the well-being of their host. Synbiotics are nutritional supplements combining probiotics and prebiotics in a synergistic form. Gut microorganisms play a role in the development of insulin resistance, hepatic steatosis, necroinflammation, and fibrosis[57], therefore, the potential benefits of probiotics and synbiotics on NAFLD/NASH have been suggested. In 2003, treatment with VSL#3 probiotic was reported to improve liver histology, reduce hepatic total fatty acid content, and decrease serum ALT levels in an animal model of NAFLD[58]. Subsequently, various studies have shown the beneficial effects of probiotics or synbiotics on animal models of NAFLD/NASH[59-61]. In contrast, in an open label pilot trial, all subjects who received one sachet of VSL#3 probiotic daily for 4 mo experienced a significant increase in liver fat content[62]. However, the study had several limitations, including a small number of subjects and use of only one dose and preparation of the probiotic compound. No subsequent clinical studies have reported a similar harmful effect of probiotics. In a randomized controlled trial, the administration of probiotic *Lactobacillus rhamnosus* strain GG (12 billion colony forming unit/d for 8 wk) significantly decreased serum ALT levels compared with the placebo in pediatric obesity-related liver disease patients[63]. In a recent randomized controlled trial, administration of a synbiotic capsule twice daily for 28 wk in addition to lifestyle modification significantly decreased serum ALT and AST levels and fibrosis scores, as determined by transient elastography compared with placebo[64]. Although promising results have been obtained in most of the previous experimental and clinical studies, the effects of probiotics and synbiotics on NAFLD/NASH need to be confirmed in larger randomized controlled

trials. In addition, the most effective preparations and dosages need to be established.

Herbal medicines/natural products

Various herbal medicines and natural products are known to possess anti-inflammatory and antioxidant properties, and their effects on NAFLD/NASH have therefore been anticipated. Japanese herbal medicines (JHMs) (Kampo medicines) are traditional Japanese medicines that are integrated into modern clinical practice. We investigated the effects of four kinds of JHMs [shosaikoto (TJ-9), inchinkoto (TJ-135), juzentaihoto (TJ-48), and keishibukuryogan (TJ-25)] on a mouse model of NASH (methionine- and choline-deficient diet-fed db/db mice), and showed that TJ-9 and TJ-48 inhibited necroinflammation and fibrosis in the liver[65] (Figure 1). We also found that TJ-9 and TJ-48, as well as TJ-135, inhibited necroinflammatory activity in another mouse model of NASH [high-fat diet (HFD)-fed db/db mice][66]. Recently, it was reported that bofutsushosan (TJ-62), an anti-obesity JHM, attenuated the progression of HFD-induced NASH in mice[67]. In a small retrospective study, TJ-25 administration led to a significant improvement in liver injury tests and blood cholesterol levels in all NAFLD patients examined[68].

Resveratrol is a polyphenol with antioxidant, anti-inflammatory, antiproliferative, and antiangiogenic effects, and plays a potentially important role in many disorders[69]. It was shown that resveratrol improves IR and NAFLD severity in rats, and this effect is suggested to be associated with activation of AMP-activated protein kinase[70,71].

In an epidemiological study, increased consumption of green tea was associated with decreased serum concentrations of total cholesterol and triglycerides and an increased concentration of HDL cholesterol, together with a decreased concentration of low- and very low-density lipoprotein cholesterol. Increased consumption of green tea is related to decreased concentrations of serum AST and ALT[72]. Green tea extracts attenuated hepatic steatosis by decreasing adipose lipogenesis and

Table 1 Mechanism of action and limitations/demerits of drugs for nonalcoholic fatty liver disease/nonalcoholic steatohepatitis

Drug	Mechanism of action	Limitations/demerits
Insulin sensitizers	Improve insulin sensitivity	Obvious side effects (heart attack with rosiglitazone; weight gain, edema, heart failure, and bone density reduction with pioglitazone)
Antioxidants (vitamin E)	Attenuate oxidative stress	Long-term safety is questionable (increased all-cause mortality and risk of prostate cancer)
Lipid-lowering drugs	Improve dyslipidemia	The efficacy has not been fully validated in clinical trials
Pentoxifylline	Inhibits synthesis of TNF-α	Randomized controlled trials led to inconsistent results. Side effects (nausea and vomiting)
UDCA	Antiapoptotic and cytoprotective properties	Most randomized controlled trials did not show positive effects
Angiotensin receptor blockers	Modulates insulin sensitivity	Randomized controlled trials are lacking. Side effects (hypotension)
n-3 PUFAs	Activate PPARα	Specific quantities and ratios of EPA and DHA are unclear in most trials
Probiotics and synbiotics	Control gut microbiota	The most effective preparation and dose have not yet been established
Herbal medicines/natural products	Anti-inflammatory and antioxidative properties	Effects have not been studied in humans

UDCA: Ursodeoxycholic acid; n-3 PUFAs: N-3 polyunsaturated fatty acids; TNF: Tumor necrosis factor; PPAR: Peroxisome proliferator-activated receptor; EPA: Eicosapentaenoic acid; DHA: Docosahexaenoic acid.

enhancing hepatic antioxidant defenses in a mouse model of NAFLD[73,74]. In addition, (-)-epigallocatechin-3-gallate, the major polyphenol found in green tea, improved plasma ALT concentrations and hepatic steatosis in HFD-fed mice[75].

The leaves of eucalyptus (*Eucalyptus globulus*) and banaba (*Lagerstroemia speciosa L.*) are used as traditional remedies for diabetes mellitus. We found that extracts of these leaves reduced lipogenesis, oxidative stress, and inflammatory cytokine expression, and thus inhibited NASH induced by excessive ingestion of fructose in rats (unpublished data). Reports on positive effects of herbal medicines and natural products on animal models of NAFLD/NASH have been increasing, and human studies are needed in the future.

CONCLUSION

As reviewed in this paper, many animal experiments and clinical studies have been performed to investigate the effects of various drugs on NAFLD/NASH. However, there is a lack of consensus regarding the most effective and appropriate pharmacotherapy for this disease. The mechanism of action and limitations/demerits of each pharmacotherapy are summarized in Table 1. Currently, insulin sensitizers (TZDs) and vitamin E seem to be the most promising. However, they cause side effects such as weight gain and increased all-cause mortality, respectively. Better understanding on the long-term safety and efficacy of these drugs is needed before they can be fully incorporated into clinical practice. Pentoxifylline, statins, angiotensin receptor blockers, and n-3 PUFAs have some promise, but their effects should be validated by large, well-designed clinical trials. Results of animal experiments suggest that herbal medicines and natural products may be promising as therapeutic agents for NAFLD/NASH. However, their efficacy and safety need to be investigated in clinical studies. Continuous clinical and preclinical studies on existing and potential drugs are needed to improve treatment for NAFLD/NASH, which is an increasingly prevalent disease.

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TOPIC HIGHLIGHT
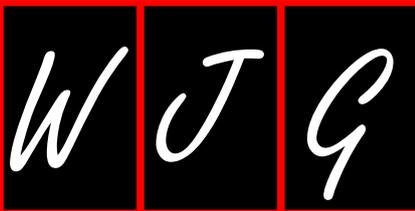
2015 Advances in hepatitis C virus

Hepatitis C virus and antiviral innate immunity: Who wins at tug-of-war?

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Abstract

Hepatitis C virus (HCV) is a major human pathogen of chronic hepatitis and related liver diseases. Innate immunity is the first line of defense against invading foreign pathogens, and its activation is dependent on the recognition of these pathogens by several key sensors. The interferon (IFN) system plays an essential role in the restriction of HCV infection *via* the induction of hundreds of IFN-stimulated genes (ISGs) that inhibit viral replication and spread. However, numerous factors that trigger immune dysregulation, including viral factors and host genetic factors, can help HCV to escape host immune response, facilitating viral persistence. In this review, we aim to summarize recent advances in understanding the innate immune response to HCV infection and the mechanisms of ISGs to suppress viral survival, as well as the immune evasion strategies for chronic HCV infection.

Key words: Hepatitis C virus; Interferon; Interferon-stimulated gene; RIG-I; Toll-like receptor; Virus-host interaction; Chronic hepatitis; Immune evasion; Cell culture system for hepatitis C virus

Core tip: The complex interaction between hepatitis C virus (HCV) and its host determines which side the balance tends to be tipped between antiviral innate response and viral immune evasion. The development of new cell culture systems and small animal models for HCV research permits increased understanding of how the host responds to viral infection and what leads to HCV evasion of innate immunity. Recent discoveries in these areas reveal many pivotal factors imparting the control of viral-induced innate immunity, and facilitate the development of novel drugs and effective vaccines for HCV infection.

INTRODUCTION

Hepatitis C virus (HCV) is a member of the Flaviviridae family that causes liver disease globally. HCV infects approximately 170 million people worldwide, with 3-4 million new infections per year[1]. Following infection, 20%-30% of infected individuals clear the virus spontaneously without any therapy during this phase, while 70%-80% of them become persistently infected[2]. The virus replicates in the liver continuously and establishes intrahepatic persistent infection progressively, putting patients at risk of hepatic fibrosis, cirrhosis and hepatocellular carcinoma[3]. Although direct acting antivirals (DAAs) have been introduced recently, PEGylated interferon-α (PEGIFN-α) plus ribavirin is still the current standard-of-care therapy for HCV in many treatment centers. However, only 40%-50% of patients infected with difficult-to-treat HCV genotypes achieve sustained viral response with PEGIFN-α treatment regimen[4]. A prophylactic anti-HCV vaccine is still lacking[5].

The first immune defense that senses HCV infection is the intrinsic innate immunity within hepatocytes. The pattern recognition receptors (PRRs) within the infected cells sense the virus as non-self and induce antiviral defenses through activation of downstream signaling cascades to clear the virus. Evolutionarily, HCV has acquired strategies to modulate and escape immune recognition by the host, which contributes to HCV persistence[6]. Furthermore, the polymorphisms in IFNL3 (also referred to as IL28B), as the host factor, influence both the outcome of infection and the response to therapy[7-11]. The mechanisms that underlie the different outcomes of viral infection are still not fully understood, but likely reflect a complex interaction between the virus and the host during the immune response. Understanding how HCV activates or evades innate immune responses is essential to develop new antiviral approaches and to design effective vaccine applications to reduce the disease burden of HCV-induced liver disease and cancer. In this review, we describe how HCV regulates the antiviral innate immune response in the hepatocytes and discuss how virus-host interactions affect the outcome of HCV infection.

LIFE CYCLE OF HCV

The HCV genome consists of an approximately 9.6 kb positive single-stranded RNA (+ssRNA)[12]. Its genome encodes a large polyprotein of about 3000 amino acids that is processed by a combination of host signal peptidases and viral proteases into 10 individual proteins, including three structural proteins (core, E1 and E2) and seven nonstructural proteins (p7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B). Based on the viral genetic variation of the Core, E1 and NS5B, HCV is classified into seven genotypes (1-7) and up to 67

different subtypes[13]. The different HCV genotypes exhibit different geographical distributions, differential responses to therapy and distinct characteristics in pathogenesis[14].

During infection of hepatocytes by HCV, much progress has been made in understanding how this enveloped RNA virus enters cells. The first described receptors for HCV were the tetraspanin CD81[15] and scavenger receptor class B type I (SR-BI)[16]. Glycosaminoglycans and low-density lipoprotein receptor mediate initial viral binding to hepatocytes before HCV envelope protein interacts with CD81 and SR-BI[14]. The cellular protein apolipoprotein E (apoE) is needed for HCV attachment to cells[17]. Using an iterative complementary DNA library screening approach, two tight junction proteins, claudin-1 (CLDN1)[18] and occludin (OCLN)[19], were identified as co-receptors for the later step of HCV entry. Moreover, the major block to HCV entry of mouse hepatocytes can be overcome by the expression of human CD81 and OCLN in the context of mouse CLDN1 and SR-BI[19]. Recently, the cholesterol absorption receptor, Niemann-Pick C1-like 1, the epidermal growth factor receptor and ephrin receptor typeA2, were reported as essential HCV entry factors that function after viral binding[20,21]. The membrane-spanning syndecan-1 and cell death-inducing DFFA-like effector B were also identified as HCV entry cofactors[22,23]. However, the next step of pH-dependent fusion and uncoating, and release of the viral genome into the cytoplasm is poorly understood[24].

After virion entry into hepatocytes, the internal ribosomal entry site (IRES) sequence located at the 5' end of the untranslated region of the viral RNA genome is recognized by ribosomes studded in host endoplasmic reticulum (ER) to mediate viral polyprotein translation[25]. The HCV nonstructural proteins, including NS3, NS4A, NS4B, NS5A, and NS5B, comprise the RNA replication machinery within ER-derived structures known as the membranous web[26]. Lipid droplets (LDs) are found in replication sites located at the ER membranous web and play a central role in viral RNA synthesis[27]. HCV particle assembly involves the coordinated action of the ER-resident E1-E2 glycoprotein complex, recruitment of LDs-associated core protein to package viral RNA, and several viral and host factors[28]. HCV uses the lipoprotein production pathway to further assemble infectious particles and to release them from the infected cells. AMP-activated protein kinase plays a key role in regulation of both lipid and glucose metabolism and is implicated in HCV replication[29,30]. Recently, based on the method of selective evolution of ligands by exponential enrichment, we screened a series of specific aptamers targeting viral proteins Core[31], E1E2[32], NS2[33], NS5A[34], respectively. The enzyme-linked oligonucleotide assay, based on core-specific aptamers, can be used to detect serum samples from

hepatitis C patients[31]. Furthermore, core-specific aptamers inhibit the production of infectious virus particles by disrupting the localization of core with LDs and NS5A, and perturbing the association of core protein with viral RNA[31]. Like core-specific aptamers, the aptamers targeting NS5A also exhibit suppression of viral production by reversing the interaction of NS5A and core[34]. Moreover, NS5A-specific aptamers repress the RNA replication of HCV[34]. The aptamers targeting E1E2 selectively inhibit HCV entry at early binding step by disrupting the association of E2 and viral receptors[32]. Interestingly, NS2-specific aptamers suppress HCV RNA replication, assembly and release[33]. These aptamers may hold promise for the investigation of the detailed mechanisms of HCV life cycle and development as therapeutic drugs for hepatitis C patients.

MODELS FOR HCV INFECTION

Cell culture models

There is a lack of efficient HCV cell culture systems; therefore, research on the virus has been impeded since the identification of HCV in 1989. The first robust HCV replicon system derived from the Con1 strain (genotype 1b) was established in Huh7 cells in 1999[35]. Until 2005, the first fully permissive cell culture system for HCV became possible using a clinic isolate (genotype 2, termed JFH1) from a Japanese patient with fulminant hepatitis C[36-39]. Similar to the JFH1-derived cell culture system, the efficient infectious culture systems based on H77 (genotype 1a) and Con1 isolates were also described, though both are less robust than JFH-1[40,41]. Continuous culture of JFH1 virus *in vitro* leads to accumulation of viral genomic mutations responsible for enhancing of virus titer[42]. Several groups reported multiple mutations residing in the p7 and NS2 regions, consistent with their important role in virus assembly[42-45]. Other viral proteins associated with HCV RNA replication, such as NS3 and NS5A, also evolve many titer-enhancing mutations with cell culture adaptation of virus[42,46-48].

Until now, most robust HCV infection has been achieved in Huh7 cells or its derived cells. However, Huh7 cells are not well suited to investigate innate immune response, virus-host interaction and HCV-associated carcinogenesis because they contain some mutant host factors and lack the majority of markers characterizing mature hepatocytes. Hence, it is desirable to develop alternative host cell systems. Indeed, some groups tried to replicate HCV in non-Huh7 cell lines such as HuH-6[49], HepG2[50,51], IMY-N9[51] and LH86[52]. Furthermore, human cell lines derived from the non-liver tissues, including HEK293[53,54], HeLa[54,55], neuroepithelioma cells[56], as well as non-human cells[55,57-61], have been reported to support HCV infection. However, the levels of viral replication in these cells are lower than Huh7 cells,

which may reflect insufficient expression of miR-122 and apoE in the non-Huh7 cells[62]. There have been significant advances in the generation of HCV culture systems based on the hepatocyte-like cells induced from pluripotent stem cells[63-65]. Importantly, Schwartz and co-workers demonstrated that induced hepatocyte-like cells mount an antiviral inflammatory response with upregulation of innate immune/inflammatory markers, including CXCL10, CXCL11, TNF-α, IL28B and IL29, by HCV infection[63]. The utilization of the hepatocyte-like cells may be limited because of a series of complicated conditions for induction and culture of cells. To maximally imitate the *in vivo* infection of virus, we and other groups isolated primary human hepatocytes (PHHs) to establish the closest cell-based *in vitro* model for HCV[66-68]. After acute HCV infection of PHHs, interferon-β (IFN-β) and interferon-stimulated genes were induced in the cells and the apoptosis of viral-infected cells were triggered by TRAIL-mediated pathway, which indicated that the innate host response is intact in HCV-infected PHHs[66]. Although PHHs can support successful HCV replication, their disadvantages, such extreme lack of donors and highly variable results, may hinder their application. Strikingly, we recently established a PHHs-like hepatoma cell line, termed HLCZ01, and found that it can support both HCVcc (HCV cell culture) and HCV clinical isolates infection and replication[69]. In comparison to Huh7 or its derived cell lines, HLCZ01 mounts an innate immune response to HCV infection[69]. In addition, HLCZ01 also supports infection and propagation of hepatitis B virus produced both in cell culture and clinically[69]. This cell line provides a powerful tool for addressing the virus lifecycle, research of antiviral innate immunity and the development of antivirals and vaccines.

Animal models

Apart from humans, chimpanzees are the only species that support natural HCV infection. The species-specific host cell factors promoting or restricting HCV replication may have led to this narrow host range[70]. Chimpanzees have played an essential role in the identification of HCV as the etiological agent of non-A non-B hepatitis[71]. In fact, the entire HCV life cycle can be achieved in chimpanzee. Viruses obtained from clinical isolates and *in vitro* tissue culture have been inoculated into chimpanzees using intravenous or intrahepatic injection[72-75]. The chimpanzee model holds promise for research of both innate and adaptive immunity, and the development or testing of new drugs and vaccines. However, studies based on this model are impeded by limited availability, high costs and ethical concerns. In addition, research on chimpanzees is now banned in most countries[76]. Therefore, alternative animal models are urgently needed. Although the tree shrew, a small squirrel-like mammal, is susceptible to HCV infection[77,78], numerous groups prefer to construct HCV-susceptible

mice models.

Humanized mice transplanted with human hepatocytes or expressing essential HCV host factors are now considered as the novel models for analyzing HCV infection and testing therapeutics. The xenotransplantation model, as one of the humanized models, must suffer from an endogenous liver injury and allow stimulated growth of human hepatocytes. The human liver chimeric Alb-uPA[79,80], MUP-uPA[81], HSV-tk[82] and FAH-/-[83] mice were reported to be infected with HCV derived from cell culture and clinic isolates. The AFC8-hu hematopoietic stem cells (HSC)/Hep mice containing both human immune system and liver tissues is a novel model for the study of the immune response against HCV[84]. However, this model is limited because of shortage of human donors to isolate HSCs and hepatocyte progenitors. Another mouse model, comprising genetically humanized mice that were transient adenovirally transfected[85] or showed stable transgenic expression of human CD81 and OCLN[86], could support HCV infection of murine hepatocytes *in vivo*. Importantly, the genetically humanized mice are immunocompetent models, which provide opportunities to study host immunity during viral infection, in spite of their low efficiency of HCV infection[85]. Although numerous studies have been done to improve the efficacy of the humanized mice, the model remains technically challenging. Currently, there is still a lack of a suitable alternative to the chimpanzee model for to study both innate and adaptive immune responses to HCV infection and to test candidate drugs and vaccines[87].

RECOGNITION OF HCV

RIG-I-like receptors detect HCV

In the host cell, several PRRs sense viruses as foreign invaders through pathogen-associated molecular patterns (PAMPs) recognition to activate the innate immune response. The major key PRRs include RIG-I (retinoic acid inducible gene-I)-like receptors (RLRs), toll-like receptors (TLRs) and other nontraditional PRRs. RLRs consist of RIG-I, melanoma differentiation-associated protein 5 (MDA5) and laboratory of genetics and physiology 2 (LGP2). RIG-I is a cytosolic RNA helicase that contains three major domains: a C-terminal domain, a central DExD/H box RNA helicase domain, and two CARD domains at the N-terminus[88,89]. Mitochondrial antiviral signaling protein (MAVS; also called Cardif, IPS-1 or VISA) is the common adaptor for RLRs. Interestingly, MAVS is located on mitochondria, peroxisomes and mitochondria-associated membranes (MAMs)[90,91] (Figure 1). Activation of MAVS leads to activation of TBK1 or IKKε, which phosphorylates downstream IFN regulatory factor 3 (IRF3) and IRF7. In addition, MAVS also activates NF-κB through the activation of the classical IKK complex.

HCV is recognized by RIG-I at the early period of infection[92]. RIG-I senses HCV PAMP that bears 5' triphosphate and 3' untranslated region of the HCV genome RNA with poly-U/UC ribonucleotides[93,94] (Figure 1). The recognition of HCV PAMP by RIG-I is dependent on the 34-nucleotide poly-uridine core within the poly U/UC region[95]. Although the 5' triphosphate and the 3' poly-U/UC region are at opposite ends of the viral genome, the interactions between the 5' and 3' ends of HCV RNA can bring both into proximity for presentation to RIG-I[96]. RIG-I is also crucial for host innate responses to other RNA viruses, including influenza virus, vesicular stomatitis virus, Sendai virus, Japanese encephalitis virus, dengue virus and West Nile virus (WNV)[97-99]. When the HCV RNA binds to RIG-I, it induces a RIG-I conformational change that promotes its oligomerization and translocation from the cytosol into intracellular membranes[100-102]. The E3 ubiquitin ligase tripartite motif-containing protein 25 (TRIM25) induces the lysine 63 (K63)-linked polyubiquitination of the CARD of RIG-I at lysine 172, which is crucial for RIG-I signaling pathway to elicit host antiviral innate immunity[103]. Recent works revealed that two other E3 ubiquitin ligases, Mex3c and Riplet (also called Reul), are also essential for K63-linked polyubiquitination of RIG-I and RIG-I-dependent innate immune responses[104-106]. The chaperone protein 14-3-3e is involved in the association of RIG-I and TRIM25, and facilitates RIG-I translocation to interact with MAVS[102]. Strikingly, unanchored K63-ubiquitin chains potently activate RIG-I by interacting with RIG-I CARD domains[107]. Furthermore, the binding of unanchored K63-ubiquitin chains to RIG-I induces the formation of a large complex comprising four RIG-I and four polyubiquitin chains, which is responsible for activation of antiviral signaling[108]. Siglec-G, a member of lectin family, promotes RIG-I degradation by E3 ubiquitin ligase c-Cbl, which inhibits the innate immune response[109].

MDA5 preferentially senses long dsRNA molecules generated during virus infection[110]. Whether MDA5 serves as a PRR for HCV infection remains to be clarified. Overexpression of MDA5 inhibits HCV infection *in vitro* and the HCV NS3/4A protein can block the transduction of MDA5 signaling[111]. In addition, suppression of MDA5 by V protein of paramyxovirus enhances HCV replication[112,113]. However, it fails to induce the IFN-β in the RIG-I^{-/-} mouse embryonic fibroblasts (MEFs) by stimulation with HCV RNA[93]. Knockdown of RIG-I in the PHHs and HLCZ01 cells impairs the production of HCV-induced IFN-β[66,114]. Moreover, the MDA5^{-/-} MEFs still produce IFN-β upon stimulation with HCV RNA[93]. These studies suggested that RIG-I is the key sensor for HCV recognition and MDA5 may be involved in regulation of innate immunity triggered by HCV infection.

TLRs sense HCV

TLRs also serve as host PRRs to detect HCV PAMPs.
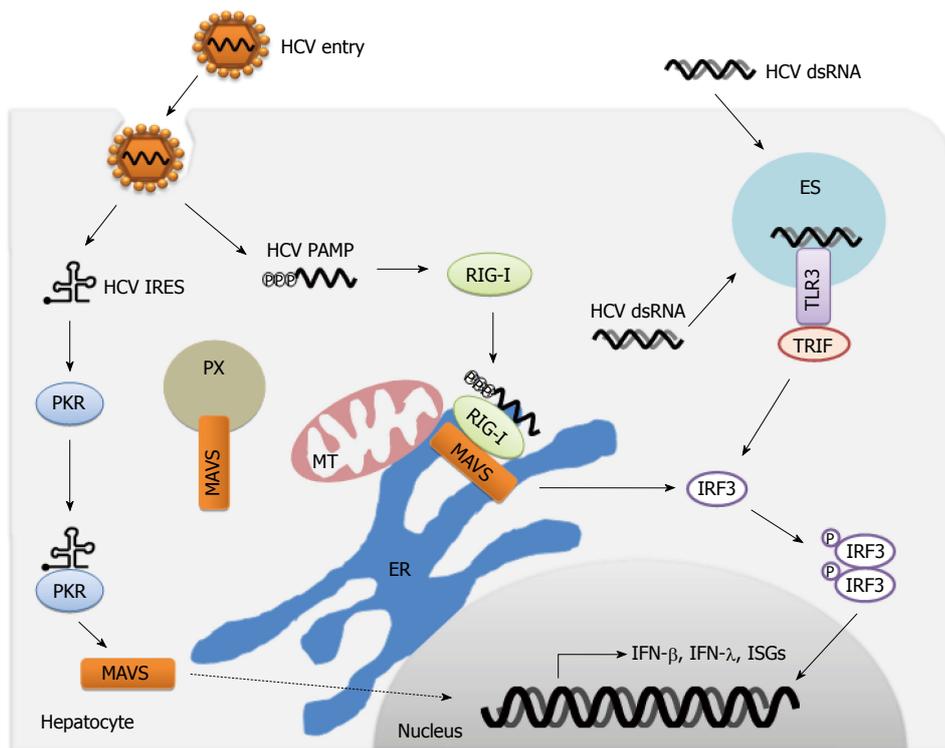
Figure 1 Immune sensing of hepatitis C virus by pattern recognition receptors. During hepatitis C virus (HCV) infection, PKR and RIG-I recognize dsRNA constructed in the HCV IRES and HCV PAMP with 5'-triphosphate plus 3'poly-U/UC region, respectively. In the late period of infection, HCV dsRNA from viral replication intermediates or extracellular dying cells is detected by TLR3. Activation of pattern recognition receptors (PRRs) leads to the transduction of signaling through adaptor protein MAVS or TRIF, and subsequent phosphorylation and dimerization of IRF3 to induce the production of IFN-β, IFN-λ, ISGs and proinflammatory cytokines. MT: Mitochondria; ER: Endoplasmic reticulum; ES: Endosome; PX: Peroxisome; MAVS: Mitochondrial antiviral signaling; PAMP: Pathogen-associated molecular pattern; IFN: Interferon; TRIF: TIR-domain-containing adaptor-inducing interferon-β.

TLR3, TLR7 and TLR9 sense viral nucleic acids, while TLR2 and TLR4 recognize viral proteins[115,116]. In addition, HCV infection can induce the expression of TLR4[117]. As an endosomal sensor of HCV dsRNA, TLR3 is expressed in many cell types within the liver, including hepatocytes and the liver-resident macrophage Kupffer cells[118,119]. TLR3 is type I transmembrane protein and comprises an amino-terminal horse shoe-shaped ectodomain that recognizes viral dsRNA, a transmembrane region, and a cytosolic carboxy-terminal Toll-IL1 receptor homology (TIR) domain that activates downstream signaling[120]. Moreover, the activation of TLR3 signaling is dependent on the acidification of endosomes[121].

TLR3 signals are transduced through the adaptor protein TIR-domain-containing adaptor-inducing interferon-β (TRIF), which activates IRF3 and NF-κB, leading to the production of type I IFN, proinflammatory cytokines and chemokines[122] (Figure 1). TLR3 also triggers the apoptosis of human cancer cells directly[123]. HCV dsRNA replication intermediates (≥ 80-100 bp) accumulated late during HCV replication and represent the HCV PAMP[124]. Actually, HCV infection activates TLR3 signaling late (3-4 d) in infection[118,124], which indicates that the incoming viral genomes or early replication products may not present as TLR3 ligands. In addition to the dsRNA accumulation from viral replication, the HCV ligands for TLR3 are probably obtained from the

uptake of extracellular HCV dsRNA (either from dying cells or from the extracellular milieu) by scavenger receptors on nearby uninfected cells[125]. Consequently, TLR3-mediated signaling may act as a secondary innate immune response or monitoring system for uninfected cells after the initial RIG-I detection of HCV.

TLR7 recognizes ssRNAs derived from HCV or other ssRNA viruses in the endosome. TLR7 is expressed mainly in plasmacytoid DCs (pDCs). Phagocytic uptake of HCV RNA triggers an innate immune response *via* TLR7 signaling in the pDCs. Activated pDCs induce type I IFNs rapidly and the levels of IFNs are more than 100-fold higher than that induced in any other blood cell type[126,127]. Exosomes can transfer HCV RNA from infected cells to pDCs and activate TLR7-dependent innate response in pDCs[128]. Exosomes from both HCV-infected cells and hepatitis C patients transmit HCV infection of human hepatoma cells[129,130]. TLR2 is expressed on the cell surface. The TLR2 pathway is involved in the induction of TNFα after stimulation by synthetic TLR2 ligands in various hepatocyte cell lines[131]. In addition, hepatic expression of TLR2 and TNF-α is associated with hepatic inflammation and liver injury in hepatitis C patients[132,133].

Other sensors recognition of HCV

The protein kinase R (PKR), an antiviral protein,

Table 1 Antiviral interferon-stimulated genes targeting different steps of the hepatitis C virus life cycle

Gene symbol	Phases of HCV life cycle targeted by ISGs	Infection or replicon for studies	Overexpression or knockdown of ISGs for tests	Ref.
IFITM1	Entry	Infection	oe/kd	[158]
	Replication	Infection	oe/kd	[159]
	Replication	Replicon	kd	[147]
IFITM3	Translation	Replicon	oe	[161]
	Replication	Replicon	oe	[160]
	Replication	Replicon	oe/kd	[147]
DDIT4	Translation	Infection/replicon	oe	[144]
IFI44L	Translation	Infection/replicon	oe	[144]
IRF1	Translation	Infection/replicon	oe	[144]
	Replication	Replicon	oe	[162]
IRF2	Translation	Infection/replicon	oe	[144]
IRF7	Translation	Infection/replicon	oe	[144]
MAP3K14	Translation	Infection/replicon	oe	[144]
MDA5	Translation	Infection/replicon	oe	[144]
NT5C3	Translation	Infection/replicon	oe	[144]
RIG-I	Translation	Infection/replicon	oe	[144]
OASL	Translation	Infection	oe	[144]
	Replication	Replicon	oe	[152]
RNaseL	Replication	Replicon	oe/kd	[147]
	Replication	Infection	oe	[153]
ADAR1	Replication	Replicon	kd	[163]
GBP-1	Replication	Replicon	oe/kd	[162]
IFI27	Replication	Replicon	oe/kd	[162]
IFI6	Replication	Replicon	oe/kd	[162]
IFIT1	Replication	Infection	oe/kd	[162]
IFIT3	Replication	Replicon	kd	[147]
IRF9	Replication	Replicon	oe	[162]
ISG20	Replication	Replicon	oe	[164]
MxA	Replication	Replicon	oe	[162]
NOS2	Replication	Replicon	oe/kd	[147]
OAS	Replication	Replicon	oe	[162]
PLSCR1	Replication	Replicon	oe/kd	[147]
TRIM14	Replication	Replicon	oe/kd	[147]
Viperin	Replication	Replicon	oe	[164]
	Replication	Replicon	oe	[165]
	Replication	Infection/replicon	oe	[155]
	Replication	Infection	kd	[156]
Tetherin	Release	Infection	oe	[166]

HCV: Hepatitis C virus; ISGs: Interferon-stimulated genes; Infection or replicon: The functions of ISGs were studied by using infectious culture system (infection) or subgenomic replicon (replicon); oe or kd: The functions of ISGs were identified by using overexpression (oe) or knockdown (kd) of indicated ISGs in cells.

is a serine/threonine kinase that regulates protein synthesis, cell proliferation, apoptosis and signal transduction. PKR is a PRR that detects HCV and activates innate immune signaling[134]. Upon binding to HCV dsRNA, PKR activates its kinase activity for phosphorylation of the α subunit of eukaryotic initiation factor 2 (eIF2α). Activated PKR results in shutting-off of the cap-dependent translation of host mRNAs, while HCV uses an IRES translation mechanism (cap-independent translation) that is insensitive to the phosphorylation of eIF2α[135-138]. PKR binding of HCV dsRNA also activates a kinase-independent signaling that induces a small subset of IFN-stimulated genes (ISGs) and IFN-β through MAVS, TNF receptor-associated factor 3, IRFs and NF-κB transduction

before RIG-I activation[134,139,140] (Figure 1). In addition, although the HCV ligand for PKR is the structured RNA at the IRES of the HCV genome[134,138], the detail course of PKR recognition remains unclear.

The Nod (nucleotide oligomerization domain)-like receptors (NLRs) belong to a large family of intracellular PRRs, including NOD1, NOD2, and NLR protein 3 (NLRP3)[141]. NLRs sense the PAMPs from RNA viruses, resulting in activation of the inflammasome[142].The inflammasome comprises a sensor protein, the adaptor protein ASC and the cellular protease caspase 1. The NLRP3 inflammasome is the most characterized inflammasome and is activated by viral dsRNA and ssRNA. NLRP3 triggers the production of proinflammatory cytokines IL-1β in macrophages during HCV infection[143].

IMMUNE EFFECTORS THAT RESTRICT HCV

The immune effectors against invading viral pathogens infection are the products of interferon-stimulated genes (ISGs). More than 300 ISGs are regulated by IFNs through the Jak-Stat signaling pathway. To identify the specific set of ISGs responsible for the inhibition of HCV replication, several screens have been conducted in vitro systems. Identified ISGs that restrict the HCV life cycle are summarized in Table 1. Using an overexpression screening approach, a recent study tested the ability of nearly 400 ISGs to inhibit the replication of several important human and animal viruses, including HCV, yellow fever virus, WNV, chikungunya virus, Venezuelan equine encephalitis virus and human immunodeficiency virus type-1[144]. For HCV, the inhibitory effectors includes IRF family members, RIG-I, MDA5 and other little characterized ISGs, such as DDIT4, IFI44L, MAP3K14, OASL and NT5C3[144,145]. In contrast to the overexpression approach, an RNA interference-based screen was performed by two groups to identify anti-HCV ISGs[146,147]. Zhao et al[146] identified 93 genes that mediate the anti-HCV effect of IFN-α, in which 23 and nine genes are involved in mRNA processing and translation initiation, respectively. Seven ISGs were identified by Metz and co-workers to contribute to the suppression of HCV replication by IFN-α or IFN-γ[147]. Although all ISGs identified in this study were upregulated by either cytokine, PLSCR1 and NOS2 were the main effectors of IFN-γ-mediated anti-HCV activity[147]. In addition to ISG proteins, the IFN-induced microRNAs (miRNAs) may also be capable of inhibiting HCV replication[148]. Similarly, Fusco and co-workers identified ISGs as well as non-transcriptionally induced genes required for the antiviral effect of IFN-α using a genome-wide siRNA screen[149]. In this screen, 120 IFN effector genes were identified that restricted HCV, and 92% of them were non-transcriptionally IFN-induced genes, which suggested an as-yet-unknown

action of the IFN pathway[149].

Although numerous ISGs have been validated in the process of IFN-mediated anti-HCV activity, the underlying mechanisms of most ISGs are unclear. Several ISGs, including OAS-RNaseL system, Viperin and the IFITM family members are relatively well characterized[150]. During viral infections, viral dsRNA activates one of the three functional human 2',5'-oligoadenylatesynthetases (OASs) to synthesize 2'-to-5'-linked oligoadenylates (2-5A), which subsequently activate RNaseL to cleave viral RNAs, as well as cellular mRNAs and rRNAs, to induce apoptosis of infected cells[151]. *In vitro* studies have shown that all three OAS proteins induce RNaseL-dependent antiviral activity against HCV[144,152,153]. Viperin (RSAD2) localizes to the ER and lipid droplets. Viperin interacts with HCV core and NS5A proteins, as well as VAP-A, an important host factor of HCV replication, in the LDs-ER membranes[154-156]. Viperin may cause an alteration of the lipid composition of the membranous web by disturbing NS5A-VAP-A interaction, leading to inhibition of HCV replication[157]. The IFN induced transmembrane proteins (IFITMs), including IFITM1, IFITM2, IFITM3 and IFITM5, contain two anti-parallel transmembrane domains, a leucin-zipper motif and a short cytoplasmic domain. IFITM1 interacts with HCV co-receptors CD81 and occludin to inhibit the process of viral entry[158]. IFITM1 and IFITM3 suppress HCV replication; however, the mechanism of replication inhibition by IFITMs remains to be determined[147,159,160]. In addition, IFITM3 inhibits the translation of both HCV polyprotein and host proteins[161]. IFI27 (also called ISG12a), a mitochondria-anchored protein, also exhibits antiviral activity through inhibition of HCV replication and by triggering apoptosis of HCV-infected cells[114,162]. Although the role of IFI27 in early control of HCV is unclear, IFI27 can mediate TRAIL-dependent apoptosis *via* induction of Noxa in the late stage of HCV infection[114]. Although single ISG exhibits anti-HCV activity, full restriction of HCV infection requires the combined activity of multiple ISGs and the transduction of other innate immune signaling pathways.

STRATEGIES FOR INNATE IMMUNITY EVASION OF HCV

Viral factors
Despite the effective recognition of HCV by many sensors to activate innate immune responses, about 80% of patients with acute HCV infection do not effectively clear the virus and develop a chronic infection[167]. HCV may have evolved several mechanisms that are responsible for viral evasion of host innate immune signaling. The key viral factor of the HCV innate immune evasion is the viral NS3/4A protease. NS3/4A, a complex of NS3 and NS4A proteins, is required for the HCV life cycle, including

viral RNA replication, polyprotein processing and viral assembly[168]. The NS3/4A complex is anchored to intracellular membranes by the N-terminal α-helix of NS3 and NS4A transmembrane domain that facilitates membrane association and cleavage of membrane-anchored substrates[169,170]. The RIG-I signaling pathway can be blocked by NS3/4A, which cleaves MAVS at Cys508 from intracellular membranes to prevent its dimerization and downstream signaling of innate immunity[92,171-175]. NS3/4A cleaves MAVS at the mitochondrial outer membrane[92,175], and MAVS also localizes to peroxisomes and MAMs[90,91]. Although NS3/4A localizes to all three kinds of membranes during HCV infection, MAM-localized MAVS is cleaved by NS3/4A rather than MAVS anchored on the outer mitochondrial membrane[91] (Figure 2). Activation of the RIG-I pathway by HCV infection may occur *via* the MAM-localized MAVS to transduce the downstream signaling. In the livers of patients with chronic HCV infection, the cleavage of MAVS by NS3/4A was also observed, which contributes to lower levels of IFN pathway induction[92,176]. Hence, the fact that NS3/4A targets and cleaves MAVS to disrupt RIG-I signaling is an effective strategy for HCV to evade the innate antiviral immunity. HCV NS3/4A also targets the E3 ubiquitin ligase Riplet and inhibits K63-linked polyubiquitination of RIG-I and its association with TRIM25 and TBK1, which provides a new mechanism for HCV evasion of host immunity[105].

TRIF, the TLR3 signaling-adaptor protein, is also cleaved by HCV NS3/4A protease at Cys372, a site containing high sequence homology to the NS4B/5A cleavage site in the HCV polyprotein[177,178] (Figure 2). The infection of hepatoma cells by JFH1 virus *in vitro* leads to substantial reduction of TRIF protein abundance, which could be partially reversed by NS3/4A inhibitor treatment[118]. Although the relative abundance of TRIF decreased, the specific TRIF proteolytic fragments have not been detected during HCV infection, probably because of technical difficulties in detecting this low abundance protein[118]. Similar to the blockage of RIG-I signaling, NS3/4A-mediated cleavage of TRIF suppresses the transduction of TLR3 signaling, as a mechanism responsible for chronic HCV infection. However, TRIF is also implicated as an adaptor protein for TLR3-independent signaling[179]. This suggests that cleavage of TRIF by NS3/4A may also inhibit other innate immune responses contributing to HCV persistence.

The dsRNA-dependent protein kinase PKR plays multiple roles in cells, including pathogen sensing and response to different stress situations[180]. Binding of HCV dsRNA to PKR activates a kinase-independent signaling that induces some early ISGs and IFN-β through MAVS[134,139,140]. Therefore, cleavage of MAVS by HCV NS3/4A protease probably suppresses the transduction of PKR signaling during HCV infection (Figure 2). Binding to dsRNA also promotes PKR homodimerization and activates the kinase domain to phosphorylate eIF2α. However, the effects of
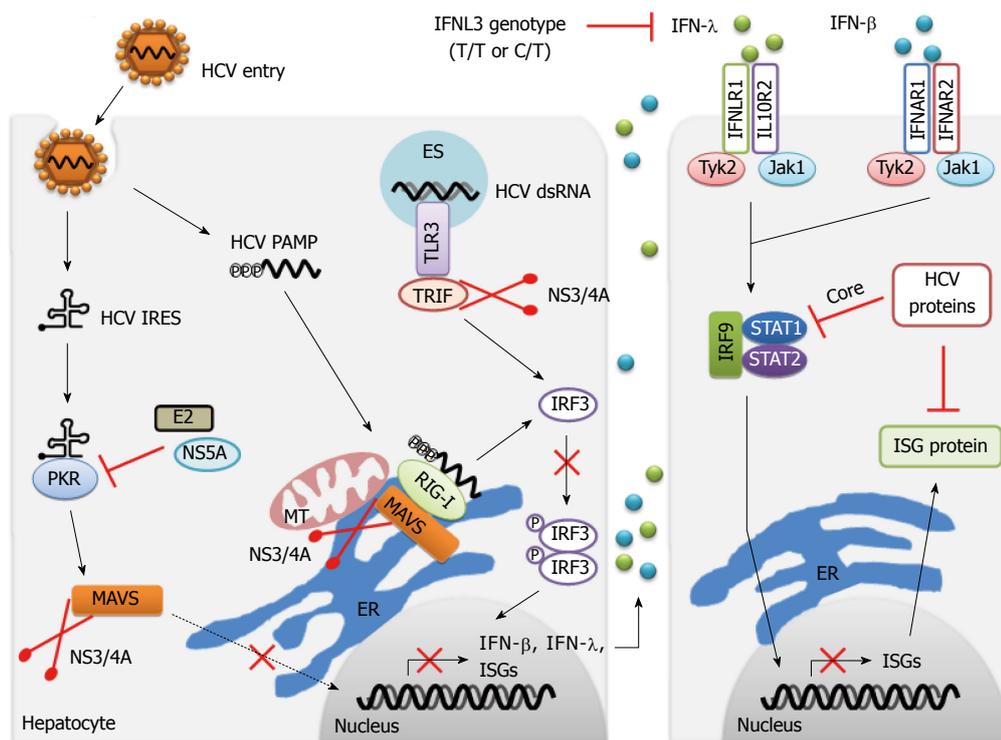
Figure 2 Innate immune evasion of hepatitis C virus infection. Multiple strategies to disrupt both IFN induction and response pathways are responsible for chronic hepatitis C virus (HCV) infection during viral infection. Notably, HCV NS3/4A protease cleaves the adaptor proteins MAVS and TRIF, contributing to the blockage of downstream signaling of PKR, RIG-I and TLR3, and prevention of IFN production. HCV E2 and NS5A proteins suppress the function of PKR by direct binding to this kinase. IFN-β and IFN-λ bind to their distinct receptors and induce the formation of IFN-stimulated gene factor-3 (ISGF3), comprising phosphorylated STAT1, STAT2 and IRF9 through the common JAK/STAT pathway. HCV core and other viral proteins inhibit the activation of STAT signaling, and therefore repress the production of ISGs. The function of specific ISG protein is also suppressed by HCV proteins. Additionally, the unfavorable IFNL3 genotype (T/T or C/T) leads to less expression of IFN-λ, which attenuates the antiviral effect of IFN-λ on HCV infection. MT: Mitochondria; ER: Endoplasmic reticulum; ES: Endosome; PAMP: Pathogen-associated molecular pattern; IRES: Internal ribosomal entry site; TLR: Toll-like receptor; IFN: Interferon; MAVS: Mitochondrial antiviral signaling; ISGs: IFN-stimulated genes; TRIF: TIR-domain-containing adaptor-inducing interferon-β.

this activation for HCV replication are controversial. During HCV infection, the phosphorylation of eIF2α by PKR inhibits cellular mRNAs translation, which contains host factors important for HCV replication and cellular growth (PKR functions as anti-HCV), while it can also suppress the translation of IFN and ISGs (PKR functions as pro-HCV)[135,138]. Two viral proteins, NS5A and E2, inhibit PKR by directly binding to the kinase[181-183] (Figure 2). NS5A represses PKR through a direct interaction with PKR at a site termed the interferon sensitivity determining region within NS5A[181]. Similarly, E2 can also target and inhibit PKR, and the interaction site in E2 bears a highly conserved amino acid sequence with remarkable sequence homology to PKR autophosphorylation and eIF2α phosphorylation sites[183]. The detailed mechanisms by which NS5A and E2 mediate inhibition of PKR are unknown. It is possible that activation and inhibition of PKR occur in a sequential manner; that is, HCV activates PKR at the early stage of infection and inhibits PKR at a later stage through high levels of synthesized NS5A and E2. Interestingly, HCV E1/E2 protein can downregulate the expression of RIG-I and TLR3 *in vitro*, which provides a new mechanism of viral evasion of innate immunity for HCV[184].

In addition to disrupting the IFN induction pathway,

HCV has evolved strategies to block the IFN response pathway. During HCV infection, HCV viral proteins (such as core protein) are implicated as negative-regulators of the IFN response pathway through blockage of JAK/STAT signaling[185]. In addition, viral proteins also inhibit specific ISGs to evade their restrictions[185] (Figure 2). Interestingly, patients infected with HCV genotype 2 or 3 show the highest response rates to therapy (70%-80%), while only 45%-60% of patients with HCV genotype 1 or 4 infection achieve SVR[186]. Actually, the patients infected with HCV genotypes 1 and 4 have high levels of ISG expression in the liver before IFN therapy, contributing to the resistance of IFN treatment[187-189]. The mechanisms by which HCV persists in the liver, despite high levels of hepatic ISGs expression, are poorly understood and future research focusing on this respect may identify novel strategies by which HCV evades innate immunity.

Host genetic factors
Host genetic factors are also involved in innate immunity evasion by HCV. In fact, the single nucleotide polymorphisms (SNPs) upstream of the IFNL3 locus have been determined as novel predictors for both successful clinical therapy of chronic hepatitis C[7,8,10,11] and spontaneous clearance of HCV infection[9,11]. The

IFNL3 locus encodes the antiviral cytokine IFNL3 (also referred to as IL28B), which belongs to type Ⅲ IFN family. The molecular mechanisms of action of this genetic variation remain unknown. One hypothesis is that these SNPs may alter the mRNA expression of IFNL3 and the expression levels of IFNL3 are probably associated with HCV clearance and therapy effect[7,8,188] (Figure 2). Several groups have verified the hypothesis that the unfavorable SNPs in the IFNL3 locus contribute to lower expression of IFNL3 within the liver, peripheral blood mononuclear cells and whole blood[7,8,189,190,191]. Patients with the unfavorable IFNL3 genotype exhibit the inhibition of innate immune function of NK cells, which suggests that the genetic variation in the IFNL3 locus directly affects antiviral immunity to HCV[192]. Further studies to address how different IFNL3 polymorphisms regulate the innate immune response to HCV infection and the outcome of IFN therapy are encouraged, which will facilitate the development of new therapeutic strategies to clear HCV.

Recently, a newly characterized gene, IFNL4, was identified[193]. IFNL4 is created by a dinucleotide variant ss469415590 (TT or ΔG) in the upstream region of IFNL3 (IL28B) on chromosome 19q13.13[193]. Compared with previously discovered variant rs12979860, ss469415590 is more strongly associated with HCV clearance in individuals of African ancestry[193]. The polymorphism in IFNL4 provides new insights into the genetic regulation of HCV clearance. It is now known that the type Ⅲ IFNs (IFN-λs) comprise IFNL1 (IL29), IFNL2 (IL28A), IFNL3 (IL28B) and IFNL4. We and other groups have validated the effect of IFN-λs on inhibiting HCV replication *in vitro*[194,195]. The restricted receptor distribution of IFN-λ makes it likely that IFN-λs could be developed as new drugs for HCV therapy, with fewer side effects than IFN-α[195].

CONCLUSION

The host innate immunity is critical for HCV sensing and subsequent viral clearance, while the dysregulation of innate immune signaling can lead to chronic viral infection. HCV triggers this immune dysregulation by evading the host innate immune response *via* numerous strategies, including viral factors and host genetic factors, which contributes to eventual viral persistence. Multiple types of cells residing in the liver microenvironment are involved in regulation of HCV-induced innate immune response. However, the detailed mechanisms of how hepatocytes and other cell types regulate each other, and how the immune modulation of cells determines the consequence of HCV infection, is still unclear. The intracellular sensors, such as RIG-I, TLR3 and PKR, are essential for HCV recognition; however the cross talk of these receptors in the detection of HCV during viral infection remains to be clarified. Although the detection of HCV by RIG-I and TLR3 is well characterized, it is unknown how and where the HCV PAMPs are presented to PKR

and NLRs for innate immune signaling. The potential mechanisms used by HCV to evade PRRs-dependent recognition should be identified. Genetic studies have provided insights into IFNL3 polymorphisms, indicating that variations of IFNL3 predict an effective immune response for both natural and IFN-induced HCV clearance. However, the IFNL3 genotype is not the best predictor of therapeutic responses to HCV infection[167]. Thus, further studies to define new host factors that determine the outcome of viral infection could help to effectively guide the individual treatment of HCV infection, together with known predictors.

Although the IFN-based therapy is still the current standard-of-care therapy for HCV in many treatment centers, significant scientific advances have enabled the development of new classes of antivirals for the treatment of HCV. In the near future, the DAAs targeting HCV NS3 protease, NS5A or NS5B polymerase may well cure virtually all hepatitis C patients with an all-oral, interferon-free regimen[196]. However, viral breakthrough and drug resistance during these therapeutic courses will need to be considered and monitored carefully[197]. Furthermore, the efficacy of these drugs for other genotypes of HCV, not genotype 1, still needs to be determined. A full understanding of how the host antiviral innate immunity responds to HCV infection, and what drives HCV evasion of this protective activity, will be important to the development of new therapeutic approaches and novel vaccines with high-efficacy for HCV. In addition, the establishment of new, efficient cell culture systems and more convenient small animal models is highly encouraged, which will facilitate basic research on viral-host interactions, innate immunity and HCV pathogenesis, leading to the development of drugs and vaccines for chronic hepatitis C.

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REVIEW

Gastrointestinal Behçet's disease: A review

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Abstract

Behçet's disease (BD) is an idiopathic, chronic, relapsing, multi-systemic vasculitis characterized by recurrent oral and genital aphthous ulcers, ocular disease and skin lesions. Prevalence of BD is highest in countries along the ancient silk road from the Mediterranean basin to East Asia. By comparison, the prevalence in North American and Northern European countries is low. Gastrointestinal manifestations of Behçet's disease are of particular importance as they are associated with significant morbidity and mortality. Although ileocecal involvement is most commonly described, BD may involve any segment of the intestinal tract as well as the various organs within the gastrointestinal system. Diagnosis is based on clinical criteria - there are no pathognomonic laboratory tests. Methods for monitoring disease activity on therapy are available but imperfect. Evidence-based treatment strategies are lacking. Different classes of medications have been successfully used for the treatment of intestinal BD which include 5-aminosalicylic acid, corticosteroids, immunomodulators, and anti-tumor necrosis factor alpha monoclonal antibody therapy. Like inflammatory bowel disease, surgery is reserved for those who are resistant to medical therapy. A subset of patients have a poor disease course. Accurate methods to detect these patients and the optimal strategy for their treatment are not known at this time.

Core tip: Behçet's disease is an uncommon subtype of inflammatory bowel disease. It can present with a wide array of clinical manifestations that may mimic other diseases including Crohn's disease. Establishing the diagnosis remains a challenge and clinicians must be aware of the relevant clinical manifestations and diagnostic considerations. The optimal medical management is limited by the lack of rigorous clinical trial data.

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INTRODUCTION

Behçet's disease (BD) is an inflammatory disorder classically characterized by recurrent oral and genital ulcers, uveitis and characteristic skin lesions. Behçet's patients can also present with arthritis, gastrointestinal lesions, central nervous symptoms and vascular lesions[1-3]. Table 1 lists the common clinical manifestations of BD. There are no pathognomonic laboratory tests for Behcet's disease. The most widely accepted criteria were published by the International Study Group (ISG) for Behcet's Disease in 1990[4]. Diagnosis requires the observation of recurrent oral ulceration (three episodes within any 12 mo period) plus any two of the following: recurrent genital ulceration, eye lesions, skin lesions or a positive pathergy test.

EPIDEMIOLOGY

Prevalence of BD is highest in countries along the ancient silk road from the Mediterranean Basin to East Asia. Prevalence estimates vary and are reported as 3.8-15.9/100000 in Italy, 7.1/100000 in France, 7.5/100000 in Spain, 7.6/100000 in Egypt, 20-420/100000 in Turkey, 15.2-120/100000 in Israel, 68/100000 in Iran, 14/100000 in China and 7.5-13/100000 in Japan[5-11]. By comparison, prevalence in North American and Northern European countries is rare: varying between 0.27-5.2 per 100000[5]. Mean age of onset is during the 3rd and 4th decades of life[12]. Male-to-female ratio varies regionally - the disease is generally more common amongst men in most Mediterranean, Middle Eastern and Asian countries; conversely, higher female prevalence has been reported in the United States, Northern European and East Asian countries[12,13].

GASTROINTESTINAL DISEASE

Gastrointestinal (GI) manifestations of Behçet's disease are of particular importance as they are associated with significant morbidity and mortality. GI manifestations usually occur 4.5-6 years after the onset of oral ulcers[14]. The most common symptoms include abdominal pain, nausea, vomiting, diarrhea and gastrointestinal bleeding[15]. Although ileocecal involvement is most commonly described, BD may involve any segment of the alimentary tract and the various GI organs[14,16]. In general, two forms of intestinal Behçet's disease exist - neutrophilic phlebitis that leads to mucosal inflammation and ulcer formation and large vessel disease (*i.e.*, mesenteric arteries) that results in intestinal ischemia and infarction[17]. The frequency of GI involvement among patients with BD varies in different countries. Lower frequency has been reported in Turkey (2.8%), India (3.4%) and Saudi Arabia (4%), moderate frequency in China (10%) and Taiwan (32%) and the highest frequency has been

reported in the United Kingdom (38%-53%) and Japan (50%-60%)[12,16,18-21]. It is imperative that the clinician caring for patients with BD is aware of the myriad of clinical manifestations, diagnostic methods and treatment strategies available for the gastrointestinal manifestations of BD.

ESOPHAGUS

Esophageal involvement in BD is uncommon; incidence rates between 2%-11% have been reported[14]. Esophageal manifestations are associated with involvement of another part of the gastrointestinal tract in more than 50% of cases[16]. Common clinical manifestations include retrosternal chest pain, dysphagia, odynophagia, melena and hematochezia[22,23]. Endoscopic findings usually consist of a single or multiple ulcers. Ulcers tend to aggregate in the middle or distal third of the esophagus. Serious complications such as stenosis and perforations may occur[24]. "Downhill" esophageal varices have also been reported in patients with obstruction of the caval veins[25,26].

BD may also affect the motility of the esophagus. A study of 25 patients with BD and dyspeptic symptoms demonstrated that 16% had esophageal motor abnormalities. Median lower esophageal pressure (LES) and LES relaxation were significantly lower in the BD group in comparison to age-matched controls[27]. Although routine endoscopy is not recommended in patients with BD[23,28], referral for upper endoscopy and/or esophageal manometry for patients with upper gastrointestinal symptoms may be appropriate.

STOMACH AND DUODENUM

The stomach is thought to be the least involved segment of the gastrointestinal tract. However, consistent with varying phenotypes of disease across different patient populations, one study of 28 patients with BD in Taiwan demonstrated a prevalence of 43% of gastroduodenal involvement amongst those of Chinese descent[29]. Dyspepsia and epigastric abdominal pain were the most common symptoms. Patients either had isolated gastric, isolated duodenal or combined gastroduodenal ulcers.

Rare manifestations in the stomach include dieulafoy's lesions and gastric non-hodgkins lymphoma[30,31]. Cases of pyloric stenosis due to edematous hypertrophy of the pyloric ring have also been reported[32,33]. Likewise, gastroparesis has also been linked with BD in a case report[34].

The prevalence of *Helicobacter pylori* does not appear to be increased in patients with BD. This was illustrated in a prospective, single center study of 45 patients with BD and upper gastrointestinal complaints. In comparison to age-matched controls there was no difference in prevalence (73.3% *vs* 75%, $P > 0.05$) and eradication rate with two weeks of triple therapy (75% *vs* 70%, $P > 0.05$)[35]. Curiously, a study

Table 1	Common clinical manifestations of Behçet's disease[12,130]
Skin, Mucocutaneous	Papulopustular lesions (Behçet's pustulosis), erythema nodosum, superficial thrombophlebitis, minor aphthous ulcers
Eyes	Anterior and posterior uveitis, retinal vasculitis
Vascular	Deep venous thrombosis, large-vein thrombosis, pulmonary artery aneurysm
Musculoskeletal	Arthralgia, arthritis (monoarticular, oligoarticular)
Gastrointestinal	Ileocecal ulcers
Genitourinary	Genital ulcers, epididymitis
Central nervous system	Meningoencephalitis, parenchymal disease (pyramidal signs, hemiparesis, behavioral changes, sphincter disturbance), intracranial hypertension secondary to dural sinus thrombosis

Table 2 Differences between intestinal Behçet's disease and Crohn's disease[39,40,48,49,52,54]

	Crohn's disease	Intestinal BD
Extra-intestinal manifestations	Iritis, episcleritis more specific	Oral and genital ulcers more common, papulopustular lesions, neurologic and arterial manifestations
Perianal disease (fistula, fissures)	Common	Rare
Strictures, fistula, abscess	Common, characteristic of disease process	Less common but possible
Serologic markers	Anti-saccharomyces cerevisiae antibody (Prevalence: 41%-76%)	IgM anti-α-enolase antibody (Prevalence: 67.5%)
Endoscopic features	Irregular, longitudinal ulcers with cobblestone appearance, may have aphthous lesions Segmental or diffuse involvement	Round or oval shaped, punched-out lesions with discrete margins, > 1 cm, Focal distribution, < 5 ulcers. No aphthous lesions
Pathognomonic lesions on histopathology	Non-caseating epithelioid granuloma	Non-specific neutrophilic or lymphocytic phlebitis with or without aortitis

BD: Behçet's disease.

of 13 patients demonstrated a statistically significant decrease in oral and genital ulcerations during the 6 mo follow-up after eradication therapy suggesting a possible etiologic role of *Helicobacter pylori*[36].

JEJUNUM, ILEUM AND COLON

Studies using video capsule endoscopy have demonstrated that BD can involve the entire small bowel[37,38]. Classically intestinal BD manifests as large (> 1 cm), round/oval shaped, deep ulcers in the ileocecal region. This was demonstrated in a landmark Korean study of 94 patients with intestinal BD in which 96% had involvement of the terminal ileum, ileocecal valve or cecum[39]. Localized single (67%) and localized multiple (27%) ulcers were the most common patterns of distribution. Multisegmental and diffuse colonic involvement were rare (6%). Eighty five percent of patients had less than 6 ulcers (67% of single ulcer, 18% of 2-5 ulcers). Ulcers were large with a mean diameter of 2.9 cm (76% of ulcers > 1 cm). Round/oval shape was most common (77%). Deep ulcers

were more common (68% *vs* 38% superficial). Of note, rectal involvement in BD is exceedingly rare and occurs in less than 1% of patients[15].

Rare complications of BD include strictures, abscess formation, fistula and perforation. One study found the rates of perforation, fistula, stricture and abscess to be 12.7%, 7.6%, 7.2% and 3.3% respectively[40]. A series of 22 patients with perforation secondary to intestinal BD demonstrated that all perforations occurred in the terminal ileum, ileocecal region or ascending colon[41]. Risk factors for perforation include age < 25 at diagnosis, history of laparotomy and volcano-shaped ulcers on colonoscopy[42].

DIFFERENTIAL DIAGNOSIS

In areas where tuberculosis and BD are endemic, it is imperative to make the correct diagnosis as the treatment differs substantially. To our knowledge, there have been no studies conducted comparing intestinal BD to intestinal tuberculosis (ITB). In a study comparing ITB and Crohn's disease (CD), multivariate analysis demonstrated that blood in stool (OR = 0.1, 95%CI: 0.04-0.5), sigmoid involvement (OR = 0.07, 95%CI: 0.01-0.3) and focally enhanced colitis on histology (OR = 0.1, 95%CI: 0.03-0.5) were more predictive of CD than ITB[43]. Chest radiography may identify pulmonary involvement in 32% of patients with ITB[44]. T-SPOT.TB can be a useful assay but with varying sensitivity and specificity of 83%-100% and 47%-100% respectively[45]. Polymerase chain reaction of endoscopic biopsies has low sensitivity (21.6%) but is highly specific (95%)[46]. A biopsy for specialized culture is definitive but time consuming and has a very low sensitivity[47]. When the diagnosis between the CD and ITB is unclear, expert opinion suggests an empiric 8 wk trial of anti-tuberculous therapy.

The more difficult distinction is between CD and BD. Both diseases typically can present in young patients, are associated with extraintestinal manifestations (EIMs), involve any area of the GI tract and have a waxing and waning course. Table 2 demonstrates the key differences between CD and intestinal BD.

CD and BD share many EIMs in common including oral ulcers, uveitis, arthritis and erythema nodosum - although oral ulcers and uveitis are more common in BD. Genital ulcers, a hallmark of BD, are rare in CD. Amongst eye findings, episcleritis and iritis are

more specific for CD whereas retinal vasculitis is more commonly associated with BD[48]. Both diseases have an increased risk of deep venous thrombosis - however, CD is not associated with other vascular manifestations such as varices, Budd-Chiari Syndrome (BCS) or arterial vasculitis. Neurologic disease, an important complication in BD, is typically not associated with CD.

Intestinal complications such as strictures, fistula and abscess occur in both diseases but are less common in BD. Jung *et al*[40] found that fistula (CD: 27.4% *vs* BD: 7.6%, $P \leqslant 0.001$), strictures (CD: 38.3% *vs* BD: 7.2%, $P \leqslant 0.001$) and abscess formation (CD: 19.6% *vs* BD: 3.3%, $P \leqslant 0.001$) were more common in CD. Perforation was more common in BD although not statistically significant (CD: 8.7% *vs* BD: 12.7%, $P = 0.114$). Perianal fistula was a rare complication in BD (CD: 39.2% *vs* BD: 2.5%, $P \leqslant 0.001$).

Serologic testing appears to be less reliable in differentiating between CD and BD. Anti-saccharomyces cerevisiae antibody, a specific marker for CD, is positive in 41%-76% of patients with CD[49] and 0%-44.3% of patients with BD[50,51] making it unhelpful in differentiating between the two diseases. The data for IgM anti-Alpha-Enolase Antibody is quite similar. The antibody is reported to be present in 67.5% of patients with intestinal BD[52] and 50% of patients with CD[53].

Colonoscopic findings can help differentiate accurately between the two diseases. A study of 235 patients with CD and intestinal BD found that round ulcer, focal single/focal multiple distribution of ulceration, less than 6 ulcers, absence of cobblestone appearance or aphthous lesions were most predictive of BD on colonoscopy in multivariate analysis[54]. Although non-epithelioid granuloma can be found in CD in 30% of patients[54], there are no other pathologic features that may help distinguish between CD and BD on intestinal mucosal biopsies.

PANCREAS

Pancreatic involvement in BD is exceptionally rare. Very few case reports of acute pancreatitis have been attributed to BD[55,56]. Chronic pancreatitis was also reported in a patient with BD - however he also had a history of heavy alcohol intake[57]. It is possible that pancreatic involvement is underreported or underdiagnosed - an autopsy series of 170 cases from Japan suggested 2.9% involvement of the pancreas[58]. Vasculitis is likely the underlying pathological process leading to pancreatic inflammation - this theory is further supported by other vasculitic syndromes such as granulomatosis with polyangiitis which has also been associated with pancreatitis[59].

LIVER

BCS is the most common manifestation of the liver in patients with BD. BCS may be a serious complication and associated with a high mortality rate. Venous thrombosis secondary to endothelial dysfunction from vasculitis has been proposed as a possible mechanism[60]. Studies have reported prevalence rates between 1.3%-3.2%[61-63]. Men are more likely to develop BCS than females. Presenting signs and symptoms include right upper quadrant abdominal pain, hepatosplenomegaly and ascites[64]. Patients can present with acute, subacute or chronic BCS. Acute BCS appears to carry a very poor prognosis[65]. Thrombosis of the hepatic veins (HV), inferior vena cava (IVC) and portal vein (PV) may occur. In a Turkish series of 14 patients with BCS secondary to BD, 2 had isolated HV involvement, 8 had HV and IVC involvement and 4 had HV, IVC and PV involvement. Six out of 8 with HV and IVC involvement and 4/4 of those with PV, HV and IVC involvement died with mean survival of 10.4 mo[62]. It appears the extent of IVC obstruction appears to be the major determinant of survival in BD patients with BCS. Notable complications from BCS include hepatosplenomegaly, ascites, lower extremity edema, esophageal varices and liver failure.

Given the relatively high prevalence and significant mortality rate of BCS in BD patients, some authors suggest that all patients with BD should be screened for BCS with duplex ultrasonography[61]. Other uncommon manifestations of BD include aseptic abscess formation in the liver, chronic hepatitis and sclerosing cholangitis[66-68].

VISCERAL ARTERIAL INVOLVEMENT

BD is a unique form of vasculitis because it can involve arteries and veins of all sizes[69]. One study of 38 BD patients with vascular involvement found that venous involvement was by far the most prevalent (88%)[70]. Incidence of vascular involvement varies between 7%-29%[71]. Males are more commonly affected[72]. Arterial manifestations include formation of aneurysms and luminal thrombi[64]. Important sites of arterial involvement include the arteries of the upper and lower extremities (radial, femoral, popliteal)[73], pulmonary artery[74] and thoracoabdominal aorta[75]. Other sites of involvement include the subclavian[76], iliac[77], carotid[78,79], renal[80] and coronary arteries[81].

Arterial involvement of the intra-abdominal organs is rare. When it occurs, patients may present with fever, abdominal pain or pulsatile mass. Complications can include intestinal infarction and gastrointestinal hemorrhage. Very few cases of visceral aortic aneurysm exist in the literature - involvement of the celiac trunk[82], superior mesenteric[83], hepatic[84], splenic[85], inferior mesenteric[86] and ileocolic artery[87] have been described.

DIAGNOSIS

Establishing the diagnosis of intestinal BD remains
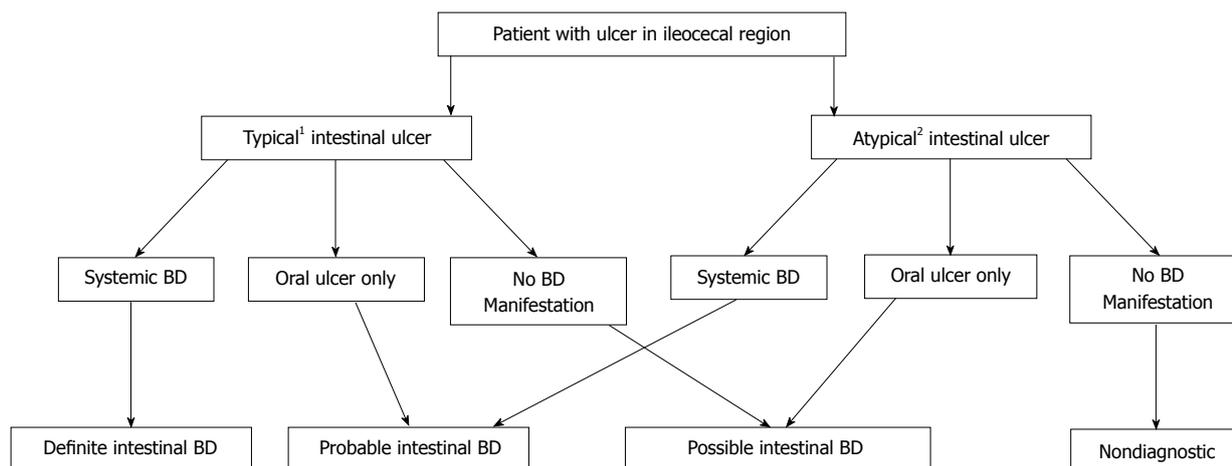
Figure 1 Algorithm for diagnosis of intestinal Behçet's disease. [1]Typical: < 5 ulcers, oval in shape, deep, discrete borders located in ileocecal region; [2]Atypical: Ulcerations that do not satisfy all criteria for typical. Adapted from Cheon *et al*[88]. BD: Behçet's disease.

Table 3 Disease activity index for intestinal Behçet's disease	
Item	**Score**
General well-being in the preceding week	
Well	0
Fair	10
Poor	20
Very poor	30
Terrible	40
Fever	
< 38 ℃	0
≥ 38 ℃	10
Extraintestinal manifestations	5 points for each manifestation[1]
Abdominal pain in the preceding week	
None	0
Mild	20
Moderate	40
Severe	80
Abdominal mass	
None	0
Palpable mass	10
Abdominal tenderness	
None	0
Mildly tender	10
Moderately or severely tender	20
Intestinal complications	10 points for each complication[2]
Number of liquid stools in the preceding week	
0	0
1-7	10
8-21	20
22-35	30
≥ 36	40
Total score	
Severity of disease	
Quiescent Intestinal BD	≤ 19
Mild intestinal BD	20-39
Moderate intestinal BD	40-74
Severe intestinal BD	≥ 75

[1]Five points are added for each type of the following manifestations: oral ulcer, genital ulcers, eye lesions, skin lesions or arthralgia. 15 points are added for each of the following: vascular involvement or central nervous system involvement; [2]Fistula, perforation, abscess or intestinal obstruction. Adapted from Cheon *et al*[89]. BD: Behçet's disease.

a challenge. Previously, the diagnosis was based primarily on the combination of clinical criteria for systemic BD with the presence of intestinal ulcer formation[88]. However, the dilemma is that not all patients with "typical" intestinal ulcers satisfy ISG criteria for systemic BD at the time of endoscopy - this often leads to delayed or misdiagnosis. As a result, Cheon *et al*[88] proposed four separate categories of classification using the modified Delphi process: definite, probable, suspected and non-diagnostic for intestinal BD (Figure 1). In their study, 145 (51.8%) of 280 patients were confirmed to have intestinal BD. Using this algorithm, the first 3 categories provide for a pooled sensitivity, specificity, positive and negative predictive values of 98.6%, 83%, 86.1% and 98.2%, respectively.

DISEASE MONITORING

A clinical scoring system called the Disease Activity Index for Intestinal BD (DAIBD) exists. Prior to its development in 2011 by Cheon *et al*[89], other IBD indices such as the Crohn's Disease Activity Index or Harvey-Bradshaw Index were frequently used. The DAIBD provides a score between 0 and 325 based on an 8-point index. It classifies disease activity as quiescent ($\leq$ 19), mild (20-39), moderate (40-74) and severe ($\geq$ 75). Laboratory and endoscopic data are not part of the scoring system making it ideal for use in the outpatient setting (see Table 3). A later study demonstrated that the correlation between DAIBD and endoscopic severity was weak (r = 0.43)[90]. It is still unclear whether endoscopic severity is superior to clinical severity in predicting prognosis.

Serum biologic markers are a helpful adjunct in monitoring disease activity in intestinal BD. Soluble triggering receptor expressed in myeloid cells-1 demonstrates the highest degree of correlation with disease activity of intestinal BD[91]. C-reactive protein

Table 4 Summary of evidence for medical management of intestinal Behçet's disease

Therapy	Level(s) of published evidence
5-ASA/sulfasalazine	Retrospective cohort study[95]
Corticosteroids	Expert opinion, European League Against Rheumatism Recommendations[114]
Thalidomide	Case reports[131-133]
Azathioprine, 6-MP	Retrospective cohort studies[97,123]
Mycophenolate	Case report[134]
Methotrexate	Case series[135]
Tacrolimus	Case report[136]
Infliximab	Single arm clinical trial[102], Retrospective cohort study[104] Case series[101]
Adalimumab	Prospective, non-placebo controlled clinical trial[110]
Etanercept	Case report[112]

(CRP) and erythrocyte sedimentation rate have also been demonstrated to correlate with disease activity. Unexpectedly, serum levels of tumor necrosis factor-α (TNF-α) are not a good biologic marker of intestinal BD activity. Stool markers of inflammation that have been shown to correlate with disease activity in patients with CD and UC including fecal calprotectin have not been studied to date in patients with BD.

MEDICAL MANAGEMENT

Management of Behçet's syndrome is challenging because of a general lack of high quality evidence[92]. Although some controlled data exists for management of arthritis, eye involvement and mucocutaneous disease, there is a considerable lack of evidence addressing treatment strategies for neurologic and vascular manifestations. Similarly, there are no internationally accepted, standardized treatment strategies for gastrointestinal BD. In order to standardize treatment, the Japanese Inflammatory Bowel Disease Research Group proposed a set of consensus statements in 2007 - they were updated again in 2014 to address growing low-level evidence supporting the use of anti-TNF-α mAb therapy[93,94]. Generally, with some exceptions, the same classes of medications that have been used for the treatment of systemic BD have also been used to treat intestinal BD and include colchicine, 5-ASA/sulfasalazine, corticosteroids (CS), immunomodulators, immunosuppressants, IFNα and anti-TNF-α mAb therapy. Table 4 summarizes the highest level of evidence for each modality of therapy.

Similar to IBD, sulfasalazine (3-4 g/d) and 5-ASA (2.25-3 g/d) have been the traditional mainstay of therapy. In a retrospective, single center Korean study of 143 patients with intestinal BD treated with 5-ASA/sulfasalazine monotherapy, 46 (32.2%) patients had a clinical relapse (defined as DAIBD $\geq$ 20)[95]. Younger age at time of diagnosis (< 35 years), elevated CRP

($\geq$ 1.5 mg/dL) and increased disease activity (DAIBD $\geq$ 60) were associated with higher relapse rates. The authors concluded that 5-ASA/sulfasalazine should be reserved for mild-to-moderate intestinal BD.

CS are usually reserved for moderate to severe disease in order to induce remission[93,94,96]. Doses of 20-100 mg prednisolone have been used depending on the severity of the disease[1]. Expert opinion recommends a weight-based approach of 0.5-1 mg/kg per day of prednisolone for 1-2 wk followed by a taper of 5 mg weekly until discontinuation[93,94]. Hospitalized patients with severe disease may require intravenous methylprednisolone therapy[15]. Intravenous pulse therapy of 1 gram/d for 3 d followed by oral prednisolone taper has been advocated[2]. At 1 mo, almost 1/2 of patients achieve complete remission, 43% attain partial remission and 11% will demonstrate no response (*i.e.*, steroid resistance). Patients who are steroid-dependent or steroid-resistant can be difficult to manage and often times require additional therapy (immunomodulators, anti-TNF-α mAb) or surgery. Maintenance therapy with CS is not appropriate and long term steroid use should be avoided given the significant systemic side effects.

Thiopurines are indicated in patients with steroid-dependent disease, enterocutaneous fistulae and maintenance of postoperative remission. Expert opinion advocates starting azathioprine at doses of 25-50 mg/d with gradual titration every 2-4 wk to 2.0-2.5 mg/kg[94]. The starting dose of 6-MP is 0.5 mg/kg and similarly is escalated every 2-4 wk to a goal dose of 1.0-1.5 mg/kg[97]. In a retrospective analysis of 272 patients with intestinal BD, 67 patients were started on thiopurines (66 on azathioprine, 1 on 6-mercaptopurine) during hospitalization for the above three indications. Of the 39 patients who were maintained on thiopurines, the relapse rates were 5.8%, 43.7% and 51.7% at one, three and five years respectively[97]. Younger age at the time of diagnosis of intestinal BD (< 25 years) and a lower hemoglobin level (< 11 g/dL) were independent risk factors for relapse on thiopurine maintenance therapy.

Anti-TNF-α mAb therapy alone or in combination with immunomodulatory therapy is also a modality of treatment for patients with steroid-dependent or steroid-resistant intestinal BD. Sfikakis *et al*[98] recommend the introduction of Anti-TNF-α mAb therapy only in patients who have failed two immunosuppressive agents and require prednisolone at a dosage > 7.5 mg/d. Infliximab (IFX) and adalimumab (ADA) are the two best studied biologic agents. The first published case reports of IFX success were in 2001; clinical response to IFX was demonstrated in 3 patients with steroid dependent BD in two separate case reports[99,100]. A larger case series of 6 patients was also promising. Four out of 6 patients who received induction and maintenance therapy (5 mg/kg) at 0, 2 and 6 wk and every 2 mo

onwards maintained remission. The other two patients had persistent ileal ulceration and required surgery - however, 1 of those two patients remained in remission on IFX after surgery[101]. Similar results were replicated in several other studies suggesting that IFX has good efficacy and tolerability for refractory intestinal BD[102,103]. A Korean multi-center retrospective study of 28 patients treated with IFX demonstrated that older age (> 40 years), female sex, longer duration of disease (> 5 years), concomitant immunomodulator use and achievement of remission within 4 wk were predictive of sustained response[104].

Remission has also been successfully demonstrated with ADA in several case reports[105-108]. ADA provides a subcutaneous option which is sometimes preferred by some patients[109]. Recently, the efficacy and safety of ADA was investigated in a prospective, non-placebo controlled, multi-center trial in Japan of 20 patients with refractory intestinal BD disease[110]. A novel composite index which combined patient reported GI symptoms in the preceding 2 wk and change in ulcer size based on endoscopic assessment was used to evaluate efficacy. Nine (45%) and 12 patients (60%) demonstrated improvement at 24 and 52 wk respectively. Four patients (20%) were able to achieve complete early and late remission at weeks 24 and 52 respectively. Furthermore, 8 of 13 patients on steroids at baseline were able to completely discontinue them during the study.

Etanercept (ETN) in a double-blinded, placebo controlled clinical trial of 40 men in Turkey demonstrated efficacy against mucocutaneous involvement - specifically oral ulcers and nodular lesions[111]. We are only aware of one case of successful treatment in a pediatric patient with refractory intestinal BD[112] - it is worth noting she was simultaneously treated with tacrolimus, prednisolone and mizoribine. Despite its use for other manifestations of BD, ETN currently has no role in the management of refractory intestinal BD and thus is not addressed in the most recent guidelines[94]. Interestingly, ETN was not shown to be effective for patients with moderate to severe CD[113].

Despite promising low-level evidence documenting success of anti-TNF-α mAb therapies, a prospective, randomized, placebo-controlled trial is necessary to validate their use. The feasibility of conducting large placebo-controlled trials is uncertain given the difficulty recruiting patients with refractory intestinal BD.

Regarding BCS, treatment options include medical therapy, interventional procedures and surgical management. Ascites can be managed with salt restriction and diuretic therapy. Endoscopy may be clinically indicated to assess and treat possible varices. Although there is limited data to guide management of major venous involvement, monthly cyclophosphamide and CS form the cornerstone of therapy for BCS[114]. Anticoagulation is controversial and is not recommended in the most recent European League Against Rheumatism guidelines[114].

Nevertheless, long term anticoagulation with warfarin is still commonplace and advocated by some authors[61,63]. IFX has been attempted but was unsuccessful in 2 patients with advanced disease refractory to monthly cyclophosphamide and CS. A third patient appeared to have regression of disease in IVC - however the development of cranial sinus thrombosis prompted the investigators to discontinue IFX[115]. It remains unclear whether anti-TNFα mAb therapy or anticoagulation has a role in BCS in BD.

Although immunosuppressive treatments are generally indicated in patients with arterial aneurysms, definitive therapy with open or endovascular repair is required because of a high risk of rupture. Treatment with prednisolone 5-60 mg/d combined with azathioprine (50-100 mg/d) and/or colchicine (1.2 mg/d) has been advocated by some experts[116].

SURGICAL MANAGEMENT

As in patients with IBD, surgical therapy is reserved for those who are refractory to medical therapy or presenting with severe gastrointestinal bleed. Other indications for surgery include perforation, fistula formation, intestinal obstruction and abdominal mass[117]. It is interesting to note that ileal disease and ocular lesions are associated with increased risk of surgical resection[118].

There is controversy over the type of surgical procedure and length of bowel to remove. Traditionally, right hemicolectomy, ileocolectomy and partial resection of the small bowel are most commonly performed. Chou *et al*[41] recommended up to 80 cm of ileal resection from the ileocecal valve at the time of right hemicolectomy More recently, authors suggest a conservative approach with removal of only the grossly involved bowel as there appears to be no relation between length of resection and rates of recurrence or reoperation[117].

For select BD patients who undergo surgery, creation of a stoma may be preferable over primary anastomosis given a high rate of intestinal leakage, perforation and fistulization at the anastomosis site[14,42]. Reoperation in those who undergo surgery is high - 30%-44% and often occurs at or near the anastomosis site (similar to CD)[117-119]. Independent predictors of reoperation include history of postoperative steroid therapy, CRP levels greater than 4.4 or endoscopic evidence of "volcano-type" deep ulcers.

If medical therapy fails in BCS, percutaneous angioplasty provides an attractive option if the segment of thrombosis in the HV or IVC is focal[120]. Alternatively, transjugular intrahepatic portosystemic shunt may be another appropriate option. It is unclear whether surgical portosystemic shunting affects survival. Orthotopic liver transplantation has been performed as a life-saving procedure in patients with BCS.

Arterial aneurysms in BD are generally treated

surgically because of a high risk of rupture. Although open surgical repair (synthetic *vs* autologous vein graft) were previously the preferred method, an endovascular approach has emerged as a more durable alternative[64]. A Korean study of 16 BD patients who underwent endovascular repair demonstrated a patency rate of 89% at 2 years[121]. A more recent study confirmed that endovascular therapy is safe and has long term durability[116]. Concomitant treatment with immunosuppressive medications is necessary to control vessel wall inflammation.

PROGNOSIS

Unlike CD whereby many patients may experience a disease flare-up and subsequent corticosteroid and/or immunosuppressive treatment at least once in their lifetime, intestinal BD generally follows a distinguishable mild or severe clinical course. This was illustrated in a 5 year retrospective study of 130 patients with intestinal BD which demonstrated that a large proportion of patients (77.1%) experienced a mild clinical course; on the other hand, 28.5% had a more severe clinical course with multiple relapses and/or chronic symptoms[122]. Other studies cite a similar recurrence rate of 24.9%-28% and 43%-49% at 2 and 5 years respectively[123-125].

Although the prognosis of intestinal BD was previously believed to be worse than CD, a retrospective cohort study of 332 CD and 276 Intestinal BD demonstrated no difference in cumulative probability of disease related surgery (P = 0.287) or hospital admission (P = 0.259) over a mean follow-up period of almost 7 years[40]. Furthermore, there was no observed difference in postoperative clinical recurrence (P = 0.724) or reoperation rates (P = 0.770).

Nonetheless, despite no significant long term difference in outcomes in comparison with CD, surgery rates still remain high. Cumulative rates of surgical interventions are 20% at 1 year, 27%-33% at 5 years and 31%-46% at 10 years after diagnosis[126-128]. Many clinical variables have been investigated as predictors of outcomes during medical and surgical therapy: young age, high disease activity at time of diagnosis, "volcano-type" ulcers on endoscopy or colonoscopy, elevated CRP and history of laparotomy confer the poorest prognosis[126].

Death from intestinal BD is uncommon. Disease-specific mortality in BD is mainly due to major vessel disease (arterial aneurysm, BCS) or neurologic involvement[129].

CONCLUSION

Behçet's disease can present with a wide array of gastrointestinal manifestations. Although ileocecal involvement is classically associated with BD, any part of the GI tract from the mouth to the anus can be involved and there may be appreciable morbidity

and mortality associated with gastrointestinal BD. Diagnosis remains a challenge with no universally accepted criteria. Management can be confusing and there are no unanimously accepted treatment algorithms. The goal of treatment is to keep patients in clinical remission, reduce relapses and prevent surgical intervention. Although endoscopic remission is a treatment goal in IBD, there is currently insufficient evidence in the literature to recommend mucosal healing as a treatment goal in BD[94]. Treatment requires cooperation across multiple specialties including the primary care physician, rheumatologist, gastroenterologist and possibly interventional radiologist and/or surgeon. Anti-TNF-α mAb therapy appears to be promising for more severe and/or refractory intestinal disease - more clinical trials are necessary to support their use. A certain subset of patients have a poor disease course and better methods to identify them early in the disease course will be an important area of study. It is unclear at this time which populations of BD patients may benefit from early aggressive therapy and whether this intervention will have an impact on the progression of disease.

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New gene therapy strategies for hepatic fibrosis

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Abstract

The liver is the largest internal organ of the body, which may suffer acute or chronic injury induced by many factors, leading to cirrhosis and hepatocarcinoma. Cirrhosis is the irreversible end result of fibrous scarring and hepatocellular regeneration, characterized by diffuse disorganization of the normal hepatic structure, regenerative nodules and fibrotic tissue. Cirrhosis is associated with a high co-morbidity and mortality without effective treatment, and much research has been aimed at developing new therapeutic strategies to guarantee recovery. Liver-based gene therapy has been used to downregulate specific genes, to block the expression of deleterious genes, to delivery therapeutic genes, to prevent allograft rejection and to augment liver regeneration. Viral and non-viral vectors have been used, with viral vectors proving to be more efficient. This review provides an overview of the main strategies used in liver-gene therapy represented by non-viral vectors, viral vectors, novel administration methods like hydrodynamic injection, hybrids of two viral vectors and blocking molecules, with the hope of translating findings from the laboratory to the patient´s bed-side.

Key words: Gene therapy; Hepatic fibrosis; Viral vectors; Non-viral vectors

Core tip: Cirrhosis is the irreversible end result of fibrous scarring and hepatocellular regeneration. Cirrhosis is a disease without effective treatment and new therapeutic strategies to accomplish healing are continuously being sought. Liver-based gene therapy has been used to improve liver function using viral and non-viral vectors. This review provides an overview of the main strategies used in liver-gene therapy, with the hope of finding a niche application in a given clinical scenario.

INTRODUCTION

The liver is the largest internal organ in the body. The main function of the liver is to take up nutrients, to store them and to provide nutrients to other organs. Cirrhosis is associated with high morbidity and mortality, and is induced by many factors, including chronic hepatitis, virus infections, alcohol and drug abuse[1].

During acute injury, the changes in liver architecture are transient and reversible. With chronic injury, there is progressive substitution of the liver parenchyma by scar tissue[2]. Despite ongoing injury, the liver has a remarkable regenerative capacity, and, as a result, patients often progress slowly to cirrhosis over decades. Substantial improvements in the treatment of chronic liver disease have accelerated interest in uncovering the mechanisms underlying hepatic fibrosis and its resolution[3].

In this setting, the present review deals with targeted gene delivery using viral and non-viral "shuttle" vectors, as a relatively novel technology that has the potential to treat both genetic and acquired disorders. The mammalian liver is an organ that can be targeted for gene transfer applications because its blood-supply can be accessed reliably using current technology. In addition, hepatocytes are long-lived cells that can sustain gene expression from episomal vectors[4]. The potential application of gene therapy protocols to human cirrhosis will depend on the successful and tissue-specific delivery of therapeutic genes to livers affected by extensive fibrosis.

In this context, experimental protocols of gene therapy directed to treat extensive

liver fibrosis have been designed to deliver specific genes to fibrotic organs. These protocols are mainly based in the use of non-viral and viral shuttle vectors. Here we describe the most important protocols published to date.

NON-VIRAL VECTORS

These delivery methods do not involve the use of viruses. Among these methods are plasmids, liposomes, conjugation with inert polymers of high molecular weight, such as diethylaminoethyl dextran, polyethylene glycol for the folding of DNA.

DNA plasmids

Plasmids are attractive vectors for direct injection into organs and tissues. Despite the relatively low expression achieved after a single plasmid administration, this expression is enough to reach physiological and therapeutics levels of the desired protein. Additionally, improvements in techniques and plasmid formulations have been performed to increase the transfection rate [5].

Augmentation of liver regeneration (ALR) is a novel cytokine, which stimulates hepatic cell proliferation and is able to block acute liver failure by inhibition of hepatic natural killer cell activity in acute liver injury[6]. ALR is an important regulator of liver regeneration, with trophic effects on the regenerating liver and potent anti-hepatitis effects. In this context, Li *et al*[7] investigated the effect of an ALR recombinant plasmid on rat hepatic fibrosis. Histological examination revealed less hepatic fibrosis in the ALR group with respect to the control group. There were also reductions in serum levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), and expression of Col-I, Col Ⅲ and tissue inhibitor of metalloproteinase-1 (TIMP-1), suggesting that ALR recombinant plasmid enhanced hepatic regeneration of injured liver cells.

Transforming growth factor-beta 1 (TGF-β1) is the most prominent cytokine implicated in hepatic fibrosis. TGF-β1 stimulates production of extracellular matrix in hepatic stellate cells in the liver. It has been reported that blockage of TGF-β1 signaling prevents hepatic fibrosis. In this context, Nakamuta *et al*[8] evaluated the effect of transfection of a plasmid containing the soluble receptor type Ⅱ TGF-β1 cDNA into skeletal muscle in an experimental model of dimethylnitrosamine (DMN) induced fibrosis in rats. This treatment decreased significantly DMN-induced hepatic fibrosis, hydroxyproline content, collagen and alpha-smooth muscle actin (α-SMA) expression. The authors suggested that this strategy may be useful for gene therapy of hepatic fibrosis.

Activation of metalloproteinases has also been evaluated. The delivery of an antisense molecule for the TIMP-1 into a plasmid to rats with hepatic fibrosis induced by pig serum injection, resulted in an increased activity of interstitial collagenase, which increased the degradation of collagen[9].

Hydrodynamic administration

A promising method of gene transfer in large animals is hydrodynamic gene transfer (HGT), which consists of the application of controlled hydrodynamic pressure in capillaries to enhance endothelial and parenchymal cell permeability. It was used for first time in the late 1990s by Budker *et al*[10], who demonstrated a successful gene transfer into rat skeletal muscle by a rapid injection of plasmid DNA solution into the femoral artery.

The first clinical trial to test HGT in humans was reported at the 9th Annual meeting of the American Society of Gene Therapy[11]. The most successful application of hydrodynamic delivery was observed in hepatocytes in rodents. This procedure involves a tail vein injection in few seconds of 8%-10% vol/body weight of physiological solution. The high DNA solution in the tail vein enters directly into the inferior vena cava and drives the injected solution into the liver in a retrograde fashion[12,13].

Huang *et al*[14] investigated the effect of recom-

binant interleukin-10 (*rIL-10*) gene by HGT on liver fibrosis progression induced by intraperitoneal administration of porcine serum (PS) in rats. Plasmid expressing rIL-10 was transferred into rats by HGT and the major organ expressing rIL-10 was evaluated by immunohistochemistry and reverse transcription (RT)-PCR. The results showed the major expression of rIL-10 in the liver after HGT. The *rIL-10* gene treatment attenuated liver inflammation and fibrosis in PS-induced fibrotic rats, decreasing collagen deposition and expression of α-SMA.

To test the efficiency between plasmid *vs* foamy virus (FV) for liver gene delivery, Zacharoulis *et al*[15] applied HGT in four juvenile pigs, to deliver the same plasmid backbone than naked FV vector particle to compare both vectors. Gene transfer efficiency and persistence of expression was assayed by PCR at 1 wk and 1 mo, respectively, after the infusions. HGT was well tolerated and no adverse reactions were observed. Plasmid injections resulted in no detectable DNA sequences at 1 wk. After 1 mo, 13% of liver sections analyzed were positive for plasmid DNA. When FV vectors were infused under identical conditions, 64.3% of liver samples were positive for vector sequences. These results indicated that the relative mild pressure obtained by hydrodynamic injection and the flooding of the liver was adequate for the entry of plasmids in hepatocytes and medium-term therapeutic levels of gene expression can be obtained with FV vectors. This effect could be attributed to the potential of HGT procedure and to the FV vector's natural affinity for hepatocytes[15].

The high efficiency and simplicity of hydrodynamic injection have raised interest among medical community towards its possible application in patients. The major focus has centered on injection volume reduction while maintaining an adequate pressure for gene transfer. A proposed strategy to reduce the injection volume is to inject directly into the vasculature of the target tissues. Zhang *et al*[16] reduced the volume to < 1.5% of body weight in rats by targeting the liver through the hepatic vein, with successful results. These results point out the possibility of liver gene delivery to hepatocytes in a human weighing 70 kg at a volume of 500-700 mL, a volume that might be clinically acceptable.

Liposomes
Many investigators have focused on the production of effective non-viral vectors gene therapeutic systems. These synthetic systems are largely based on polycationic structures, because of their ability to interact with negatively charged nucleic acids[17].

Within this group, liposomes are considered as a novel strategy for delivery of drugs and genes to cells. Use of liposome formulations for gene delivery *in vivo* is valuable for gene therapy and would avoid several problems associated with viral delivery[18]. Ueki *et al*[19] transfected the human hepatocyte growth factor (*HGF*)

gene, incorporated in liposomes, into skeletal muscles in rats with cirrhosis induced by dimethylnitrosamine. HGF is a potent mitogen for hepatocytes with anti-apoptotic activity and is essential for hepatic regeneration. The vector comprised liposomes containing the hemagglutinating virus of Japan with mixed liposomes (HVJ liposomes). This strategy induced a high plasma level of HGF, which binds and induces tyrosine phosphorylation of the HGF/c-Met receptor and suppression of TGF-β1 inhibiting fibrogenesis and hepatocyte apoptosis, resulting in fibrosis resolution.

VIRAL VECTORS FOR GENE DELIVERY
Viral vectors are the most frequently used method to deliver genes into living organisms. Delivery of genes by a virus is termed transduction and the infected cells are described as transduced. Comparing the different vectors for gene therapy, viral vectors can ensure that nearly 100% of cells are infected, without severely affecting cell viability. The first modified virus for gene therapy was constructed in 1970 by Paul Berg. He modified the simian virus-40 (SV40) virus by addition of the bacteriophage lambda DNA and infected monkey kidney cells successfully *in vitro*[20]. Since then, the use of viral vectors has been reported by several authors in experimental and clinical protocols. To date, there has been no clinical protocol for liver fibrosis. However, several important strategies of gene therapy in animal models have shed light on this issue, which promise to lead to the use of viral vectors in humans in the not too distant future. Here we describe the most significant progress in this area.

Adenoviruses-based shuttle vectors
Adenoviruses have been shown to be the most efficient vector in fibrotic liver models, overpassing technological hurdles and showing high hepatic tropism. In this context, several authors have used specific strains of adenovirus to deliver therapeutic genes, with promising results. Arias *et al*[21] cloned the adenoviral construct Ad5- cytomegalovirus (CMV)-AS-TGF-β1 expressing an antisense complementary to the 3'-portion of rat *TGF-β1* mRNA and a control virus expressing the reporter gene green fluorescent protein (GFP). Both transgenes were driven by the human CMV promoter and were fused to the SV40 early mRNA polyadenylation signal. The authors found that transduction with Ad5-CMV-AS-TGF-β1 induced significantly more mRNA production than the endogenous gene. In cirrhotic rats with ligature of the common bile duct (BDL), the adenoviral vector abrogated production of collagen and α-smooth muscle actin, but had no significant impact on serum levels of AST, ALT, or bilirubin. The authors concluded that transfer of the TGF-β1 antisense was sufficient to abolish ongoing liver fibrogenesis, but did not interfere with the injury *per se*[21].

A different strategy was used by Salgado *et al*[22], where an adenoviral vector carrying a modified cDNA coding for a non-secreted form of human urokinase plasminogen activator (Ad-ΔhuPA) was administered to rats with liver fibrosis. The non-secreted uPA was chosen to diminish the risk of bleeding, which is an important problem in cirrhotic animals that may have preexisting coagulopathy, and because huPA is known as a potent activator of latent hepatic collagenases, which in turn would promote the degradation of extracellular matrix deposited in cirrhotic livers. Salgado *et al*[22] demonstrated that a single application of Ad-ΔhuPA through the iliac vein of severely cirrhotic rats induced profound beneficial changes, such as a significant reversion in carbon tetrachloride (CCl₄)-induced hepatic fibrosis. Salgado *et al*[22] showed that Ad-ΔhuPA treated rats had an enormous improvement at day 10 *via* reduction of α-SMA, increase of MMP-2 and stimulation of liver regeneration with 40% more presence of PCNA. HGF expression was increased, which correlated with its cognate receptor c-Met. Furthermore, an improvement in functional hepatic tests was indicted by reduced ALT, AST and ALP levels.

Metalloproteinases gene delivery in adenovirus for liver fibrosis

Metalloproteinases (MMPs) play a crucial role in the pathogenesis of liver fibrosis and may represent an important therapeutic target in the design of anti-fibrotic strategies for chronic liver diseases. In this context, several types of MMPs can digest fibrillar collagens. The most potent MMPs against these kinds of collagens are: metalloproteinase-1 (MMP1) metalloproteinase-8 (MMP8) and metalloproteinase-13 (MMP13).

Delivery of collagenases has been reported in experimental models of cirrhosis by several authors.

Iimuro *et al*[23] delivered a cDNA encoding human pro-MMP-1 in an adenoviral vector into established liver fibrosis in rats, supposing that the manipulation of the imbalance between collagenases and their inhibitors (TIMPs) might attenuate liver fibrosis. After Ad-MMP-1 transduction, they found that liver fibrosis was significantly attenuated, as indicated by Masson's trichrome staining. Notably, the area of α-SMA positive cells (a marker of activated HSC) dramatically decreased. Active MMP1 protein was detected by western blotting, indicating that expressed pro-MMP-1 protein was activated *in vivo*. Hepatocytes proliferation was also induced by MMP-1 expression in the liver. Degradation of fibrillar collagens could affect the interaction between ECM and hepatocytes. This modification possibly stimulates several growth factors bound to extracellular matrix, thereby improving liver function.

Meanwhile, in cirrhotic mice induced by CCl₄ intoxication, Kim *et al*[24] delivered a plasmid containing an internal ribosome entry site, the gene of the green fluorescent reporter protein and *MMP-13* gene into a vector cationic polymer, which has a relatively high transfection efficiencies and prolonged gene expression. Intravenous injection of pMMP3 in the vector cationic polymer increased the level of the *MMP-13* mRNA by 25 times in liver tissue, slowing liver fibrosis, reducing collagen Ⅰ deposition and restoring plasma AST levels compared with the control group mice treated with empty vector.

Considering all the experimental data reported by several authors, gene delivery of collagenases seems promising for the treatment of advanced cirrhosis in humans.

However, persistent overexpression of collagenases in the liver might digest normal architectures in addition to pathologically deposited ECM. Therefore, precise, controlled delivery of active interstitial MMPs may be necessary to develop a treatment for clinical use. In this context, our group cloned MMP-8, a neutrophil collagenase, which degrades type Ⅰ collagen preferentially, under the transcriptional control of a phosphoenol pyruvate carboxykinase (*PEPCK*)-gene promoter, which contains the regulatory sites for hormonal regulation of expression in the liver. Experiments were conducted in HepG2 to demonstrate that addition of glucagon resulted in MMP-8 over-expression compared with the control using a plasmid without *PEPCK* gene promoter. These results showed that expression of a therapeutic gene like MMP-8 could be controlled at will, allowing modulation of the quantity of the extracellular matrix according to the body's needs[25].

In a different approach, Siller-López *et al*[26] showed that adenoviral administration containing the *MMP-8* gene promoted *in situ* degradation of extracellular matrix proteins in liver fibrosis induced by CCL₄ intoxication and bile duct ligation in rats, releasing hepatic growth factors, and freeing up space for hepatic cell proliferation. Furthermore, they used a single application of 3 × 10¹¹ VP/kg of AdMMP8 *via* the iliac vein in severely cirrhotic rats, obtaining *in situ* production of the cognate protein. AdMMP8-treated rats had a variable, yet remarkable, degree of hepatic fibrosis resolution by day 14 after adenovirus vector administration, the authors proposed that degradation of fibrotic tissue could also be taking place *via* activation of latent tissue gelatinases.

The systemic administration of adenovirus allows at least some of them to be introduced in different organs to the liver; therefore, Liu *et al*[27] cloned a cDNA of the truncated active *MMP-8* in a hepatitis B virus vector. This vector was fused with an adenovirus to create a chimeric vector, with the aim of increasing liver tropism and transduction efficiency simultaneously. Rats with thioacetamide-induced liver cirrhosis were injected with this vector to evaluate therapeutic efficacy. They observed beneficial effects of this vector on hepatic fibrosis and hepatocyte regeneration.

The imbalance between MMPs and TIMPs is

considered a crucial parameter of deposition and breakdown of the extracellular matrix. TIMP-1, the most important endogenous inhibitor of MMPs, plays a crucial role in the pathogenesis of liver fibrosis, and may represent an important therapeutic target in the design of anti-fibrotic strategies for chronic liver disease. TIMP-1 expression is upregulated in cirrhotic rats compared with normal liver. TIMP-1 binds to MMPs and inhibits their activity. Roderfeld *et al*[28] had already shown *in vitro* that an inactive MMP-9 (MMP-9-H401A) inactivated TIMP-1 by binding to it. Thus, they investigated the potential anti-fibrotic effect of WT-MMP-9 and MMP-9 mutants delivered by adenovirus vector to cirrhotic mice in a CCl4 model. They showed that inactive MMP-9 mutants delivered by adenovirus inhibited hepatic fibrogenesis, collagen-1 gene expression and hepatic stellate cells activation associated with decreased TIMP-1. This was the first work using an inactivated enzyme acting as a TIMP-1 scavenger as a therapeutic agent against fibrosis. The authors concluded that application of *MMP-9* mutants as TIMP-1 scavengers opens up a new avenue for the treatment of hepatic fibrosis.

Delivery of additional therapeutic genes with adenovirus

Several authors have reported that liver fibrogenesis involves a disturbance in mineral physiological concentrations, in particular zinc. The availability of zinc affects the activities of the zinc-dependent enzymes like MMPs. In this context, several studies have shown the beneficial effect of zinc supplementation on liver fibrosis. Metallothionein is a protein involved in the regulation of zinc homeostasis. For this reason, Jiang *et al*[29] delivered adenovirus containing the human MT- Ⅱ gene (*Ad-MT2A*) through intravenous injection, to study the effect on liver fibrosis induced by CCl4 in mice. Ad-MT2A reversed fibrosis along with increased hepatocyte regeneration. MT was associated with increased activities of liver collagenases. This study indicated that MT makes an important contribution in the resolution of chemical-induced hepatic fibrosis and could be a therapeutical outcome in patients with liver fibrosis of certain etiologies.

Otherwise, Marquez-Aguirre *et al*[30] constructed a recombinant adenovirus containing the truncated receptor for TGFβ1 (Ad-TβRIIΔcyt). They administrated a single injection of Ad-TβRIIΔcyt (5×10^{11} vp/kg) *via* the iliac vein in rats with TAA-induced cirrhosis. This single injection diminished significantly hepatic fibrosis and the expressions of fibrogenic genes, such as collagen α1, TGF-β1, PAI-1, and MMP-2. Ad-TβRII Δcyt also increased the expression of anti-fibrotic transcriptional factor SnoN in sinusoidal cells. There was also a significant difference in serum levels of AST and total bilirubin between cirrhotic rats and cirrhotic rats transduced with TβRIIΔcyt. The results suggested that delivery of TβRIIΔcyt in an adenovirus is effective to express this therapeutic gene. Blocking

of TGF-β1 signal with Ad-TβRIIΔcyt could upregulate the transcriptional repressor SnoN, which antagonizes TGF-β1 signaling (TGF-β/Smad-pathway inhibitor) and downregulated profibrogenic genes expression[30].

Increased intrahepatic vascular tone in cirrhosis has been attributed to a decrease of hepatic nitric oxide (NO), secondary to alterations in the post-translational regulation of the enzyme eNOS[31]. Low activity of superoxide dismutase contributes to a reduction of NO bioavailability in cirrhotic livers. Thus, Laviña *et al*[32] investigated whether the removal of NO by a superoxide dismutase could improve endothelial dysfunction and reduce portal pressure in cirrhotic rats. To achieve this, they delivered an adenoviral vector expressing extracellular superoxide dismutase or beta-galactosidase (Ad-βgal) *via* the tail vein to CCl4-induced cirrhotic rats. This transduction to fibrotic livers reduced O^{2-} levels significantly, increasing cGMP and decreasing liver nitrotyrosinated proteins, which are associated with a significant improvement in vasodilatation. Portal pressure was also significantly decreased in comparison with control rats. The authors suggested that scavenging of O^{2-} might be a good therapeutic strategy in the management of portal hypertension in cirrhosis[32].

On the other hand, the bone morphogenic protein 7, a member of the TGF-β1 superfamily, has been reported to counteract the profibrogenic actions of TGF-β1 Kinoshita *et al*[33] examined if adenovirus-mediated overexpression of bone morphogenetic protein-7 (BMP-7), administered *via* the tail vain, could antagonize the effect of TGFβ1 in an experimental model of fibrosis induced by thioacetamide in rats. They found that hydroxyproline content and Sirius red stained areas were significantly reduced compared with the control.

Dual delivery of therapeutic genes

Qiu *et al*[34] used adenovirus for dual gene transfer, human IL-10 and human hepatocyte growth factor, to rats with liver fibrosis induced by CCl4. This strategy protected hepatocytes from damage by reducing hepatocyte degeneration, hepatic fibrosis, and intra-hepatic inflammatory cell infiltration, thereby preserving liver function. The authors concluded that this liver protection could be the consequence of the regulation of the immune response caused by IL-10 and that this dual gene expression vector constitutes one of the most promising current strategies for liver gene therapy.

Meanwhile, Lin *et al*[35] used a combinatorial delivery of urokinase-type plasminogen activator (uPA) and *HGF* genes to investigate the effect of these two genes on hepatic fibrosis. Ad vectors expressing uPA (Ad-uPA), HGF (Ad-HGF) or uPA + HGF (Ad-uPA + HGF) were generated and injected into rats with hepatic fibrosis. Extracellular matrix and collagen type Ⅰ and type Ⅲ expression in the fibrotic liver decreased

significantly more in the dual-gene transduction group compared with the individual AdHGF and AduPA groups, indicating that combinatorial gene delivery had a larger effect on reversion of hepatic fibrosis than mono-gene therapy, probably by a synergistic effect of these two genes on hepatic fibrosis resolution.

A different strategy was devised by Ozawa *et al*[36], who used the combination of truncated type II TGF-β1 receptor (*TβTR*) gene and *HGF* in an adenoviral vector (AdTβTR + AdHGF) to analyze the effect on liver fibrosis induced by chronic administration of dimethylnitrosamine in rats. The body and rats-liver weight treated with the combination and hepatocyte proliferation increased, while the grading of fibrosis was significantly less compared with an irrelevant vector AdLacZ or the single administration of either AdTβTR or AdHGF, supporting the premise that the combination of two therapeutics genes for liver fibrosis treatment is more effective than individual delivery.

Adeno-associated vectors

Similar to adenovirus, adeno-associated viral (AAV) vectors have been shown to be efficient in experimental cirrhosis models. They have high cellular tropism, can achieve long-term gene expression and are now feasible for use in human gene therapy, because they do not awaken an exacerbated cellular immune response. For these reasons, AAV has emerged as an attractive vector for gene therapy. Production and purification of AAV has been improved recently, and it is now possible to produce high yields of vector, free from contaminating cellular and helper virus proteins. Eventually, tissue specific vectors to evade the immune response will be manufactured[37].

Some experiments have focused on demonstrating that AAV can efficiently transduce livers with fibrosis. Sobrevals *et al*[38] compared the ability of AAV to transduce normal and cirrhotic rat livers. They injected AAV serotype-1 (AAV1) encoding the reporter luciferase gene (*AAV1Luc*) through the hepatic artery, portal vein, into the biliary tree of normal and cirrhotic rats. They found that AAV1Luc allowed long-term and constant luciferase expression in rat livers. Interestingly, intra-portal administration led to higher expression levels in healthy livers compared with cirrhotic livers, whereas the opposite occurred when using intra-arterial injection. Intra-hepatic administration led to similar transgene expression in both animal groups, whereas intra-biliary infusion was the least effective route. After 70% partial hepatectomy, luciferase expression decreased in the regenerating liver, suggesting a lack of efficient integration of *AAV1* DNA into the host genome. Transgene expression was found mainly in hepatocytes.

Different protocols using AAV have been developed for the treatment of hepatic fibrosis. In 2005, Chen *et al*[39] constructed a recombinant AAV vector encoding human IFN-gamma (rAAV-IFN-γ), and took the primary rat hepatic stellate cells and carbon tetrachloride-injury induced rats as the experimental hepatic fibrosis model *in vitro* and *in vivo*. Histological examination revealed that rAAV-IFN-γ could inhibit the progression of hepatic fibrosis, hydroxyproline content, and serum AST and ALT levels were decreased compared with the fibrosis control group. mRNA expressions of *TIMP-1*, *TGF-β1* and *MMP-13* were decreased.

In the same year, Tsui *et al*[40], demonstrated that rAAV exhibit high efficiency in transduction of a homeostatic gene, heme oxygenase-1 (HO-1), to activated stellate cells, where the binding of rAAVs to HSCs increased significantly after serum-stimulated activation compared with the quiescent state. Portal injection of rAAVs to normal or CCl4-induced liver fibrosis showed a distinct distribution of rAAV binding. The majority of injected rAAVs bound to the cells in fibrotic areas that were associated with higher expression levels of fibroblast growth factor receptor-1alpha at 2 h after administration. Isolation of different types of cells from CCl4-induced fibrotic livers showed predominant expression of the transgene in stellate cells after rAAV/HO-1 administration on day 3 and remained stable for 12 wk. In addition, HO-1-transduced stellate cells showed reduced transcript levels of type 1 collagen and impaired proliferative ability compared with controls.

In 2007, Suzumura *et al*[41] constructed an AAV vector expressing HGF (AAV5-HGF) and examined its effect in two mouse hepatic fibrosis models: CCl4 administration and BDL in Balb/c mice. Mice that received AAV5-HGF achieved stable HGF expression both in the serum and liver for at least 12 wk. In both models, significant improvement of liver fibrosis was observed in all mice receiving AAV5-HGF, based on Azan-Mallory staining. Suppression of HSCs was confirmed by immunohistochemistry. Expressions of TGF-β1, collagen I and α-SMA mRNAs were significantly suppressed in the liver of AAV5-HGF transduced mice damaged with CCl4 or BDL. Expression of the inhibitor of matrix metalloproteinases, TIMP-1, was significantly suppressed in livers of AAV5-HGF-transduced mice in both animal models[41].

In 2009, with the aim of investigating the effects of TGFβ3 on rat hepatic fibrosis, Liu *et al*[42] cloned the *TGFβ3* cDNA into the rAAV2 vector. TGFβ3 is an anti-fibrotic cytokine that inhibits collagen production. Rats were randomly divided into four groups: normal control group, model group, negative control group and TGFβ3 group. Hepatic fibrosis was induced by hypodermic injection of 40% CCl4. Recombinant AAV2-TGFβ3 viral particles were injected *via* caudal vein one week before CCl4 treatment. Rats were sacrificed 8 wk after CCl4 treatment, and global histological changes were observed after HE staining, indicating that collagen fibers were reduced in the TGFβ3 group. Masson staining showed that collagen fibers deposited around the blood vessels, portal area and the perisinusoidal

space in liver tissues of TGFβ3 group were significantly decreased.

New approaches have been tested with novel administration methods that are less invasive and easier to perform. For example, Hao *et al*[43] used the BMP-7, a potent antagonist of TGF-β1 and an antifibrotic factor. In that study, they generated a recombinant AAV carrying BMP-7 (AAV-BMP-7) and tested its ability to suppress CCl4-induced hepatic fibrosis in mice. The results showed that ectopic expression of BMP-7 in gastrointestinal (GI) mucosa caused by AAV-BMP-7 administration led to long-term elevation of serum BMP-7 concentrations and resulted in drastic amelioration of CCl4-induced hepatic fibrosis in BALB/c mice. Immunostaining for α-SMA and desmin demonstrated that AAV-BMP-7 inhibited HSCs activation and promoted hepatocyte proliferation. The authors suggested that oral AAV-BMP-7 could be developed into a safe, simple, and effective therapy for hepatic fibrosis (Figure 1).

NOVEL VIRAL VECTORS FOR GENE THERAPY

Even though some serotypes of Ads and AAVs, such as Ad-5 or AAV-5/8, have high hepatic tropism, scientists continue the search for the best vector. Several investigations involved the usage of other recombinant viruses. In 2006, Merle *et al*[44], proved the principle of a lentiviral gene transfer in the Long-Evans cinnamon (LEC) rat, an animal model of Wilson disease. Rats were treated either by systemic application of lentiviral vectors or by intrasplenic transplantation of LEC-rat hepatocytes lentivirally transduced with *ATP7B* gene. *ATP7B* gene encodes a copper transport protein that plays a key role in incorporating copper into ceruloplasmin and moving excess copper out of the liver. *ATP7B* gene expression was analyzed by RT-PCR and its hepatic expression was detected at different time-points post-treatment and lasted for up to 24 wk (end of experiment). Liver copper levels were lowered in all treatment groups compared with untreated LEC rats. Twenty-four weeks after treatment, the area of the examined liver-tissue sections occupied by fibrosis was significantly minor, only with small fibrous septa in rats treated with cell therapy compared with untreated rats.

In the same manner Hamada *et al*[45], assessed the usefulness of oncostatin M (*OSM*) gene therapy in liver regeneration. They examined whether the introduction of OSM cDNA could enhance regeneration of livers damaged by DMN in rats. They enclosed the cDNA of OSM in hemagglutinating virus of Japan envelope into the spleen, resulting in the exclusive expression of OSM protein in Kupffer cells of the liver, which was accompanied by increases in body weight, liver weight, and serum albumin levels and reduction of serum liver injury parameters (bilirubin, AST and ALT) and a serum fibrosis parameter (hyaluronic acid). Histological examination showed that *OSM* gene therapy reduced centrilobular necrosis and inflammatory cell infiltration, and augmented hepatocyte proliferation. Apoptosis of hepatocytes and fibrosis were suppressed by *OSM* gene therapy.

Different sorts of vectors have been tested, such as SV40, an icosahedral papovavirus, which has recently been modified to serve as a gene delivery vector. Recombinant SV40 vectors (rSV40) are good candidates for gene transfer, as they display some unique features: they are non-replicative vectors, easy-to-make, and can be produced in high titers. They also efficiently transduce both resting and dividing cells, deliver persistent transgene expression to a wide range of cell types, and are non-immunogenic.

In 2007, Vera *et al*[46], analyzed the efficacy of a rSV40 encoding IGF-I (rSVIGF-I) to prevent cirrhosis progression. The transgenic expression of luciferase was evaluated in mice. The results showed long-term hepatic expression of the transgene, with luciferase expression increased significantly in CCl4-damaged livers and upon IGF-I administration. Thus, liver injury and IGF-I expression from rSVIGF-I should favor transgene expression. rSVIGF-I therapeutic efficacy was studied in rats where cirrhosis was induced by CCl4 inhalation during 36 wk. At the end of the study, hepatic levels of IGF-I and IGF-binding protein 3 were higher in rSVIGF-I-treated rats than in control cirrhotic animals.

This vector was also used in experiments performed by Sobrevals *et al*[47], where they found that injection through the hepatic artery with an SV40 vector encoding insulin growth factor (SVIGF-I) in cirrhotic rats increased hepatic levels of IGF-I, improved liver function tests, and reduced fibrosis associated with diminished α-SMA expression, upregulation of MMPs and decreased expression of tissue inhibitors of MMPs, TIMP-1 and TIMP-2. SVIGF-I therapy induced downregulation of TGF-β1, amphiregulin, platelet-derived growth factor (PDGF), connective tissue growth factor (CTGF), vascular endothelium growth factor (VEGF) and induction of the antifibrogenic and cytoprotective protein, HGF.

Some virus have a high natural tropism for the liver, one of them is the hepatitis B virus (HBV). However, HBV vectors have a limited insertion capacity and are replication-defective. Conversely, in an HBV infected cell, vector replication may be rescued in trans by the resident virus, allowing conditional vector amplification and spread. Capitalizing on a resident pathogen to help in its elimination and/or in treating its pathological consequences would represent a novel strategy. However, resident HBV may also reduce susceptibility to HBV vector super-infection. Thus, a size-compatible truncated MMP-8 (tMMP8) gene was cloned into an HBV vector, which was then used to generate a chimeric Ad-HBV shuttle vector that was

not subject to super-infection exclusion. Rats with TAA-induced extensive liver fibrosis were injected with this chimera to evaluate therapeutic efficacy. The data demonstrated that infectious HBV vector particles could be obtained *via* trans-complementation by wild-type virus, and that tMMP8 HBV vector could efficiently be shuttled by an Ad vector into cirrhotic rat livers. In the liver, it exerted a comparable beneficial effect on fibrosis and hepatocyte proliferation markers as a conventional full-length MMP-8 Ad vector[27].

HBV also contain numerous overlapping open reading frames and regulatory *cis*-elements, which have hampered early attempts to harness HBV into a gene-transfer vector by simple insertion of foreign sequences. HBV vectors obtained in this way selectively accumulate in the liver after inoculation into peripheral vessels, efficiently infected quiescent hepatocytes, and successfully transduced genes for GFP and type Ⅰ interferon (IFN-γ) These data suggested that HBV-based vectors may become useful against other liver diseases[27].

Gene therapy of hepatic stellate cells

HSCs play a central role in hepatic fibrosis and their elimination is a crucial step towards the resolution and reversion of liver fibrosis. Arabpour *et al*[48], investigated the potential application of a fused protein of an anti-epidermal growth factor receptor scFv antibody-TNF-α (scFv425- sTRAIL) delivery by an Ad vector on the targeted elimination of activated HSCs in cell culture. Treatment with Ad-scFv425-sTRAIL induced a reduction of around 100% in HSC viability, a 60% reduction in ECM production, and decreased caspase inhibition, where no effect was observed on hepatic parenchymal cells. The authors suggested that this strategy may represent a new therapeutic strategy against liver fibrosis.

In a different study, other authors developed a CCl₄-induced micronodular cirrhosis model to study the effect of rAAV/HO-1 administration, where expression of HO-1 by rAAV/HO-1 significantly increased the HO enzymatic activities in a stable manner. The development of micronodular cirrhosis was significantly inhibited in rAAV/HO-1-transduced animals. Portal hypertension was markedly diminished in rAAV/HO-1-transduced animals compared with controls, and no significant changes in systolic blood pressure were noted. These findings were accompanied with improved liver biochemistry, fewer infiltrating macrophages and fewer activated HSCs in rAAV/HO-1-transduced livers[49].

In a different study, Reetz *et al*[50] described a novel method to deliver genes for HSC in fibrotic liver, ablating the native tropism to liver. This paper was based on the concept that the expression of P75 neutrophin receptor (P75NTR) is increased in HSC in liver fibrosis, compared with low expression in quiescence HSCs and no expression in hepatocytes. Nerve growth factor (NGF) is a neutrophin that binds P75NTR. According to this premise, NGF was conjugated to the Ad surface using an adapter derived from a single chain antibody *via* polientilenglicol. The Ads carried the reporter gene *GFP*. This vector was injected systemically in mice and GFP expression was evaluated. The authors showed that the *GFP* expression was detected in the liver, but not in other organs like the lung and brain. Liver expression was selective and increased in HSCs of liver fibrosis compared to normal cells. There was no expression in hepatocytes. The authors concluded that this strategy might provide an effective mechanism for direct therapeutic gene delivery into activated HCSs without affecting hepatocytes[50].

To reduce liver fibrosis, Narmada *et al*[51] delivered *HGF* specifically to activated HSCs in fibrotic livers using vitamin A-coupled liposomes by retrograde intrabiliary infusion to bypass capillarized hepatic sinusoids. The HSC-targeted transgene enhanced the antifibrotic effect by reduction of α-SMA and collagen genes.

BLOCKING MOLECULES FOR THE INHIBITION OF DELETERIOUS GENES

Technology based in the delivery of short DNAs or RNAs is a revolutionary tool employed to silence the expression of specific genes in cells with no toxic response. These molecules are delivered to the cells or produced by them using expression cassettes, which are introduced into cells through viral vectors, plasmids or DNA constructs[52]. In this context, several molecules, such as decoys, antisense oligonucleotides, short interfering RNAS (siRNAs) and mircoRNAs (miRNA), have been investigated to evaluate their effect on expression inhibition of important genes for cirrhosis development.

Decoy molecules

The usage of decoy technology has been recently reported. A synthetic double-stranded oligodeoxynucleotide (ODN) containing the consensus binding sequence of the transcription factor Sp1, which regulates the inflammation-repair process and suppresses expression of several genes including TGF-β1, collagen type Ⅰ, VEGF, to block its activity, was used by Park *et al*[53] in a CCl₄-liver fibrosis model. They injected 10 μg of this ODN through tail vein in mice. The decoy molecule for Sp1 reduced gene expression of TNF-α, IL-1β, IL-6, VEGF and MCP-1 and also decreased the production of pro-fibrogenic proteins like fibronectin, α-SMA, TGFβ-1 and TIMP-1.

Antisense oligonucleotides

In 2005, Cheng *et al*[54], developed an anti-gene
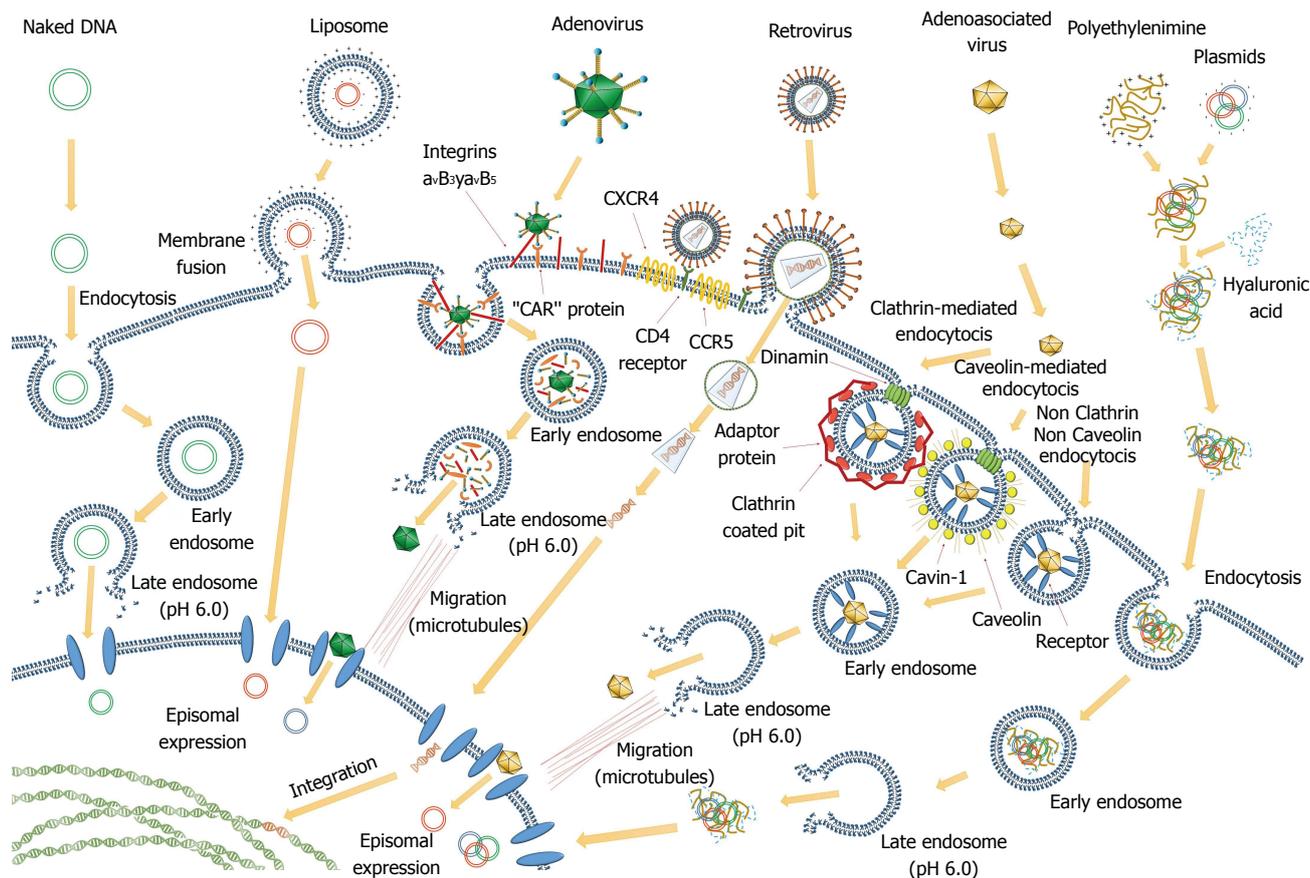
Figure 1 Several cell-targeted methods employing different vectors routinely used in gene therapy approaches. Some of them remain episomal and their expression is transient. Others enter into the nucleus and integrate their DNA into the cellular genome and their expression is permanent.

approach using a type alpha1 (I) promoter specific triplex-forming oligonucleotide (TFO) to inhibit collagen gene expression. In this report, biodistribution and hepatic cellular and subcellular localization of the 25-mer antiparallel phosphorothioate TFO were determined after intravenous injection into rats. TFOs distributed to all the major organs, with higher uptake in the liver, kidney and spleen. Competition studies with polyinosinic acid and dextran sulfate suggested the involvement of scavenger receptors in the hepatic uptake of TFO. Intrahepatic cellular distribution by Kupffer, endothelial and HSCs accounted for almost 70% of the liver uptake of P-TFO, while only 30% was associated with hepatocytes. The level of liver nuclei-associated TFO was much lower relative to that found in the cytoplasm at 2 and 4 h post-injection. However, the TFO inhibited collagen expression, as evidenced by sirius red staining of the liver section of fibrotic rats. In conclusion, systemic delivery of the TFO against type alpha1 (I) collagen gene promoter may be used for the treatment of liver fibrosis.

In the same year, Jiang *et al*[9], constructed a rat antisense TIMP-1 recombinant plasmid that could be expressed in eukaryotic cells. The recombinant plasmids were encapsulated with glycosyl-poly-*L*-lysine and injected into rats suffering from pig serum-induced liver fibrosis. The expression of the exogenous transfected plasmid was assessed by northern blotting, RT-PCR and western blotting. The antisense construct was successfully expressed *in vivo* and could block the gene and protein expression of TIMP-1. Active and latent hepatic interstitial collagenase activities were elevated. The hepatic hydroxyproline content and the accumulation of collagen types Ⅰ and Ⅲ were lowered, and liver fibrosis was alleviated in the antisense TIMP-1 group compared with the model group.

The use of these kinds of molecules has increased in recent years and a large number of molecules have been tested. Lu *et al*[55] constructed a recombinant plasmid for a rat antisense RNA for CTGF, which could be expressed in eukaryotic cells. The recombinant plasmids were encapsulated with lipofectamine and then transduced into a CCl$_4$-induced rat liver fibrosis model. The gene and protein expression of CTGF were significantly decreased in the fibrotic liver transfected with antisense-CTGF compared with the control group. Index fibrosis and collagens type Ⅰ and type Ⅲ were also significantly minimized in this group.

Small interfering RNA
SiRNAs are a recent powerful tool for post-trans-criptional gene silencing and have opened up new

avenues in gene therapy. The problems of lack of cell specificity *in vivo* and the subsequent occurrence of side effects, has hampered their development for hepatic fibrosis treatment. To overcome these shortcomings, several targeted strategies using siRNA for cirrhosis treatment have been developed. For example, in 2007 George *et al*[56] used a CTGF siRNA to prevent the progression of NDMA-induced hepatic fibrosis. The serial administration of NDMA resulted in activation of HSCs, upregulation of CTGF and TGF-β1 at both the mRNA and protein levels, and well-developed hepatic fibrosis. Immunostaining, western blotting and semiquantitative real-time RT-PCR studies showed downregulation of CTGF and TGF-β1 after treatment with the CTGF siRNA. These results demonstrated that CTGF gene silencing through siRNA reduced the activation of hepatic stellate cells, prevented upregulation of *CTGF* and *TGF-β1* gene expression, and inhibited accumulation of liver connective tissue proteins.

Three years later, another working group also tested the anti-fibrogenesis properties of a single intra-portal vein injection CTGF siRNA in a rat model of liver fibrosis. The authors observed that in CTGF siRNA-treated cirrhotic rats, protein expression of CTGF and α-SMA, and the number of active HSC, decreased compared with the model group. Attenuation of liver fibrosis was also observed[57].

In 2008, Chen *et al*[58], constructed a PDGFR-β siRNA expression plasmid and investigated its effect on the activation of HSCs. A hydrodynamics-based transfection method was used to deliver PDGFR-β subunit-siRNA to rats with hepatic fibrosis. PDGFR-β-siRNA significantly downregulated PDGFR-β expression, and suppressed HSCs activation and proliferation *in vitro*. The progression of fibrosis in the liver was significantly suppressed by PDGFR-β siRNA in two animal models of fibrosis: DMN intoxication and BDL. The authors suggested that the plasmid could be delivered into activated HSCs by the hydrodynamics-based transfection method, and remarkably improved liver function in cirrhotic rats.

In the same year, Cheng *et al*[59], designed a siRNA and short hairpin RNA (shRNA) targeting different regions of *TGF-β1* mRNA, and measured the silencing effect after transfection into immortalized rat liver HSC (HSC-T6). There was not only a significant decrease in TGF-β1, TIMP-1, α-SMA and type I collagen after transfection with TGF-β1 siRNAs, but also synergism in gene silencing when siRNAs targeting two different start sites were used as a pool for transfection. The two siRNA sequences, which efficiently inhibited *TGF-β1* gene expression, were converted to shRNAs *via* cloning into the pSilencer1.0. In conclusion, both siRNA and shRNA showed sequence-specific and dose dependent *TGF-β1* gene silencing and have the potential to treat liver fibrosis.

MicroRNAs

MiRNAs are short, endogenous, noncoding RNA molecules that regulate gene expression at a post-translational level. MiRNAs have been recognized in the regulation of physiological conditions. Moreover, awareness of the association between dysregulated miRNAs and human diseases is increasing, which consequently brings miRNAs into the frontline of novel therapeutic strategies.

This technology is effective, as demonstrated by the work of Yang *et al*[60]. They investigated the antifibrotic effects of an artificial miRNA targeting CTGF, using the ultrasound-targeted cationic liposome-bearing microbubble destruction gene delivery system. Plasmids carrying the most effective artificial miRNA sequences were delivered by this method to rats with hepatic fibrosis. The results showed that this method of gene delivery effectively transported the plasmids into the rat liver. The artificial miRNA reduced hepatic fibrosis, pathological alterations, and the protein and mRNA expressions of *CTGF* and *TGF-β1*. Furthermore, *CTGF* gene silencing decreased the levels of type I collagen and α-SMA. These data suggested that delivery of an artificial miRNA targeted against CTGF using ultrasound-targeted cationic liposome-bearing microbubble destruction may be an effective therapeutic method to ameliorate hepatic fibrosis.

CONCLUSION

Gene therapy represents a novel alternative for the treatment of those diseases that currently have no satisfactory cure. In recent years, gene therapy has been directed to the treatment of mortal chronic degenerative diseases to offer the patient a better quality of life. For those diseases caused by lack of control in the expression of certain genes, such hepatic fibrosis, delivery of genes that counteract this overexpression should be an excellent strategy to control it. Thus, numerous articles have been published in the experimental field of gene therapy, addressing innovative strategies that demonstrate that hepatic fibrosis could be treated with gene therapy, supporting its use in a near future in cirrhotic patients.

A large number of vectors used in gene therapy have been implemented, each with its advantages and disadvantages. The use of gene therapy in humans has been controversial because the delivered genes could potentially be integrated into the cell genome, causing an insertional mutation that might result in cancer, especially if the vector used is a virus. As mentioned earlier, adenoviruses are the most commonly used viral vectors for therapeutic gene delivery to the liver[61], with the advantage that they recognize the CAR receptor present in hepatocytes, do not integrate into the cell genome, and the risk of insertional mutation is zero[62]. Other vectors, like liposomes, plasmids and

adeno-associated viral vectors, have been proved; however, the ideal vector to deliver genes to a diseased liver remains to be established. Some clinical trials using gene therapy against hepatocarcinoma and hepatitis C infection are being implemented[61]; however, there are only experimental models directed to liver fibrosis at this moment. These experimental approaches have been demonstrated as effective to decrease and prevent experimental fibrosis. We await their implementation in humans in the near future.

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REVIEW
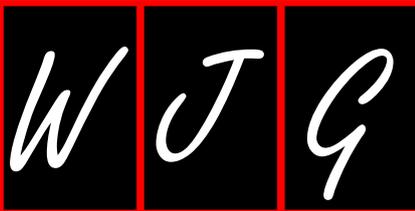
Current management of hepatocellular carcinoma: An Eastern perspective

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Abstract

Hepatocellular carcinoma (HCC) is one of the leading causes of cancer death, especially in Eastern areas. With advancements in diagnosis and treatment modalities for HCC, the survival and prognosis of HCC patients are improving. However, treatment patterns are not uniform between areas despite efforts to promote a common protocol. Although many hepatologists in Asian countries may adopt the principles of the Barcelona Clinic Liver Cancer staging system, they are also independently making an effort to expand the indications of each treatment and to combine therapies for better outcomes. Several expanded criteria for liver transplantation in HCC have been developed in Asian countries. Living donor liver transplantation is much more commonly performed in these countries than deceased donor liver transplantation, and it may be preceded by other treatments such as the down-staging of tumors. Local ablation therapies are often combined with transarterial chemoembolization (TACE) and the outcome is comparable to that of surgical resection. The indications of TACE are expanding, and there are new types of transarterial therapies. Although data on drug-eluting beads, TACE, and radioembolization in Asian countries are still relatively sparse compared with Western countries, these methods are gradually gaining popularity because of better tolerability and the possibility of improved response rates. Hepatic arterial infusion chemotherapy and radiotherapy are not included in Western guidelines, but are currently being used actively in several Asian countries. For more advanced HCCs, appropriate combinations of TACE, radiotherapy, and sorafenib can be considered, and emerging data indicate improved outcomes of combination therapies compared with single therapies. To include these paradigm shifts into newer treatment guidelines, more studies may be needed, but they are certainly in progress.

Core tip: This article describes the current status of the management of hepatocellular carcinoma, focusing on the changing trends of treatment modalities in Eastern countries. Newly adopted therapies as well as emerging combination strategies are discussed based on recent data.

INTRODUCTION

Worldwide, hepatocellular carcinoma (HCC) is the sixth most prevalent cancer[1]. More than 600000 people are newly diagnosed every year and approximately the same number die due to HCC annually. The main etiology of HCC is liver cirrhosis caused by chronic hepatitis B or C, alcohol, fatty liver diseases, or less commonly, autoimmune or genetic metabolic liver diseases[2]. The incidence, characteristics, and prognosis of HCC vary from region to region according to the prevalence of underlying chronic liver diseases as well as the screening and treatment strategies for HCC. Currently, efforts are being made to promote the use of common protocols, but the patterns of treatment are still not uniform as the therapeutic approach to HCC mainly depends on the availability of treatment modalities as well as the preferences of physicians[2-7]. As three-quarters of HCC cases occur in East Asia, the experiences and data in this area should have been substantially accumulated, and the treatment trends would have characteristic features. This article aims to review the current status of the management of HCC from an Eastern perspective. The first section introduces the principles and current trends of different treatment modalities, and the second section summarizes the findings on multidisciplinary treatments based on recently available data.

MAIN TREATMENT MODALITIES: EVOLVING ROLES AND CHANGING TRENDS

For decisions regarding initial treatments, the Barcelona Clinic Liver Cancer (BCLC) staging system from Western guidelines is frequently applied[3,4]. This system has very strict guidelines for treatments; only very early stage and early stage HCCs are indicated for the curative therapies, and only one treatment option is assigned to each of intermediate stage and advanced stage disease. Furthermore, no combination therapy is recommended according to the BCLC algorithm. Hence, despite the worldwide use of the BCLC guidelines, debates regarding their practicality are ongoing. The Asian Pacific Association for the Study of the Liver has guidelines for HCC treatment similar to those in the BCLC system[5]; both consider hepatic function as well as tumor size, number, and its extent, and the treatment options are not much different from BCLC. However, indications of pre-existing therapies are expanding and newly emerging therapies are currently being implemented (Figure 1). In addition, alternative therapies or combination therapies for each stage are available as determined by clinical situations in real practice. In this section, current status of surgical, interventional, medical, and radiation therapies are reviewed with newly available data, particularly, from Asian countries.

Surgical therapies

Liver transplantation: Transplanting a healthy liver provides the most favorable survival outcomes in HCC patients[8]. If the patients have underlying decompensated liver cirrhosis, no other option exists. However, the availability of organs limits access to this best curative therapy. The annual incidence of deceased organ donors does not exceed 5 per million in most Asian countries[9]. Compared with those in Western countries, patients with HCC in Asia have a low probability of receiving a deceased donor liver transplantation (DDLT) in a timely manner and thus have a higher risk of drop-out because of tumor progression[10]. For this reason, living donor liver transplantation (LDLT) has been promoted. In Korea, the proportion of adult LDLT recipients with HCC has increased to 30%-40% of all HCC liver transplant recipients[11]. Despite technical complexity, LDLT is replacing DDLT in other Asian countries as well[8]. Donor mortality and morbidity rates of LDLT were 0.2% and 24%, respectively, according to a report of a worldwide survey[12]. Most LDLT centers develop their own criteria for maximizing donor safety[13]. Although the right lobe is the most suitable graft for the recipient, its procurement is limited by size of donor liver. When the right lobe cannot be used alone, a dual graft from 2 donors containing the left lobe can be utilized[14]. Despite this method, the donor pool has not significantly expanded because of the technical complexity of the surgery and ethical concerns. To further overcome organ shortage, ABO-incompatible LDLT was attempted and became successful after the implementation of rituximab, which decreased antibody-mediated rejection rates from 23.5% to 6.3%, as shown in a Japanese multicenter study[15]. The Milan criteria (solitary tumor < 5 cm, 2 or 3
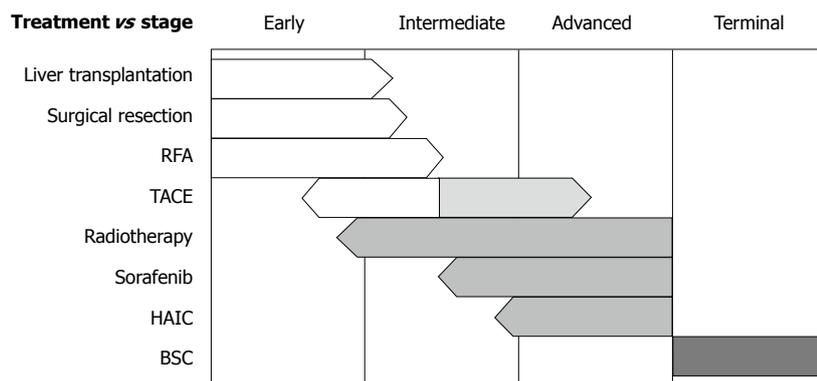
Figure 1 Expanding indications of treatment modalities for hepatocellular carcinoma. Indications of each treatment are currently expanding and treatments may be combined as determined by clinical situations. RFA: Radiofrequency ablation; TACE: Transarterial chemoembolization; HAIC: Hepatic arterial infusion chemotherapy; BSC: Best supportive care.

Table 1 Expanded criteria for liver transplantation proposed in Eastern countries

Criteria (city, country, reference)	Tumor number	Tumor diameter (cm)	Additional criteria	Overall survival within criteria	
Hong Kong, China[20]	1	≤ 6.5	No diffuse type,	3 yr	78%
	≤ 3	≤ 4.5	no vascular invasion	5 yr	66%
Hangzhou, China[21]	NC	Total ≤ 8	Histopathologic grade Ⅰ or Ⅱ with	3 yr	70.7%
			AFP ≤ 400 ng/dL if tumor > 8 cm	5 yr	70.7%
Seoul (AMC), Korea[22]	≤ 6	≤ 5	No gross vascular invasion	3 yr	87.5%
				5 yr	81.6%
Seoul (CMC), Korea[23]	≤ 7	≤ 7	NC	5 yr	86.3%
Tokyo, Japan[24]	≤ 5	≤ 5	NC	3 yr	82%
				5 yr	75%
Kyoto, Japan[25]	≤ 10	≤ 5	PIVKA-Ⅱ ≤ 400 mAU/mL	5 yr	87%

AMC: Asan medical center; CMC: Catholic medical center; AFP: Alfa-fetoprotein; PIVKA Ⅱ: Protein induced by vitamin K absence or antagonist-Ⅱ; NC: Not commented.

tumors < 3 cm each, and absence of vascular invasion and extrahepatic metastasis) have been applied for the selection of candidates for liver transplantation[16-18]. However, these criteria have been criticized because many patients missed opportunities for transplants because of the strictness of the criteria. Therefore, Yao et al[19] proposed their own set of criteria, permitting the listing of patients with somewhat larger-sized tumors. In Asia, several independent criteria have also been proposed, expanding indications without increasing the risk of HCC recurrence significantly[20-25] (Table 1). Five-year survival rates were as high as 80% after transplantation using these criteria. However, these criteria should be applied very carefully to DDLT candidates until a consensus is achieved.

Current issues related to "bridging therapy" and "downstaging" are discussed in the section on multidisciplinary treatments.

Hepatic resection: As hepatic resection is a potentially curative therapy, it has been considered a first-line option for HCC patients with well-preserved hepatic function, especially when there is only one tumor or when tumors are confined to a single lobe. To assess hepatic function, a Japanese group measured the indocyanine green retention rate at 15 min (ICG15)[26]. Feasibility and the extent of the resection are decided according to the degree of retention of the dye[27]. Although the BCLC algorithm mandates Child-Pugh A liver function without portal hypertension for hepatic resection, selective resection has been attempted in HCC patients exhibiting upper Child-Pugh B liver function or mild portal hypertension in Asian countries, with reference to the ICG15 value[6,26].

Prognosis after hepatic resection is determined by number and size of tumor, vascular invasion, and level of alpha-fetoprotein[28-30]. Five-year survival rates are > 50% after the resection of solitary tumors, whereas rates of 20%-30% have been reported for 3 or more nodules[28-30]. With respect to tumor size, 5-year survival rates for patients with HCCs < 2 cm, 2-5 cm, and > 5 cm are 66%, 52%, and 37%, respectively[28-30]. However, in selected cases with proper hepatic function, large single HCCs can be surgically removed with favorable long-term survival outcomes[29]. More advanced stages of HCCs have been resected in 511 Chinese patients, yielding a 5-year survival rate of 30.5%[31]. The presence of vascular invasion or extrahepatic metastasis resulted in poor outcomes[31].

Recently, laparoscopic liver resection has been implemented for the treatment of HCC. This is a minimally invasive surgery, so postoperative morbidity and duration of hospitalization are reduced with no changes in surgical margin status, tumor recurrence, and overall survival[32]. This technique is successfully being applied for the resection of large tumors between 5 and 10 cm and lesions at difficult-to-approach locations[33-35] as well as intra-abdominal metastatic HCCs in Asian countries[36].

Interventional therapies

Local ablative therapies: Local ablation can be categorized as chemical or thermal. Chemical ablation includes percutaneous ethanol injection (PEI) and acetic acid injection, whereas thermal ablation includes radiofrequency ablation (RFA), the use of microwaves, cryotherapy, and high-intensity focused ultrasound[3,5]. As these are considered potentially curative therapies, patients with early stage HCCs are the candidates, especially when surgical treatments are not available. Among these modalities, RFA is currently the most commonly used. Excellent long-term results of RFA, up to 10 years, were reported in Korean HCC patients meeting the Milan criteria[37]. The results at 5 and 10 years were as follows: cumulative local tumor progression rates, 27.0% and 36.9%; cumulative intrahepatic distant recurrence rates, 73.1% and 88.5%; and overall survival rates, 59.7% and 32.3%, respectively[37]. Comparison of the efficacy of RFA with other local therapies showed that RFA was substantially superior to PEI, especially in tumors with a diameter > 2 cm[38,39]. Nevertheless, PEI is associated with a necrosis rate of 90%-100% in tumors < 2 cm and is still useful in selected patients when RFA is not technically feasible[40-42]. Recently, it was reported that in cases where the tumor is located under the diaphragm or near the surface of the liver, creating artificial ascites or pleural effusion is helpful in performing RFA and avoiding burns on adjacent organs[43,44]. This technique is being applied in several Asian countries with good results[43,44].

Several randomized controlled trials compared the efficacy of RFA with that of resection in Asian patients with HCC meeting the Milan criteria[45,46]. Pooled data demonstrated no significant differences in overall survival or recurrence-free survival between the treatments at 1 and 3 years. The 5-year overall survival [relative risk (RR), RR = 0.72, 95% confidence interval (CI): 0.60-0.88] and recurrence-free survival (RR = 0.56, 95%CI: 0.40-0.78) rates were higher in the resection group[46]; however, the 5-year data were provided by only one study, which advocated surgery. Complication rates were lower and hospitalization period shorter in patients who received RFA rather than resection[46]. Although the efficacy of RFA appears to be comparable to that of hepatic resection with lower complication rates, additional data may be

needed and the need for long-term surveillance should be re-enforced.

Transarterial chemoembolization: Patients with either large tumors or multinodular tumors and a good performance status are candidates for transarterial chemoembolization (TACE). However, the presence of decompensated liver disease, severe hepatic dysfunction, portal vein thrombosis, or extrahepatic tumor spread precludes TACE. Although TACE is associated with a complete response rate of only 40%, it improved survival compared with supportive treatment in 2 independently performed randomized controlled trials in Eastern and Western countries[47,48]. A meta-analysis of 7 trials that included 545 HCC patients showed similar results [odds ratio (OR), OR = 0.42, 95%CI: 0.20-0.88][49]. Importantly, when the tumor size is ≤ 2 cm, prognosis is even better; a Korean study of TACE in small HCCs reported cumulative survival rates of 93.4%, 75.4%, 63.1%, and 51.1% at 1, 3, 5, and 8 years, respectively, for TACE, which were not significantly different from those of 97.6%, 86.7%, 74.5%, and 60.0%, respectively, for RFA[50]. Therefore, TACE may have a potential role as a curative therapy for small HCCs when surgical or local ablative therapies are not feasible.

Although TACE has been contraindicated in cases of HCC with portal vein invasion, multiple studies reported that it can be safely performed and may have better survival benefits than supportive care in patients with compensated liver function[51-54]. Notably, when a tumor is nodular and restricted to 1 lobe or 1-2 segments and hepatic function is classified as Child-Pugh class A, median survival after TACE is as long as 22-30 mo even in the presence of main portal vein tumor thrombosis[51,52]. When compared with sorafenib, which is a current standard treatment for advanced HCC, median overall survival rates for TACE were not significantly different from those of sorafenib (9.2 and 7.4 mo, respectively; P = 0.377)[55]. Therefore, TACE could be an alternative therapeutic option for advanced HCC.

TACE was originally intended to maintain intra-tumoral concentrations of chemotherapeutic agents by transiently obstructing supply vessels and thus minimizing systemic exposure. The strategy for TACE was recently refined after the introduction of microspheres that can increase the duration of drug retention in the tumor without blocking blood flow, which reduces hepatic derangement and systemic toxicity[56]. In a multicenter phase Ⅱ randomized study of 201 HCC patients, TACE with drug-eluting beads (DEB) was compared with conventional TACE, and hepatic toxicity and drug-related adverse events were significantly less observed in the DEB-TACE arm[57]. Although this study showed a nonsignificant trend toward better antitumoral effects with DEB-TACE, a case-control study conducted in Korea reported a

significantly better objective response rate with DEB-TACE (85%) than with conventional TACE (30%, *P* < 0.01) as assessed by modified Response Evaluation Criteria in Solid Tumors. A systemic review of the published data demonstrated the superiority of DEB over conventional TACE in terms of overall disease control, especially in patients with more advanced stage disease[58]. To summarize, the indications of TACE are expanding, and new types of transarterial therapy are currently available in Eastern areas.

Medical therapies

Cytotoxic chemotherapies: Cytotoxic chemotherapy has been attempted continuously since treatment of HCC began but has failed to improve overall survival in most clinical trials to date[59,60]. The main problem of cytotoxic chemotherapy in HCC is the co-existence of liver cirrhosis. Cirrhosis can delay the metabolism of chemotherapeutic agents and may enhance their toxicity[61]. In addition, HCC is relatively chemoresistant to most cytotoxic anticancer drugs. An early randomized trial of doxorubicin conducted in Hong Kong showed a tumor response of less than 10% and borderline improvement in overall survival (10.6 wk) compared with no treatment (7.5 wk, *P* = 0.036)[61]. Notably, 25% of patients died due to doxorubicin-related complications, including septicemia and cardiotoxicity. The antitumor activity of other cytotoxic agents such as gemcitabine[62,63], oxaliplatin[64], and capecitabine[65] in clinical and retrospective studies was modest with objective responses of < 20%. In randomized controlled trials, combination therapies such as PIAF (cisplatin, interferon, adriamycin, fluorouracil) and FOLFOX (5-fluorouracil, folic acid, and oxaliplatin) did not significantly improve survival compared with doxorubicin[59,60]. Moreover, a high rate of myelotoxicity was reported in the PIAF group[59]. Therefore, no cytotoxic chemotherapy regimen has provided strong evidence of improving the survival of HCC patients, and regular practice of chemotherapy is not advised. Nonetheless, a current retrospective study in Korea indicated that ECF (epirubicin, cisplatin, and 5-fluorouracil) combination therapy prolonged overall survival in sorafenib-refractory patients with metastatic HCC if a tumor response was observed; overall survival periods were 20.4 mo in responders and 4.9 mo in nonresponders (*P* < 0.001)[66]. Thus, ECF may be an alternative or rescue therapy for patients who failed sorafenib therapy, but further prospective evaluations will be needed.

Hepatic arterial infusion chemotherapy (HAIC) has been used for treatment of advanced HCC with portal vein tumor thrombosis in Asian countries[67-72]. Traditionally, the presence of tumor thrombus is assumed to aggravate ischemic injuries after TACE, so alternative modalities were sought. HAIC does not use embolic material, and the chemotherapeutic agent is infused into the hepatic artery *via* an implanted

catheter, which reduces systemic side effects by first-pass effects and maximizes drug delivery to the tumor. Although this is considered an experimental treatment modality and is not recommended for treatment of HCC in Western countries, a large amount of clinical data on HAIC have been accumulated in Eastern countries[67-72]. A small retrospective study showed survival benefits of HAIC using low doses of cisplatin and 5-fluorouracil compared with systemic cytotoxic chemotherapy or supportive care (median survival, 6, 4, and 2 mo, respectively; *P* = 0.003) in cases of advanced HCC with portal vein tumor thrombosis[73]. A subsequent prospective study showed better efficacy of HAIC when a higher dose of cisplatin was used[74]. Importantly, a recent retrospective study by the same group in Korea compared HAIC and sorafenib in advanced HCC patients with portal vein tumor thrombosis and showed better overall survival (7.1 and 5.5 mo, respectively; *P* = 0.011) and longer median time to progression (3.3 and 2.1 mo, respectively; *P* = 0.034) in the HAIC group[75]. These findings are consistent with those of a Japanese study[76]. Although well-designed prospective studies are warranted to confirm these results, HAIC at least appears to be an alternative therapy for patients with portal vein tumor thrombosis when sorafenib is not available or is intolerable. Further research is also needed regarding the use of HAIC as salvage therapy in patients with advanced HCC who do not respond to standard therapy.

Molecular target therapies: Sorafenib is the only approved systemic agent for the treatment of advanced HCC. It is a multikinase inhibitor whose targets include Raf-1 and B-Raf serine/threonine kinases, vascular endothelial growth factor receptor (VEGFR) and platelet-derived growth factor receptor (PDGFR) tyrosine kinases, and c-kit receptors[77]. The Sorafenib Hepatocellular Carcinoma Assessment Randomized Protocol trial, which enrolled 602 patients with advanced stage HCC, showed improved median overall survival in the sorafenib group compared with the placebo group (10.7 and 7.9 mo, respectively; *P* < 0.001)[78]. A subsequent study conducted in the Asia-Pacific region showed a similar trend (overall survival of 6.5 and 4.2 mo in the sorafenib and placebo groups, respectively; *P* = 0.014)[79]. On the basis of the results of these trials, sorafenib became the standard treatment for advanced HCC with well-preserved liver function. The most significant adverse effects were diarrhea and hand-foot skin reactions[78,79]. Interestingly, these toxicities were associated with better survival in patients receiving sorafenib[80,81]. Therefore, despite the occurrence of adverse reactions, the use of sorafenib should not be discouraged when tolerable.

Sorafenib had not been compared with other treatment modalities before its approval. Currently, its efficacy in a real-life setting was compared with

Table 2 Randomized controlled trials with molecular target therapies[92]

	Status
First line	
Comparison with placebo	
Sorafenib (SHARP, Asian-Pacific)	Proven benefit
Sorafenib in Child B (BOOST)	Phase III Ongoing
Comparison study between sorafenib and single agent (head to head)	
Sunitinib -> endpoint not met	Terminated
Brivanib (BRISK-FL) -> endpoint not met	Failed
Linifanib -> endpoint not met	Terminated
Lenvatinib	Phase III ongoing
Combination of sorafenib and another agent	
Sorafenib + Erlotinib (SEARCH) -> endpoint not met	Failed
Sorafenib + Doxorubicin (CALGB-80802)	Phase III ongoing
Sorafenib + Everolimus	R-Phase II; Failed
Second line	
Sorafenib failure	
Brivanib (BRISK-PS) -> endpoint not met	Failed
Brivanib (BRISK-APS)	Terminated
Everolimus (EVOLVE-1) -> endpoint not met	Failed
Ramucirumab (REACH)	Phase III ongoing
Regorafenib (RESORCE)	Phase III ongoing
Cabozantinib (CELESTAL)	Phase III ongoing
Tivantinib (Metiv-HCC)	Phase III ongoing
Combination or addition to standard therapies	
Adjuvant setting after surgery or RFA:	
Sorafenib (STORM)	Failed
Combination with TACE:	
Sorafenib (SPACE) -> endpoint not met	Failed
Brivanib (BRISK-TA) -> endpoint not met	Failed
Sorafenib (TACTICS)	R-Phase II ongoing

RFA: Radiofrequency ablation; TACE: Transarterial chemoembolization.

the efficacy of other treatments (TACE, radiation, and cytotoxic chemotherapy) in Korean patients with advanced HCC[82,83]. Overall survival times were 8.4 and 8.2 mo for sorafenib and other treatments, respectively, and the difference was not significant[82,83]. To improve the efficacy of sorafenib, combination therapy or a multidisciplinary approach may be needed[84].

Several newer molecular target therapeutic agents were evaluated in clinical trials. Sunitinib, an orally administered multikinase inhibitor of receptor tyrosine kinases, showed modest activity against HCC. Although an overall survival time of 9.8 mo was observed in a phase II study[85], sunitinib did not outperform sorafenib in a phase III randomized study (overall survival, 8.1 and 10.0 mo, respectively; $P = 0.0019$)[86]. Brivanib, a selective dual inhibitor of fibroblast growth factor (FGF) and vascular endothelial growth factor (VEGF) signaling, was associated with a median overall survival of 10 mo in a phase II trial[87] and was considered a promising new drug for advanced HCC. However, the primary endpoint of brivanib not being non-inferior to sorafenib was not met in a subsequent phase III trial (overall survival, 9.5 and 9.9 mo, respectively, P value is nonsignificant)[88]. The efficacy

of brivanib in advanced HCC patients who were intolerant to sorafenib or failed to respond to sorafenib previously was also tested. The results of this study showed no significant improvement in overall survival compared with placebo (9.4 and 8.2 mo for brivanib and placebo, respectively)[89]. Linifanib (ABT-869), a receptor tyrosine kinase inhibitor targeting VEGFRs, also failed to significantly improve survival compared with sorafenib in a phase III trial (overall survival, 9.1 and 9.8 mo, respectively)[90]. The reasons for most of these novel agents failing to improve survival may be diverse and include lack of understanding of critical drivers of cancer progression, unpredicted toxicity, and marginal antitumor effects[91]. To overcome these obstacles, the clinical trial design should be modified to focus on biomarker-based subpopulation targeting strategies, and thereby, personalized therapies should be pursued in the future. In addition, efficacy and toxicity need to be evaluated in detail in phase I and II studies before moving to phase III studies[91]. Currently, several novel molecular targeting agents are being evaluated in phase III trials as first-line or second-line therapies including lenvatinib [VEGFR1-3, FGF receptor (FGFR) 1-3, PDGFR-β, RET, KIT], ramucirumab (VEGFR2), regorafenib (VEGFR, TIE-2, PDGFR-β, FGFR, KIT, RET, RAF), cabozantinib (MET, VEGFR-2), and tivantinib (MET)[92]. Table 2 summarizes the current status of the randomized controlled trials of molecular target therapies.

In summary, there are no currently available first-line molecular targeted agents other than sorafenib and no standard second-line treatments for patients intolerant or nonresponsive to sorafenib. If underlying liver function is well preserved, novel molecular target therapies, HAIC, or systemic cytotoxic chemotherapy may have a role as second-line treatment, but further studies are warranted. If a patient with advanced HCC has poor hepatic function, aggressive anticancer treatments are not indicated.

Radiotherapies and emerging therapies
External radiation therapies: Radiotherapy techniques for the treatment of HCC have substantially evolved over the past decades. Delivery of radiation energy became more precise, which enabled the exposure of tumors to higher doses of radiation, while saving non-tumorous liver parenchyma[93]. In the past, the role of radiation therapy was limited to alleviation of bone pain due to bone metastasis and to emergency use in spine and brain metastasis[94-96]. Radiation therapy has currently been adopted as a definitive therapy with curative intent if the tumor is at an early stage. Particularly, stereotactic body radiation therapy can achieve high rates of locoregional tumor control as it can deliver high doses of radiation in a single treatment session or in a small number of fractions[97,98]. In locally advanced HCCs, radiation therapy can be used to relieve obstruction and improve

portal blood flow if the tumor invades the biliary tree or portal vein[99,100]. A large multicenter study in Korea of 994 HCC patients with portal vein tumor thrombosis showed a median survival of 9.2 mo[101]. This was a relatively longer survival time than that of advanced HCC patients who did not receive any treatment in previous trials[78,79]. Studies from Japan and China also reported the efficacy of radiotherapy for HCC with portal vein thrombosis, and overall survival was significantly better in patients receiving radiotherapy than in patients receiving sorafenib (10.9 and 4.8 mo, respectively; $P = 0.025$) or undergoing surgery (12.3 and 10.3 mo, respectively; $P = 0.029$)[102,103]. Although these studies were retrospective, they suggest the usefulness of radiotherapy in advanced HCC. However, radiotherapy has not been incorporated into the international guidelines for HCC despite its efficacy. This may be attributed to the paucity of well-designed randomized controlled studies, which are urgently needed. In addition, guidelines for optimal dose fractionation and protocols for avoiding radiation toxicity should be further established[93].

Proton beam therapy (PBT) can dramatically reduce damage to surrounding liver tissue by modulation of the Bragg peak of protons in energy and time, and thereby, maximizes the effects of radiation on the tumor. In Eastern areas, studies of PBT in HCC patients have been reported mainly by Japanese groups[104-106]. A retrospective study of PBT in 162 surgically unresectable patients reported a local control rate of 89% and an overall survival rate of 23.5% at 5 years[106]. Although the tumor stages of the patients were diverse and TACE or PEI may have also been administered, the overall efficacy seems quite favorable. PBT showed a good response rate even for large tumors (> 10 cm) and HCCs with main portal tumor thrombosis[107,108].

Radioembolization: Radioembolization is a modality involving the use of a transarterial approach to the hepatic tumor and subsequent infusion of radioactive substances. The rationale for this approach is that the efficacy of external beam radiation therapy is limited by the low tolerability of cirrhotic livers leading to radiation hepatitis or decompensation. To avoid exposure of non-tumorous parenchyma to radiation, microspheres emitting high-energy and low-penetration radiation are selectively delivered to the tumor[109]. The most commonly used radioembolic agents are iodine-131 and yttrium-90 glass beads, both of which showed favorable antitumoral effects with an acceptable safety profile[109,110]. The benefits of radioembolization over the other types of transarterial therapies still need to be validated. A retrospective analysis showed no significant differences in efficacy between radioembolization and TACE for intermediate stage HCC; median survival times were 15.0 and 14.4 mo, respectively[111]. However, patients receiving radioembolization needed less hospitalization and fewer treatments. Fewer treatment sessions should improve quality of life and reduce the possibility of liver derangement; therefore, in these respects, radioembolization is considered better than conventional TACE. The efficacy of radioembolization in patients with advanced HCC patients has also been evaluated. Sixty- three patients with portal vein thrombosis were analyzed from an European HCC cohort according to underlying liver function[112]. Median overall survival and time to progression were 13.8 and 5.6 mo, respectively, for Child-Pugh A patients and 6.5 and 4.9 mo, respectively, for Child-Pugh B patients[112]. Although these data appear very promising, there are still no randomized controlled trials comparing radioembolization with standard treatments for each stage. Data from Asian countries are limited, but a multicenter prospective study in Korea showed a median time to progression of 18 mo and a 3-year survival rate of 75%[113]. This is an improved result compared with data from Western countries[114,115], but future well-designed studies are needed.

Emerging therapies: Recently, the oncolytic and immunotherapeutic vaccinia virus has been reported to induce antibody-mediated, complement-dependent cancer cell lysis in humans[116]. Immunotherapy may benefit patients with advanced stage HCC who do not have further treatment options. The results of a phase III trial need to be confirmed.

Currently, several target delivery systems has been exploited for the treatment of HCC. New formulations including polymeric nanoparticles, nanocapsules, liposomes, nanoemulsions, microsphere, and polymeric micelles have been reported[117,118]. Novel drug delivery systems are expected to improve treatment efficacy and to decrease toxicity by drug targeting to the specific site of action[118]. For example, the asialoglycoprotein (ASPG) receptor is expressed on hepatocyte, and a synthetic ligand, lactosylated liposomes can be used for effective delivery vehicles of doxorubicin in HCC therapy[119]. In a previous report, lactosylated liposomes encapsulating doxorubicin showed stronger anti-tumor response than the non-targeted liposomal doxorubicin and free doxorubicin. A galactose ligand with chitosan modifications, galactosylated chitosan, is also a promising carrier of chemotherapeutic agent, such as 5-fluorouracil, to the ASPG receptor, and its *in vitro* and *in vivo* efficacy was well described[120]. It is thought that efficacy of anticancer therapy utilizing target delivery system will be more synergized by combination of molecular target therapy. Further studies are warrantied.

MULTIDISCIPLINARY TREATMENT BEYOND TREATMENT ALGORITHMS

As treatment modalities for HCC are very diverse,

not only hepatologists but also surgeons, intervention radiologists, medical oncologists, and radiation oncologists should jointly discuss the best treatment options for HCC patients. Treatment may not necessarily be a sole modality; combinations of multiple treatments can be considered. Although current guidelines do not recommend multiple treatments, emerging data indicate better outcomes with multidisciplinary treatments for HCC. Furthermore, several newer clinical trials aim to properly evaluate such strategies. In this context, multimodality treatment options based on currently available evidence, especially from Eastern countries, are described in this section, according to HCC stage.

Very early or early stage

Guidelines recommend liver transplantation, hepatic resection, or RFA/PEI for very early or early stage HCC[3-5]. However, treatment may be diversified according to the status of the patient or the tumor. In addition to the single treatments described above, the following treatments can be considered as an adjuvant or a combination.

Bridging therapy for liver transplantation: Although the best outcome can be achieved with liver transplantation, HCCs may progress while patients are on the waiting list. To avoid dropout, the rate of which approaches 20%, bridging therapies may be needed[121]. Most commonly applied therapies are RFA, TACE, and surgical resection, although data from Asian countries are rather sparse. If the tumor is within the Milan criteria and liver function is not decompensated, RFA should be the first bridging therapy attempted because its post-procedural intratumoral necrosis rate is higher than that of other locoregional therapies, and it is associated with the lowest drop-out rate[122]. PEI appears to be less efficacious than RFA, but can be chosen if the lesions are close to adjacent organs, where RFA is dangerous to perform. If the tumor size is > 3 cm, TACE or TACE plus RFA may be favored as tumors become more vascularized and the effect of RFA may be diminished[121]. Surgical resection can precede liver transplantation, and salvage transplantation can be performed in the event of recurrence, without a decrease in overall post-transplant survival[123]. However, most data on bridging therapy data are uncontrolled, so it is difficult to strongly recommend this therapeutic strategy, especially if patients are eligible for LDLT.

Adjuvant therapy after resection: Hepatic resection is the preferred treatment for patients with early stage tumors and well-compensated liver function, but recurrence is the main obstacle to improving long-term prognosis. To reduce the recurrence rate, various neoadjuvant and adjuvant therapies were evaluated, but they failed to demonstrate any benefits

to recurrence-free survival[124-126]. Recently, sorafenib was tested for prevention of recurrence after curative therapy, including resection and ablation (the STORM study), but again no benefits to recurrence-free survival were observed[127]. A Japanese group previously showed positive effects of acyclic retinoids and vitamin K analogues on recurrence-free survival, but overall survival was not improved and large-scale studies were not performed appropriately[128,129]. Interferon has been suggested as an adjuvant therapy after resection[130]. According to a large current database in Taiwan, antiviral therapies reduce the recurrence of HCC after surgery in patients with chronic hepatitis B or C[131,132]. In agreement, a randomized controlled trial conducted in China showed better recurrence-free survival (RR = 0.651, 95%CI: 0.451-0.938) and overall survival (RR = 0.420, 95%CI: 0.271-0.651) in patients receiving antiviral therapy, especially in terms of prevention of late recurrence[133]. It would be reasonable to recommend nucleoside or nucleotide analogues or interferon therapy to patients with hepatitis B and pegylated interferon-based therapy to patients with hepatitis C after curative hepatic resection[130-133].

RFA/PEI combined with TACE: Local ablative therapies, which are curative modalities for HCC as mentioned previously[3,5], have been very useful in the treatment of patients reluctant to undergo or ineligible for surgery because of issues other than liver diseases. As the size of the tumor limits the efficacy of RFA or PEI, the combination of RFA or PEI and vaso-occlusive therapies such as TACE has been attempted to overcome the limitations of interventional therapies and to maximize synergistic effects[134]. A retrospective study conducted in Korea evaluated the therapeutic efficacy of RFA plus TACE in patients with medium-sized (3.1-5.0 cm) HCCs and found that it significantly lowered the local tumor progression rate compared with RFA alone (55% and 86% at 5 years, respectively; *P* < 0.001)[135]. Subsequently, several randomized controlled trials compared RFA and RFA plus TACE in Japan and China[136,137], and a meta-analysis of these studies showed that the combined treatment was significantly associated with higher overall survival (OR = 1.85, 95%CI: 1.26-2.71) and recurrence-free survival (OR = 2.13, 95%CI: 1.41-3.20) rates[138]. The benefits of the combination therapy could be attributed to the avoidance of the heat sink effect and the subsequent increase in the size of the thermal coagulation zone. In addition, synergism between hyperthermia and high concentrations of chemotherapeutic agents may enhance the destruction of microscopic satellite lesions. Recently, a non-randomized controlled study compared RFA plus TACE and surgical resection for the treatment of single HCCs ranging in size from 2 to 5 cm[139]. The study showed that the combination therapy was as effective as resection in terms of recurrence-

free survival (69.4% and 65%, respectively, at 4 years, *P* value is nonsignificant) and overall survival (78.4% and 80.3%, respectively, at 4 years, *P* value is nonsignificant). Collectively, the available data suggest that RFA plus TACE provides better outcomes than RFA alone and may be as efficacious as surgical resection for medium-sized HCCs.

PEI may be also combined with TACE, thereby peripheral micrometastasis will be better controlled and diffusion of the ethanol can be more facilitated compared with PEI alone[140]. This combination has been reported to be associated with superior efficacy in terms of local control, but no survival benefits compared with PEI monotherapy have been confirmed[141].

Intermediate stage

Most HCCs beyond the Milan criteria correspond to intermediate stage HCCs if vascular invasion and distant metastasis are absent. TACE is the recommended therapy for this stage[3,5], but the beneficial effects of TACE on long-term survival are limited. Therefore, further treatment would be necessary even in the presence of an initial tumor response.

Liver transplantation after downstaging: As liver transplantation is associated with the best treatment outcome of HCC, listing patients for transplantation should be considered whenever available; patients in the intermediate stage will be eligible if effective treatment was achieved and their HCC status was shifted to meet the Milan criteria[142]. TACE is the most commonly used modality for downstaging, and local ablative therapies may be combined[142]. The expected 5-year overall survival rate in patients who received liver transplants after downstaging is comparable to that of HCC patients who met the Milan criteria without downstaging[143]. However, the 5-year disease-free survival rate is lower in the downstaged patients[143]. Stricter follow-ups would be necessary for these patients.

TACE combined with RFA or radiotherapy: Combination therapy with RFA or PEI and TACE is being used to treat early stage HCC as mentioned earlier. This therapy can also be applied to intermediate stage HCC. However, tumor may be too extensive or multiple to combine ablative therapies in intermediate stages. For best results, modalities commonly used for more advanced stage HCC can be adopted in combination with TACE (*e.g.*, sorafenib and radiotherapy). The efficacy of TACE plus radiotherapy has been studied in 12 non-randomized and 5 randomized controlled trials in Korea, Japan, and China. A meta-analysis of these trials showed significantly improved survival at 1 year (OR = 2.23, 95%CI: 1.76-2.83) and 5 years (OR = 4.47, 95%CI: 2.08-9.61) and a better tumor response

(OR = 2.58, 95%CI: 1.64-4.06) in patients receiving TACE plus radiotherapy compared with patients receiving TACE alone[144]. In this analysis, most although not all patients in the individual studies had intermediate stage HCC. Therefore, the combination of TACE and radiotherapy should be beneficial for this stage, but consensus is needed for routine recommendation in practice guidelines.

TACE combined with sorafenib: TACE may upregulate circulating VEGF, which is associated with vascular invasion, tumor growth, metastasis, and poor survival. Therefore, control of VEGF signaling and of other tumor growth factors is necessary to prevent the progression and recurrence of HCC in patients receiving TACE[145]. A randomized controlled trial was conducted in Japan and Korea to assess the effects of TACE plus sorafenib[146]. In that study, time to progression was significantly longer in Korean patients receiving TACE plus sorafenib than in those receiving TACE alone, but not in Japanese patients. To clarify the clinical results, a meta-analysis of 6 studies was performed, and the pooled results showed that overall survival [hazard ratio (HR) = 0.65, 95%CI: 0.47-0.89] and time to progression (HR = 0.68, 95%CI: 0.52-0.87) were significantly longer in patients who received TACE plus sorafenib than in patients who received TACE only[147]. Another recent meta-analysis of 9 studies mostly from China reached the same conclusions[148]. These results indicate that appropriate combination therapies will improve clinical outcomes in patients with unresectable HCCs.

Advanced stage

This stage encompasses locally advanced HCCs with vascular invasion and HCCs with extrahepatic metastasis. Whether the tumor has advanced locally or distantly, the BCLC guidelines uniformly recommend treatment with sorafenib. Although survival benefits were observed compared with no treatment, the efficacy of sorafenib is limited[78,79]. Therefore, it would be appropriate to search for more effective methods. For instance, sorafenib may be combined with other type of therapies (*e.g.*, TACE, radioembolization, and external radiation) and TACE or HAIC may be combined with radiotherapy in patients with advanced HCC.

Sorafenib combined with TACE: A relatively large retrospective study compared the efficacy of TACE plus sorafenib and sorafenib alone in 355 advanced stage HCC patients (164 and 191 patients, respectively)[149]. Overall survival was significantly longer in the combination group than in the sorafenib monotherapy group (8.9 and 5.9 mo, respectively; *P* = 0.009) as was median time to progression (2.5 and 2.1 mo, respectively; *P* = 0.008). The difference in time to progression was still significant after

propensity score matching, whereas the difference in overall survival was not. Another study compared the efficacy of TACE plus sorafenib and TACE alone in 246 advanced stage HCC patients (82 and 164 patients, respectively) after propensity score matching. Overall survival was significantly longer in the combination group than in the TACE monotherapy group (7.0 and 4.9 mo, respectively; *P* = 0.003) as was time to progression (2.6 and 1.9 mo, respectively; *P* = 0.001)[150]. These data suggest that the combination of TACE and sorafenib is most likely more efficacious than either therapy alone in advanced HCC. Other types of transarterial therapies, including DEB-TACE or radioembolization, are emerging modalities for the treatment of advanced HCC as mentioned above[112,151]. To potentially improve their efficacy, combining new modalities with sorafenib are being evaluated in clinical trials[152,153]; a phase Ⅱ study which combined DEB-TACE with sorafenib showed objective response rate of 58% and disease control rate of 100% in advanced HCC patients[152]. The combination is a promising HCC treatment strategy considering the current data, but its benefits compared with monotherapy needs to be confirmed in a future phase Ⅲ trial.

Sorafenib combined with radiotherapy: Sorafenib was reported to enhance the radiosensitivity of human HCC cell lines by inhibiting radiation-induced activation of VEGFRs, a downstream kinase (extracellular signal-regulated kinase), and nuclear factor-κB and by increasing radiation-induced apoptosis[154]. Therefore, combining sorafenib and radiotherapy, in the form of either radioembolization or external beam radiation, is expected to be synergistic. A multicenter phase Ⅱ study evaluated safety and efficacy of combining sorafenib therapy and radioembolization in several Asian-Pacific countries[155]. Sorafenib was administered after radioembolization, and the median overall survival time was 8.6 mo in patients with advanced stage HCC[155]. Most of toxicities were associated with sorafenib therapy. Considering phase Ⅲ Asian-Pacific trial data of sorafenib which showed median survival time of 6.5 mo in advanced HCC, the data of radioembolization plus sorafenib combination therapy appears favorable[79]. Data of sorafenib plus external beam radiation are emerging, recently. A phase Ⅱ study of sorafenib therapy plus external beam radiation reported an initial complete or partial response rate of 55% and a 2-year overall survival rate of 32% in 40 Taiwanese patients with advanced HCC[156]. These efficacy data seem encouraging, but further investigations are warranted.

TACE combined with radiotherapy: As mentioned in the intermediate stage section, TACE plus radiotherapy is an effective synergistic strategy. Most of the previous randomized and non-randomized clinical trials of TACE plus radiotherapy included both intermediate stage and advanced stage HCC[100,144], whereas studies of advanced stage HCC only are few[157,158]. A retrospective study assessed outcome of patients with locally advanced HCC; 27 patients who were treated with TACE plus radiotherapy and another 27 patients who received sorafenib alone were compared after propensity score matching. Interestingly, overall survival was better in the former group than in the latter one (6.7 and 3.1 mo, respectively; *P* < 0.001)[158]. Although this was a small study, the results iterate that universal application of sorafenib for advanced stage patients may not be the best option. Further well-designed studies are warranted.

HAIC combined with radiotherapy: To facilitate the efficacy of HAIC for treatment of advanced HCC with portal vein thrombosis, radiotherapy may be combined. In a previous pilot clinical trial, infusion of 5-fluorouracil was performed at 1st and 5th wk of radiotherapy, and then continued every 4 wk. Objective response rate was 45% and median survival time was 13.1 mo[99]. More recently, HAIC combined with radiotherapy was compared with HAIC in advanced HCC patients, and the combination therapy was shown to be better than HAIC monotherapy in terms of time to progression (5.0 and 2.7 mo, respectively; *P* = 0.0024) and overall survival (8.6 and 5.0 mo, respectively; *P* = 0.0002), particularly, among the HAIC non-responders[159]. Although, these studies are retrospectively performed, HAIC combined with radiotherapy appears to have more benefits than monotherapy, suggesting synergistic effects of the therapies.

Sequential therapy with metastasectomy: In cases of extrahepatic metastasis, radiotherapy is considered if the lesions cause severe symptoms[160]. Metastatic lesions are often surgically removed if: (1) liver function is well preserved; (2) intrahepatic lesions are adequately controlled by surgery or locoregional therapy; and (3) extrahepatic metastatic lesions are confined to a single organ[161-164]. Studies from Asian countries showed 5-year survival rates ranging from 26% to 37% in HCC patients with lung metastasis who underwent metastasectomy[162-164], which are surprising survival data at the patients' tumor stage despite the selection of surgical candidates. Although sorafenib became currently the first-line treatment for HCC with distant metastasis, uniform application of sorafenib monotherapy does not seem to be the best way because of its low objective response rate. Therefore, when possible, treating intrahepatic and metastatic lesions *via* metastasectomy, locoregional therapy or radiotherapy before the administration of sorafenib would be a reasonable plan[165]. However, because no data exist regarding this strategy, it needs to be evaluated further in the future.

Terminal stage
At this stage, best supportive care (BSC) is recommended. BSC includes management of cirrhotic

complications such as ascites, hepatic encephalopathy, variceal hemorrhage, and hepatorenal syndrome. Another important aspect would be management of cancer pain. Indeed, pain management has been frequently neglected at many tertiary hospitals in Asian countries. However, a systematic approach to cancer pain control is important.

Non-opioid drugs (paracetamol) and mild opioids (codeine, tramadol, and dihydrocodeine) may be useful for mild to moderate pain if administered on a regular basis[166]. Nonsteroidal ant-inflammatory drugs, which can cause renal derangement, should be avoided. Transdermal patches (fentanyl and buprenorphine) are considered if patient's requirements of opioid are stable[166]. Breakthrough pain can be managed with rapid-acting rescue therapies administered *via* intravenous or subcutaneous routes. Strong opioids (morphine, oxycodone, hydromorphone, oxymorphone, and fentanyl) are used to control severe pain[166], but expose the patient to the risk of developing hepatic encephalopathy. Hence, close monitoring is essential. Emotional and nutritional support is also important for terminal stage care, so collaboration between the hospice team and the clinical nutrition team would be helpful[167]. Until the final round, a multidisciplinary approach should be maintained.

Need of updated staging system

With the advancement of therapeutic modalities and aggressive treatment by either mono- or combination therapy as reviewed so far, the prognosis of HCC has improved remarkably; survival benefits are better observed in more advanced stage diseases. In this regard, reevaluation of preexisting staging systems and refinement of the best-fit models have been performed in Korea, Japan, Taiwan, and China[168-171]. Most recently, a newer staging system was pronounced from a single center in Hong Kong, reflecting recent improved survival outcomes in subsets of intermediate and advanced stage patients with more radical therapies[172]. In addition, the Hong Kong Liver Cancer staging was better than BCLC staging in stratifying HCC patients with different prognostic groups. Although further validation may be needed in non-Asian patients, the system will be helpful for identifying patients who are suitable for more aggressive treatments than what BCLC staging system recommends.

CONCLUSION

There is an increasing demand that international HCC treatment guidelines should be updated properly. Still, combinations of treatment modalities have not been incorporated into recent guidelines, and there are several unmet needs. Treatment intervals, strategies in the event of recurrence, and the timing of retreatment have not been properly studied, and no established

recommendations are available. Therefore, to further improve the outcomes of HCC patients, strategies for surveillance, diagnosis, initial treatment, recurrence monitoring, and treatment after recurrence should be more organized. Close collaboration between specialists in multiple fields is of utmost importance in achieving these aims.

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REVIEW

Hepatic artery infusion chemotherapy for advanced hepatocellular carcinoma

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Abstract

Hepatocellular carcinoma (HCC) is one of the most common cancers worldwide. Surgery, percutaneous ablation and liver transplantation are the only curative treatment modalities for HCC. However, the majority of patients have unresectable disease at diagnosis. Therefore, effective treatment options for patients with advanced HCC are required. In advanced HCC, according to current international guidelines, sorafenib, a molecular targeted agent, is the standard treatment. However, alternative treatment modalities are required because of the low response rates and unsuitability of molecular agents in real practice. In various treatment modalities, mostly in Asia, hepatic arterial infusion chemotherapy (HAIC) has been applied to advanced HCC with a view to increasing the therapeutic efficacy. HAIC provides direct drug delivery into the tumor feeding vessels and also minimizes systemic toxicities through a greater first-pass effect in the liver. However, the sample sizes of studies on HAIC have been small and large randomized trials are still lacking. In this article, we describe the treatment efficacy of HAIC for advanced stage HCC and discuss future therapeutic possibilities.

Key words: Hepatocellular carcinoma; Advanced stage hepatocellular carcinoma; Sorafenib; Hepatic arterial infusion chemotherapy; Treatment efficacy

Core tip: Sorafenib is the standard of treatment for advanced hepatocellular carcinoma (HCC). However, the suitability of sorafenib is limited by its low response rates, and unsuitability for patients with poor liver function. Therefore, other treatment modalities are required. Hepatic arterial infusion chemotherapy (HAIC) has the advantages of delivering high levels of chemotherapeutic drugs directly into tumor-associated hepatic arterial branches and repeat injections are relatively simple to carry out. Thus the local therapeutic level is increased and systemic adverse effects are decreased. In the future, HAIC may be a promising treatment strategy for the management of advanced HCC.

INTRODUCTION

Hepatocellular carcinoma (HCC) is the fifth most common malignancy and shows high cancer-related mortality worldwide[1]. The incidence of HCC is increasing with the prevalence of major risk factors such as hepatitis B, hepatitis C, alcohol and nonalcoholic steatohepatitis. Despite surveillance programs in high-risk patients, most patients with HCC are diagnosed at an advanced stage. In limited patients (fewer than 30%), curative treatments, including resection, liver transplantation, or radiofrequency ablation, can be applied[2]. The prognosis of patients with HCC is still poor, and life expectancy is difficult to predict[3]. Furthermore, advanced HCC patients may show heterogeneous clinical features, from single nodules associated with limited portal vein thrombosis, to multiple intrahepatic metastasis associated with extrahepatic spread[4,5].

Sorafenib, the multi-tyrosine kinase inhibitor, was reported to show survival benefits and is the current standard treatment in advanced HCC[6,7]. Sorafenib treatment has shifted the treatment strategy towards molecular targeted therapies for advanced HCC[8]. However, other alternative treatment modalities are required because of low response rates[9] and the unsuitability of molecular agents in real clinical practice.

In other alternative therapies, hepatic arterial infusion chemotherapy (HAIC) has been applied to advanced stage HCC with a view to increasing the therapeutic efficacy in Japan and Korea. HAIC provides direct delivery of chemotherapeutic agents into tumor feeding vessels and also minimizes systemic toxicities through a greater first-pass effect in the liver[10,11]. Therefore, the purpose of this article was to describe the treatment efficacy of HAIC for advanced stage HCC and discuss future therapeutic possibilities.

HEPATIC ARTERY INFUSION CHEMOTHERAPY

HAIC has been applied to treat advanced HCC patients with tumors that are unresectable, refractory to TACE in single or multiple tumors, the infiltrative type or those with portal vein thrombosis. Theoretically, HAIC shows better efficacy than systemic chemotherapy in advanced HCC because the infusion of the chemotherapeutic agents through the hepatic artery provides direct delivery of high concentrations of drugs to the feeding arteries of HCC. In addition, HAIC also minimizes systemic toxicities through a greater first-pass effect in the liver, reflecting the lower the systemic levels of the drugs compared to systemic infusion. HAIC has been applied in advanced stage HCC with a view to improving the therapeutic indexes in Asia, especially Japan and Korea. However, there is no evidence for a survival benefit of HAIC compared with sorafenib.

Various chemotherapeutic agents based on 5-fluorouracil (5-FU) and cisplatin are commonly used and have been investigated for HAIC[12,13]. The mechanisms of the 5-FU are the disruption of RNA synthesis and inhibition of the nucleotide synthetic enzyme thymidylate synthase by active metabolites of 5-FU, including fluorodeoxyuridine monophosphate (FdUMP), fluorodeoxyuridine triphosphate and fluorouridine triphospate[14]. Cisplatin also shows cytotoxic effects and reinforces the effect of 5-FU[8,15]. The mechanism of cisplatin is the direct inhibition of DNA replication and interruption of methionine transport in cancer cells. Furthermore, cisplatin increases levels of intracellular folic acid, which is important for binding of FdUMP, an active metabolite of 5-FU to thymidylate synthetase[16,17]. Therefore, the synergistic effect of cisplatin and 5-FU is the basis of HAIC. While various treatment regimens based on 5-FU and cisplatin have been tried in HAIC[18,19], few comparative trials for these regimens have been evaluated in advanced HCC patients.

TECHNICAL ASPECT

To evaluate hepatic artery vascularization and patency of portal vein, angiography of the celiac trunk and superior mesenteric artery were performed through access to the femoral artery. Any reflux of anti-cancer drugs into the gastrointestinal tract, and out of the liver, is a contraindication for HAIC. If necessary, embolization of non-tumor feeding vessels is performed to prevent the reflux of cytotoxic drugs into both uninvolved liver parenchyma and extrahepatic organs, such as the stomach and duodenum. After selection of the tumor feeding artery, the catheter was inserted at the proper hepatic or common hepatic artery and connected to the port system. The port device in a subcutaneous pocket was implanted in the right or left iliac fossa. An infusion pump is necessary to prevent the reflux of chemotherapeutic agents because of implantation of the infusion port in the hepatic artery (Figure 1).

TREATMENT OUTCOME

In 1995, Toyoda et al[20] reported the treatment outcome of HAIC in HCC patients with portal vein thrombosis as first. Transarterial chemoembolization (TACE) has long been used as a palliative therapy for unresectable HCC in real clinical practice. However, HAIC has shown favorable outcomes in patients with intractable, advanced HCC compared with TACE (Figures 2 and 3).

Sumie et al[21] reported a comparative study between TACE and HAIC in advanced HCC. The tumor response rates (objective response) of HAIC and TACE groups were 56.3% and 23.8%, respectively. In advanced HCC (TNM stage IV or the tumor maximal diameter > 5 cm), patients tended to show better
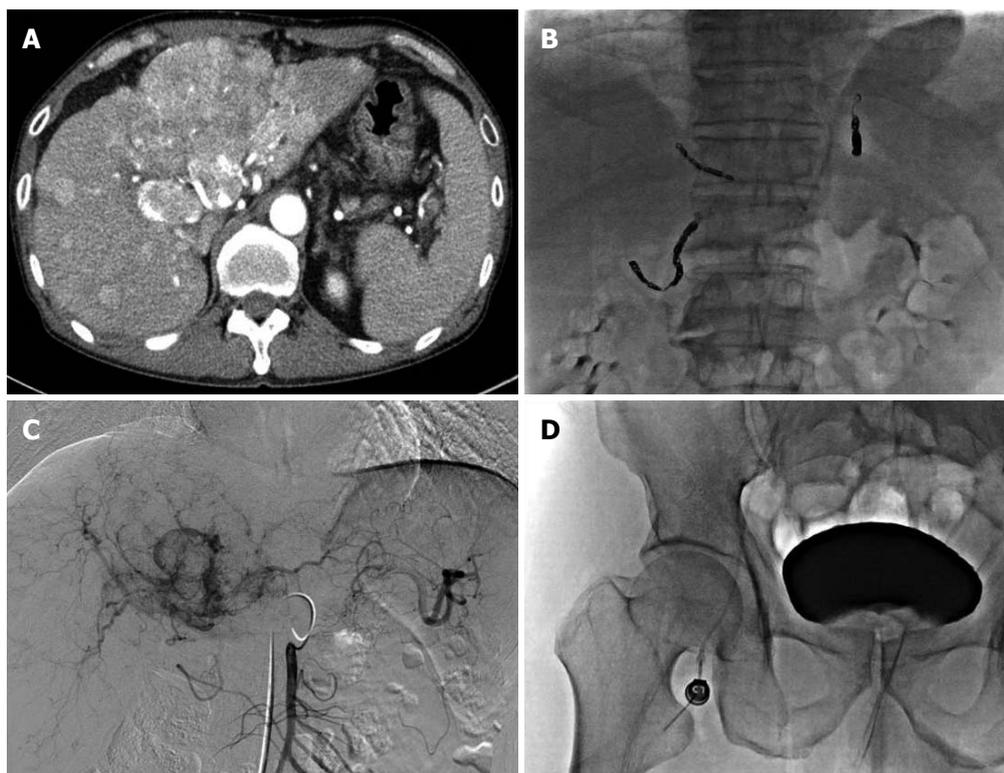
Figure 1 Technical aspects of hepatic artery infusion chemotherapy. A: Liver dynamic computed tomography showing multinodular hepatocellular carcinoma (HCC) with portal vein thrombosis; B: The embolization of non-target vessels to minimize the flow of chemotherapeutic agents into both uninvolved liver parenchyma and extrahepatic tissues; C: After finding HCC in the feeding artery, the tip of the catheter was located at the proper hepatic or common hepatic artery, chemotherapeutic agents were infused through a pump; D: The proximal end of the catheter was connected to the injection port, which was implanted in a subcutaneous pocket in the right iliac fossa.

survival benefits in the HAIC group than in the TACE group, although the overall survival rates between the two groups showed no significant difference. Kim et al[22] also reported that the objective response rate and overall survival in HAIC showed better than TACE group (16.7% vs 0%, P = 0.030, median survival; 193 vs 119 d, P = 0.026, respectively). In terms of adverse events, there were no significant differences between the HAIC and TACE group.

The reported overall response rate was 15%-56% (Table 1). Various combination regimens based on 5-FU and cisplatin have been investigated for HAIC. Hamada et al[23] reported treatment response and survival in HAIC using cisplatin (10 mg) and 5-FU (250 mg). The objective response rate was 17% (CR 1%, PR 16%). The median survival time was 19.5 mo. Lin et al[24] prospectively evaluated the effect of HAIC of the combination of cisplatin, mitomycin C, 5-FU and leucovorin. The treatment regimen consisted of cisplatin (10 mg/m^2), mitomycin C (2 mg/m^2), leucovorin (15 mg/m^2) and 5-FU (100 mg/m^2) for 5 consecutive days. The objective response was 28.3% (CR 9.4%, PR 18.9%). The patients with treatment response showed longer survival benefits than the patients without treatment response (24.6 vs 8.7 mo, P < 0.001). Hwang et al[25] evaluated the efficacy of HAIC using the FEM (5-FU, epirubicin, mitomycin C) regimen for advanced HCC. The regimen consisted of

5-FU (330 mg/m^2, every week), epirubicin (30 mg/m^2, every 4 wk) and mitomycin-C (2.7 mg/m^2, every 2 wk). The objective response was 38.9% and the median survival was 8 mo.

While various treatment regimens based on 5-FU and cisplatin have been tried in HAIC, few comparative trials for these regimens have been evaluated in advanced HCC patients. Recently, in a prospective study in Korea, Woo et al[26] reported a comparative study between high dose HAIC (5-FU, 500 mg/m^2 for 3 consecutive days and cisplatin, 60 mg/m^2 on day 2) and low dose HAIC (5-FU, 170 mg/m^2 and cisplatin, 7 mg/m^2 on days for 5 consecutive days). The objective response rate in the high-dose HAIC showed significantly better efficacy than the low-dose HAIC (16.7% vs 0%, P = 0.024). The median time to progression and overall survival showed more favorable trends in the high-dose HAIC group than in the low-dose HAIC group (145 vs 90 d, P = 0.095, 193 vs 153 d, P = 0.108, respectively). Furthermore, Kim et al[17] showed a better long-term outcome of high dose HAIC. During the follow-up period, overall survival and time to progression were 9.5 and 6.0 mo, respectively. These results seem comparable to the reported outcome of sorafenib.

A randomized phase Ⅱ trial by Yamashita et al[27] compared the response rates to treatment with interferon combined with HAIC using 5-FU and
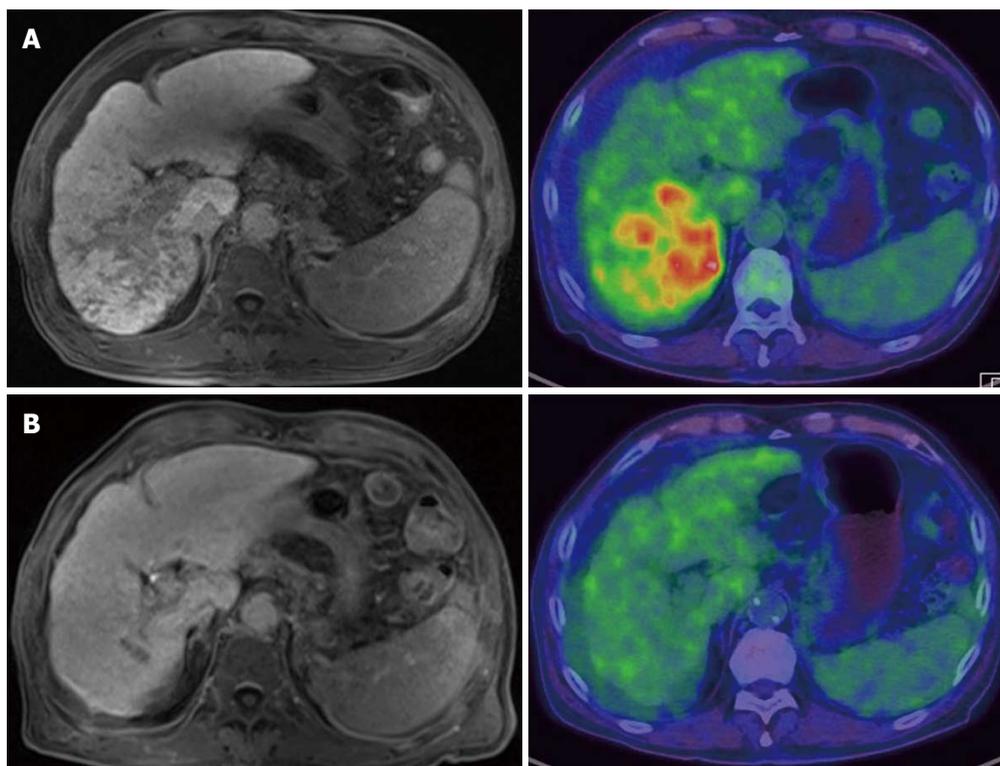
Figure 2 Favorable outcome of patient with infiltrative hepatocellular carcinoma treated by hepatic artery infusion chemotherapy. A: Patient with infiltrative type hepatocellular carcinoma (HCC) with portal vein thrombosis in liver dynamic magnetic resonance imaging (MRI) showed high FDG uptake; B: After hepatic artery infusion chemotherapy, this patient showed no viable HCC except focal portal vein thrombosis in a follow-up liver MRI and positron emission tomography/computed tomography.
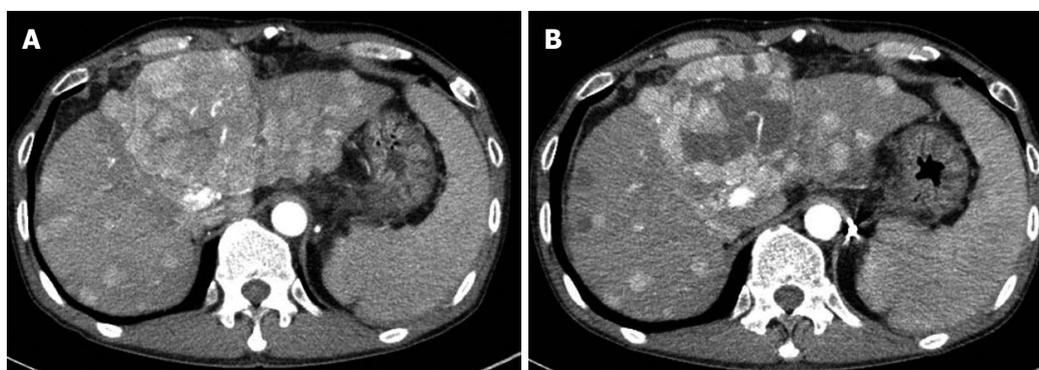
Figure 3 Favorable response of patient with multinodular hepatocellular carcinoma treated by hepatic artery infusion chemotherapy. A: Patient with multinodular type hepatocellular carcinoma (HCC) with portal vein thrombosis in baseline liver dynamic computed tomography (CT); B: After hepatic artery infusion chemotherapy, this patient showed partial necrosis of HCC in a follow-up liver dynamic CT.

cisplatin (IFN/FU + CDDP) or 5-FU (IFN/FU) alone. The response rates were 45.6% for the IFN/FU + CDDP group and 24.6% for the IFN/FU group ($P = 0.030$). Although there was no significant difference in overall survival, the progression free survival showed a better outcome in the IFN/FU+CDDP compared with the IFN/FU group (6.5 mo vs 3.3 mo, respectively, $P = 0.0048$).

Recently, Nouso et al[28] evaluated the efficacy of HAIC of 5-FU and cisplatin for advanced HCC in a nationwide survey in Japan. The outcome of 476 patients with HCC who underwent HAIC was compared with 1466 patients who did not receive active therapy.

In propensity score-matched analysis, median survival in patients with HAIC was longer than that in patients with supportive care (14.0 vs 5.2 mo, respectively, $P < 0.0001$). Song et al[29] reported a comparative study between sorafenib and HAIC. The median overall survival in the HAIC treatment group was better than that in the sorafenib group (7.1 vs 5.5 mo, $P = 0.011$). Therefore, HAIC might show a survival benefit as well as reducing the tumor burden. However, most previous reports were retrospective designs. The small sample size and disparity in the treatment response of each institution are limitations.

Table 1 Treatment response and survival rate of hepatic arterial infusion chemotherapy

Group	No.	Treatment regimen	Response rate (CR + PR)	Median survival
Toyoda et al[20]	21	Cisplatin: 5-10 mg 5-FU: 500 mg	14.3%	36-549 d
Sumie et al[21]	16	Group 1: Cisplatin 10 mg + 5-FU : 250 mg (5 d)	56.3%	2.7 yr
	21	Group 2: TACE with epirubicin	23.8%	1.7 yr
Kim et al[22]	36	Group 1: 5-FU 500 mg/m^2 on D 1-3 + Cisplatin 60 mg/m^2 on D2	16.7% (PR16.7%)	193 d
	31	Group 2: TACE with doxorubicin (10-60 mg)	0%	119 d
Hamada et al[23]	88	Cisplatin: 10 mg 5-FU: 1000 mg	17% (CR1%/PR16%)	19.5 mo
Lin et al[24]	53	Cisplatin: 10 mg, 5-FU 100 mg, Mitomycin 2 mg Leucovorin 15 mg	28.3%	NA
Hwang et al[25]	18	5-FU 330 mg/m^2 every week Epirubicin 30 mg/m^2 Mitomycin-C 2.7 mg/m^2 every 2 wk	38.9% (PR 38.9%)	8 mo
Sim et al[30]	67	Group 1: Cisplatin: 80 mg/m^2 (1 d)	20% (CR2.5%/PR17.5%)	5 mo
	36	Group 2: Cisplatin: 60 mg/m^2 (1 d) + 5-FU 500 mg/m^2 (3 d)	19.2% (CR 3.8%/PR15.4%)	8.5 mo
Lim et al[31]	40	Group 1: Cisplatin 10 mg + 5-FU 250 mg (5 d)	3.8%(PR 3.8%)	5 mo
	39	Group 2: Conservative care	0%	3 mo
Woo et al[26]	36	Group 1 (High dose): 5-FU 500 mg/m^2 on D 1-3 + Cisplatin 60 mg/m^2 on D2	16.7% (PR 16.7%)	193 d
	32	Group 2 (Low dose): 5-FU 170 mg/m^2 on D 1-5 + Cisplatin 7 mg/m^2 on D 1-5	0%	153 d
Yamashita et al[27]	57	Group 1 (IFN/FU): 5-FU 300 mg/m^2 per day for 5 d for 1st 2 wk + IFNα-2b 3M U IM 3 times/wk for 4 wk	45.6% (CR 1.7%/PR 43.9%)	10.5 mo
	57	Group 2 (IFN/FU + Cisplatin): IFN/FU + Cisplatin 20 mg/m^2 on day 1, 8	24.6% (CR 5.3%/PR 19.3%)	17.6 mo
Nouso et al[28]	476	Group 1: Cisplatin + 5-FU	40.5%	14.0 mo
	1466	Group 2: No therapy	(CR 4.0%/PR 36.5%)	5.2 mo
Song et al[29]	50	Group 1: Cisplatin + 5-FU ± epirubicin	24.0%	7.1 mo
	60	Group 2: Sorafenib	13.3%	5.5 mo

NA: Not available; 5-FU: 5-fluorouracil.

SAFETY AND COMPLICATION

The complications of using HAIC were reported as fever, jaundice, GI complication (nausea, vomiting or abdominal pain) and complication of port insertion site (infection and thrombosis). The rates of these post embolization complications in HAIC are lower than TACE. In particular, TACE may induce the following adverse effects: hepatic artery injury (stenosis or obstruction) or the development of collaterals, such as periportal or inferior phrenic artery. HAIC could be precluded by these complications. In some cases, hepatic or renal failure was reported. These cases may have been caused by underlying liver disease or disease progression rather than toxicity of HAIC.

CONCLUSION

Molecular targeting agents, including sorafenib, have been used and investigated clinically to treat advanced HCC. Although sorafenib treatment has shifted to the treatment strategy of molecular targeted therapies for advanced HCC, other alternative therapies are required because of the low response rates and unsuitability for patients with poor liver function. These studies may suggest the possibility of HAIC as an alternative therapy for advanced HCC. In particular, the indications for HAIC were unresectable, refractory to TACE in single or multiple tumors, infiltrative type or tumor with portal vein thrombosis. The advantages of HAIC are the delivery high doses of chemotherapeutic drugs directly into the hepatic arterial branches relating with the tumors and the ability to repeat the injection relatively simply, consequently increasing the local therapeutic level and decreasing systemic adverse effects. Therefore, HAIC may be a promising treatment strategy for the management of advanced HCC.

However, the limitations of this study on HAIC are that the sample size was small and that large randomized trials are still lacking. Further study to determination of appropriate treatment regimen in HAIC is important. As sorafenib is the standard treatment for advanced HCC, a comparative study between sorafenib and HAIC is needed. Currently, randomized controlled trials between HAIC and sorafenib in advanced HCC are ongoing to validate the overall clinical benefits of HAIC.

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MINIREVIEWS

Adjuvant therapy for gastric cancer: What have we learned since INT0116?

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Abstract

Gastric cancer is one of the main cancer-related causes of death worldwide. The curative treatment of gastric cancer consists of tumor resection and lymphadenectomy. However, surgical treatment alone is associated with high recurrence rates. Adjuvant treatment strategies have been studied over the last decades, but there have been controversial results from the initial studies. The pivotal INT0116 study demonstrated that the use of adjuvant chemoradiotherapy with 5-fluorouracil increases relapse-free and overall survival, and it has been adopted across the Western world. The high toxicity of radiochemotherapy and suboptimal surgical treatment employed, with fewer than 10% of the patients submitted to D2 lymphadenectomy, were the main study limitations. Since its publication, other adjuvant treatment modalities have been studied, and radiochemotherapy is being refined to improve its efficacy and safety. A multimodal approach has been demonstrated to significantly increase relapse-free and overall survival, and it can be offered in the form of perioperative chemotherapy, adjuvant chemoradiotherapy or adjuvant chemotherapy, regardless of the extent of lymphadenectomy. The objective of the present review is to report the major advances obtained in the last decades in the adjuvant treatment of gastric cancer as well as the perspectives of treatment based on recent knowledge of the molecular biology of the disease.

Key words: Stomach neoplasms; Adjuvant radiotherapy; Adjuvant chemotherapy; Histology; Genes erbB-2

Core tip: Adjuvant therapy of gastric cancer significantly improves overall survival. The most accepted adjuvant therapy in the Western world is chemoradiotherapy according to the pivotal INT0116 study. However, in the time following its publication, other adjuvant treatment modalities have been discussed, and significant improvements have been obtained in our understanding of the multimodal approach of gastric cancer. The present review reports on the major advances obtained in the last decades in the adjuvant treatment of gastric

cancer as well as the perspectives of treatment based on recent knowledge of the molecular biology of the disease.

INTRODUCTION

Gastric cancer is the fourth most common malignant neoplasm in the world, and it ranks at the same position as a cancer-related cause of death[1]. Its carcinogenesis is intimately related to environmental factors, especially those involving diet, as reflected by the geographic distribution of the disease[2]. Eastern countries, Eastern Europe and Latin America are high risk areas for the development of gastric cancer, whereas Southeast Asia, North America and Australia are low risk areas[1].

The risk factors involved in the development of gastric cancer vary according to the histological type of the tumor[3]. The Laurén intestinal-type is closely related to infection with *Helicobacter pylori*[4], especially the cagA+ subtype, gastroesophageal reflux disease and obesity[3]. The Laurén diffuse-type does not involve clearly defined environmental risk factors. The Laurén diffuse-type is associated with a mutation of the CDH1 gene, which is responsible for e-cadherin expression, and is the histological type that is usually detected in genetic syndromes associated with gastric cancer[5].

The curative treatment for gastric cancer consists of tumor resection and lymphadenectomy[6]. However, surgical treatment alone is associated with high recurrence rates[7,8]. A multimodal approach has been demonstrated to significantly increase relapse-free and overall survival[9], and it can be offered in the form of perioperative chemotherapy, adjuvant chemoradiotherapy or adjuvant chemotherapy. The use of adjuvant chemotherapy in the treatment of gastric cancer is estimated to reduce the risk of death by approximately 20%[9].

Adjuvant treatment strategies have been studied over the last decades, but initial studies have generated controversial results due to the methodological limitations and use of toxic chemotherapy regimens. In 2001, the pivotal INT0116 study demonstrated that the use of adjuvant chemoradiotherapy with 5-fluorouracil increases relapse-free and overall survival, albeit with high toxicity[10]. The main limitation of the study was the type of surgical treatment employed, which was considered to be suboptimal considering that fewer than 10% of the patients underwent D2 lymphadenectomy, the standard of care[11].

The INT0116 study represented a milestone in the treatment of gastric cancer and has been adopted across the Western world. Since its publication, other adjuvant treatment modalities have been discussed, and chemoradiotherapy is being refined to improve its efficacy and safety.

The objective of the present review is to report on the major advances in the last decades for the adjuvant treatment of gastric cancer as well as the perspectives of treatment based on our recent knowledge of the molecular biology of the disease.

SURGICAL TREATMENT

Surgical treatment is the therapeutic modality with the possibility of a cure for patients with gastric cancer[6]. It consists of tumor resection with wide margins and lymphadenectomy. D2 lymphadenectomy is the most recommended nodal dissection, which is related to its lower rate of locoregional recurrence and lower death rate from gastric cancer, but it may involve a higher risk of postoperative complications[11,12].

The risk of recurrence after surgical treatment depends on the initial stage of the disease, histological type and surgical radicality[6]. The risk of recurrence is higher if the tumor invasion is deeper into the gastric wall, especially when the serosa is involved, as well as with the presence of nodal involvement. It is also known that the Laurén diffuse-type and the presence of microscopic residual disease are associated with a higher risk of recurrence[6]. Studies have reported variable data according to the investigated population, but the largest series in the literature demonstrated recurrence risks ranging from 20% to 40% after resections performed for curative purposes[7,8]. The pattern of recurrence also varies according to the sample studied. Patients who undergo more limited nodal dissection and who have microscopic residual disease tend to have a higher risk of locoregional and peritoneal recurrence. The presence of poorly differentiated tumors with more extensive nodal involvement is associated with a higher risk of distant metastases. Strategies for adjuvant treatment have been planned based on the analysis of the risk and pattern of disease recurrence.

ADJUVANT CHEMORADIOTHERAPY

Since the publication of the INT0116 study, which demonstrated an improvement in the overall survival with adjuvant chemoradiotherapy, the treatment strategy published in that report was adopted as one of the major therapeutic options in Western countries. Before the publication of this study, the standard treatment was gastrectomy alone due to the methodological limitations and use of highly toxic

chemotherapy regimens in previous clinical studies, which has raised doubts about the benefits of adjuvant treatment.

The major limitation of the INT0116 study is the surgical treatment that was adopted[10]. The authors performed D2 lymphadenectomy in fewer than 10% of the patients, which is likely to leave microscopic residual nodal disease in most patients and would justify the survival benefit with the addition of radiotherapy. However, a potential benefit when evaluating the role of adjuvant treatment in patients who did not undergo a standardized surgical technique would be that we can reflect on the results for when D2 lymphadenectomy is not routinely performed. The update of the study after a median follow-up of 10 years supported the initial data with convincing results[13]. A gain of 8 mo in the relapse-free survival (27 mo *vs* 19 mo; HR = 1.51, 95%CI: 1.25-1.83) and in the overall survival (35 mo *vs* 27 mo; HR = 1.32, 95%CI: 1.10-1.60) has convinced the medical community to adopt this approach as one of the preferred adjuvant treatments.

A negative aspect of the treatment proposed by the INT0116 study is its toxicity. The incidence of grade 3 or higher hematologic toxicity in 54% and gastrointestinal toxicity in 33% of the patients, with a treatment-related mortality of 1%, has indicated the need for improving the treatment safety.

The absence of standardization for lymphadenectomy raises questions about the efficacy of combined treatment for patients who undergo D2 lymphadenectomy, even though the study did not detect differences in the benefit of adjuvant treatment according to the type of lymphadenectomy. Retrospective studies have supported the findings of the INT0116 study and have demonstrated the efficacy of fluoropyrimidine-based chemoradiotherapy in patients who undergo D2 lymphadenectomy[14,15].

Aiming to improve the safety and efficacy of chemoradiotherapy treatment, the RTOG 0114 study compared two therapeutic regimens, which were both associated with 45 Gy radiotherapy, *i.e.*, paclitaxel and cisplatin (PC) *vs* paclitaxel, cisplatin and 5-fluorouracil (PCF)[16]. Two chemotherapy cycles were applied, which was followed by radiotherapy concurrent with PC or PCF. The patients treated with the triple regimen had an incidence of 59% for a toxicity of grade 3 or higher, which led to the premature closure of this study arm. The patients treated with the PC regimen showed a 2-year disease-free survival of 52%, which was lower than the results of the INT0116 study. Therefore, the RTOG 0114 study did not increase the gains compared to standard treatment.

The most relevant study about the role of chemoradiotherapy that has been published in the time following the INT0116 study was the ARTIST trial, which compared adjuvant chemotherapy alone, consisting of six cycles of capecitabine and cisplatin (XP), to combined treatment with capecitabine and radiotherapy at the dose of 45 Gy in 25 fractions, with two cycles of XP before and after the combined phase[17]. All 458 patients underwent D2 lymphadenectomy. The arms of the study showed similar 3-year disease-free survival (HR = 0.74; 95%CI: 0.52-1.05) and overall survival (HR = 1.13; 95%CI: 0.77-1.64). However, combined treatment was superior to chemotherapy alone when the subgroup of patients with positive lymph nodes was analyzed (HR = 0.70; 95%CI: 0.49-0.99), which was also true when the subgroup of patients with intestinal-type histology was evaluated (HR = 0.44; 95%CI: 0.23-0.84). This result supports the hypothesis of the benefit of radiotherapy in the adjuvant treatment of gastric cancer.

Another interesting finding of the ARTIST trial is related to the safety of the therapeutic regimen used. When the chemoradiotherapy and chemotherapy groups were compared with respect to grade 3 and 4 toxicity, 48% *vs* 40% neutropenia, 12% *vs* 12% nausea, 3% *vs* 3% vomiting and 1% *vs* 2% diarrhea were observed, respectively. These rates are more favorable when compared to the INT0116 study findings. It should be emphasized that 5-HT^3 inhibitors for the prophylaxis of nausea and vomiting were not used in the INT0116 study because they were not yet available at the time of study initiation. The greater hematological and gastrointestinal toxicity of bolus 5-fluorouracil (5-FU) is well known compared to infusional 5-FU and capecitabine[18]. Replacement with capecitabine may have been responsible for the greater tolerance observed in the ARTIST study. Therefore, even though partially favorable, the efficacy and safety data indicate that the XP regimen with capecitabine and concurrent with radiotherapy could be a regimen adopted for the adjuvant treatment of gastric cancer.

In addition to the ARTIST trial, two other randomized trials and a meta-analysis have tried to elucidate the comparison of chemoradiotherapy to adjuvant chemotherapy in patients with gastric cancer who undergo D2 lymphadenectomy[19-21]. A phase Ⅲ, single institution Korean study with 90 patients, which was prematurely stopped due to low recruitment, demonstrated a reduced risk of locoregional relapse with the addition of radiotherapy to chemotherapy and an equivalent overall survival[19]. The therapeutic regimens followed the guidelines of the INT0116 study, including the use of 5-FU and folinic acid alone in the arms treated with adjuvant chemotherapy. Both treatments showed a similar toxicity profile.

A phase Ⅲ Chinese study on 404 patients, which used 5-FU according to the INT0116 study, while associating radiotherapy with the intensity-modulated radiotherapy technique also demonstrated a reduced risk of relapse (HR = 1.35; 95%CI: 1.03-1.78); the study's authors reported a median relapse-free survival of 50 mo in the combined treatment group *vs* 32 mo in the chemotherapy group, but there was no impact

Table 1 Randomized clinical trials comparing adjuvant chemoradiotherapy *vs* chemotherapy						
Ref.	Treatment	*n*	D	DFS	OS	Toxicities G3-G4
Park *et al*[17]	XP *vs* XP/XRT/XP	458	D2	0.74 (0.52-1.05)	1.13 (0.77-1.64)	N 12%, V 3% in both groups
Kim *et al*[19]	FL/FL + RT/FL *vs* FL	90	D2	60.9% *vs* 50.0%	65.2% *vs* 54.6%	Hem 25%, GI 11.4% *vs* Hem 19.6%, GI 17.4%
Zhu *et al*[20]	FL/FL + IMRT/FL *vs* FL	404	D2	1.35 (1.03-1.78)	1.24 (0.94-1.65)	Leuco 7.5%, N 2.7% *vs* Leuco 7.3%, N 0%

D: Lymphadenectomy; DFS: Disease-free survival; OS: Overall survival; XP: Capecitabine + Cisplatin; FL: 5-Fluorouracil + Leucovorin; Hem: Hematological; GI: Gastrointestinal; N: Nausea; V: Vomiting; Leuco: Leucopenia.

on overall survival (HR = 1.24; 95%CI: 0.94-1.65)[20]. There was no difference between groups with respect to toxicity. The most frequent adverse effects were neutropenia (31% *vs* 25% in the chemoradiotherapy *vs* chemotherapy group, respectively) and diarrhea (38% *vs* 30%) (Table 1).

A meta-analysis evaluating the comparison of adjuvant chemoradiotherapy *vs* chemotherapy in patients with gastric cancer who underwent D2 lymphadenectomy demonstrated an increase in relapse-free survival (HR = 0.72; 95%CI: 0.59-0.89) and locoregional relapse-free survival (HR = 0.53; 95%CI: 0.32-0.87) in favor of combined treatment[21]. Three randomized studies were selected[17,19,20], including a total of 895 patients, who were all from Asian countries, and there was no benefit in terms of the distant metastasis-free survival and overall survival. There was no difference in the toxicity between groups.

A second meta-analysis evaluating the same comparison, but with a higher number of patients (6 trials with a total of 1171 patients), had similar results[22]. Chemoradiotherapy was associated with an increase in disease-free survival (HR = 1.48; 95%CI: 1.08-2.03). However, there was no difference in overall survival (HR = 1.27; 95%CI: 0.95-1.71). An analysis of five trials demonstrated no statistically significant differences in the toxicities between the two groups.

ADJUVANT CHEMOTHERAPY

Studies evaluating the role of adjuvant chemotherapy in gastric cancer used to be characterized by small sample sizes of patients, low recruitment, highly toxic chemotherapy regimens, and methodological limitations. With improvement in the clinical studies and treatment regimens, it has been possible to observe the benefits of adjuvant therapy. A meta-analysis based on individual data for 3838 patients demonstrated an 18% relative risk reduction in death (HR = 0.82; 95%CI: 0.76-0.90) with the use of adjuvant chemotherapy[9]. Group analysis did not identify differences when treatment modalities were analyzed, *i.e.*, monotherapy or polychemotherapy. Therefore, it is not possible to identify the best chemotherapy regimen. However, considering only one study included did not have fluoropyrimidines in its regimen, the recommendation is that when adjuvant chemotherapy alone is chosen, the regimen should

include 5-FU or oral fluoropyrimidines.

The practice of adjuvant chemotherapy is more common in Eastern countries. Since the publication of the INT0116 study, the relevant studies that have evaluated the role of adjuvant chemotherapy were conducted on this population. The reproducibility of these data in the Western population is being debated in view of the distinct surgical practices, different biological characteristics of the tumors and different patterns of recurrence between populations.

The use of S-1, an oral fluoropyrimidine, for adjuvant treatment was investigated in the ACTS-GC study, which involved 1059 patients with disease stages Ⅱ and Ⅲ who were submitted to curative resection associated with D2 lymphadenectomy; one group received surgical treatment followed by systemic therapy for 1 year, and a second group was treated with surgery alone[23,24]. The use of adjuvant S-1 permitted a 34% relative risk reduction of death (HR = 0.66; 95%CI: 0.54-0.82) as well as a 5-year overall survival of 71.7%, compared to 61.1% for the group that underwent surgical treatment alone[24]. Together with this marked survival gain, treatment safety was observed, including low rates of grades 3 and 4 toxicity (6.0% anorexia, 3.7% nausea, and 3.1% diarrhea)[23].

As in the ACTS-GC study, the CLASSIC trial evaluated the use of adjuvant oral fluoropyrimidines in patients who underwent curative resection in combination with D2 lymphadenectomy[25]. Only patients from South Korean, Chinese and Taiwanese centers participated in the study, and relapse-free survival was the primary endpoint. The study randomized 1035 patients to surgical treatment alone and to surgical treatment followed by 6 mo of adjuvant XELOX (oral capecitabine 1000 mg/m^2 twice daily on days 1 to 14 plus intravenous oxaliplatin 130 mg/m^2 on day 1 of each cycle). The systemic treatment was associated with a 44% relative risk reduction of relapse within 3 years (HR = 0.56; 95%CI: 0.44 to 0.72), which is a higher value than the 35% reduction reported in the ACTS-GC study (HR = 0.65; 95%CI: 0.53-0.79). The XELOX regimen resulted in higher grades 3 and 4 toxicity (56% *vs* 6% for the group with surgery alone) as well as 22% neutropenia, 8% nausea and 8% thrombocytopenia (Table 2).

Extrapolation of data obtained in studies of metastatic disease shows that the combination of fluoropyrimidines and platins has greater therapeutic activity than monotherapy. The comparison of 5-FU

Table 2 Randomized clinical trials comparing adjuvant chemotherapy *vs* observation

Study	Treatment	n	D	DFS	OS	Toxicities G3-G4
ACTS-GC[23,24]	S-1 *vs* observation	1059	D2-3	0.65 (0.53-0.79)[a]	0.66 (0.54-0.82)[a]	Anorexia 6%, N 3.7%
CLASSIC[25]	XELOX *vs* observation	1035	D2	0.56 (0.44-0.72)[a]	NR	Leuco 22%, N 8%

[a]*P* < 0.05, adjuvant chemotherapy *vs* observation. D: Lymphadenectomy; DFS: Disease-free survival; OS: Overall survival; XELOX: Capecitabine + Oxaliplatin; N: Nausea; Leuco: Leucopenia; NR: Not reported.

and leucovorin to 5-FU, leucovorin and adjuvant oxaliplatin (FOLFOX4) was performed in a randomized, single-institution study of only 80 patients[26]. The combined regimen led to an increase in relapse-free survival (30.0 mo *vs* 16.0 mo, *P* < 0.05) and overall survival (36.0 mo *vs* 28.0 mo, *P* < 0.05), and there were similar rates of adverse events, except for a higher incidence of peripheral neuropathy in the FOLFOX4 group.

Therefore, even though meta-analysis data do not demonstrate the superiority of polychemotherapy over adjuvant monotherapy in gastric cancer, recent studies that were not included in the cited meta-analysis suggest that as for metastatic disease, the combination of fluoropyrimidines and platins has a potentially greater reduction in the risk of death than fluoropyrimidines alone[26].

The absence of data on the overall survival in the CLASSIC study does not prevent the adoption of this regimen in clinical practice in view of data favoring disease-free survival as a surrogate endpoint of the overall survival in the adjuvant treatment of gastric cancer[27].

PERIOPERATIVE CHEMOTHERAPY

The high recurrence rates associated with the exclusive surgical treatment of gastric cancer are explained by the early occurrence of micrometastases. The combination of systemic and surgical treatment is justified by the imperative need to treat micrometastases. The start of systemic treatment before the surgical procedure is intended to provide early treatment for micrometastases as well as have the potential benefit of increasing the rates of resection by reducing the tumor size; additional goals are a complete pathological response, evaluation of therapeutic sensitivity *in vivo* and better tolerability of the systemic treatment in the absence of postoperative complications.

Based on these principles, the strategy of perioperative systemic treatment was proposed, including the use of treatment regimens known to be active for treating advanced disease and the use of chemotherapy both before and after surgical treatment. The main clinical study evaluating this strategy is the MAGIC study, involving 503 patients with gastric or distal esophagus adenocarcinoma[28]. The patients were randomized to a group of perioperative treatment (three cycles of the ECF regimen - epirubicin, cisplatin

and 5-fluorouracil - before and after surgery) and to a group of surgery alone. The use of perioperative treatment was associated with a reduced risk of relapse (HR = 0.66; 95%CI: 0.53-0.81) and of death (HR = 0.75; 95%CI: 0.60-0.93). The group of patients who underwent perioperative treatment had a higher rate of curative resection (79% *vs* 70%, *P* = 0.03), smaller tumors (T1-T2: 51% *vs* 36%, *P* = 0.002) and lower nodal involvement (N0-N1: 84% *vs* 70%, *P* = 0.01) upon anatomopathological study. The main adverse effects related to chemotherapy were myelotoxicity (23% and 27% grades 3 and 4 neutropenia during the preoperative and postoperative phase, respectively), nausea and vomiting.

An aspect that reflects the difficulty of perioperative treatment with the ECF regimen is that only 41% of the patients randomized to this group were able to complete the entire treatment schedule proposed. The administration of 5-FU in an infusional regimen lasting 21 d per cycle is difficult to execute in clinical practice. However, the recent availability of capecitabine and oxaliplatin, combined with the demonstration of the equivalent efficacy of the ECF regimen and of regimens in which replacement with these more recent drugs is possible, has increased the feasibility of perioperative treatment in clinical practice[29].

The ACCORD-07 study followed the MAGIC study and evaluated the efficacy and safety of perioperative treatment consisting only of platins and fluoropyrimidines, without the addition of anthracycline agents, in 224 patients[30]. Only 25% of the patients in this study had gastric cancer. The remaining patients had esophageal or esophagogastric junction tumors. The patients received 2 or 3 cycles of CF (cisplatin and infusional 5-FU) preoperatively and 3 or 4 cycles postoperatively, resulting in a total of 6 cycles. As also observed in the MAGIC study, perioperative treatment with the CF regimen was associated with a reduced risk of relapse (HR = 0.65; 95%CI: 0.48-0.89) and a reduced risk of death (HR = 0.69; 95%CI: 0.50-0.95). The patients who underwent perioperative chemotherapy also presented with higher rates of curative resection (87% *vs* 74%, *P* = 0.004), although there was no significant difference between the groups in terms of the pathological stage. The CF regimen showed the expected grades 3 and 4 toxicity as well as 20% neutropenia and 9% nausea and vomiting in the preoperative phase (Table 3).

Table 3 Randomized clinical trials comparing perioperative chemotherapy *vs* observation

Study	Treatment	n	D	DFS	OS	Toxicities G3-G4
MAGIC[28]	ECF *vs* observation	503	D1-2	0.66 (0.53-0.81)[a]	0.75 (0.60-0.93)[a]	Leuco 27.8%, N 12.3%
ACCORD-07[30]	CF *vs* observation	224	NR	0.65 (0.48-0.89)[a]	0.69 (0.50-0.95)[a]	Leuco 20.2%, N 9.2%

[a]$P < 0.05$, perioperative chemotherapy *vs* observation. D: Lymphadenectomy; DFS: Disease-free survival; OS: Overall survival; XELOX: Capecitabine + Oxaliplatin; N: Nausea; Leuco: Leucopenia; NR: Not reported.

INDIVIDUALIZED TREATMENT

HER2 and adjuvant treatment

HER2 overexpression and/or amplification is a controversial prognostic factor in gastric cancer, but its predictive value for the use of trastuzumab, an anti-HER2 monoclonal antibody, was demonstrated in the ToGA study, which involved patients with locally advanced or metastatic disease[31]. The addition of trastuzumab to cisplatin plus fluoropyrimidines in HER2-positive patients reduced the relative risk of death by 26% (HR = 0.74; 95%CI: 0.60-0.91), permitting an increase in overall survival from 11.1 to 13.8 mo. In exploratory analysis, the risk reduction was more pronounced in the HER2-enriched population, with 3+ or 2+ immunohistochemistry and FISH-positive status. In this population, the addition of trastuzumab increased survival from 11.8 to 16.0 mo (HR = 0.65; 95%CI: 0.51-0.83). The ToGA study was the first to permit the inclusion of a monoclonal antibody in the treatment of advanced gastric cancer, leading to the approval of the drug in several countries. No published prospective studies have evaluated the use of anti-HER2 therapies in the adjuvant treatment of gastric cancer. Ongoing phase Ⅱ trials are evaluating the combination of capecitabine, oxaliplatin and trastuzumab in the neoadjuvant and adjuvant setting of HER2-positive gastric cancer patients (clinicaltrials.gov NCT 01748773, NCT01130337). The potential predictive value of HER2 expression to adjuvant therapies was obtained through the use of an exploratory analysis of relevant clinical trials.

The INT0116 study, by performing an immunohistochemical evaluation of 148 patients and FISH in 258 of the 556 patients, failed to identify the prognostic value of HER2 expression and/or amplification[32]. Among the patients with HER2 amplification ($n = 28$), there was no survival benefit with the use of adjuvant chemoradiotherapy (HR = 1.44; 95%CI: 0.44-4.75). In patients without HER2 amplification ($n = 230$), adjuvant chemoradiotherapy resulted in a significant increase in overall survival (HR = 1.58; 95%CI: 1.17-2.14), which was also demonstrated in the general population. In view of the small patient sample, the absence of a benefit from adjuvant treatment based on 5-fluorouracil concurrent with radiotherapy in patients with HER2 amplification should be interpreted with caution.

The evaluation of the HER2 status in the MAGIC study revealed data similar to that of the INT0116 study[33]. Of the 503 patients included in the study, 415 had their specimens evaluated for HER2 status. The hypothesis that HER2 overexpression and/or amplification would influence the sensitivity to the adjuvant therapy regimen based on anthracyclines was not confirmed. The HER2 status was not a prognostic factor in the MAGIC study nor was it a predictive factor for response to the ECF regimen. HER2-positive and HER2-negative patients had similar benefits after exposure to perioperative chemotherapy (interaction, $P = 0.77$).

An exploratory analysis of the ACTS-GC study also did not demonstrate an influence of the expression and/or amplification of HER2 on the prognosis for a population of patients with gastric cancer, and the benefit obtained with the administration of adjuvant S-1 did not vary according to HER2 status[34]. Of the 1059 patients included in the study, 829 were retrospectively evaluated in terms of the expression and/or amplification of HER2. A total of 113 patients (13.6%) were considered to be HER2-positive and, within this group, the use of adjuvant S-1 reduced the relative risk of death with the same magnitude as that observed in the HER2-negative group (HR = 0.63; 95%CI: 0.48-0.83 in HER2-negative; and HR = 0.63; 95%CI: 0.33-1.19 in HER2-positive).

Histological type and adjuvant treatment

Despite the recognized existence of distinct histological subtypes in gastric adenocarcinoma, with different risk factors and carcinogenesis, there is no individualized adjuvant therapy approach according to histological type.

The updates to the INT0116 study suggested, in the subgroup analysis, the absence of a benefit of adjuvant chemoradiotherapy in patients with the Laurén diffuse-type[10]. However, the interaction test did not show statistical significance.

The ARTIST trial demonstrated similar findings in a recent update, which revealed the absence of a benefit of chemoradiotherapy in patients with Laurén diffuse-type[17], which was also the case in a subgroup analysis. These findings, together with the INT0116 study, allow for the development of a hypothesis that patients with Laurén diffuse-type histology have little to no benefit from adjuvant chemoradiotherapy. However, given the known statistic limitations of the subgroup analysis, it cannot be generalized to clinical practice.

An ongoing phase Ⅱ/Ⅲ study (clinicaltrials.gov NCT01717924) is evaluating therapeutic strategies in patients with signet ring cell gastric adenocarcinoma. The patients will be randomized to perioperative treatment (3 cycles of ECF before and after surgical treatment) or to primary surgical treatment followed by 6 cycles of adjuvant ECF. This study may help clarify the best treatment approach for this subgroup of patients with gastric cancer.

Disease stage and adjuvant treatment

To date, there are no randomized studies that support the adjuvant treatment of gastric cancer directed for each stage of the disease. Through the lessons learned from breast and colon cancers, each stage of disease is expected to derive different benefits from adjuvant therapy. Based on the subgroup analysis of relevant clinical trials, greater disease stages may have lower benefits from adjuvant therapy, which is not statistically significant[13,23].

Ongoing clinical trials are trying to propose a stage-specific directed therapy. A phase Ⅲ study evaluating only stage IB gastric cancer patients randomized patients to adjuvant capecitabine *vs* observation (clinical trials.gov NCT01917552). Three randomized clinical trials are currently underway to evaluate adjuvant therapy only in patients with stage Ⅲ disease (clinicaltrials.gov NCT01618474, NCT01935778, NCT00182611). Adjuvant intraperitoneal chemotherapy with mitomycin C has been investigated together with systemic chemotherapy in a phase Ⅲ study in serosa-positive disease (clinical trials.gov NCT02205008).

POTENTIAL BIOMARKERS

Gene amplification is the most common genetic alteration in gastric cancer[35]. Most of these targetable driver mutations involve human receptor tyrosine kinases. Clinical studies evaluating the prognostic role of these overexpressed receptors and the use of tyrosine kinases inhibitors have been conducted in recent years. While these studies have been performed for advanced disease, the receptors are potential therapeutic targets in the adjuvant setting.

Fibroblast growth factor receptor (FGFR) is a transmembrane receptor tyrosine kinase family[35,36], which is represented by four members (FGFR1-4) that are involved in cell signaling by interacting with fibroblast growth factors (FGFs). The activation of FGFRs by FGFs leads to the autophosphorylation and activation of several downstream signaling pathways, including mitogen-activated protein kinase and phosphoinositide 3-kinase/akt/mTOR/p70S6kinase, which are crucial effectors in oncogenic signaling[35]. Studies have demonstrated FGFR2 amplification in 4% to 6% of gastric cancer patients, and it seems to be a prognostic factor in gastric cancer because patients harboring this genetic alteration have a poor survival rate[37,38]. FGFR2 inhibitors, such as ponatinib[39], dovatinib[40] and AZD4547[41], have activity against FGFR2-amplified cell lines *in vitro*. A randomized phase Ⅱ trial comparing AZD4547 to paclitaxel as a second-line treatment of advanced gastric cancer harboring FGFR2 polysomy or amplification is currently underway (clinicaltrials.gov NCT01457846).

The mesenchymal-epithelial transition (MET) receptor is also a transmembrane receptor tyrosine kinase that belongs to the hepatocyte growth factor receptor family[35]. It is estimated that 2% to 4% of gastric cancer patients have MET-amplification[41-43], which seems to confer poor prognosis[42]. In a report of four gastric cancer patients with advanced disease and MET-amplification, two responded to crizotinib, but the response had a limited duration[42]. Also disappointing was the use of foretinib in MET-amplified gastric cancer patients; none of the 69 patients treated with foretinib responded to this tyrosine kinase inhibitor[43]. One promising strategy for targeting MET is through monoclonal antibodies that bind to the MET receptor or to the circulating ligands for MET, such as hepatocyte growth factor. Onartuzumab, a MET antibody, is currently being tested with mFOLFOX6 in advanced gastric cancer patients who are HER2-negative and MET-positive based on immunohistochemistry (clinicaltrials.gov NCT01662869).

Epithelial growth factor receptor (EGFR) is a member of the HER receptor family that is over-expressed in a variable proportion of patients with gastric cancer[44-47], but gene amplification was found in only a small proportion of patients (2%)[44,47]. The strategy of inhibition of the EGFR pathway through both monoclonal antibodies and tyrosine kinase inhibitors in gastric cancer has been frustrating. Randomized trials using cetuximab[48], panitumumab[49], nimotuzumab[50] and erlotinib[51] in an unselected population of patients showed no clinical benefit. However, an evaluation in an enriched population may reveal new data.

PERSPECTIVES

The recent molecular characterization, including the identification of driver mutations in malignant neoplasms over the last decades and the resulting significant therapeutic impact, may contribute to modifying the adjuvant treatment of gastric cancer in the coming years. Monoclonal antibodies and tyrosine-kinase inhibitors are currently limited to advanced disease and are not used in adjuvant therapies, except for imatinib in GIST and trastuzumab in breast cancer. The recent incorporation of trastuzumab in the treatment of HER2-positive patients with advanced gastric cancer is currently being evaluated in the adjuvant treatment of early disease (clinicaltrials. gov NCT01130337, NCT01748773). Other anti-HER2 drugs, such as pertuzumab, which has recently been incorporated into the treatment for breast

cancer, are being investigated in advanced gastric cancer (clinicaltrials.gov NCT01774786) and, if they demonstrate a beneficial effect, they may be investigated in adjuvant treatment.

Currently, the association of systemic and surgical treatment has been incorporated into clinical practice, but the existence of distinct therapeutic options raises the question of which one is the best and when they should be used. The strategy of adjuvant chemotherapy has been rarely investigated in western countries and merits reproduction of the studies conducted in eastern countries, where this therapeutic modality has been evaluated more extensively.

Studies comparing the three most frequently adopted treatment strategies (adjuvant chemoradiotherapy, perioperative chemotherapy and adjuvant chemotherapy) are currently underway and will bring interesting updates in the next few years (clinicaltrials. gov NCT00407186, NCT01534546, NCT01761461, NCT01989858, NCT01516944, NCT00591045, NCT01665274, and NCT01640782).

In recent years, the chemotherapy regimen used in the INT0116 study has been criticized because bolus infusion of 5-FU is in disuse due to its greater toxicity compared to infusional 5-FU or capecitabine. When adjuvant chemoradiotherapy is performed, the recommendation is to replace bolus 5-FU with infusional regimens or with capecitabine or to follow the therapeutic regimen adopted in the ARTIST study, which includes the combination of cisplatin and capecitabine concurrent with radiotherapy. Studies that are currently underway are investigating the increased efficacy of chemoradiotherapy when, potentially, more effective regimens are used (clinicaltrials.gov NCT00052910).

CONCLUSION

Since the publication of the pivotal INT0116 study in 2001, important contributions have been made to the adjuvant treatment of gastric cancer. The main limitation of the original study, suboptimal surgical treatment, was corrected in later studies demonstrating that chemoradiotherapy prolongs the survival of patients who undergo D2 lymphadenectomy. The equivalent efficacy of chemotherapy and chemoradiotherapy that was demonstrated in the ARTIST study, albeit with better performance of the combined treatment in the subgroup of patients with positive lymph nodes and in the Laurén intestinal-type histology, shows that more than ten years after the original report, treatment based on chemoradiotherapy continues to be one of the main options of adjuvant treatment. The therapeutic options have also been expanded on the basis of studies evaluating the role of perioperative chemotherapy and adjuvant chemotherapy.

There is a need to investigate and identify the prognostic and predictive factors in early gastric cancer

to obtain the benefits already achieved in treating breast and colon cancer, for which there is a greater therapeutic individualization of adjuvant treatment with distinct benefits of adjuvant treatment according to tumor specificities. Therefore, randomized clinical trials in gastric cancer that consider the heterogeneity of gastric adenocarcinoma are needed.

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Antiviral therapies for hepatitis B virus-related hepatocellular carcinoma

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Abstract

Chronic hepatitis B virus (HBV) infection is a critical risk factor for the carcinogenesis and progression of hepatocellular carcinoma (HCC). It promotes HCC development by inducing liver fibrogenesis, genetic and epigenetic alterations, and the expression of active viral-coded proteins. Effective antiviral treatments inhibit the replication of HBV, reduce serum viral load and accelerate hepatitis B e antigen serum conversion. Timely initiation of antiviral treatment is not only essential for preventing the incidence of HCC in chronic hepatitis B patients, but also important for reducing HBV reactivation, improving liver function, reducing or delaying HCC recurrence, and prolonging overall survival of HBV-related HCC patients after curative and palliative therapies. The selection of antiviral drugs, monitoring of indicators such as HBV DNA and hepatitis B surface antigen, and timely rescue treatment when necessary, are essential in antiviral therapies for HBV-related HCC.

Key words: Chronic hepatitis B; Hepatocellular carcinoma; Antiviral therapy

Core tip: This review provides an overview of recent studies and practice guidelines on antiviral treatments for hepatitis B virus (HBV)-related hepatocellular carcinoma (HCC) and emphasizes their significance. HBV infection promotes HCC development by inducing liver fibrogenesis, genetic and epigenetic alterations, and the expression of active viral-coded proteins. Timely initiation of antiviral treatment is not only essential for preventing the incidence of HCC in chronic hepatitis B patients, but also important for reducing HBV reactivation, improving liver function, reducing or delaying HCC recurrence, and prolonging overall survival of HBV-related HCC patients after curative and palliative therapies.

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INTRODUCTION

Liver cancer is the fifth most common cancer in men (523000 cases/year, accounting for 7.9% of all cancers) and the seventh most common cancer in women (226000 cases/year, accounting for 6.5% of all cancers) worldwide. Hepatocellular carcinoma (HCC) is the most common form of liver cancer. Approximately 90% of HCC cases are associated with a known risk factor. According to statistics, approximately 5% of the world's population (*i.e.*, 350-400 million people) has chronic hepatitis B virus (HBV) infection, 75% of them are Asian, and approximately 60% of the HCC cases in Asia are associated with chronic HBV infection. The relative risk of HCC development is 100-fold for those who are infected with HBV *vs* those who are not. The risk is even higher for those with HBV infection and cirrhosis. A longer duration of infection and an increased degree of viremia also increase the rate of HCC occurrence. The incidence of HCC in subjects with chronic HBV infection in East Asian countries is estimated to be 0.2 per 100 person years in inactive carriers, 0.6% person-years in chronic hepatitis B (CHB) patients without cirrhosis, and 3.7% person-years in those with cirrhosis[1-3]. Therefore, it is worth focusing on antiviral therapy in patients with HBV-related HCC. This has been clarified in the recently published Chinese Expert consensus of antiviral treatment for HBV-related hepatocellular carcinoma[4]. This review provides an overview of recent studies on antiviral treatments for HBV-related HCC and emphasizes their significance.

HBV INFECTION IS AN IMPORTANT RISK FACTOR FOR HCC

Pathogenic mechanism of HBV-related HCC

In the liver of CHB patients, the immune reaction in response to persistent HBV infection may lead to long-term inflammation and injury, followed by hepatocyte regeneration, fibrogenesis and scar formation. The prolonged fibrogenic response is accompanied by regional hypoxia, angiogenesis and the distortion of tissue architecture, ultimately resulting in irreversible structural alterations in the liver and decompensated cirrhosis. During this process, HBV DNA consistently replicates and is integrated into the host genome, adding to the coexistence of metabolic disorders, inflammatory responses and oxidative injuries, which induce genetic instability and an imbalance of cell growth and apoptotic tolerance signals. These are all biologic driving forces for HCC development in CHB patients[2].

HBV DNA may integrate into host hepatocellular DNA and induce genetic alterations such as chromosomal instability, and modify host gene expression. It may also cause random genetic and chromosomal damage, chromosomal rearrangements, the activation of cellular oncogenes, and the inactivation of tumor suppressor genes, leading to dysregulation of cell growth, differentiation and apoptosis. HBV insertion, which is as frequent as 70%, may occur in genes encoding for proteins that are important in the control of cell signaling, proliferation, and viability (*e.g.*, telomerase). With repeated hepatocellular regeneration, the X, pre-S and S genes of HBV may increasingly integrate into host DNA, resulting in increased expression of intracellular HBV-encoded proteins. The viral proteins such as hepatitis B virus X protein may sensitize the host to chemical carcinogens or alter cellular oncogenes such as c-myc, and transactivate a number of cellular promoters by acting on cis-acting regulatory elements. The alteration of host gene expression associated with oncogenesis of HCC in CHB may also be mediated by epigenetic changes, which include aberrant DNA methylation, histone modifications, chromatin remodeling, transcriptional control, and the differential expression of noncoding RNAs[2,5,6].

In patients with HBV infection, the risk factors for HCC include progression to cirrhosis, longer duration of HBV infection, higher serum viral load, males with advanced age, ethnic groups native to regions of East Asia and sub-Saharan Africa, the virus genotype (genotype A in African population or genotype C in Asian population), co-infection with hepatitis C, D, or human immunodeficiency viruses, and a family history of liver cancer. Cirrhosis is the most important independent risk factor for HCC. Up to 70%-90% of primary liver cancers occur in patients with cirrhosis[1,3].

Antiviral treatment prevents the occurrence of HBV-related HCC

Effective antiviral treatment inhibits HBV replication, reduces serum viral load and accelerates hepatitis B e antigen (HBeAg) serum conversion, which may therefore alleviate liver damage and reduce the development of cirrhosis. At present, the nucleoside and nucleotide analogs (NAs) and interferon (IFN) are common clinically used antiviral drugs. NAs can be structurally grouped into (1) L-nucleosides which include lamivudine (LAM) and Telbivudine; (2) acyclicnucleotide phosphonates which include adefovir dipivoxil (ADV) and tenofovir disoproxil (TDF); and (3) D-cyclopentanes, which include Entecavir (ETV). This categorization reflects the drug's pattern of resistance. ETV and TDF are two NAs which have been recommended as first-line anti-HBV drugs by the updated consensus and recommendations on the management of CHB due to their high efficacy and high barrier to drug resistance[7-9].

The results of a meta-analysis showed that the median cumulative incidence of HCC in CHB patients treated with antiviral therapy for 3 and 5 years was lower than that without treatment (1.5% *vs* 4.0%; 5.1% *vs* 12%, respectively). Antiviral therapy significantly reduced the 3- and 5-year cumulative

incidence of HCC by 2.8% (95%CI: 0.5-5.1; P = 0.0162) and 7.1% (95%CI: 4.1-10.2; P < 0.0001), respectively[10]. Whereas in another meta-analysis which included 8 randomized controlled trials (RCTs), 8 prospective cohort studies and 19 case-control studies, the prospective cohorts and case-control series showed opposing results[11]. Although sensitivity analyses showed that antiviral therapy reduced the risk of HCC among patients with cirrhosis (RR = 0.74; 95%CI: 0.57-0.96), the strength of the evidence does not allow for extrapolation to clinical practice as the research design plays an essential role in the overall assessment.

Long-term studies on CHB patients at various stages, including asymptomatic patients, those without and with cirrhosis, showed that effective LAM and ADV treatments consistently reduced the incidence of HCC. In contrast, the development of drug resistance by HBV mutation, for example YMDD mutation due to alternations on the P region of HBV DNA and mutations on enhancer Ⅱ/basal core promoter/precore (EnhII/BCP/PC), has been proven to increase HCC risk. However, it is noteworthy that on-therapy virologic remission did not completely halt the incidence of HCC, which still developed in some CHB patients within 30 mo after the start of treatment. This phenomenon was considered to be associated with the early integration of HBV into the host genome, and the patients had already developed cirrhosis, which is an independent risk factor for HCC[12].

Long-term follow-up studies of peg-IFN-α therapy showed inconsistent results. The beneficial effect was observed mainly in CHB patients with preexisting cirrhosis. Some studies also suggested that HCC incidence was lower in patients with sustained virological response (SVR) than in both non-responders and those without treatment[12]. A retrospective cohort study indicated that combination therapy with IFN-α and ribavirin significantly reduced the risk of HCC (HR = 0.76, 95%CI: 0.59-0.97), liver-related mortality (HR = 0.47, 95%CI: 0.37-0.6) and all-cause mortality (HR = 0.42, 95%CI: 0.34-0.52) in HCV-HBV dually-infected patients[13].

A retrospective review based on two prospective surveillance cohorts showed that the survival rate of patients who started antiviral therapy in the surveillance period was dramatically higher than those without any history of antiviral therapy, or those who initiated therapy after the diagnosis of HCC. The 5-year survival rates were 23.9% and 57.8%, respectively (HR = 0.472, 95%CI: 0.25-0.89, P = 0.0191)[14].

EFFECTS OF ANTIVIRAL TREATMENT ON THE PROGNOSIS OF HBV-RELATED HCC PATIENTS

Staging and treatment of HCC

As recommended by the guideline of the American Association for the Study of Liver Diseases (AASLD)[15], the Clinic Liver Cancer staging system classifies HCC patients into very early (single HCC ≤ 2 cm, performance status (PS) 0, Child-Pugh A, without portal hypertension), early (single HCC ≤ 5 cm or up to three nodules < 3 cm, PS 0, Child-Pugh A or B), intermediate (single or multifocal HCC > 5 cm, PS 0 to 2, Child-Pugh A or B), advanced (with symptoms and/or vascular invasion or extrahepatic spread, PS 1 to 2, Child-Pugh A or B) and end-stage (PS 3 to 4, Child-Pugh C) according to their liver function, tumor status and PS. Patients at different stages are managed by corresponding treatments.

Both surgical resection and liver transplantation (LT) are curative treatments for HCC. Other treatment options include: (1) local ablation, *e.g.*, radiofrequency ablation (RFA), percutaneous ethanol injection (PEI), microwave ablation and cryoablation; (2) transcatheter arterial chemoembolization (TACE); (3) radiation therapy, *e.g.*, three-dimensional conformal radiotherapy (3D-CRT), intensity-modulated radiation therapy and stereotactic radiotherapy; (4) radio-embolization; (5) systemic chemotherapy; (6) molecularly targeted therapies; (7) traditional Chinese medicine; (8) biotherapy; and (9) symptomatic supportive treatment. Of these, PEI and RFA are highly effective and may even be curative for patients with small HCC[15-18].

HCC patients at very early and early stages should be considered for potentially curative options such as surgical resection and RFA/PEI. The early-stage patients are also recommended for LT whenever possible. A favorable prognosis can usually be achieved by these treatments. HCC patients at intermediate stages may benefit from TACE, which has been shown to induce extensive tumor necrosis in more than 50% of patients. Studies on the effects of TACE in combination with molecular-targeting agents such as sorafenib, which has been shown to inhibit tumor proliferation and angiogenesis, are underway. There is no effective therapy for HCC patients at advanced stages. Several agents have been compared to sorafenib which is unequivocally effective in improving survival. The median overall survival of sorafenib-treated patients was 10.7 mo *vs* 7.9 mo in those treated with placebo (P < 0.001). Patients with end-stage HCC have a poor prognosis and may only receive symptomatic supportive treatment[15-17].

The guideline on the diagnosis and treatment of primary liver cancer of China[18] indicates that surgical resection and LT are the first choice for the treatment of HCC if applicable. Local ablation may be an alternative therapy in patients with early-stage HCC, and may be used as part of palliative treatment in some situations. TACE is recommended for HCC patients who can not receive surgery. Modern precise radiotherapy provides a treatment option for local tumors that can not be excised by surgery. It is also used as a palliative treatment for distant metastasis.

Systemic treatment including molecular targeted drugs and systemic chemotherapy are used in patients with advanced HCC. Multi-disciplinary comprehensive treatment has been recommended in HCC patients, especially those at intermediate and advanced stages.

HBV reactivation is an important risk factor that impacts on the prognosis of HBV-related HCC after conventional therapy

HBV reactivation is defined as either a greater than 10-fold increase in serum HBV-DNA load when compared with the baseline level in HBV carriers, or the reappearance of hepatitis B surface antigen (HBsAg) or serum HBV-DNA level greater than 200 IU/mL in baseline HBsAg-negative patients. HBV reactivation is a frequent complication of systemic chemotherapy, especially in patients with detectable serum HBV DNA before chemotherapy, and those who have received intensive chemotherapy. Impaired host immunity is considered to allow active HBV replication to occur, since it can also occur after the use of other immunosuppressive therapies. The clinical consequences of HBV reactivation include provoking active hepatitis, thereby causing massive hepatic necrosis, liver failure, and even death[19].

A variety of metabolic and endocrine responses induced by surgery and anesthesia may result in an extensive immunosuppressive status in the immediate postoperative period. It has been reported that the incidence of HBV reactivation after liver resection is 28% in HBV infected patients. In patients with HBV-related HCC, the occurrence of HBV reactivation after partial hepatectomy may reach 19.1% within one year even in those with low preoperative viral load (HBV DNA < 2000 IU/mL). The incidence of postoperative hepatitis and mortality due to liver failure in HBV reactivated patients is significantly higher than those without HBV reactivation (76.3% *vs* 2.0%, *P* < 0.001, and 11.8% *vs* 6.4%, *P* = 0.002, respectively). The 3-year disease-free survival (DFS) and OS rates after resection were significantly lower in patients with HBV reactivation than those without reactivation (34.1% *vs* 46.0%, *P* = 0.009; and 51.6% *vs* 67.2%, *P* < 0.001, respectively)[20]. During a median follow-up period of 29.4 mo, 23.1% and 16.6% of HBV-related HCC patients with LT treatment experienced HBV relapse or HCC recurrence, respectively. It was also observed that HBV relapse was closely associated with HCC recurrence (*P* = 0.004), which led to an unfavorable OS[21].

Previous studies have indicated that anti-HBV therapies inhibit HBV replication, reduce serum HBV load, improve liver function, and render the patients more tolerant of conventional treatments for HCC. The treatments also reduce the incidence and severity of potentially life-threatening HBV reactivation. Early loss of HBV-DNA has been correlated with a better prognosis, delayed and reduced recurrence of HCC, and prolonged OS[22].

Antiviral treatments and surgery: In a study on HBV-related HCC patients with HBV-DNA level less than 2000 IU/mL, HBeAg positive, detectable preoperative HBV-DNA level, high Ishak inflammatory score, preoperative TACE, longer operating time, and blood transfusion were identified as independent risk factors for HBV reactivation after HCC surgery. Prophylactic antiviral therapy was found to be a protective factor. The rate of HBV reactivation in the preoperative HBV-DNA negative group was lower than that in the HBV-DNA positive group (16.7% *vs* 29.4%, *P* < 0.001)[20].

A two-stage longitudinal study showed that high viral load ($\geqslant 10^4$ copies/mL) significantly predicted unfavorable OS and relapse-free survival (RFS) after hepatectomy for HCC, whereas antiviral treatment significantly improved both types of survival. The RCTs on postoperative antiviral treatment with LAM, ADV or ETV showed that anti-viral treatment significantly decreased HCC recurrence and reduced HCC-related death (HR = 0.48; 95%CI: 0.32-0.70, and HR = 0.26; 95%CI: 0.14-0.50, respectively). Antiviral treatment also significantly decreased early recurrence (HR = 0.41; 95%CI: 0.27-0.62) and improved liver function at 6 mo after surgery when compared with the controls (*P* < 0.001). The treated patients with normalized liver function had a higher 2-year RFS rate than those without improvement (*P* = 0.003)[23].

With regard to anti-viral treatment with IFN, a recent randomized, observation-controlled, phase Ⅲ trial which enrolled HBV-related HCC patients with curative resection suggested that adjuvant IFN-α-2b treatment only temporarily inhibited viral replication without a prominent effect on reducing HCC recurrence or mortality. At a median follow-up period of 63.8 mo, 57.5% of patients experienced tumor recurrence, and 31.3% were deceased. The cumulative 5-year RFS and OS rates of the intent-to-treat cohort were 44.2% and 73.9%, respectively. The median RFS of HBV-related HCC patients in the treatment group and the control group were 42.4 and 49.1 mo, respectively (*P* = 0.828)[24]. Meta-analyses also suggested that there was little evidence of benefit for adjuvant IFN therapy in reducing the recurrence rate or prolonging OS in patients with hepatitis B virus-related HCC after curative therapy. Further study is needed due to lack of stratified assessment for SVR[25,26].

Antiviral treatments and TACE: Based on a recent study of HBsAg positive HCC patients who underwent hepatectomy or TACE, HBV reactivation rates in the antiviral treatment group were 0% and 1.5%, and the rates of liver function deterioration were 2.4% and 1.5%, respectively. Whereas in the non-antiviral-treated control group, the rates of HBV reactivation were 15.7% and 17.5%, and the rates of liver function

deterioration were 4.1% and 8.1%, respectively. For TACE treatment in HBV-related HCC patients, the absence of antiviral treatment was a risk factor for HBV reactivation (OR = 0.083). The occurrence of HBV reactivation, baseline alanine aminotransferase (ALT) and alpha-fetoprotein levels were closely associated with the exacerbation of liver function after TACE (OR = 3.550, 1.031 and 2.832, respectively), indicating that antiviral treatment reduced the risk of HBV reactivation and protected the patients against liver failure, especially in patients undergoing TACE[27].

Antiviral treatments and radiotherapy: A retrospective study showed that the cumulative rate of HBV reactivation in patients who underwent 3D-CRT was significantly greater in patients without LAM therapy than in those with LAM therapy (21.8% *vs* 0%, *P* = 0.048) or the control group without any specific treatment (*e.g.*, 3D-CRT or LAM) (21.8% *vs* 2.3%, *P* = 0.047). Prophylactic antiviral therapy should be considered as 3D-CRT may induce HBV reactivation in patients with HBV-related HCC[28].

Selection of antiviral drugs for patients with HBV-related HCC

The Chinese Expert consensus of antiviral treatment for HBV-related hepatocellular carcinoma has indicated that antiviral therapy is an important secondary precaution for preventing the incidence of HBV-related HCC in CHB patients[4]. Indicators such as HBV DNA and HBsAg should be monitored in HBV-related HCC patients under comprehensive treatments for HCC, and NAs should be initiated as soon as possible if needed. The treatment should be individualized and the concrete regimen of NAs should refer to the guideline for CHB. The patients who are treated with TACE, radiotherapy or chemotherapy should be screened for HBsAg routinely. NAs should be administered before HCC treatments in those who are HBsAg positive, even if they have negative HBV DNA and a normal ALT level. The antiviral drugs should be continued for 6 mo after chemotherapy. Long-term antiviral treatment should be considered once a positive conversion of HBV DNA has occurred. For HBV DNA-negative patients who undergo surgery or ablation, clinicians must be vigilant for HBV reactivation. If HBV DNA is detectable during the monitoring period and remains positive after an interval of 2 wk, long-term antiviral treatment is recommended. Patients with detectable HBV DNA who undergo LT should start the antiviral treatments 1 to 3 mo before surgery.

In patients with decompensated cirrhosis who undergo LT, the aim of antiviral therapies is to lower the risk of HBV re-infection, and delay the deterioration of cirrhosis and its complications. A substantial improvement in liver function achieved by antiviral treatment in some patients may even result in their withdrawal from the transplantation list. Currently, the combination of NAs and Hepatitis B immune globulin (HBIG) is considered to be a standard care against HBV recurrence after LT. A systemic review showed that HBV recurrence in patients undergoing HBIG and ETV or TDF combination therapy was similar (1.5% *vs* 0%, *P* > 0.05), and was significantly lower than that in patients undergoing combination therapy with HBIG and LAM (1.0% *vs* 6.1%, *P* < 0.001). There were no significant differences between ETV/TDF mono-prophylaxis after discontinuation of HBIG and the combination of HBIG plus ETV or TDF (3.9% *vs* 1.0%, *P* > 0.05), or the combination of HBIG plus LAM (3.9% *vs* 6.1%, *P* > 0.05)[29].

Long-term LAM mono-therapy has a much higher rate of viral resistance due to YMDD mutations, which are 24% at year 1, and may reach up to 70% at year 5. Therefore, close monitoring and timely rescue therapies are necessary. ETV and TDF are potent anti-HBV NAs with high barriers to drug resistance, thus are recommended worldwide as first-line mono-therapies for antiviral treatments, especially when long-term antiviral treatment is required. The cumulative HCC incidence at 5 years was 3.7% for the ETV and 13.7% for the control groups, respectively (*P* < 0.001). Cox proportional hazard regression analysis, after adjustment for the number of known HCC risk factors, showed that patients in the ETV group had a lower risk of HCC than those in the control group (HR = 0.37, 95%CI: 0.15-0.91; *P* = 0.03). Further analysis suggested that the treatment effect was greater in patients with a high risk of HCC (the risk scores were based on age, gender, cirrhosis status, levels of ALT, HBeAg, baseline HBV DNA, albumin, and bilirubin). In sub-analyses, the incidence of HCC at year 5 was lower in ETV-treated than non-rescue LAM treated cirrhosis patients (*P* = 0.043)[30].

A study of antiviral therapy naive patients with HBV-related advanced HCC has reported treatment outcomes during the follow-up period of 3, 6 and 12 mo, including virological, biochemical and serologic responses and the appearance of antiviral resistance which were similar in the LAM and ETV groups (all *P* > 0.05). The median OS in the LAM group was 9.6 mo, lower than that in the ETV group (13.6 mo), but not significantly different (*P* = 0.493). Thus, LAM might still be an option for antiviral treatment in HBV-related advanced HCC when the anticipated treatment time is short[31].

For hepatitis-B-related HCC patients with LAM resistance, a recent study suggested that the antiviral efficacy of LAM plus ADV combination therapy was comparable in HCC and non-HCC CHB patients. The virological response rates at months 12, 24, 36 and 48 were 33.3%, 58.3%, 50% and 33.3%, respectively, whereas the biochemical response rates were 55.6%, 58.3%, 70.0% and 58.3%, respectively. Therefore, LAM plus ADV combination therapy may be a rescue treatment for LAM-resistant HBV-related HCC patients[32].

CONCLUSION

Chronic infection with HBV is the main cause of HCC and is associated with an unfavorable prognosis. Many studies have demonstrated that timely initiation of antiviral treatment is not only essential for preventing the incidence of HCC in chronic hepatitis B patients, but also important for reducing HBV reactivation, improving liver function, reducing or delaying HCC recurrence, and prolonging overall survival of HBV-related HCC patients after curative and palliative therapies. ETV and TDF with high efficacy and a high barrier to drug resistance are recommended as first-line anti-HBV drugs. Close monitoring is essential during antiviral treatment and rescue therapy should be administered as soon as possible once drug resistance occurs.

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ORIGINAL ARTICLE

Basic Study

Insulin-like growth factor-1 mRNA isoforms and insulin-like growth factor-1 receptor mRNA expression in chronic hepatitis C

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Abstract

AIM: To evaluate the expression of different insulin-like growth factor (IGF)-1 mRNA isoforms and IGF-1 receptor (IGF-1R) mRNA in hepatitis C virus (HCV)-infected livers.

METHODS: Thirty-four liver biopsy specimens from chronic hepatitis C (CH-C) patients were obtained before anti-viral therapy. Inflammatory activity (grading) and advancement of fibrosis (staging) were evaluated using a modified point scale of METAVIR. The samples were analyzed using quantitative real-time PCR technique. From fragments of liver biopsies and control liver that were divided and ground in liquid nitrogen, RNA was isolated using RNeasy Fibrous Tissue Mini Kit according to the manufacturer's instruction. Expression levels of IGF-1 mRNA isoforms (IGF-1A, IGF-1B, IGF-1C, P1, and P2) and IGF-1R mRNA were determined through normalization of copy numbers in samples as related to reference genes: glyceraldehyde-3-phosphate

dehydrogenase and hydroxymethylbilane synthase. Results on liver expression of the IGF-1 mRNA isoforms and IGF-1R transcript were compared to histological alterations in liver biopsies and with selected clinical data in the patients. Statistical analysis was performed using Statistica PL v. 9 software.

RESULTS: The study showed differences in quantitative expression of IGF-1 mRNA variants in HCV-infected livers, as compared to the control. Higher relative expression of total IGF-1 mRNA and of IGF-1 mRNAs isoforms (P1, A, and C) in HCV-infected livers as compared to the control were detected. Within both groups, expression of the IGF-1A mRNA isoform significantly prevailed over expressions of B and C isoforms. Expression of P1 mRNA was higher than that of P2 only in CH-C. Very high positive correlations were detected between reciprocal expressions of IGF-1 mRNA isoforms P1 and P2 ($r = 0.876$). Expression of P1 and P2 mRNA correlated with IGF-1A mRNA ($r = 0.891$; $r = 0.821$, respectively), with IGF-1B mRNA ($r = 0.854$; $r = 0.813$, respectively), and with IGF-1C mRNA ($r = 0.839$; $r = 0.741$, respectively). Expression of IGF-1A mRNA significantly correlated with isoform B and C mRNA ($r = 0.956$; $r = 0.869$, respectively), and B with C isoforms ($r = 0.868$) ($P < 0.05$ in all cases). Lower expression of IGF-1A and B transcripts was noted in the more advanced liver grading (G2) as compared to G1. Multiple negative correlations were detected between expression of various IGF-1 transcripts and clinical data (*e.g.*, alpha fetoprotein, HCV RNA, steatosis, grading, and staging). Expression of IGF-1R mRNA manifested positive correlation with grading and HCV-RNA.

CONCLUSION: Differences in quantitative expression of IGF-1 mRNA isoforms in HCV-infected livers, as compared to the control, suggest that HCV may induce alteration of *IGF-1* splicing profile.

Key words: Chronic hepatitis C; Insulin-like growth factor-1 receptor; Insulin-like growth factor-1 mRNA isoforms; Quantitative polymerase chain reaction

Core tip: Hepatitis C virus (HCV) may induce alteration of insulin-like growth factor (IGF)-1 splicing profile. A quantitative polymerase chain reaction analysis confirmed higher relative expression of total IGF-1 mRNA and of IGF-1 mRNAs isoforms P1, A, and C in HCV-infected livers as compared to the control. An increase in inflammatory activity (grading) of HCV-infected livers was linked to decreased IGF-1 mRNA expression, an altered profile of mRNA isoforms, and to an increase in IGF-1R mRNA expression. Decreased expression level of IGF-1 mRNA isoforms and an increased liver expression of IGF-1R mRNA were associated with indicators of liver damage (*e.g.*, grading, staging, steatosis, and liver serum enzyme activity), and may be of prognostic significance.

INTRODUCTION

Hepatitis C virus (HCV) is the major causative agent of hepatocellular carcinoma (HCC), mainly through the indirect pathways of chronic inflammation, cell death, and proliferation[1,2]. The function of the insulin-like growth factor (IGF) system in the promotion of cell growth and neoplastic transformation has been previously described[3,4]. The IGF ligands (IGF-1 and -2) bind to the receptors, which include: IGF-1 receptor (IGF-1R), IGF-2R, insulin receptor, and hybrid receptors (IGF-1R/insulin receptor)[3]. The involvement of IGF receptors in the maintenance of the transformed hepatocyte phenotype was also described[5-9]. Most studies documented an increase in IGF-1R expression from preneoplastic lesions to developed HCC[5,9,10]. Other studies found no significant difference in the expression of IGF-1R mRNA between HCC and a control[8]. Similarly, an increase in expression of IGF-1R could not be noted in cultured human hepatocytes[11]. In a few studies on patients with liver cirrhosis and chronic hepatitis C (CH-C), the amounts of IGF-1R transcript were found to increase as compared to the normal liver[12,13]. A more thorough recognition of the role played by IGF system in hepatic carcinogenesis is thought to improve HCC therapy[3,9,14].

The six exons of *IGF-1* are alternatively spliced into multiple transcripts, encoding specific circulating and tissue-specific isoforms of the IGF-1 peptide. At the 5'end of the gene, different promoters (P1 and P2), in combination with alternative transcription start sites and differential splicing, generate the mutually-exclusive class 1 and class 2 IGF-1 isoforms[15-17]. At the 3'end of the gene, alternative splicing gives rise to at least three subsets of RNA transcripts, each encoding three distinct C-terminal portions of the unique E-peptide, as well as the 3'-UTR[15,18,19]. Exon 3 encodes parts of the signal peptide and the mature peptide common to all isoforms, while exon 4 encodes the rest of the mature peptide and the proximal part of the E domain. The composition of nucleotides in exons 5 and 6 determines the formation of isoforms A (Ea), B (Eb), and C (Ec) within classes 1 and 2[20]. The biochemical mechanism which controls use of *IGF-1* promoters 1 or 2 in alternate splicing remains poorly recognized[16,21-23]. Studies on human liver RNA have demonstrated that IGF-1 transcript undergoes alternate splicing that contains exons 3 and 4, as well as 49 bp of exon 5 and exon 6 (exon

4-5-6)[24]. The role of *IGF-1* alternate splicing has been best recognized in skeletal muscular tissue[25-28] and nervous tissue[29,30]. A differential profile of IGF-1 mRNA isoforms was demonstrated in different tumors[31-34]. It remains unknown as to whether HCV and its oncogenic proteins (C, NS3, and NS5A) *in vivo* may induce alterations in the profile of hepatic IGF-1 gene expression[35].

This study aimed to evaluate the expression of various IGF-1 mRNA isoforms (P1, P2, 1A, 1B, and 1C) and IGF-1R mRNA in chronically HCV-infected livers. Herein we examined if IGF-1 alternative splicing is associated with the degree of liver damage (grading and staging) caused by HCV virus. Results on liver expression of the IGF-1 mRNA isoforms and IGF-1R transcript were compared to histological alterations in liver biopsies and with selected clinical data in the patients. Data concerning changes in IGF-1 alternative splicing in CH-C have not been published up to now. The relationship between liver expression of mRNA IGF-1 isoforms and progression of CH-C to HCC is unclear.

MATERIALS AND METHODS

Patients

The examined group consisted of 34 patients (age: 18-63 years; 18 men and 16 women) with CH-C who were diagnosed and treated in the Department of Infectious Diseases, Poznan University of Medical Sciences in Poznan from 2010 to 2012. Patients were referred to an anti-viral treatment and not previously treated. Infections with other hepatotropic viruses (HBV, HCMV, or EBV) or other reasons of liver damage were excluded (*e.g.,* alcohol abuse, autoimmune hepatitis, NASH, drugs, or history of anti-cancer therapy). Patients with diabetes mellitus, kidney failure or any hormones disturbances were not included in the group. In the study, we used basic clinical data on HCV-infected patients, as well as other results involving biochemical tests on peripheral blood, results of ELISA tests (glucose, insulin), and histopathological examination of liver biopsies. Presence of HCV-specific antibodies was tested using chemiluminescence and ARCHITECT Anti-HCV kits (ABBOTT, Wiesbaden, Germany) in ARCHITECT and 2000 analyzers (ABBOTT). Infection with HCV was confirmed by estimating serum HCV-RNA *via* the application of GeneProof HEPATITIS C VIRUS HCV tests (GeneProof a.s., Brno, Czech Republic) and manifesting sensitivity of 50 IU/mL. In all patients, HCV genotype was estimated (VERSANT HCV GENOTYPE 2.0 ASSAY, LiPA).

Negative tissue control (*n* = 7) (patient age: 35-72 years; 4 men, 3 women) involved liver fragments with no morphological traits of organ pathology that were perioperatively sampled from the vicinity of the dissected focal lesion in the liver and a single liver biopsy taken from a patient to diagnose the reasons for elevated aminotransferase activity. Said patient proved to be HCV-negative (absence of specific antibodies and of HCV RNA). The remaining control material also originated from HCV- and HBV-negative patients. Age and sex of the patients were known. The control material was obtained from the Chair and Department of General Surgery, Gastroenterological Oncology and Plastic Surgery, Poznan University of Medical Sciences in Poznan.

Tissue material

Liver biopsy was done in all cases as a routine procedure before antiviral therapy. Basing on USG tests and alpha fetoprotein (AFP) levels, none of the patients had neoplastic growth (HCC). Written informed consent was obtained from every patient before liver biopsy and approval for the study was granted by the institution's ethical committee (No. 22/09). The excised liver fragment (HCV-infected and control) was divided, cutting off its terminal 0.5 cm fragment, which was immersed in RNA Stabilization Solution (RNAlater®, Applied Biosystems) at -80 ℃ until use. The remaining part of the fragments obtained from patients were fixed in a buffered 10% solution of formalin and embedded in paraffin. About 5 μm-thick preparations were stained with hematoxylin and eosin and then silver impregnated using standard techniques. Inflammatory activity (grading) and advancement of fibrosis (staging) were evaluated using a modified point scale of METAVIR[36]. This score is composed of a two-letter and two-number scoring system: histological activity (grading: G0 - no activity, G1 - mild activity, G2 - moderate activity, G3 - severe activity) and fibrosis (staging: S0 - no fibrosis, S1 - portal fibrosis without septa, S2 - portal fibrosis with rare septa, S3 - numerous septa without cirrhosis, S4 - cirrhosis). Fatty degeneration of the liver was evaluated using a point scale, in which grade 0 corresponded to an absence of fatty degeneration, while grades 1 and 2 corresponded to < 30% and ≥ 30%-70% hepatocytes showing traits of fatty degeneration, respectively.

Technique of quantitative real-time PCR

RNA was isolated using RNeasy Fibrous Tissue Mini Kit (QIAGEN), according to the manufacturer's instructions, from fragments of liver biopsies and control liver that were divided and ground in liquid nitrogen. In the course of the procedure, traces of DNA contamination were eliminated using DNases. Total RNA was dissolved in RNase-free water. Quality of RNA preparations was consecutively checked using electrophoresis in an agar-formaldehyde gel. RNA content was quantitated by spectrophotometry. Every RNA sample was subjected to additional digestion with DNase using RNase-Free DNase Set (QIAGEN) in order to avoid contamination with genomic DNA. Subsequently, 1 μg RNA from every sample was subjected to reverse transcription using QuantiTect Reverse Transcription Kit (QIAGEN) and cDNA was
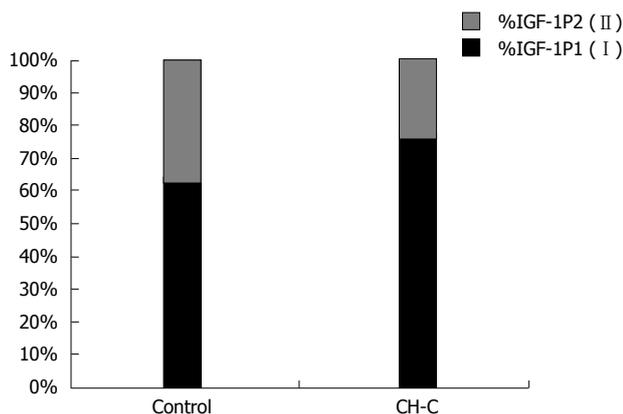
Figure 1 Shares (%) of studied insulin-like growth factor-1 mRNA isoforms of class 1 (Ⅰ) and class 2 (Ⅱ) (depending upon employed promoter P1 or P2) as related to the total mRNA for both insulin-like growth factor-1 promoters (100%) in chronic hepatitis C patients and in the control. IGF: Insulin-like growth factor; CH-C: Chronic hepatitis C.

obtained. Analysis of gene expression was performed using primers specific for individual mRNA IGF-1 isoforms (as previously described)[34] and selected reference genes: GAPDH (glyceraldehyde-3-phosphate dehydrogenase) and HMBS (hydroxymethylbilane synthase)[37]. The primer sequences for IGF-1R were: forward 5'-GGGAATGGAGTGCTGTATG-3', reverse 5'-CACAGAAGCTTCGTTGAGAA-3', GAPDH forward 5'-AAGGTCGGAGTCAACGGATTT-3', reverse 5'-ACCAGAGTTAAAAGCAGCCCTG-3', HMBS forward 5'-TGCAACGGCGGAAGAAAA-3', and reverse 5'-ACGAGGCTTTCAATGTTGCC-3'. The reactions were performed in a final volume of 10 µL. Each sample contained 15 ng/µL cDNA and a mixture of reagents forming the SYBR Green PCR master mix (Applied Biosystems, United Kingdom), with each primer pair at a concentration of 0.25 mmol/L. The reaction was conducted using an automated fluorimeter (Rotor-Gene 6000, Corbett Research). The PCR program was as follows: (1) preliminary denaturation, 95 ℃, 10 min; (2) denaturation, 95 ℃, 10-15 s; (3) primer annealing, 53-67 ℃, 15-35 s; and (4) elongation, 72 ℃, 15-40 s. The number of cycles was 40-50. The initial quantity of the product was calculated in relation to the standard curve. Presence of an appropriate product was evaluated by determination of the melting point for a specific PCR product. All samples were amplified in duplicate or triplicate and, in cases in which the results varied by more than 15%, the reactions were repeated. Expression levels of IGF-1 mRNA isoforms (IGF-1A, IGF-1B, IGF-1C, P1, and P2), and IGF-1R mRNA were determined through normalization of copy numbers in samples as related to reference genes (housekeeping genes). In the normalization, reference genes were accepted to include liver-specific GAPDH and HMBS genes, according to literature data[37].

Statistical analysis
At the first stage of statistical analysis, the consistency

of all results with the normal distribution of Gauss was verified by using the Shapiro-Wilk test. Parameters of descriptive statistics were subsequently calculated (arithmetic mean, standard deviation, median value, and minimum and maximum value). Data related to quantitative gene expression of IGF-1 mRNA isoforms, IGF-1R mRNA were compared to data for normal liver (negative control) using Mann-Whitney's test (a non-parametric test for unlinked variables for two groups). In cases of linked variables, the Wilcoxon test was used. For comparing more than two groups, Kruskal-Wallis and multiple comparison Dunn tests were employed. For comparison of the percentage shares of IGF-1 mRNA isoforms, the test of differences between two structural indices was employed. Pearson's correlation and Spearman's rank correlation were used to correlate values of variables. Effect of age was also analyzed to determine any correlation between IGF-1 mRNA isoforms, and IGF-1R transcript, and selected clinical data. The results were thought to be statistically significant at $P < 0.05$. Statistical analysis was performed using Statistica PL v. 9 software (Statsoft, Inc., Tulsa, OK, United States).

RESULTS

Expression of mRNA class 1 (I) and 2 (II) as related to the used promoter P1 or P2
Both among the HCV-infected patients and in the control, transcription of IGF-1 from the first promoter (P1) (class 1) prevailed. In the CH-C group, the shares of transcripts from class 1 (from P1) (76.4%) and class 2 (from P2) (23.6%) were similar to those in the control (62.7% and 37.3%, respectively) ($P > 0.05$) (Figure 1).

Expression of mRNA isoforms: IGF-1A, IGF-1B, and IGF-1C - shares in percentages
In the CH-C group, expression of the IGF-1A (A) splicing form of mRNA prevailed (92% of all transcripts). Similarly, in the control group, the highest expression was manifested by the A isoform, followed by B and C (Figure 2). Expression of the A isoform was significantly higher in the CH-C group than in the control (92% *vs* 85%), while expression of isoform B was lower than in the control (5.2% *vs* 12.9%). No significant differences could be demonstrated between the two compared groups in shares of expressed P1 and P2 transcripts (Figure 3).

Quantitative analysis of IGF-1 mRNA isoforms in the CH-C and control groups
Detailed analysis of the relative expression manifested by each IGF-1 mRNA isoform, normalized against reference genes (GAPDH and HMBS), demonstrated a significantly higher expression of IGF-1A and IGF-1C mRNA isoforms, of P1 transcripts (class 1), and of total IGF-1 mRNA in the CH-C group as compared to
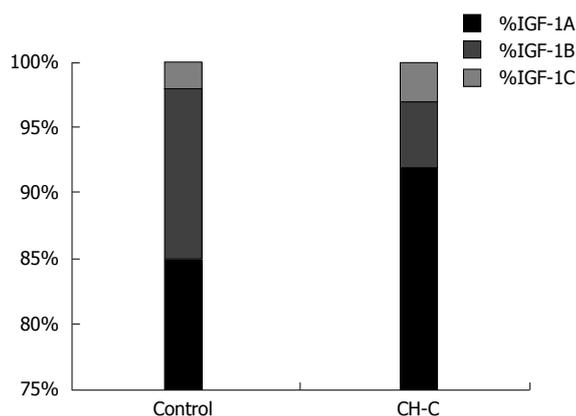
Figure 2 Shares (%) of splicing mRNA isoforms for insulin-like growth factor-1 (1A, 1B, 1C) as related to the total amount of mRNA for all three insulin-like growth factor-1 isoforms (100%) in chronic hepatitis C patients and in the control. IGF: Insulin-like growth factor; CH-C: Chronic hepatitis C.
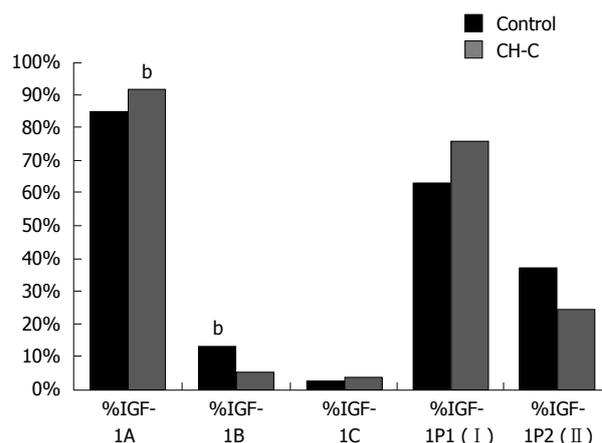
Figure 3 Comparison of shares (%) in expression of insulin-like growth factor-1 mRNA isoforms in patients chronically infected with hepatitis C virus and in the control. [b]*P* < 0.01 *vs* control.

Table 1 Quantitative expression of insulin-like growth factor-1 mRNA isoforms, total mRNA insulin-like growth factor-1, and insulin-like growth factor-1R mRNA (mean ± SD) normalized in relation to housekeeping genes in chronic hepatitis C and in control organ

mRNA	Control (*n* = 7)	CH-C (*n* = 34)	*P* value	CH-C		*P* value
				Women (*n* = 16)	Men (*n* = 18)	
class 1 (P1)	0.19 ± 0.21	0.45 ± 0.45	*P* = 0.025	0.63 ± 0.59	0.28 ± 0.17	*P* = 0.003
class 2 (P2)	0.08 ± 0.07	0.15 ± 0.17	*P* = 0.164	0.20 ± 0.15	0.11 ± 0.18	*P* = 0.006
IGF-1A	1.30 ± 1.31	5.28 ± 9.94	*P* = 0.009	7.88 ± 13.83	2.97 ± 3.32	*P* = 0.027
IGF-1B	0.18 ± 0.18	0.28 ± 0.40	*P* = 0.486	0.35 ± 0.45	0.22 ± 0.35	*P* = 0.042
IGF-1C	0.04 ± 0.05	0.21 ± 0.57	*P* = 0.031	0.35 ± 0.82	0.08 ± 0.06	*P* = 0.046
Total IGF-1	1.52 ± 1.52	5.77 ± 10.85	*P* = 0.014	8.58 ± 15.09	3.26 ± 3.70	*P* = 0.027
IGF-1R	0.26 ± 0.18	0.14 ± 0.04	*P* = 0.016	0.14 ± 0.05	0.14 ± 0.04	*P* = 0.295

IGF-1: Insulin-like growth factor 1; CH-C: Chronic hepatitis C.

Table 2 Tissue expression of insulin-like growth factor-1 mRNA isoforms and insulin-like growth factor-1R mRNA (mean ± SD) as related to grading and staging in hepatitis C virus-infected livers

mRNA	Grading[1] 1 (*n* = 15)	Grading 2 (*n* = 16)	*P* value	Staging[1] 1 (*n* = 21)	Staging 2 (*n* = 8)	*P* value
class 1 (P1)	0.63 ± 0.61	0.33 ± 0.20	*P* = 0.037	0.50 ± 0.55	0.41 ± 0.23	*P* = 0.905
class 2 (P2)	0.25 ± 0.22	0.09 ± 0.06	*P* = 0.009	0.19 ± 0.20	0.11 ± 0.07	*P* = 0.549
IGF-1A	8.67 ± 14.34	2.42 ± 1.45	*P* = 0.008	6.55 ± 12.47	3.01 ± 1.77	*P* = 0.943
IGF-1B	0.44 ± 0.55	0.15 ± 0.10	*P* = 0.012	0.34 ± 0.49	0.18 ± 0.12	*P* = 0.720
IGF-1C	0.36 ± 0.84	0.08 ± 0.06	*P* = 0.093	0.27 ± 0.72	0.09 ± 0.07	*P* = 0.582
Total IGF-1	9.47 ± 15.66	2.65 ± 1.58	*P* = 0.006	7.16 ± 13.61	3.29 ± 1.91	*P* = 0.830
IGF-1R	0.12 ± 0.04	0.16 ± 0.03	*P* = 0.025	0.14 ± 0.04	0.16 ± 0.04	*P* = 0.198

[1]Parameters evaluated in a semi-quantitative scale (see Material and Methods). IGF-1: Insulin-like growth factor 1.

the control. A significantly higher expression of total IGF-1 and all isoforms and classes of IGF-1 mRNA was detected in women as compared to men (Table 1).

Analysis of IGF-1 mRNA isoforms in HCV-infected livers as related to activity of inflammation (grading) and of liver fibrosis (staging)

A liver with more intense inflammatory lesions (grading 2, G2) contained a lower expression of all IGF-1 isoforms as compared to their expression in a liver with (G1), with the exception of the IGF-1C isoform. No

significant differences could be determined between the expression of all IGF-1 mRNA isoforms and the different stages of fibrosis (*P* > 0.05 in all the cases) (Table 2).

Expression of IGF-1R mRNA

In the CH-C group, expression of IGF-1R mRNA was significantly lower than in the control. No sex-related differences were detected in the expression of the IGF-1R transcript (Table 1). Expression of IGF-1R mRNA was higher in livers with higher grading (G2) as

Table 3 Values of Spearman's coefficient for correlation between expression of insulin-like growth factor-1, insulin-like growth factor-1R mRNAs, and clinical data in hepatitis C virus-infected patients

	Class 1 (P1)	Class 2 (P2)	IGF-1A	IGF-1B	IGF-1C	Total IGF-1	IGF-1R
Age (yr)	-0.545[1]	-0.644[1]	-0.582[1]	-0.654[1]	-0.532[1]	-0.596[1]	0.372[1]
BMI	-0.527[1]	-0.452[1]	-0.468[1]	-0.414[1]	-0.489[1]	-0.468[1]	0.060
Grading	-0.431[1]	-0.569[1]	-0.446[1]	-0.430[1]	-0.285[1]	-0.457[1]	0.456[1]
Staging	-0.223	-0.346[1]	-0.182	-0.187	-0.160	-0.190	0.264
Steatosis (%)	-0.415[1]	-0.571[1]	-0.412[1]	-0.408[1]	-0.454[1]	-0.422[1]	0.125
ALT (U/L)	-0.538[1]	-0.616[1]	-0.540[1]	-0.577[1]	-0.533[1]	-0.550[1]	0.181
AST (U/L)	-0.434[1]	-0.569[1]	-0.378[1]	-0.400[1]	-0.342[1]	-0.392[1]	0.212
AFP (ng/mL)	-0.291	-0.390[1]	-0.168	-0.271	-0.240	-0.180	0.311
HCV RNA (IU/mL)	-0.330	-0.388[1]	-0.357[1]	-0.244	-0.096	-0.345[1]	0.403[1]
Total protein (g/dL)	0.302	0.251	0.324	0.375[1]	0.366[1]	0.319	0.141
Albumins (g/dL)	0.293	0.381[1]	0.303	0.349[1]	0.222	0.295	0.123
Gamma globulins (g/dL)	-0.061	-0.164	0.039	0.045	0.174	0.044	0.138
Blood platelets (G/L)	0.273	0.372[1]	0.235	0.265	0.138	0.232	-0.099
Cholesterol (mg/dL)	0.386[1]	0.286	0.409[1]	0.405[1]	0.372	0.404[1]	0.048
GGTP (U/L)	-0.384	-0.603[1]	-0.284	-0.399	-0.367	-0.307	0.330
Blood glucose (mg/dL)	-0.083	-0.226	-0.190	-0.112	-0.084	-0.201	0.152
HOMA-IR	-0.067	-0.157	-0.237	-0.140	-0.184	-0.234	-0.019
Insulin (μU/mL)	-0.020	-0.082	-0.145	-0.079	-0.125	-0.135	-0.045

[1]The numbers indicate values of *r* coefficient for which the *P* < 0.05. IGF-1: Insulin-like growth factor 1; HCV: Hepatitis C virus; AFP: Alpha fetoprotein.

compared to livers with G1 (Table 2).

Expression of various transcripts of IGF-1 and IGF-1R vs the clinical data

Isoforms of IGF-1 mRNA: Expression of total IGF-1 mRNA and of all mRNA isoforms manifested a very strong negative correlation with patient age and BMI value. Expression of all isoforms also demonstrated negative correlations with liver steatosis. Highly negative correlations were detected with activity of ALT, and slightly less pronounced ones (though still negative) with activity of AST. Expression of mRNA isoforms A, B, P1, and P2 showed negative correlations with liver grading. Very poor correlation was detected between liver fibrosis and expression of P2 mRNA. For expression of P2 mRNA, a relatively poor negative correlation was documented, with AFP concentration and HCV viral load. Similarly low were the negative correlations between HCV viral load and expression of IGF-1A mRNA and total IGF-1 mRNA in HCV-infected liver (Table 3).

Transcripts of IGF-1R: Expression of IGF-1R mRNA manifested poor positive correlations with age of HCV-infected patients. Moreover, positive correlations were detected between tissue expression of IGF-1R mRNA, grading, and HCV-RNA (Table 3).

Reciprocal correlations between expressions of IGF-1 mRNA isoforms and IGF-1R mRNA

Very high positive Spearman's correlations were detected between reciprocal expressions of IGF-1 mRNA isoforms P1 and P2 (*r* = 0.876), P1 and A (*r* = 0.891), P1 and B (*r* = 0.854), P1 and C (*r* = 0.839); P2 and A (*r* = 0.821), P2 and B (*r* = 0.813), P2 and C (*r* = 0.741), A and B (*r* = 0.956), A and C (*r* = 0.869), and B and C (*r* = 0.868) (*P* < 0.05 in all cases)

in patients with HCV infection. In livers with CH-C, additional significantly weak negative correlations were detected between expression of IGF-1R mRNA and quantities of mRNAs for isoforms of IGF-1A (*r* = -0.397), IGF-1B mRNAs (*r* = -0.419), and for total expression of IGF-1 mRNA (*r* = -0.397) (*P* < 0.05 in all cases).

In control livers, we confirmed very high positive Spearman's correlation between reciprocal expression of all IGF-1 mRNA isoforms (data not shown). No significant relationships were detected between expression of IGF-1 mRNA isoforms and IGF-1R mRNA in control livers (data not shown).

DISCUSSION

Studies on expression of various IGF-1 mRNA isoforms have been performed for the first time on livers with CH-C. Our results point to the prevalent expression of mRNA from P1 promoter of *IGF-1*, both on the control liver and in liver with chronic HCV infection. This confirms the involvement of P1 in the production of 60%-65% and of P2 in the production of approximately 25% of IGF-1 transcripts, which was originally demonstrated in rat liver[17,22]. The percentage shares of the remaining IGF-1 mRNA isoforms (A, B, and C) in the control and HCV-infected liver also demonstrated the prevalence of mRNA isoform A overexpression of mRNAs of the remaining isoforms. In the literature data, there are no references to such results. In studies on human papilloma virus-positive and -negative tissues of uterine cervix carcinoma, a significant prevalence was demonstrated of IGF-1B share over remaining isoforms in tissues with uterine cervix carcinoma as compared to the remaining stages of carcinogenesis, suggesting that this form of the transcript may lead to the formation of the peptide Eb

with strongly mitogenic properties[33].

Analysis of the relative values of expression manifested by various IGF-1 mRNA isoforms in the livers of the two examined groups confirmed the quantitative prevalence of isoform A over the remaining variants of IGF-1 transcripts. Prevalence of expression for IGF-1A over IGF-1B was also documented by Ohtsuki *et al*[38] in such organs as the uterus, ovaries, liver, and kidneys in mice. The authors accentuated organ-specific control of transcription manifested by the gene in the course of development. The prevalence (as high as tenfold) of IGF-1A over IGF-1B mRNA transcripts, both in *in vivo* conditions (human liver) and in culture (hepatoma cells, macrophage-like cells, and fibroblasts) using RT-PCR technique was also demonstrated by Nagaoka's team[39]. The dominant expression of IGF-1A transcripts among other transcripts in the liver itself has been described by other authors[16,40].

A detailed quantitative analysis confirmed the higher expression of mRNAs for A and C isoforms (and of total IGF-1 mRNA) in our HCV infected patients as compared to the control. Expression of all studied IGF-1 transcripts has also been significantly higher in women as compared to men with HCV infection. Koczorowska *et al*[33] demonstrated a quantitatively higher expression of total IGF-1 mRNA in the precancerous stages of the uterine cervix, as well as the activity of both gene promoters at the stage of intraepithelial neoplasia. Brokaw *et al*[31] demonstrated a significant relationship between higher expression of IGF-1A isoform expression and progression of ovarian carcinoma. Nevertheless, the role of IGF-1 isoform A remains unclear. It has been argued that peptide Ea arising from it may exert mitogenic effects[41] and inhibit growth of neoplastic cells[42].

The quantitative prevalence of expression manifested by IGF-1 isoforms A and C in HCV-infected livers, as compared to the healthy organ, may point to the influence of HCV on alterations in the splicing profile of the gene in humans. Our other observations suggest the influence of mainly non-structural proteins (NS3 and NS5A) on an increase of IGF-1 protein expression in HCV-infected livers[35] and on the augmentation of IGF-1 P1 and P2 mRNA expression mRNA (unpublished data). Another explanation may involve a compensatory increase in the production of IGF-1 mRNA (particularly that of the IGF-1A isoform) under the effect of locally acting growth factors, proinflammatory cytokines, and an increased regeneration of the organ in CH-C. An increased production of two principal IGF-1 transcripts in rabbit skeletal muscle [*i.e.*, muscle L.IGF-1 (resembling isoform A in the liver) and of mRNA mechano-growth factor (MGF, isoform IGF-1B in rabbits; a homologue of IGF-1C in humans)] was detected under muscle stretching and electrical stimulation[43]. Mechanisms of differential IGF-1 mRNA stability were also described[44,45], including the effects of various RNA-binding proteins (*e.g.,* Hu and hnRNP families) on this process[46]. In the patients analyzed in this study, hepatic expression of IGF-1 mRNA

isoforms has been significantly lower upon higher activity of inflammation. The relationships have not been as spectacular as those related to fibrosis. We may have examined an insufficient number of patients with more advanced staging (3 patients with staging 3, one with staging 4). Studies have also documented a negative relationship between expression of all IGF-1 mRNA isoforms, steatosis, and ALT activity. The results indicate a coexistence of the more pronounced inflammatory/necrotic lesions in the liver and a lower hepatic production of IGF-1 mRNA isoforms. Since the literature data contains no references to the role of IGF-1 mRNA isoforms in the progression of HCV-related hepatic diseases, the results seem to be pioneering. The other few reports on the subject mainly concern human tumors[31-34]. In the case of hepatoma and HCV-associated HCC, expression of IGF-1 mRNA was studied, but with no references to specific isoforms[8,13,47]. Su *et al*[47], using Northern blotting, demonstrated numerous IGF-1 transcripts of various sizes in hepatoma cells and in lines of other neoplastic cells (HepG2, Huh-7, PLC/PRF/5, and Hep3B). Each fragment of neoplastic tissue showed a lower expression of IGF-1 mRNA as compared to control tissue. Using the quantitative technique of real-time RT-PCR (similarly to this study), Tovar *et al*[8] demonstrated a decrease in IGF-1 mRNA expression at the early stages of HCV infection as compared to control, but with no significant differences between preliminary and advanced stages of HCC in patients. Stefano *et al*[13], using the RT-PCR technique, demonstrated a comparable quantity of IGF-1 mRNA in patients with CH-C and in the control. Another study on cultured rat hepatocytes, also using the RT-PCR technique, demonstrated a 50-fold increase in the expression of IGF-1B mRNA isoform (a homologue of human isoform C) in the cells of obese animals as compared to the hepatocytes of lean individuals[48]. Armakolas *et al*[32], examining alternative splicing of IGF-1 in prostate carcinoma, demonstrated overexpression of IGF-1C (MGF) in the cells, thereby suggesting the role of the IGF-1 isoform in the stimulation of cell proliferation.

Expression of IGF-1R transcript proved to be lower in our HCV-infected livers than that in the control, and was accompanied by an increase in at least a portion of studied IGF-1 transcripts in the liver. It is known from earlier studies that, even if normal liver represents an organ with the highest production of IGF-1, it contains almost undetectable levels of IGF-1R mRNA[10]. Moreover, marked expression of the receptor used to be noted mainly in Kupffer, vascular endothelial, and stellate cells, but not in hepatocytes[49,50]. In this study, expression of IGF-1R mRNA in the CH-C group has manifested positive correlation with grading. Therefore, it seems that local production of IGF-1R mRNA does not increase until HCV-associated hepatic lesions become pronounced, which was noted to be accompanied by a reduced production of total IGF-1 mRNA (and of certain isoforms). A lowered expression of IGF-1, coexisting with an increased

production of IGF-1R in uterine carcinoma, was also described[33]. In studies on more advanced stages of liver carcinogenesis, an increase in IGF-1R expression was already detected in preneoplastic focal lesions in the liver, in HCC itself, and in cell lines of human hepatoma[5,10]. No such increase was detected in human hepatocyte cultures[11]. Studies by Price *et al*[6] on the rat model demonstrated a higher expression of IGF-1R mRNA in the control liver as compared to HCC, and it was only the cooperation of two proteins [IGF-1 and hepatocyte growth factor-scatter factor (HGF-SF)] that stimulated mitogenesis of hepatocytes in the animals. In patients with liver cirrhosis and CH-C, increased amounts of IGF-1R transcript were detected as compared to normal liver[12,13]. Tovar *et al*[8] failed to detect a significant difference in the expression of IGF-1R mRNA in hepatocellular carcinoma as compared to a control in human HCC.

Differences in quantitative expression of IGF-1 mRNA isoforms in HCV-infected livers, as compared to the control, suggest that HCV may induce alteration of the *IGF-1* splicing profile. An increase in the grading of HCV-infected livers was linked to decreased IGF-1 mRNA expression, an altered profile of mRNA isoforms, and to an increase in IGF-1R mRNA expression. The demonstration of increased tissue expression of IGF-1R mRNA and the decreased expression level of IGF-1 mRNA isoforms, accentuated in line with increasing liver damage, may be of a prognostic significance.

COMMENTS

Background
Insulin-like growth factor 1 (IGF-1) represents a well-recognized pro-proliferative factor.

Research frontiers
No studies are available on the role played by local expression involving various IGF-1 mRNA isoforms in chronic hepatitis C *in vivo*.

Innovations and breakthroughs
A quantitative polymerase chain reaction analysis used in the study confirmed the higher expression of total IGF-1 mRNA and of IGF-1 mRNAs isoforms A and C in hepatitis C virus-infected livers as compared to the control.

Applications
The demonstration of decreased expression levels of IGF-1 mRNA isoforms and an increased tissue expression of IGF-1R mRNA, associated with indicators of liver damage (*e.g.,* grading, staging, steatosis, and liver serum enzyme activity), may be of prognostic significance.

Peer-review
This paper is intriguing but presents important clinical limitations, mostly relating to the low number of patients with chronic hepatitis C investigated, to the low prevalence of elderly patients with severe forms of the disease, and to the insufficient number of controls.

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ORIGINAL ARTICLE

Basic Study

Fucosylation is a common glycosylation type in pancreatic cancer stem cell-like phenotypes

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Abstract

AIM: To evaluate/isolate cancer stem cells (CSCs) from tissue or cell lines according to various definitions and cell surface markers.

METHODS: Lectin microarray analysis was conducted on CSC-like fractions of the human pancreatic cancer cell line Panc1 by establishing anti-cancer drug-resistant cells. Changes in glycan structure of CSC-like cells were also investigated in sphere-forming cells as well as in CSC fractions obtained from overexpression of CD24 and CD44.

RESULTS: Several types of fucosylation were increased under these conditions, and the expression of fucosylation regulatory genes such as fucosyltransferases, GDP-fucose synthetic enzymes, and GDP-fucose transporters were dramatically enhanced in CSC-like cells. These changes were significant in gemcitabine-resistant cells and sphere cells of a human pancreatic cancer cell line, Panc1. However, downregulation of cellular fucosylation by knockdown of the GDP-fucose transporter did not alter gemcitabine resistance, indicating that increased cellular fucosylation is a result of CSC-like transformation.

CONCLUSION: Fucosylation might be a biomarker of CSC-like cells in pancreatic cancer.

Key words: Anti-cancer drug resistance; Cancer stem cells; Fucosylation; Glycosylation; Pancreatic cancer; Sphere formation

Core tip: Fucosylation is one of the most important glycosylation events involved in cancer and inflammation. In the present study, we investigated oligosaccharide modifications in pancreatic cancer stem cell (CSC)-like cells. Using several models of

CSC-like cells, we found that fucosylation is a common type of glycosylation change in pancreatic cancer CSC-like cells. CSCs are known to be preferentially resistant to many current therapies, including various chemotherapeutic agents and radiation treatment. Our present study suggests that the identification of fucosylated glycoproteins derived from pancreatic cancer cells could lead to novel biomarker development for anticancer drug resistance.

INTRODUCTION

Increasing evidence indicates that many tumors are composed of significant functionally and mor-phologically heterogeneous cells. Tumor initiation and selected growth are driven by a small subset of cancer stem cells (CSCs) or tumor-initiating cells that give rise to a large population of differentiated progeny, and comprise the bulk of tumors[1,2]. Several lines of research indicate that CSCs are preferentially resistant to many current therapies, including various chemotherapeutic agents and radiation treatment[3,4]. Therapeutic strategies that effectively target CSCs could significantly impact patient survival. Recent progress in CSC research includes advances in isolation methods, markers, and biologic functions of CSCs. CSCs exhibit the following general biologic characteristics: slow and asymmetric growth, anti-cancer drug resistance, increased tumorigenicity in immunodeficient mice, and sphere formation.

Pancreatic cancer is the fourth leading cause of cancer-related mortality, with an overall five-year survival rate of only 1%-4%, and a median survival period of 4-6 mo[5,6]. It is usually diagnosed at a late stage with metastasis and is resistant to chemotherapy and radiotherapy. CSCs of pancreatic cancer were reported to appear at the chronic pancreatitis stage, and the detection of CSCs at this stage can help overcome pancreatic cancer completely[7].

Glycans are branched oligosaccharide moieties that often become attached to proteins and lipids, and are then structurally and functionally modified. Because glycan structures differ among cell types, glycans can considered as the characteristic face of the cell. Recently, we reported that sialylated glycans are useful markers for CSC-like cells in hepatoma cell lines[8]. CSC-like fractions, isolated using a combination of CD133 antibody and *Sambucus sieboldiana agglutinin* lectin, showed higher tumorigenicity in athymic mice,

greater spheroid formation ability, and resistance to 5-fluorouracil (5-FU) treatment[8]. Therefore, it appears that sialylation is a characteristic glycan modification for CSC-like cells of hepatoma cells. The biologic functions of oligosaccharides, however, differ in various cancer types. For example, although increased *N*-glycan branching by *N*-acetylglucosaminyltransferase V plays a pivotal role in cancer metastasis and high expression is associated with poor prognosis in some cancers[9-11], it is a favorable prognosis marker in other cancers[12]. Thus, characteristic glycan structures for CSC-like cells could differ among various cancer types.

In the present study, we performed lectin mic-roarray analysis on CSC-like fractions of the human pancreatic cancer cell line Panc1 by establishing anti-cancer drug-resistant cells in order to examine the characteristic glycan structures of CSC-like cells in pancreatic cancers. Changes in glycan structure of CSC-like cells were also investigated in sphere-forming cells as well as in CSC fractions obtained from overexpression of CD24 and CD44, which are conventional CSC markers for pancreatic cancer cells.

MATERIALS AND METHODS

Cell culture

Pancreatic cancer cell lines, Panc1, MIA PaCa-2, PSN-1, Capan-1, and BxPC-3 were obtained from the American Type Culture Collection (ATCC, Manassas, VA, United States). A pancreatic cancer cell line, PK59 was purchased from RIKEN BioResource Center (Tsukuba, Japan). All cell lines except Capan-1 cells were cultured in RPMI-1640 (Sigma-Aldrich, St. Louis, MO, United States) with 10% fetal bovine serum (FBS), 100 units/mL penicillin, and 100 μg/mL streptomycin at 37 ℃ under 5% CO_2 in humidified air. Capan-1 cell line was cultivated with Iscove's Modified Dulbecco's Medium (Gibco of Thermo Fisher Scientific, Waltham, MA, United States) supplemented with 20% FBS and the same antibiotics. To establish gemcitabine-resistant Panc1 cells, the cells were treated step-wise with 1-200 ng/mL gemcitabine (Sigma-Aldrich) for 5 mo, and the resulting gemcitabine-resistant Panc1 cells were named Panc1-RG. Parental Panc1 cells were also cultured for the same period without gemcitabine, and designated as Panc1-P. In the case of short-term gemcitabine treatment in five pancreatic cancer cell lines (PK59, MIA PaCa-2, PSN-1, Capan-1, and BxPC-3), the cells were incubated with IC50 gemcitabine (in the case of PK59, 1 μg/mL) for 2 d.

Lectin microarray

Total patterns of oligosaccharide structures in Panc1-P and Panc1-RG cells were investigated using evanescent-field fluorescence-assisted lectin microarray. Forty-five kinds of lectin were immobilized on a glass slide in triplicate. This procedure has been previously described in detail by Kuno *et al*[13].

Genes	Analysis	5'-Sequence-3'
FUT1	RT-PCR	F: AGACTTTGCCCTGCTCACAC
		R: TGAAGTTGGCCAGGTAGACAG
FUT2	RT-PCR	F: CAGATGCCTTTCTCCTTTCC
		R: ACTCCCACATGGCTTGAATC
FUT3	RT-PCR	F: CTGGATCTGGTTCAACTTGG
		R: CGGTAGGACATGGTGAGATTG
FUT4	RT-PCR	F: GGGTTTGGATGAACTTCGAG
		R: AGCCATAAGGCACAAAGACG
FUT8	RT-PCR	F: ATCCTGATGCCTCTGCAAAC
		R: GGGTTGGTGAGCATAAATGG
GMDS	RT-PCR	F: TGGAGGCTATGTGGTTGATG
		R: CAAATTCCCGGACACTATGG
FX	RT-PCR	F: ACACGTCATCCATCTTGCTG
		R: AGGACGTTGTCGTTCATGTG
SLC35C1	RT-PCR	F: GGTGTGGCCTTCTACAATGTG
		R: ATGATGATACCGCAGGTGAG
HNF4α	RT-PCR	F: CATCTTCTTTGACCCAGATGC
		R: CGTTGATGTAGTCCTCCAAGC
IL-6	RT-PCR	F: ATGCAATAACCACCCCTGAC
		R: GCGCAGAATGAGATGAGTTG
RPL4	RT-PCR	F: GACTTAACACACGAGGAGATGC
		R: GCATGCTGTGCACATTTAGG
FUT1	shRNA	GGTAATCAGATGGGACAGTAT
Negative control	shRNA	GGAATCTCATTCGATGCATAC
SLC35C1	shRNA	CUACUUAUAGACCCAAUCAUU
Negative control	shRNA	UCUUAAUCGCGUAUAAGGCUU

Lectin blotting and Western blotting

Cells were harvested from culture dishes in PBS. After precipitation by centrifugation at 400 × *g* for 5 min at 4 ℃, cells were suspended in TNE buffer [10 mmol/L Tris-HCl (pH 7.8), 1% NP40, 0.15 mol/L NaCl, 1 mmol/L EDTA] containing a protease inhibitor cocktail (Roche, Basel, Switzerland), and then placed on ice for 30 min to allow for solubilization. Samples were centrifuged at 20000 × *g* for 15 min at 4 ℃, and supernatants were collected. Cell lysates were quantitated using a bicinchoninic acid protein assay kit (Thermo Fisher Scientific). Ten micrograms of total cellular protein were subjected to 10% SDS-polyacrylamide gel electrophoresis under reducing conditions, and then transferred to a nitrocellulose membrane (Millipore, Billerica, MA, United States). After blocking with PBS containing 3% bovine serum albumin overnight at 4 ℃, the membrane was incubated with biotinylated *Aleuria aurantia* lectin (AAL; J-Oil Mills, Tokyo, Japan) or *Aspergillus oryzae* lectin (AOL; unbiotinylated [Tokyo Chemical Industry, Tokyo, Japan], biotinylated with the Biotin Labeling Kit-NH₂, [DOJINDO Molecular Technologies, Kumamoto, Japan]). The membrane was washed with Tris-buffered saline containing 0.05% Tween 20 (pH 7.4) and incubated with diluted avidin-peroxidase conjugates (ABC kit, Vector Laboratories, Burlingame, CA, United States). Signals were detected using RX-U X-ray film (Fujifilm, Tokyo, Japan) with Immobilon Western Chemiluminescent HRP Substrate (Millipore) according to the manufacturer's protocol.

RNA extraction and quantitative real-time reverse transcription PCR

Total RNA was extracted from cells using the ReliaPrep RNA Cell Miniprep System (Promega Corp., Madison, WI, United States). The RNA concentration was determined spectrophotometrically, and samples were then stored at -80 ℃ until use. RNA samples (500 ng) were reverse-transcribed into complementary DNA (cDNA) using SuperScript Ⅲ reverse transcriptase with oligo(dT), dNTPs, and RNaseOUT (Invitrogen of Thermo Fisher Scientific). The cDNA was then diluted five-fold and specific PCR product amplification was performed with SYBR Premix Ex TaqⅡ (TAKARA Bio, Shiga, Japan). Primers were used at 625 nmol/L each in a 20-μL reaction volume. The cycle parameters were: denaturation at 95 ℃ for 2 min, and 40 cycles composed of 15-s denaturation at 95 ℃, 10-s annealing at 59 ℃, and 25-s polymerization at 72 ℃. Total RNA from each sample was analyzed in triplicate for each target RNA in separate wells. Quantitative real-time reverse transcription PCR (qRT-PCR) was performed on a Mx3000P Real-Time QPCR System (Agilent, Santa Clara, CA, United States). Primer sequences used in this study are provided in Table 1. Expression levels of the genes of interest were normalized to ribosomal protein L4 and calculated based on the $^{\Delta\Delta}$CT method[14]. The results are expressed relative to those of Panc1-P as control.

Sphere formation assay

Panc1 and PSN-1 cells were seeded on 10 cm culture dishes (AGC Techno Glass, Tokyo, Japan) and cultured in serum-free medium consisting of DMEM/F-12 medium (Invitrogen) supplemented with 20 ng/mL epidermal growth factor and 20 ng/mL basic fibroblast growth factor (Peprotech, Rocky Hill, NJ, United States), B27 (Invitrogen), 5 μg/mL insulin, and 2.75 μg/mL transferrin (Sigma-Aldrich). Sphere cells were passaged every 3 d. Sphere cells cultured for 6 d were collected and analyzed. Sphere cell-forming ability was calculated with the BZ Analyzer Ⅱ software equipped with fluorescence microscope (KEYENCE Corporation, Osaka, Japan). Briefly, 5×10^5 pancreatic cancer cells were seeded in 60 mm culture dishes and after 3 d culture, the area of the formed sphere colonies were estimated in each cell line.

Flow cytometry

Cells were harvested with PBS containing 0.5 mmol/L EDTA and fixed with Cytofix/Cytoperm solution (BD Biosciences, Franklin Lakes, NJ, United States) for 20 min on ice. The cells were incubated with fluorescein isothiocyanate-labeled AAL, *Pholiota squarrosa* lectin (PhoSL, J-Oil Mills), or *Ulex europaeus* agglutinin Ⅰ (UEA-l, J-Oil Mills) for lectin flow cytometry analyses. To investigate the expression of CSC markers, Panc1 cells were incubated with allophycocyanin-conjugated anti-human CD24 (Miltenyi

Biotec GmbH, Germany) and phycoerythrin-conjugated anti-human CD44 (BD Biosciences) in PBS containing 0.1% bovine serum albumin for 20 min on ice. Isotype-matched mouse IgG (BD Biosciences) was used as a control. Cells were washed three times with PBS, and flow cytometric analysis was performed using a FACSCalibur flow cytometer operated with CellQuestPro software version 5.2, (BD Biosciences). Ten thousand events were acquired in each sample. For FACS cell sorting, 5-10 × 10^6 living cells were stained with anti-human CD24 and CD44 antibodies, and then sorted using FACSAria Ⅱ (BD Biosciences). Doublet cells were eliminated using FSC-A/FSC-H and SSC-A/SSC-H. Dead cells were also excluded by gating staining with 7-amino-actinomycin D (BD Biosciences).

RNA interference
For *FUT1* knockdown, an expression vector carrying small hairpin RNA (shRNA) against human *FUT1* was purchased from Qiagen (Venlo, Limburg, Netherlands), and transfected into Panc1-RG cells with NEON Transfection System (Invitrogen). At 24 h, the medium was changed to complete medium with Hygromycin B (Invitrogen) at 500 μg/mL for selection. To knock down the GDP-fucose transporter gene, Panc1-RG cells were transfected with shRNA against *SLC35C1 via* retroviral introduction. Small interfering oligonucleotides specific for *SLC35C1* were designed using an online design tool (Takara Bio). The oligonucleotides were annealed and then ligated into the *Bam*HI/*Cla*I sites of the pSINsi-hU6 DNA vector (Takara Bio). Retroviral supernatant was obtained by transfection into human embryonic kidney 293T cells, using a Retrovirus Packaging Kit Ampho (Takara Bio) according to the manufacturer's instructions. Panc1-RG cells were infected with the viral supernatant, and the cells were then selected with 5 μg/mL puromycin for 2-3 wk to obtain stable transfectant cells.

Cell survival assay
Cell growth was assessed by the *WST*-8 assay (2-[2-methoxy-4-nitrophenyl]-3-[4-nitrophenyl]-5-[2,4-disulfophenyl]-2H-tetrazolium, monosodium salt; Nacalai Tesque, Kyoto, Japan) following the manufacturer's protocol. Briefly, 10 μL of WST-8 reagent was added to the cells in each well and incubated at 37 ℃ for 3 h. After incubation, sample absorbance was determined using an iMark Microplate Reader (Bio-Rad, Tokyo, Japan) at 450 nm.

Enzyme-linked immunosorbent assay for interleukin-6
Panc1 cells [Panc1-P, Panc1-RG, monolayer cells (same as Panc1-P), and sphere cells] were seeded at a density of 2.4 × 10^5 cells/60 mm culture dish, and cultivated in RPMI-1640 supplemented with 10% FBS, 100 units/mL penicillin, and 100 μg/mL streptomycin or in sphere conditioned medium (described in sphere formation assay). After 48 h, the supernatants were

collected and interleukin (IL)-6 levels were quantified using Enzyme-linked immunosorbent assay system (SRL Inc., Tokyo, Japan). The IL-6 concentrations were normalized to cell number at the time of collection.

Statistical analysis
Statistical analysis was conducted using JMP Pro 10.0 software (SAS Institute Inc., Cary, NC). The results are expressed as mean ± SD. Groups of data were compared by the Wilcoxon test for non-parametric data. Differences were considered statistically significant for *P* < 0.05.

RESULTS

Gemcitabine-resistant Panc1-RG cells are enriched with CSC-like cells
By exposing Panc1 cells stepwise to increasing gemcitabine concentrations over 5 mo, we established gemcitabine-resistant Panc1 cells, (Panc1-RG). Panc1-RG cells were 37.5-fold more resistant to gemcitabine treatment compared with Panc1-P cells [Figure 1A; 25% inhibitory concentration (IC$_{25}$) values: 80 and 3000 ng/mL for Panc1-P and Panc1-RG, respectively]. Next, we performed flow cytometry analysis to investigate whether CSC markers for pancreatic cancer are increased on the Panc1-RG cell surface. In general, CD24 and CD44 are often used as cell surface markers of pancreatic CSC fractions. As expected, Panc1-RG cells exhibited an increased population of CD24$^+$/CD44$^+$ cells (98.30%) compared with Panc1-P cells (56.40%; Figure 1B). These results indicate that CSC-like cells are enriched in Panc1-RG cells following long-term gemcitabine treatment.

Identification of characteristic glycan structure in Panc1-RG cells
To determine glycan structures unique to Panc1-RG cells, lectin microarray analysis was performed (Figure 2A). Panc1-P and Panc1-RG cells exhibited several differences in lectin binding; in particular, three fucosylated glycan-recognizing lectins (UEA-I, AAL, AOL) showed substantially higher intensities in Panc1-RG than in Panc1-P cells. UEA-I specifically recognizes α1,2-fucosylation, AAL recognizes all types of fucosylation such as α1,2, α1,3/α1,4 and α1,6 linkages, and AOL recognizes α1,2- and α1,6-fucosylation[15]. Lectin blot analyses using AAL or AOL also revealed increased cellular fucosylation in Panc1-RG cells (Figure 2B). Furthermore, flow cytometric analysis showed increased cell surface expression of fucosylated proteins recognized by UEA-I and AAL lectins, but not PhoSL lectin, which recognizes α1,6-fucosylated glycans[16]. These results indicate an increase in α1,2- and α1,3-/α1,4-fucosylation, but not in α1,6-fucosylation, in Panc1-RG cells (Figure 2C).

To investigate the role of various regulatory
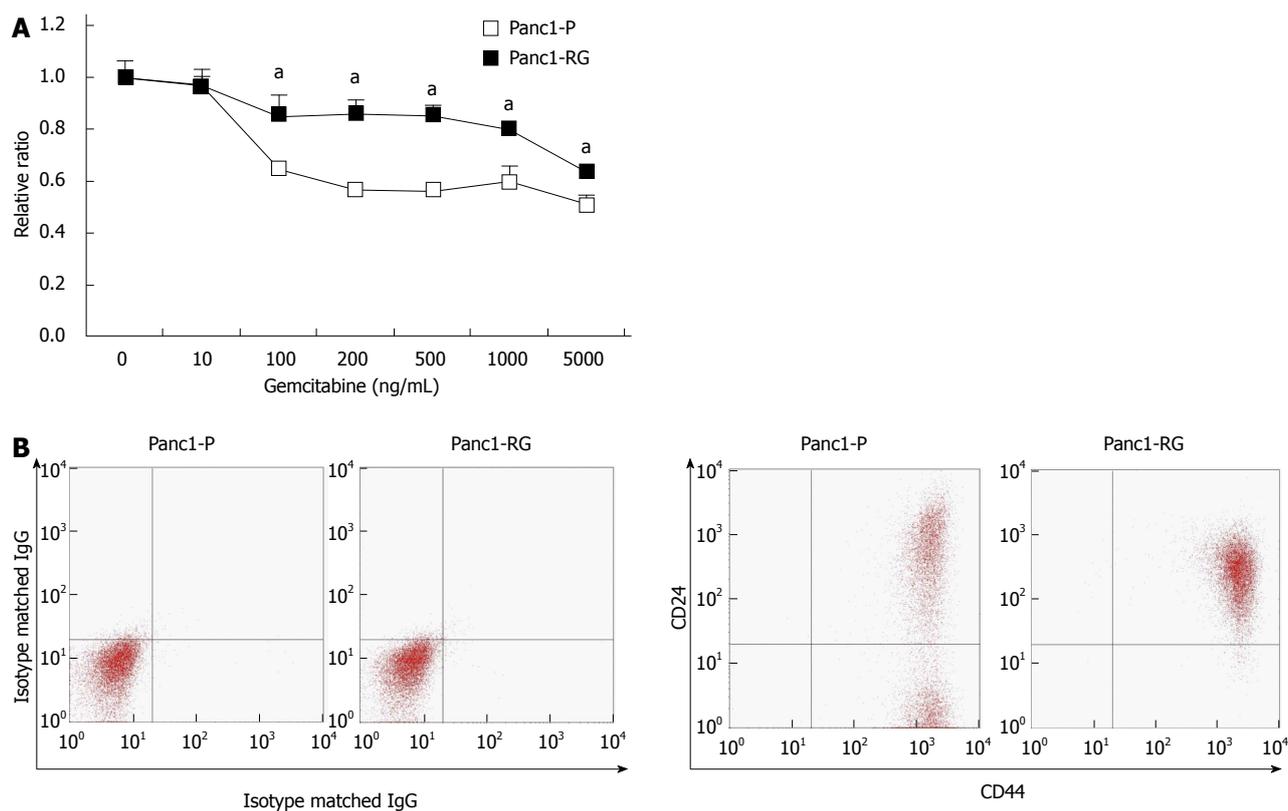
Figure 1 Cancer stem cells are enriched in established gemcitabine-resistant cells. A: Panc1-RG cells were established by long-term step-wise gemcitabine treatment. WST assay showed that Panc1-RG cells exhibited gemcitabine resistance compared with Panc1-P cells; B: Flow cytometric analysis showing a higher ratio of CD24^{+}/CD44^{+} cells in Panc1-RG cells compared with Panc1-P cells. All results are expressed as mean ± SD; [a]$P < 0.05$ *vs* Panc1-P cells.
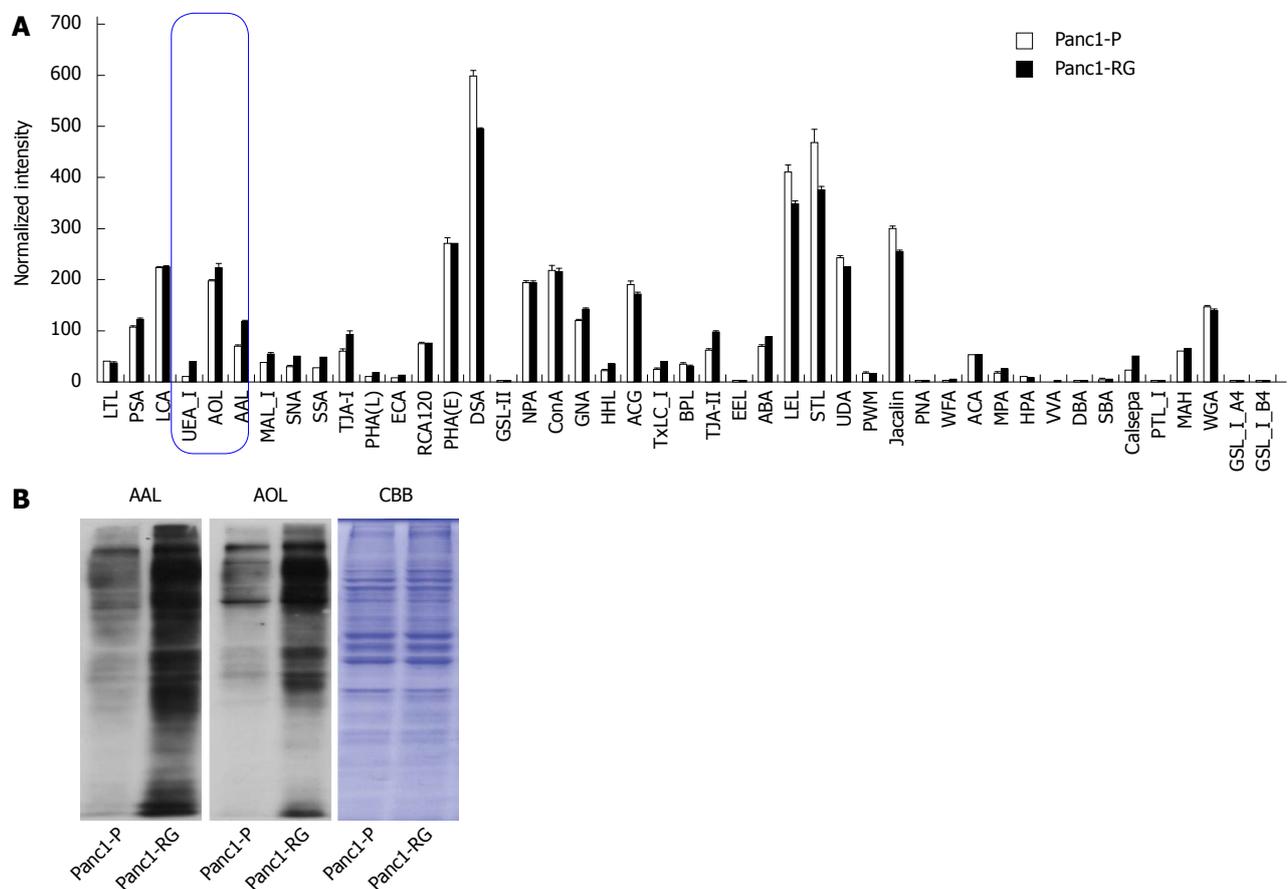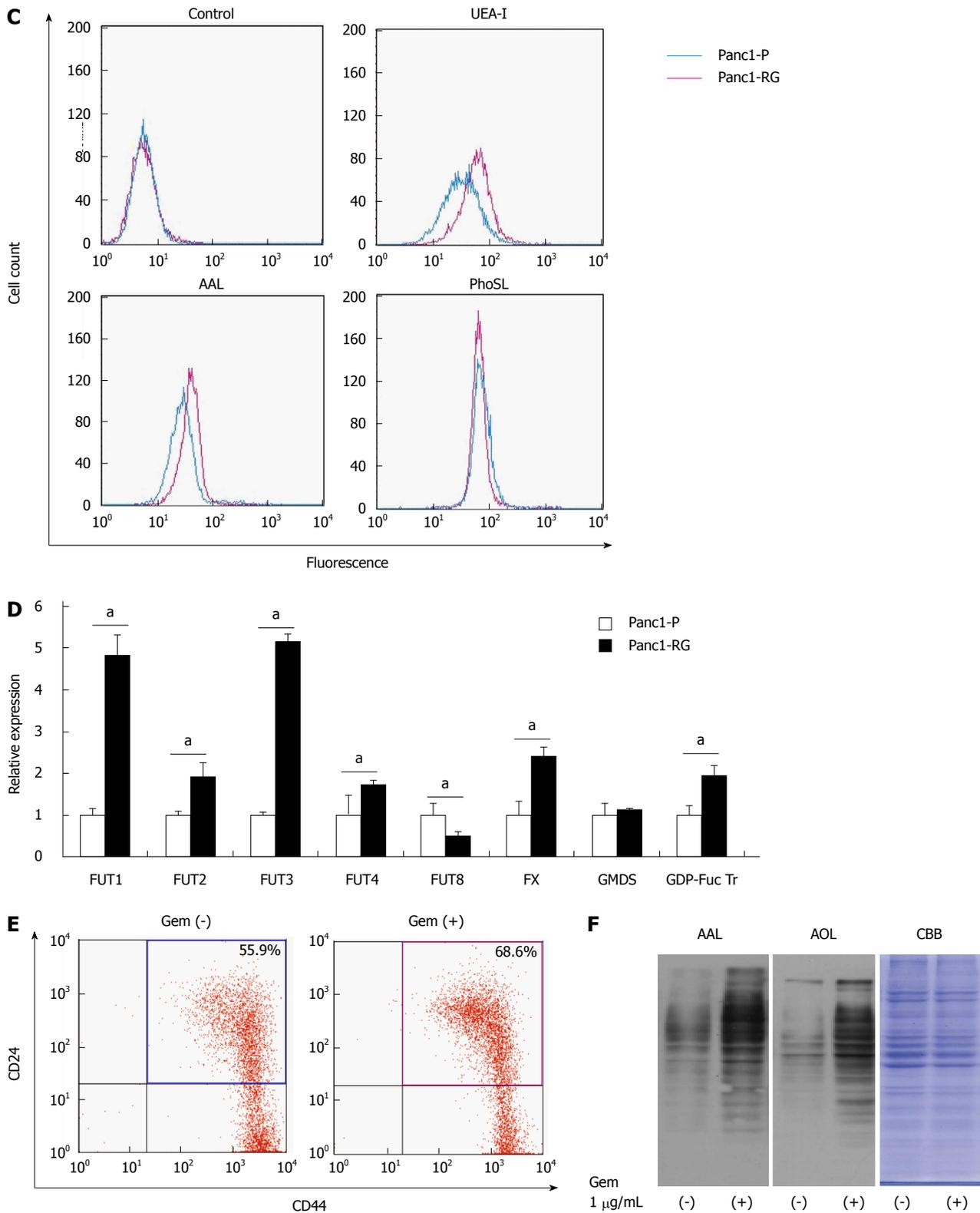
Figure 2 Fucosylation is enhanced in gemcitabine-treated pancreatic cancer cells. A: Differential glycan profiles of Panc1-P and Panc1-RG cells were analyzed by lectin microarray. The fluorescence intensity of each lectin was normalized to the intensity of wheat germ agglutinin; B: *Aleuria aurantia* lectin (AAL) or *Aspergillus oryzae* lectin (AOL) lectin blot of Panc1-P and Panc1-RG. Coomassie brilliant blue (CBB) staining confirms equal protein loading among lanes; C: Cell-surface fucosylated glycoproteins in Panc1-P and Panc1-RG cells were assessed by flow cytometry using *Ulex europaeus* agglutinin (UEA)-I, AAL, and *Pholiota squarrosa* lectin (PhoSL) lectins; D: Fucosylation-related gene expression, determined by real-time reverse transcription PCR in Panc1-P and Panc1-RG cells, and expressed relative to that of Panc1-P cells; E: Flow cytometric analysis represented gemcitabine treatment increased the ratio of CD24^{+}/CD44^{+} cells in PK59; F: AAL or AOL lectin blot of PK59 cells with (+) or without (-) gemcitabine treatment. All results are expressed as mean ± SD; [a]$P < 0.05$ *vs* Panc1-P cells.

factors in the increased fucosylation, we compared the mRNA expression levels of GDP-mannose-4,6-dehydratase, FX, GDP-fucose transporter (GDP-Fuc Tr), fucosyltransferase (FUT) 1, FUT2, FUT3, FUT4, and FUT8 between Panc1-P and Panc1-RG cells by real-time RT-PCR analysis (Figure 2D). FUT1, FUT2, FUT3, FUT4, FX, and GDP-Fuc Tr expression levels in Panc1-RG cells were significantly higher than those in Panc1-P cells (Ps < 0.05). These results corroborate the results of lectin blot analysis and lectin flow cytometry, as FUT1 and FUT3 catalyze the formation of $\alpha 1,2$ and $\alpha 1,3/\alpha 1,4$ linkages, respectively. Moreover, FX and GDP-Fuc Tr increase the amount of GDP-Fuc substrate in the Golgi apparatus.

We established Panc1-RG cells for long-term gemcitabine treatment and this cell line contained more CSC like cells than Panc1-P. However, Hermann *et al*[17] showed short-term gemcitabine treatment also works in CSC-like cells concentration. Therefore, we analyzed the fucosylation status in other cell lines such as PK59, MIA PaCa-2, PSN-1, Capan-1, and BxPC-3, which were treated with gemcitabine for a short time. Expectedly, we found that short-term gemcitabine treatment also caused concentration of CSC-like cells and enhanced fucosylation in mutliple cell lines. Representative results in PK59 cells are shown in Figure 2E and F.

Sphere-forming cells and CD24^high^/CD44^high^ cancer stem-like cells from Panc1-P showed high expression of fucosylated glycans

Because numerous studies demonstrated that the formation of spheres in serum-free medium is one of the characteristics of CSCs[17,18], Panc1-P cells were cultured under stem cell-selective conditions. After 6 d, Panc1 cells aggregated into floating spheroid clusters (Figure 3A). To examine CD24 and CD44 expression in sphere and monolayer cells, we performed flow cytometry analysis. A larger CD24+/CD44+ population was observed in sphere cells (97.13%) compared with monolayer cells (54.93%). Subsequently, we performed western blot analysis using AAL or AOL lectins to examine whether fucosylated glycans were increased in sphere cells. Increased binding to both lectins was observed in sphere cells as compared with monolayer cells (Figure 3C). Furthermore, the expression pattern of fucosylation regulatory genes in sphere cells was similar to that seen in Panc1-RG cells (data not shown). It is already known that CSC markers for pancreatic cancer, such as CD24 and CD44, regulate cellular behavior[19,20]. To evaluate the relationship between the extent of fucosylation and CD24 and CD44 expression, we further studied cellular fucosylation in the CD24^high^/CD44^high^ fraction, and compared this with the CD24^low^/CD44^low^ fraction. The region of each fraction is indicated in the Figure 3B "monolayer". The CD24^high^/CD44^high^ fraction exhibited higher cellular fucosylation than the CD24^low^/CD44^low^

fraction (Figure 3D). These results indicate increased fucosylation in cancer stem-like phenotype cells.

Panc1-RG cells contained more CSC like cells than parental Panc1-P cells (Figure 1B). This observation suggests that Panc1-RG forms more sphere colonies than Panc1-P. To clarify this possibility in Panc1-RG, we performed sphere-forming assay using Panc1-P and Panc1-RG cells. We found sphere formation was significantly increased in Panc1-RG compared with in Panc1-P (P < 0.01) (Figure 3E).

Next, we investigated whether or not sphere formation enhances cellular fucosylation in other pancreatic cancer cell lines (PK59, MIA PaCa-2, PSN-1, Capan-1, and BxPC-3). Among these cell lines, sphere formation was observed only in PSN-1 cells, and we confirmed fucosylation was increased in sphere formation of PSN-1 cells (Figure 3F).

Fucosylation does not directly contribute to gemcitabine resistance

Previously, Cordel *et al*[21] reported that FUT1 is associated with 5-FU resistance in rat colon cancer cells. To examine whether or not $\alpha 1,2$-fucosylation by FUT1 is involved in gemcitabine resistance in Panc1 cells, shRNA against FUT1 was transfected into Panc1-RG cells and stable transfectants were selected with hygromycin B. The shFUT1 transfectants exhibited an approximately 30% decrease in FUT1 mRNA expression compared with the shRNA-negative control (Figure 4A). $\alpha 1,2$-fucosylation levels were lower in shFUT1-transfected Panc1-RG cells, as evidenced by AOL lectin blot as well as UEA-I flow cytometry (Figure 4B and C). However, there was no change in gemcitabine resistance among these cells (Figure 4D). Next, to investigate whether total cellular fucosylation is directly involved in gemcitabine resistance, GDP-Fuc Tr, a key regulator of cellular fucosylation, was knocked down in Panc1-RG cells[22]. The shRNA transfectants showed significantly decreased GDP-Fuc Tr mRNA expression compared to negative control (P < 0.05) (Figure 5A). Cellular fucosylation also decreased dramatically in shRNA transfectant cells, as evidenced by lectin blot analyses using AAL or AOL lectins (Figure 5B). However, decreased fucosylation had no effect on gemcitabine sensitivity (Figure 5C). In other words, cellular fucosylation does not directly contribute to gemcitabine resistance in Panc1 cells; increased fucosylation is merely a marker for CSC-like cells in pancreatic cancer.

Panc1-RG cells secreted higher levels of IL-6, compared to Panc1-P cells

In this study, we found that fucosylation was increased under several CSC-like cell transformation conditions. Some bioactive substances are related to the increased fucosylation in CSC-like cells. Previously, we reported that IL-6 treatment of human hepatoma cell lines increased fucosylation with a marked increase in fucosylation regulatory genes[23]. Therefore, we also
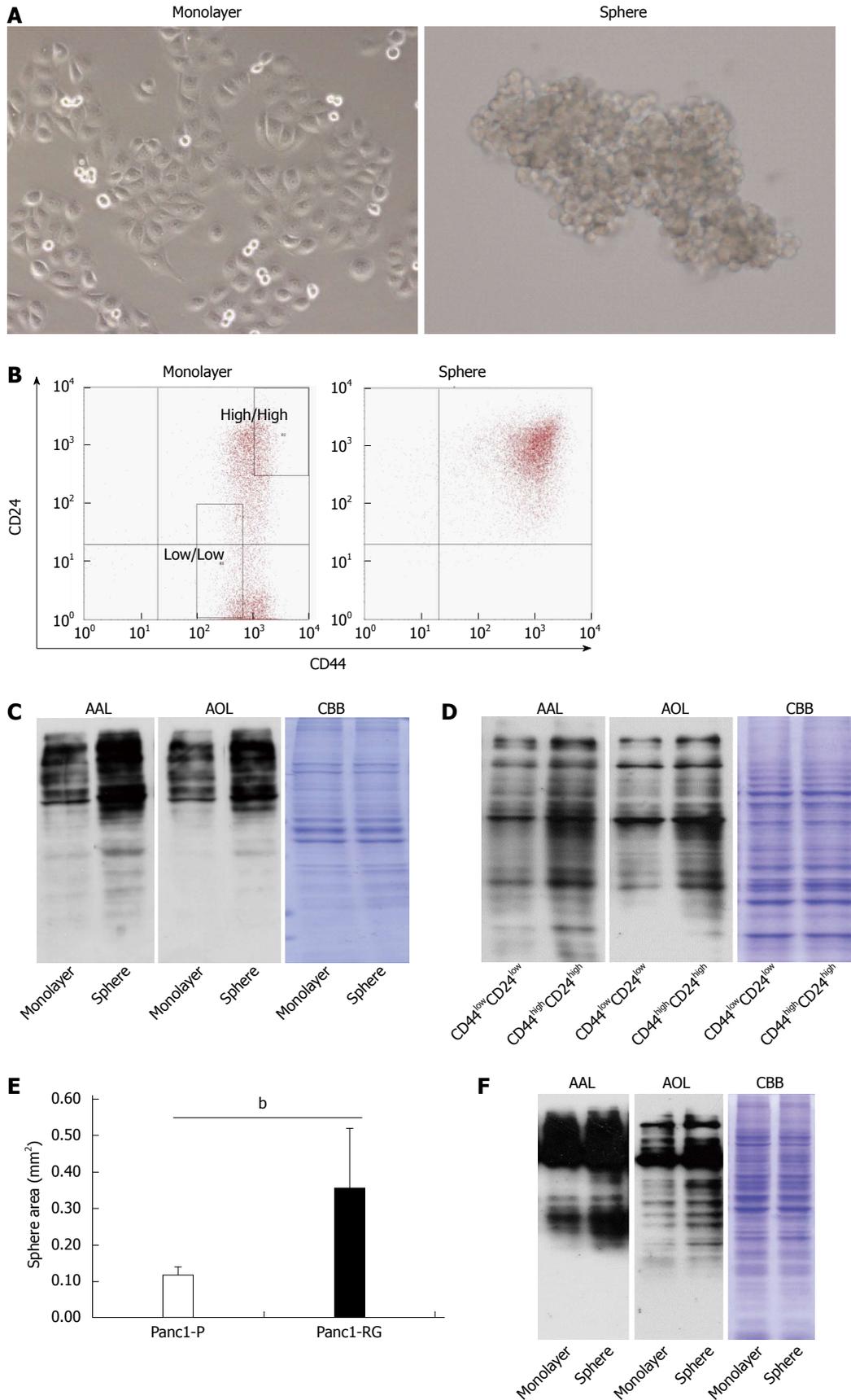
Figure 3 Fucosylation is enhanced in cancer stem cell-like cells fractions of pancreatic cancer cells. A: Phase-contrast images of monolayer (left panel) and sphere cells (right panel) derived from Panc1 cells; B: Results of flow cytometric analysis in monolayer and sphere Panc1 cells using CD24 and CD44 antibodies. CD24$^+$/CD44$^+$ cells were increased in sphere cells compared with monolayer cells; C: *Aleuria aurantia* lectin (AAL) or *Aspergillus oryzae* lectin (AOL) lectin blot of monolayer and sphere Panc1 cells; D: AAL or AOL lectin blot of CD24^{high}/CD44^{high} or CD24^{low}/CD44^{low} Panc1 cells obtained by cell sorting. Each region is indicated as a square field in (B); E: Comparison of sphere cell-forming ability between Panc1-P and Panc1-RG; F: AAL or AOL lectin blot of monolayer and PSN-1 cells. Results are expressed as mean ± SD; $^b P < 0.01$ *vs* Panc1-P cells. CBB: Coomassie brilliant blue.
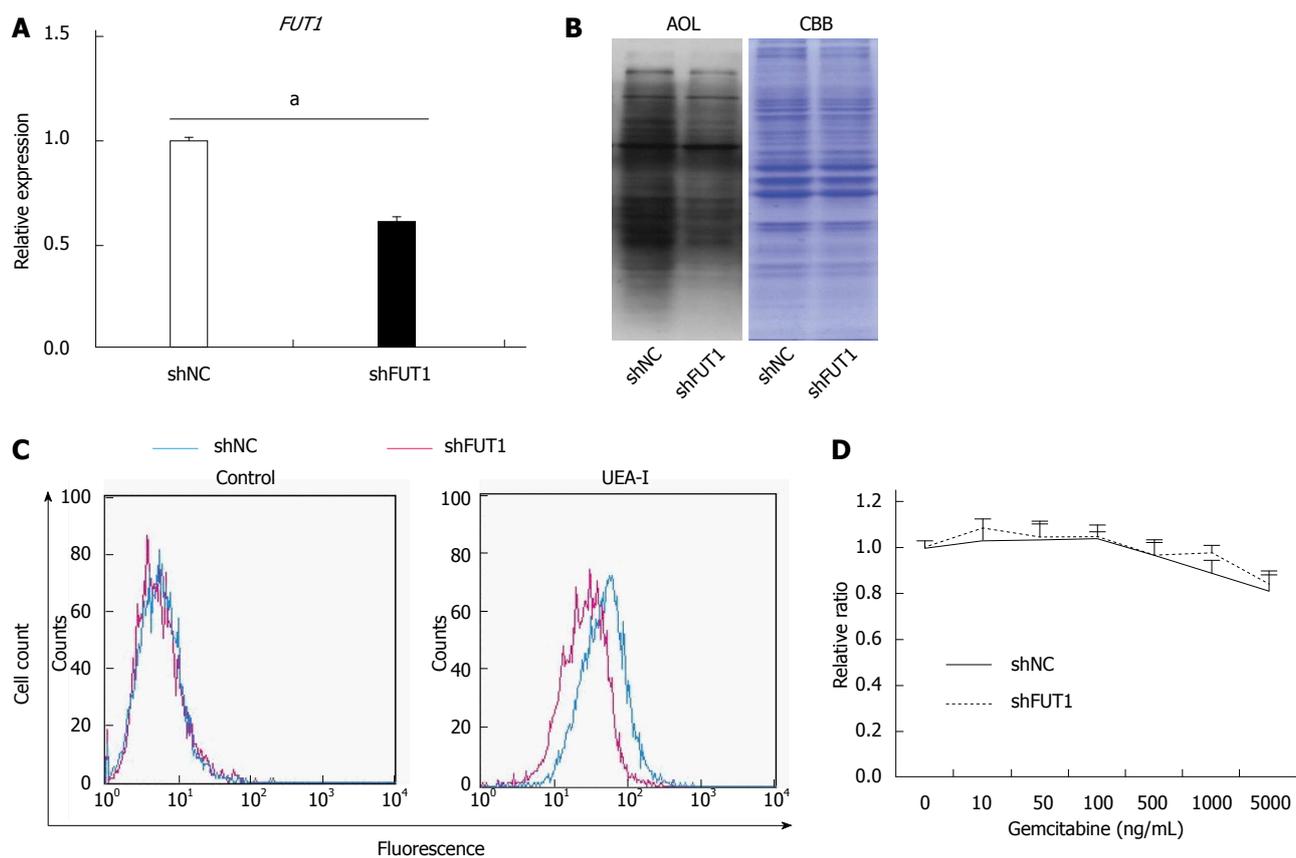
Figure 4 *FUT1* knockdown in Panc1-RG cells does not alter gemcitabine resistance. A: Expression of fucosyltransferase (FUT)1 mRNA in Panc1-RG cells stably expressing small hairpin (sh)RNA; B: *Aspergillus oryzae* lectin (AOL) blot of shNC- or shFUT1-expressing Panc1-RG cells; C: Flow cytometric analysis using *Ulex europaeus* agglutinin (UEA)-I; D: WST assay results of transfectant cells treated with various gemcitabine concentrations. Results are expressed as mean ± SD; [a]$P < 0.05$ *vs* shNC. CBB: Coomassie brilliant blue; NC: Negative control.
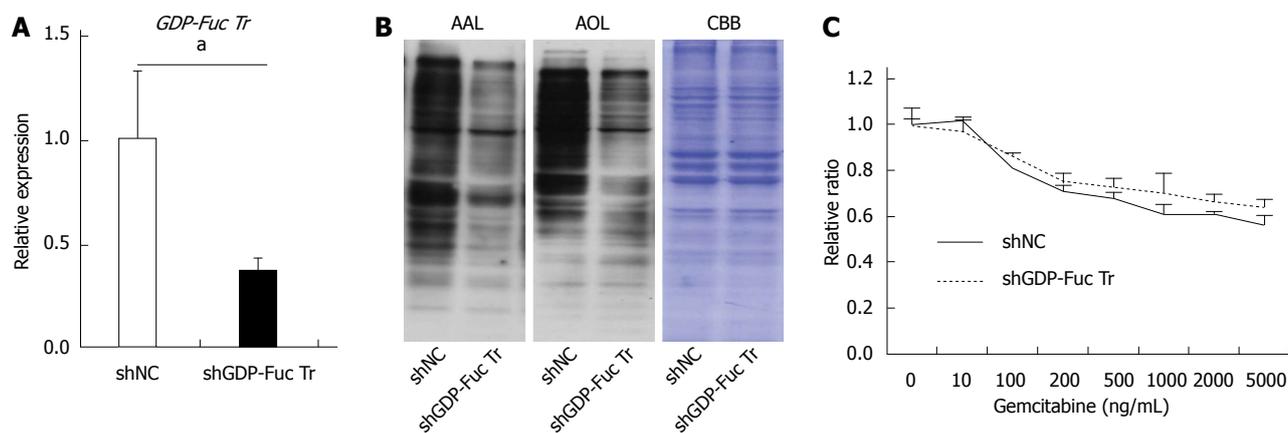
Figure 5 GDP-fucose transporter gene knockdown does not affect gemcitabine resistance in Panc1-RG cells. A: Expression of GDP-fucose transporter (GDP-Fuc Tr) mRNA in small hairpin (sh)RNA-expressing Panc1-RG cells; B: *Aleuria aurantia* lectin (AAL) or *Aspergillus oryzae* lectin (AOL) blots for shRNA transfectant cells; C: WST assay results for cells treated with various gemcitabine concentrations. Results are expressed as mean ± SD; [a]$P < 0.05$ *vs* shNC. CBB: Coomassie brilliant blue; NC: Negative control.

investigated the IL-6 production between Panc1-P and Panc1-RG cells in this study. Expression of IL-6 mRNA was significantly higher in Panc1-RG than that in Panc1-P ($P < 0.05$) (Figure 6A). The concentration of IL-6 in conditioned media from Panc1-RG cultures was also substantially higher than that from Panc1-P

(Figure 6B). In addition, the concentration of IL-6 in conditioned media from sphere Panc1-P cells was also higher than those from monolayer Panc1-P cells (Figure 6C). These results indicate that both anti-cancer drug resistance and sphere formation enhanced IL-6 production in Panc1-P cells.
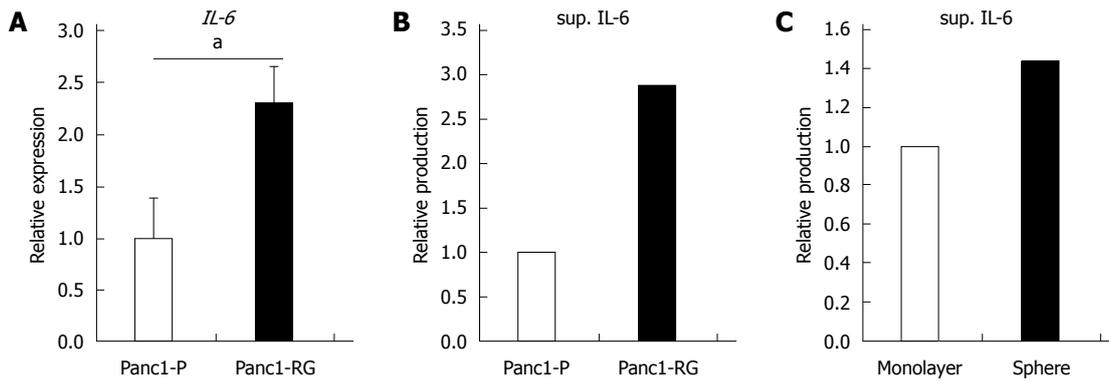
Figure 6 Increased interleukin-6 production in anti-cancer drug-resistance and sphere-forming cells. A: Expression of interleukin (IL)-6 mRNA was measured by real-time reverse transcription PCR in Panc1-P and Panc1-RG; B: The concentration of IL-6 in conditioned media from Panc1-P or Panc1-RG cultures was assessed by enzyme-linked immunosorbent assay (ELISA); C: The concentration of IL-6 in conditioned media from monolayer or sphere-forming Panc1-P cells was measured by ELISA. All results are expressed as mean ± SD; [a]$P < 0.05$ *vs* Panc1-P cells.
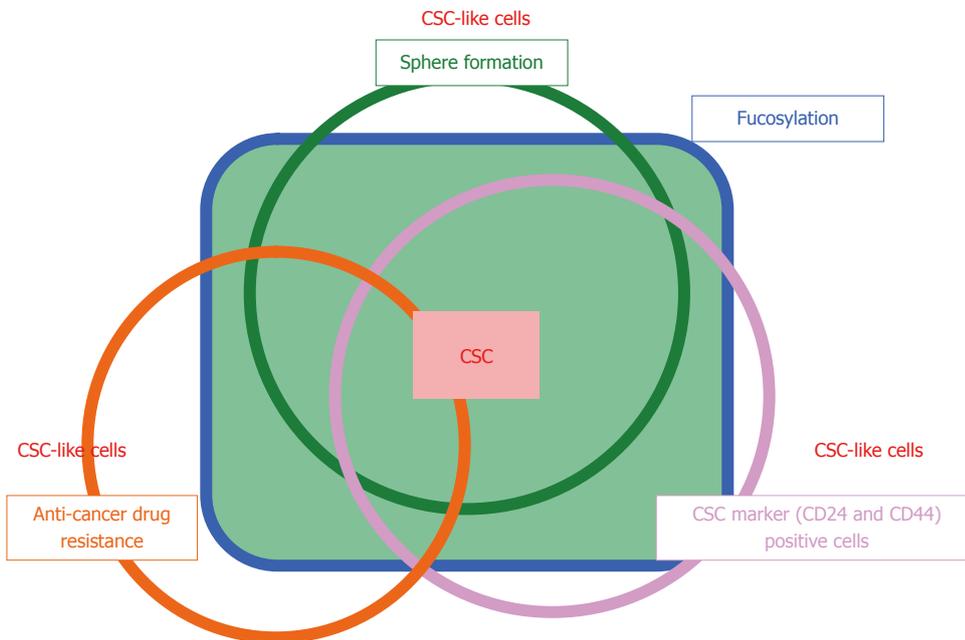
Figure 7 Overview of the relationship between fucosylation and three types of cancer stem cell-like cells. Cancer stem cells (CSCs) are a limited fraction of cancer cells, which show asymmetric and slow growth, anti-cancer drug resistance, and sphere formation. CSC-like cells are differentiated from and have similar biologic characteristics of CSCs. Fucosylation is common type of glycosylation in the CSC-like phenotype of pancreatic cancer under various conditions.

DISCUSSION

In this study, we found that fucosylation is a common glycosylation marker for three cancer stem cell-like phenotypes: gemcitabine-resistant cells, sphere-forming cells, and CD24^{high}/CD44^{high} CSC fractions. Specifically, gemcitabine-resistant cells and sphere-forming cells exhibited very similar fucosylation patterns. In our present study, the fucosylation patterns varied, likely dependent on the differing conditions of cells in culture. The protein expression patterns were also different in each condition. Although α1-2- and α1-3/1-4-fucosylation increased, α1-6-fucosylation did not change upon downregulation of *FUT8* expression and upregulation of FX and GDP-Fuc Tr genes. These results suggest that CSC-like cells, similar to sphere-forming

cells, were condensed during long-term exposure to gemcitabine. Induced pluripotent cells and embryonic stem cells have been shown to exhibit increased α1-2-fucosylation and decreased α1-6-fucosylation[24]. Although α1-6-fucosylation was unchanged in Panc1-RG cells compared with Panc1-P cells, *FUT8* knockdown in Panc1-RG cells decreased α1-6-fucosylation, accompanied by a slight enhancement in gemcitabine resistance (data not shown).

Next, we investigated whether other types of fucosylation are directly involved in gemcitabine resistance. Downregulation of α1-2-fucosylation in Panc1-RG cells using shFUT1 RNA did not alter gemcitabine resistance. This result is inconsistent with the observation that FUT1 is involved in 5-FU resistance in rat colon cancer cell lines[21]. This might be due to

differences between cancer cell lines and/or anti-cancer agents. Our insufficient FUT1 knockdown efficiency, in spite of repeated attempts, might have caused the discrepancy in gemcitabine resistance. Therefore, we focused on GDP-Fuc Tr, the most important regulatory factor for all types of fucosylation[22].

GDP-Fuc Tr was reduced to approximately 1/4 of its original expression level; this resulted in a dramatic decrease in total cellular fucosylation, but no change in gemcitabine resistance. This indicates that fucosylation does not directly contribute to gemcitabine resistance, and might be merely a marker for pancreatic cancer CSC-like cells. The molecular mechanisms underlying increased fucosylation in Panc1-RG cells remain unknown. Recently, Lauc *et al*[25] reported that hepatocyte nuclear factor (HNF)1α and its downstream molecule HNF4α are master transcription factors controlling cellular fucosylation. High HNF4α mRNA expression was observed in Panc1-RG cells compared with Panc1-P cells (data not shown). IL-6 production in pancreatic cancer has been associated with cancer aggressiveness and poor prognosis in patients with pancreatic cancer[26,27]. We previously reported that treatment of human hepatoma cell lines with IL-6 caused a marked increase in fucosylation regulatory genes[23]. Interestingly, secreted IL-6 levels as well as IL-6 mRNA levels were significantly higher in Panc1-RG cells than in Panc1-P cells. These results strongly suggest that long-term stimulation with high-dose IL-6 induces increasing fucosylation in Panc1-RG cells. Yamada *et al*[28] showed that IL-6 is involved in resistance to anticancer drugs. These findings suggest that IL-6 plays pivotal and independent roles in increased cellular fucosylation and gemcitabine resistance. Taken together, increased cellular fucosylation is a common glycan change in three CSC-like phenotypes in pancreatic cancer (Figure 7), and the identification of fucosylated glycoproteins derived from pancreatic cancer cells could lead to novel biomarker development for anticancer drug resistance.

COMMENTS

Background
Fucosylation is one of the most important glycosylation events involved in cancer and inflammation.

Innovations and breakthroughs
In the present study, authors investigated oligosaccharide modifications in pancreatic cancer cancer stem cell (CSC)-like cells. Using several models of CSC-like cells, they found that fucosylation is a common type of glycosylation change in pancreatic cancer CSC-like cells.

Applications
CSCs are known to be preferentially resistant to many current therapies, including various chemotherapeutic agents and radiation treatment. The present study suggests that the identification of fucosylated glycoproteins derived from pancreatic cancer cells could lead to novel biomarker development for anticancer drug resistance.

Peer-review
The manuscript reported an interesting finding that fucosylation is a common oligosaccharide modification in pancreatic cancer CSC-like cells, and increased cellular fucosylation is correlated with drug (such as gemcitabine) resistance.

Therefore, the identification of fucosylated glycoproteins derived from pancreatic cancer cells could lead to novel biomarker development for anticancer drug resistance. Overall, the experiments were well done and properly interpreted.

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ORIGINAL ARTICLE

Basic Study

Esophageal variceal pressure influence on the effect of ligation

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Abstract

AIM: To explore the effect of *in vitro* porcine esophageal variceal pressure on complete ligation degree for polycyclic ligators.

METHODS: An *in vitro* model of experimental porcine venous vessels was used to test various venous pressures. Three treatment groups were designated according to the preset pressure range: P_1 = 25-30 cmH_2O; P_2 = 35-40 cmH_2O; P_3 = 45-50 cmH_2O. The effect of pressure on ligation was assessed and compared among the groups.

RESULTS: Complete ligation was achieved at a rate of 56.25% (18/32) in group P_1, 37.5% (12/32) in group P_2, and 33.33% (11/33) in group P_3 (χ^2 = 3.6126; P = 0.0573).

CONCLUSION: Higher variceal pressures impair the ligation completion rate. Therefore, measuring variceal pressure may help predict the effect of endoscopic ligation and guide treatment choice.

Key words: Endoscopic variceal ligation; Mold of animal; Variceal pressure

Core tip: This study aims to explore the factors influencing rebleeding after endoscopic variceal ligation, and provide the theoretic basis for prevention and reduction of rebleeding after the procedure.

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INTRODUCTION

An increase in esophageal variceal pressure is a key factor leading to vascular rupture bleeding. Therefore, the measurement of esophageal variceal pressure is of great significance to evaluate and predict the risk of hemorrhage. Our previous *in vitro* preliminary work utilizing experimental ligation has shown that the ligation degree significantly differs among groups with various diameters of esophageal varices[1,2], though these experiments lacked evaluation of vascular pressure effects on the polycyclic ligator. Therefore, the aim of the present follow-up study was to explore the factors influencing rebleeding after endoscopic variceal ligation (EVL), in order to provide a theoretic basis for prevention and reduction of post-procedure rebleeding.

MATERIALS AND METHODS

Instrumentation and equipment

General surgical instruments were used in the procedures, including multi-band-ligators (Boston Scientific Corporation, Marlborough, MA, United States), glass column burettes, three-way stopcocks, and sodium chloride methylene blue solution. An Olympus GIF-Q260 gastroscope (Olympus Corporation, Tokyo, Japan) with a main engine and aspirator was also used.

Constructing venous pressure model from in vitro porcine vein vessels

The piglets were sacrificed, and exploratory laparotomy was performed to select the inferior vena cava, portal vein, and superior mesenteric vein as previously described[2]. Three pressure groups were included in the *in vitro* study: group P_1: 25-30 cm H_2O; group P_2: 35-40 cm H_2O; and group P_3: 45-50 cmH_2O. The "0" point of the liquid level in the glass burette was calibrated before each reading (Figure 1).

Making the in vitro porcine esophageal varices model

The piglet was sacrificed, the chest cavity was opened, and a section the esophagus (40 cm long) was removed and divided into three segments. The esophageal inner membrane was inverted, and a blunt dissection of the submucosal soft tissue was performed with hemostatic forceps, forming the porcine esophageal submucosal tunnel (Figure 2). Hemostatic forceps were used to pull one end of a porcine vein through the esophageal submucosal tunnel, creating the model of esophageal varices with

Figure 1 Measuring venous pressure.

Figure 2 Established submucosal tunnel.

different pressures.

In vitro ligation model of pig esophageal varices and judgment of the effect

A Speedband Super 7 multiple band ligator was mounted on an Olympus upper endoscope aimed at a varicose vein with continuous negative pressure suction (0.03-0.05 MPa)[3]. When the endoscopic view was completely blue, the bands were released at the handle, the negative pressure suction was stopped, and a rubber band was firmly ligated on the lesion base[4]. After cutting off the esophageal mucosa after band ligation, the submucosal ligated varix was stripped, and the effect of endoscopic variceal ligation was observed: complete ligation (100%) the ligation effect is reliable and complete, and bands do not easily fall off; partial or incomplete ligation (50%) indicates that the ligation effect is not reliable, and bands can fall off early; no ligation (0%) indicates that the ligation failed, and esophageal varices were not ligated (Figure 3).

Statistical analysis

SPSS 13.0 software (SPSS Inc., Chicago, IL, United States) was used for data management and statistical analysis. Using the linear trend χ^2 test, $P < 0.05$ was considered as statistically significant.
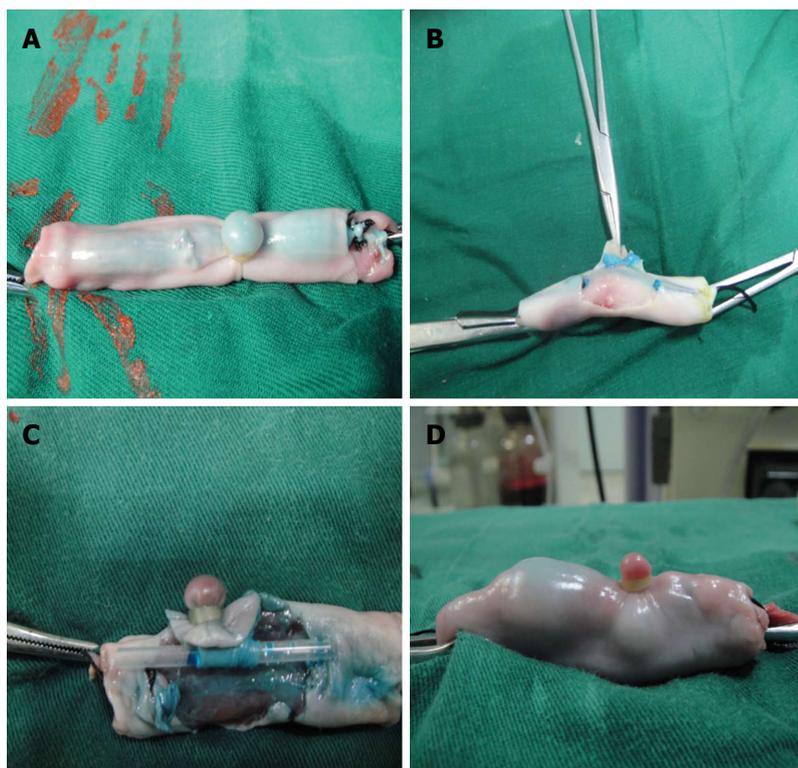
Figure 3 *In vitro* ligation model of pig esophageal varices and judgment of the effect. A: Ligation model; B: Complete ligation (100%); C: Partial ligation (≤ 50%); D: No ligation (0%).

RESULTS

A total of 97 *in vitro* models of esophageal varices were included in the study. For group P_1, complete ligation was achieved 56.25% (18/32), whereas 6.25% (2/32) showed incomplete ligation and 37.50% (12/32) showed no ligation. In group P_2, 37.50% (12/32) showed complete ligation, 12.50% (4/32) showed incomplete ligation, and 50.00% (16/32) showed no ligation. In group P_3, complete ligation was achieved in 33.33% (11/33), incomplete ligation was observed in 3.03% (1/33), and 63.64% (21/33) showed no ligation. Univariate analysis revealed that variceal pressure is an interference factor in predicting the degree of ligation (χ^2 = 3.6126, P = 0.0573).

DISCUSSION

Interest in esophageal variceal manometry has been increasing in recent years[5]. This study indicates that variceal pressure, rather than hepatic venous pressure gradient, may more directly predict the risk of bleeding, as measuring esophageal variceal pressure is particularly important for determining the effect of bleeding prevention and treatment[6-9]. Nevens *et al*[10] reported that variceal pressure is the most important predicting index of bleeding.

Normal portal vein pressure is 13-24 cmH$_2$O (1.27-2.35 kPa), when portal hypertension occurs, the pressure can increase to 30-50 cmH$_2$O (2.94-4.90 kPa). When the pressure exceeds 25 cmH$_2$O (2.45 kPa), gastroesophageal varices are prone to rupture and hemorrhage. Currently, endoscopic sclerotherapy and ligation are the primary methods for treating variceal bleeding, which can be effective for emergency hemostasis and occlusion of varicose veins, though some patients still exhibit recurrent bleeding. Portal pressure and hepatic venous pressure gradient do not correlate well and do not accurately predict variceal bleeding[11,12]. Whether intravariceal pressure influences the effect of variceal ligation and correlates with the other suggested endoscopic predictors is not clearly known.

The purpose of the current study was to explore the hemostatic effect after EVL in an effort to reduce the incidence of rebleeding and improve the outcome from esophageal varices rupture or hemorrhage. Rebleeding after EVL may occur because the variceal surface of band ligation is not strong, which can easily lead to band dropping off early. Strict control of EVL indications and contraindications will help to reduce the incidence of postoperative bleeding. Moreover, gastroesophageal varices are significantly related with portal pressure; the higher the portal pressure, the more serious the esophageal gastric varices[13], and increased portal pressure is a necessary prerequisite for esophageal variceal bleeding. A highly significant positive correlation was seen between variceal pressure and bleeding, indicating that patients with higher pressures bled more often. Intravariceal pressure was the most important variable in predicting variceal bleeding[14,15].

The experiments in the current study demonstrate that venous pressure parameter variation in the model is similar to that in humans[16]. We found that when the variceal pressure is within range of 25-30 cmH2O, the rate of complete ligation is the highest, whereas the efficacy is reduced with increasing pressures. Although the results did not achieve statistical significance, the marginal *P* value (*P* = 0.0573) indicates that venous pressure might be an interference factor in predicting the degree of complete ligation. Additional studies with larger sample sizes are needed to further confirm these findings. Therefore, the search for the mechanisms of variceal rupture and the factors influencing variceal bleeding continues[17-29]. In other words, there are other variables influencing variceal bleeding, such as the thickness of the variceal wall; the wall tension increases disproportionate to the rise in pressure in blood vessels[30]. This is because a rise in the pressure causes an increase in the radius and a decrease in the wall thickness[31]. Thus, measuring variceal pressure is expected to be helpful in correctly predicting the effect of variceal ligation; however, investigation of other variables influencing the effect of EVL should continue.

COMMENTS

Background

Endoscopic variceal ligation is the primary method for the treatment of esophageal variceal bleeding. However, the effect of ligation on hemostasis in acute bleeding from esophageal varices was not been thoroughly investigated. The main problem for ligation in acute esophageal variceal bleeding is the accumulation of large amounts of blood in the gastrointestinal lumen, which may obscure endoscopic visualization. Therefore, the aims of this study were to understand the impact and the role of different degrees of variceal pressure on achieving complete ligations.

Research frontiers

Using porcine veins and esophagus, *in vitro* ligation of esophageal varices, is an innovative method to evaluate efficacy of variceal ligation treatments.

Innovations and breakthroughs

This study evaluated porcine esophageal variceal pressure and complete ligation degree using an animal model to guide endoscopic variceal treatment. The article has better practicability, and there are currently no relevant reports.

Applications

Variceal pressure may provide a valuable predictor for variceal bleeding following endoscopic variceal ligation.

Peer-review

This manuscript about esophageal variceal pressure influence is very interesting. In this manuscript, the authors explored the effect of *in vitro* porcine esophageal variceal pressure on complete ligation degree for polycyclic ligator. Three groups were studied. The results are interesting. Based on the results, the authors concluded that when variceal pressure is higher, the effect on ligation is worse.

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ORIGINAL ARTICLE

Basic Study

Caffeic acid phenethyl ester inhibits liver fibrosis in rats

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Abstract

AIM: To investigate the hepatoprotective effects and antioxidant activity of caffeic acid phenethyl ester (CAPE) in rats with liver fibrosis.

METHODS: A total of 75 male Sprague-Dawley rats were randomly assigned to seven experimental groups: a normal group ($n = 10$), a vehicle group ($n = 10$), a model group ($n = 15$), a vitamin E group ($n = 10$), and three CAPE groups (CAPE 3, 6 and 12 mg/kg, $n = 10$, respectively). Liver fibrosis was induced in rats by injecting CCl_4 subcutaneously, feeding with high fat forage, and administering 30% alcohol orally for 10 wk. Concurrently, CAPE (3, 6 and 12 mg/kg) was intraperitoneally administered daily for 10 wk. After that, serum total bilirubin (TBil), aminotransferase (ALT) and aspartate aminotransferase (AST) levels were measured to assess hepatotoxicity. To investigate antioxidant activity of CAPE, malondialdehyde (MDA), glutathione (GSH) levels, catalase (CAT) and superoxide dismutase (SOD) activities in liver tissue were determined. Moreover, the effect of CAPE on α-smooth muscle actin (α-SMA), a characteristic hallmark of activated hepatic stellate cells (HSCs), and NF-E2-related factor 2 (Nrf2), a key transcription factor for antioxidant systems, was investigated by immunohistochemistry.

RESULTS: Compared to the model group, intraperitoneal administration of CAPE decreased TBil, ALT, and AST levels in liver fibrosis rats ($P < 0.05$), while serum TBil was decreased by CAPE in a dose-dependent manner. In addition, the liver hydroxyproline contents in both the 6 and 12 mg/kg CAPE groups were markedly lower than that in the model group ($P < 0.05$ and $P < 0.001$, respectively). CAPE markedly decreased MDA levels and, in turn, increased GSH levels, as well as CAT and SOD activities in liver fibrosis rats compared to the model group ($P < 0.05$). Moreover, CAPE effectively inhibited α-SMA expression while increasing Nrf2 expression compared to the model group ($P < 0.01$).

CONCLUSION: The protective effects of CAPE against liver fibrosis may be due to its ability to suppress the activation of HSCs by inhibiting oxidative stress.

Key words: Caffeic acid phenethyl ester; Liver fibrosis; Oxidative stress; NF-E2-related factor 2; α-smooth

muscle actin

Core tip: It has been demonstrated that caffeic acid phenethyl ester (CAPE) has several biological and pharmacological properties. The aim of this study was to evaluate the hepatoprotective effects and antioxidant activity of CAPE in rats with liver fibrosis, as well as the underlying mechanism.

INTRODUCTION

Caffeic acid phenethyl ester (CAPE), a flavonoid-like compound, is one of the major components of honeybee propolis. Honeybee propolis possesses a highly complex and variable chemical composition, and approximately 300 components have been identified[1,2]. CAPE has been widely accepted as a hepatoprotective folk medicine for many years. Its protective effects are attributed to its cytoprotective, anti-oxidant, anti-proliferative, and anti-inflammatory effects, and it has been shown to inhibit both lipoxygenase activity and lipid peroxidation[3-9]. CAPE can also inhibit phorbol ester-induced H_2O_2 production and tumor promotion[10,11], and it was reported that many CAPE activities were related to transcription factor inhibition of nuclear factor-kappa B (NF-κB)[10,12-14].

Liver fibrosis results from chronic damage to the liver in conjunction with the accumulation of extracellular matrix proteins, which is a characteristic of many chronic liver diseases[15]. Oxidative stress can cause excessive damage to hepatocytes through lipid peroxidation and protein alkylation. In addition, oxidative stress can liberate mediators and assist in the activation of hepatic stellate cells (HSCs), which are the predominant fibrogenic cell type producing collagen type I in the liver[16,17]. Therefore, oxidative stress has been recognized as a fundamental factor in the pathological process of liver fibrosis. NF-E2-related factor 2 (Nrf2) is a key transcription factor that regulates the induction of genes encoding antioxidant proteins and phase 2 detoxifying enzymes, which is involved in drug metabolism, detoxification and antioxidant defenses[18,19]. In nuclear protein extracts from normal and activated HSCs, Nrf1 and Nrf2 proteins were identified as part of the NAD(P)H quinine oxidoreductase 1 antioxidant response element (ARE) DNA/protein complex. Concomitant activation of the ARE defense and enhanced cell proliferation may confer protection against electrophile-mediated HSC toxicity during liver fibrosis[20]. All of these factors have led us to propose the following hypothesis: CAPE is more effective in inhibiting liver fibrosis and oxidative stress by activating Nrf2 expression compared to the effects of vitamin E (vit E), which is one of the well-known antioxidant agents.

MATERIALS AND METHODS

Chemicals
CAPE was obtained from Yuancheng Technology Co. (Wuhan, China; purity 98%). Vit E was obtained from Sigma Chemical Co. (St. Louis, MO). Serum total bilirubin (TBil), alanine aminotransferase (ALT), and aspartate aminotransferase (AST) were measured with an auto chemistry analyzer (Hitachi Co., Japan). Antibodies against α-smooth muscle actin (α-SMA) and Nrf2 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Malondialdehyde (MDA), superoxide dismutase (SOD), catalase (CAT), glutathione (GSH) and hydroxyproline detection kits, CCl_4, olive oil, and alcohol were also obtained from commercial sources.

Animals and treatment
Seventy-five male Sprague-Dawley rats (254.50 ± 16.22 g) were obtained from Xi'an Jiaotong University Medical College. The rats were reared in a controlled environment at 21 ± 2 ℃ and 50% ± 5% relatively humidity under a 12-h dark/light cycle and were acclimatized for at least 1 wk prior to use. The experimental procedures were performed according to the animal care guidelines of the National Institutes of Health and performed under the Xi'an Jiaotong University Guidelines for Animal Experimentation.

The rats were randomly assigned to seven experimental groups as follows: a normal group that was given water and a standard chow diet ad libitum ($n = 10$); a vehicle group that was treated by intraperitoneal injection of 10% alcohol (vehicle for CAPE) and subcutaneous injection of olive oil (vehicle for CCl_4; $n = 10$); a model group (10% alcohol *via* the intraperitoneal route, once a day; $n = 15$); a vit E group (vit E dissolved in olive oil was administered intraperitoneally at 10 mg/kg, once daily; $n = 10$); and three CAPE groups (CAPE dissolved in 10% alcohol was administered intraperitoneally at 3, 6 or 12 mg/kg, once daily, respectively; $n = 10$). Except for the normal and vehicle groups, the other groups were injected with CCl_4 dissolved in 40% v/v olive oil at 1 mg/kg body weight (2 mg/kg for the first time) twice a week *via* the subcutaneous route, fed rich-fat forage, and given alcohol (30%) orally for 10 wk.

At the conclusion of the experiment, all rats were fasted for 12 h before sacrifice. Liver, kidneys and spleen were removed and weighed, and blood was collected for serum isolation. Liver tissue samples (0.5 g) were homogenized (1:10, w/v) in ice-cold normal

saline (NS). Homogenates were centrifuged at 5000 rpm for 10 min to remove debris. The supernatants, including the cytosolic fraction, were obtained and used for the analyses of select parameters. Protein concentrations of supernatants were measured by the Coomassie brilliant blue method[21].

Determination of TBil, ALT and AST in serum
Serum TBil, ALT and AST levels were measured to assess hepatotoxicity. Briefly, blood samples were kept overnight in a 4 ℃ refrigerator before centrifugation at 3000 *g* for 10 min. Afterwards, serum samples were stored in a -80 ℃ freezer before analysis. TBil, ALT and AST in serum were evaluated by an autoanalyzer (Hitachi-7170, Hitachi, Japan).

Determination of liver hydroxyproline
Liver samples were frozen at -20 ℃ before analysis. Hepatic collagen content was evaluated by hydroxyproline (Hyp) quantification according to the method described and validated by Edwards and O'Brien[22] with some modifications. Briefly, liver tissues (75 to 100 mg) were homogenized in 1 mL of phosphate-buffered saline and hydrolyzed in 6 mol/L HCl overnight at 120 ℃. Next, 5 μL of hydrolysates was mixed with 5 μL of citrate acetate buffer, and then 100 μL of chloramine T solution was added. After 20 min of incubation at room temperature, 100 mL of Ehrlich's solution was added. The mixture was incubated for 15 min at 65 ℃, and absorbance was read at 550 nm. Recovery of known standard amounts was performed using similar liver samples to provide quantification. The hepatic hydroxyproline content is expressed as g/g wet liver weight.

Determination of lipid peroxidation and GSH levels
Hepatic lipid peroxidation levels were determined by measuring MDA (a thiobarbituric acid reactive substance) levels[23]. Briefly, 10% hepatic homogenate samples were mixed with a thiobarbituric acid (TBA) reagent consisting of 0.375% TBA and 15% trichloroacetic acid in 0.25 mol/L hydrochloric acid. The reaction mixtures were placed in a boiling water bath for 40 min and then centrifuged at 4000 *g* for 5 min. Supernatant absorbance was measured at 535 nm. Results are expressed as nmol/mg protein. Hepatic GSH level was estimated by a colorimetric method using 5,5'-dithio bis-(2-nitrobenzoic acid) (DTNB)[24]. Samples were mixed with sodium phosphate buffer and DTNB. The mixture was incubated for 5 min at room temperature. Absorbance was measured at 405 nm. GSH content was determined from a standard curve generated with known concentrations of GSH. Results are expressed as μmol/mg protein.

Determination of antioxidant enzymes
CAT and SOD assays were performed according to the manufacturer's instructions as previously described[25].

Briefly, CAT activity was defined as the enzyme concentration required to decompose 1 μmol H_2O_2 within 1 min. This reaction was initiated by addition of 1.0 mL of freshly prepared 20 mmol H_2O_2. The rate of H_2O_2 decomposition was measured spectrophotometrically at 240 nm for 1 min. To measure SOD activity, xanthine and xanthine oxidase were used to generate superoxide radicals that react with 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyl tetrazolium chloride to form a red formazan dye. Absorbance was then measured at 550 nm. CAT and SOD activities are expressed as UI/mg protein.

Histological examination
Fresh liver tissues that had been trimmed to a thickness of 3 mm were placed in plastic cassettes and immersed in neutral buffered formalin for 24 h. Fixed tissues were processed routinely, embedded in paraffin, sectioned, deparaffinized, and rehydrated using standard techniques. The extent of CCl4-induced necrosis was evaluated by assessing the morphological changes in liver sections stained with hematoxylin-eosin (HE) and van Gieson (VG). Fibrosis was scored semi-quantitatively as described by Zhang *et al*[26]. Accordingly, 0 was accepted as normal liver; 1, as thickened perivenular collagen and thin collagen septa; 2, as thin septa with incomplete bridging between portal regions; 3, as thin septa and extensive bridging; and 4, as thickened septa with complete bridging of portal regions and nodular appearance.

Immunohistochemistry
Immunohistochemistry for α-SMA was performed to identify HSC activation. Nrf2 expression in liver tissue was confirmed by immunohistochemistry, and the density of positive cells was measured with Image-Pro plus 6.0 software. Integrated optical density of positive staining of α-SMA and Nrf2 in each photograph was measured, and the average of all photographs in each group was calculated[27].

Immunohistochemical staining was performed on formalin-fixed paraffin-embedded liver sections (thickness, 5 mm). Following dehydration through xylene and a graded series of alcohol, the sections were washed with phosphate buffered saline (PBS; pH 7.4) and treated with 0.3% H_2O_2 for 20 min to block endogenous peroxidase activity. Tissue antigen retrieval was then accomplished by microwave heating for 2 min. After three washes with PBS, 10% goat non-immune serum was applied to liver sections for 30 min to prevent non-specific antibody binding. The sections were incubated with a primary mouse antibody (α-SMA or Nrf2, 1:100) in a humidified chamber at 37 ℃ for 3 h. A biotinylated secondary antibody and streptavidin peroxidase complex (ZSGB-BIO, Histostain-Plus Kit, Cat No: 85-9043) were consecutively applied at 37 ℃ for 15 min each, and three PBS washes were performed between applications. Antibody binding

A

B

C
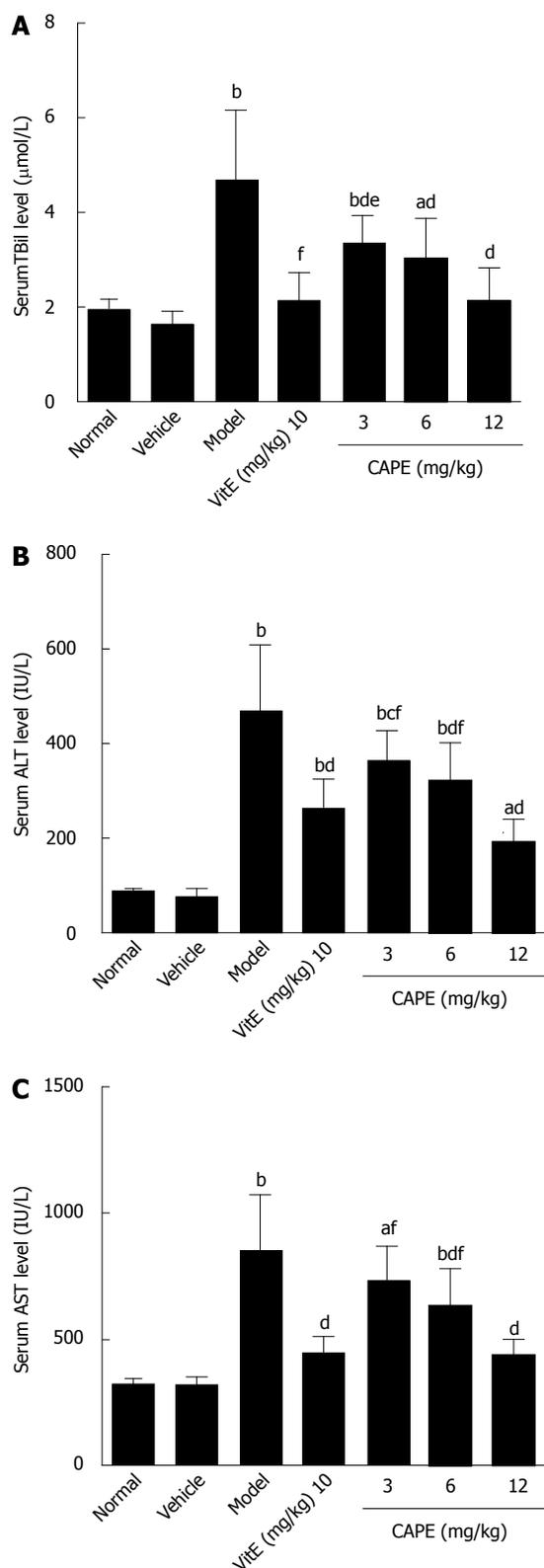
Figure 1 Effects of caffeic acid phenethyl ester on serum TBil (A), ALT (B), and AST (C) levels in rats. Bars and error bars represent mean ± SD, *t* = 2.741-10.5, [a]*P* < 0.05, [b]*P* < 0.01 *vs* normal group, respectively; *t* = 2.9-7.371, [c]*P* < 0.05, [d]*P* < 0.01 *vs* model group, respectively; *t* = 3.225-5.229, [e]*P* < 0.05, [f]*P* < 0.01 *vs* CAPE (12 mg/kg) group, respectively. CAPE: Caffeic acid phenethyl ester; Vit E: Vitamin E.

Figure 2 Liver hydroxyproline contents in the groups. Bars and error bars represent mean ± SD. *t* = 4.959-10.38, [b]*P* < 0.01 *vs* normal group; *t* = 3.090-5.148, [c]*P* < 0.05, [d]*P* < 0.01 *vs* model group, respectively. CAPE: Caffeic acid phenethyl ester; Vit E: Vitamin E; Hyp: Hydroxyproline.

counter-stained with Mayer's hematoxylin and rinsed with tap water, and the slides were mounted and observed under a light microscope.

Statistical analysis

The GraphPad.prism version 5.0 software package was used for statistical analysis. Results of measurement data are expressed as mean ± SD and tested by analysis of variance followed by the Tukey-Kramer test. Results of ranked data were statistically analyzed by the nonparametric Kruskal-Wallis *H* test followed by the Wilcoxon test for paired comparisons. Differences were considered significant if the *P*-value was less than 0.05.

RESULTS

Effects of CAPE on experimental hepatotoxicity

The biochemical results of analyses are presented in Figure 1. Serum TBil, ALT, and AST levels were significantly higher in the model group compared to the normal group (*P* < 0.01). Intraperitoneal administration of CAPE significantly reduced (*P* < 0.05) TBil, ALT, and AST levels in liver fibrosis rats when compared to the model group, and serum TBil was decreased by CAPE in a dose-dependent manner.

Liver hydroxyproline

As demonstrated in Figure 2, liver hydroxyproline content in the model group was 1.5 times higher than that in the normal group, and the liver hydroxyproline contents in both the 6 and 12 mg/kg CAPE groups were significantly lower than that in the model group (*P* < 0.05 and *P* < 0.001, respectively).

Tissue levels of MDA and GSH

To evaluate the effect of CAPE on liver lipid pero-

was visualized by color development using 3, 3 diaminobenzidine/H_2O_2. Finally, the sections were
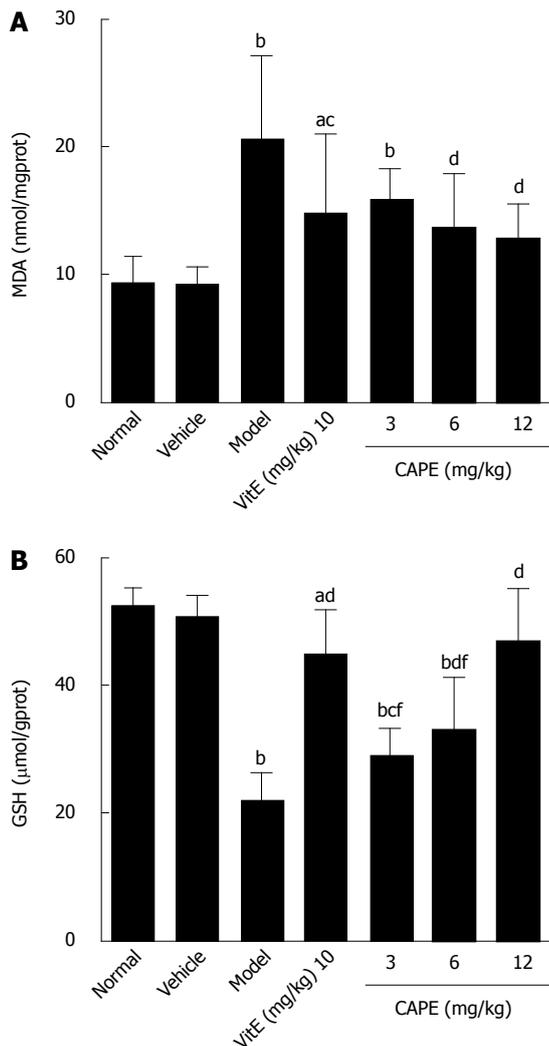
Figure 3 Tissue levels of malondialdehyde (A) and glutathione (B) in the groups. Bars and error bars represent mean ± SD. t = 2.767-9.292, [a]P < 0.05, [b]P < 0.01 *vs* normal group, respectively; t = 2.721-9.551, [c]P < 0.05, [d]P < 0.01 *vs* normal group, respectively; t = 5.154-6.903, [e]P < 0.05, [f]P < 0.01 *vs* CAPE (12 mg/kg) group, respectively. CAPE: Caffeic acid phenethyl ester; Vit E: Vitamin E; MDA: Malondialdehyde; GSH: Glutathione.

Figure 4 Superoxide dismutase and catalase activities in liver tissue. Bars and error bars mean ± SD. t = 4.552-11.06, [b]P < 0.01 *vs* normal group; t = 2.876-8.689, [c]P < 0.05, [d]P < 0.01 *vs* model group, respectively; t = 3.393-7.503, [f]P < 0.01 *vs* CAPE (12 mg/kg) group, respectively; t = 3.969-4.997, [g]P < 0.05, [h]P < 0.01 *vs* vit E group, respectively. CAPE: Caffeic acid phenethyl ester; Vit E: Vitamin E; SOD: Superoxide; CAT: Catalase.

xidation, MDA levels (an indicator of oxidative damage and one of the principal products of lipid peroxidation) were monitored. GSH is an important part of the non-enzymatic antioxidant system and is known to play an important role in liver injury; therefore, GSH levels were also determined in this study. The results of MDA and GSH are shown in Figure 3. In the CAPE (6 and 12 mg/kg) treated rats, the MDA levels were significantly lower, and the levels of GSH were significantly higher than those in the model group. Moreover, MDA and GSH concentrations in groups treated with 12 mg/kg CAPE were not significantly different from those in the normal group.

Antioxidant enzymes activities
As shown in Figure 4, in a similar manner to GSH, CAT and SOD activities were significantly decreased in liver fibrosis rats induced with CCl₄, alcohol and rich-fat forage. CCl₄, alcohol and rich-fat forage administration significantly depleted antioxidant enzyme (CAT and SOD) activities, whereas CAPE (6 and 12 mg/kg) treatment significantly protected against the depletion of CAT and SOD, and the effects of treatment with 12 mg/kg CAPE were more obvious compared to 10 mg/kg vit E.

Pathological changes
Histopathological changes in all of the groups are shown in Figures 5 and 6 (HE and VG staining). In microscopic examinations of the samples, liver sections in the normal and vehicle groups showed normal parenchymal cells, portal system, and blood sinusoids (no histopathological changes). Administration of CCl₄, alcohol and rich-fat forage induced severe alterations in liver histopathology. The model group liver sections showed peri-portal apoptosis, lymphocytic infiltration,
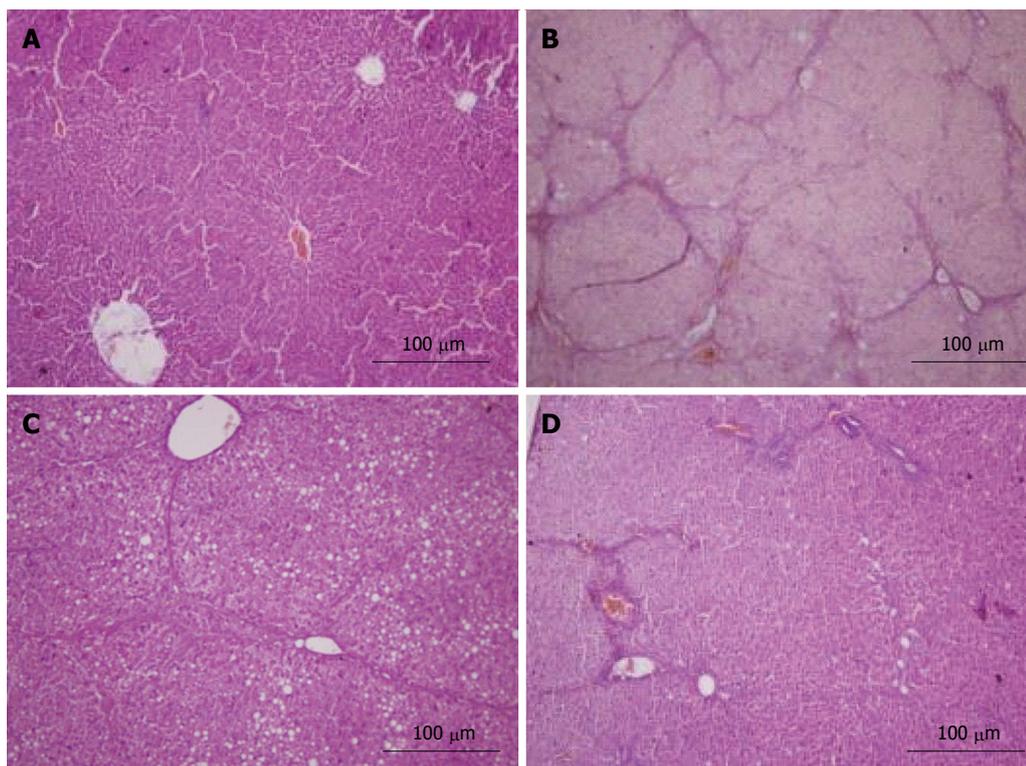
Figure 5 HE staining. All sections were stained with hematoxylin and eosin (× 100). A: Liver sections of the normal group did not show histopathological changes; B: Liver sections of the model group showed liver tissue structural disorder, apoptotic cells, lymphocytic infiltration and typical pseudo-lobule; C: Liver sections of the vitamin E group showed steatosis, edema and swelling of hepatocytes, but no typical pseudo-lobule can be observed; D: Liver sections of the caffeic acid phenethyl ester (12 mg/kg) group showed spotty necrosis, steatosis, and blood sinusoids with mild congestion. The results of the other groups are not shown here.
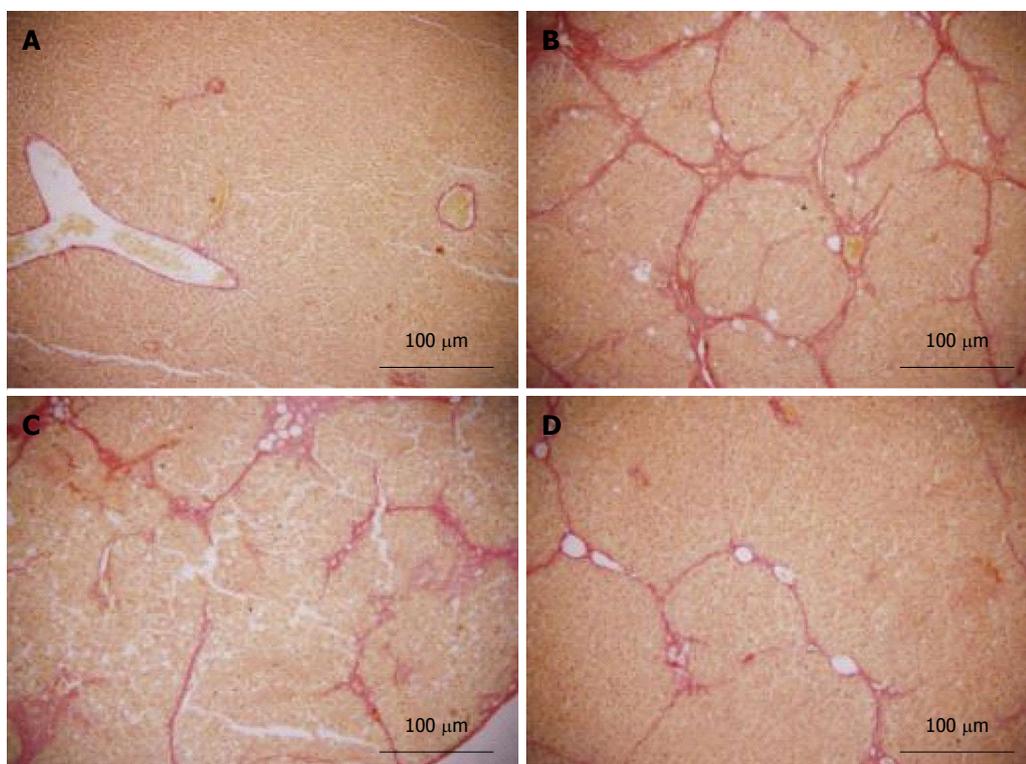
Figure 6 Van Gieson staining. All sections were stained with van Gieson (× 100). A: Liver sections of the normal group did not show fibroplasia; B: Liver sections of the model group showed thick bundles of proliferous collagenous fibers stained with eosin and typical pseudo-lobule; C: Liver sections of the vitamin E group showed a small amount of collagenous fibers; D: Liver sections of the caffeic acid phenethyl ester (12 mg/kg) group showed a small amount of fibroplasia, which was less than that in the vitamin E group. The results of other groups are not shown here.

Table 1 Degree of liver fibrosis in the groups (*n* = 6 for each group)

Group	0	1	2	3	4
Normal	6	0	0	0	0
Vehicle	6	0	0	0	0
Model	0	0	0	3	3
Vit E	0	4	2	0	0
CAPE (mg/kg)					
3	0	0	2	3	1
6	0	1	3	2	0
12	0	5	1	0	0

H = 46.12. Model group vs normal group, χ^2 = 9.778, P < 0.05; Vitamin E (Vit E) group *vs* model group, χ^2 = 8.899, P < 0.05; Caffeic acid phenethyl ester (CAPE; 12 mg/kg) *vs* model group, χ^2 = 9.209, P < 0.05. The results were statistically analyzed by the nonparametric Kruskal-Wallis H test followed by Wilcoxon test for paired comparisons.

dilated central veins, thick bundles of proliferous collagenous fibers stained with eosin and typical pseudo-lobule. The liver did not show significant improvement in either the 3 or 6 mg/kg CAPE group, whereas the 12 mg/kg group had similar recovery as in the vit E positive control group. However, steatosis, edema and swelling of hepatocytes (cobble stone appearance), spotty necrosis and a small amount of collagenous fibers still could be observed in the CAPE (12 mg/kg) and Vit E groups. The degree of liver fibrosis in the groups is shown in Table 1.

α-SMA expression
In the model group, immunohistochemistry localized the α-SMA protein predominantly to the fibrous septa, inflamed area and portal area, and we observed a significantly higher (P < 0.001) expression level than in the normal group (Figure 7). CAPE treatment could down-regulate the α-SMA expression in liver tissue. The expression level of α-SMA in the 12 mg/kg CAPE group was significantly lower than those in the model group and the vit E group (Figure 8).

Nrf2 expression
As shown in Figures 9 and 10, it was observed that Nrf2-positive cells detected by immunohistochemistry in liver tissue were increased in the model group compared to the normal group, and the expression levels of Nrf2 were increased in the 6 and 12 mg/kg CAPE groups compared with the model group. In addition, expression level of Nrf2 in the 12 mg/kg dose group was highest (P < 0.05) in all the groups.

DISCUSSION
CCl4 induced hepatotoxicity derives from the reductive dehalogenation of CCl4 that is catalyzed by CYP450 in the endoplasmic reticulum of liver cells to generate unstable trichloromethyl (CCl_3^+) and trichloromethyl peroxyl ($CCl_3O_2^-$) radicals. Similarly, it was reported
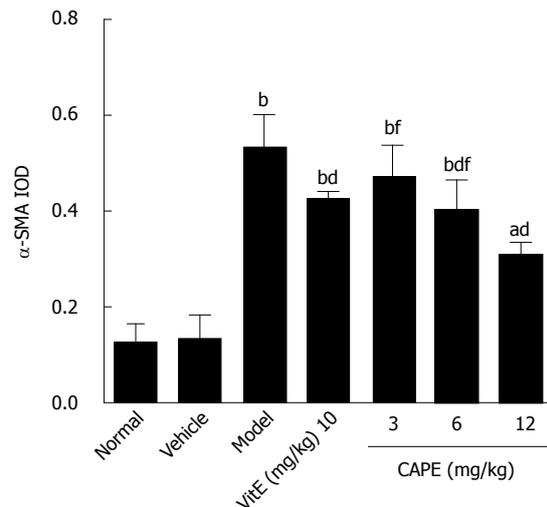
Figure 7 **α-smooth muscle actin expression level in liver tissue.** Bars and error bars represent mean ± SD. t = 6.557-14.487, [a]P < 0.05, [b]P < 0.01 *vs* normal group, respectively; t = 3.784-7.913, [c]P < 0.05, [d]P < 0.01 *vs* model group, respectively; t = 3.284-5.718, [f]P < 0.01 *vs* CAPE (12 mg/kg) group, respectively; t = 4.129, [g]P < 0.05 *vs* vit E group, respectively. CAPE: Caffeic acid phenethyl ester; Vit E: Vitamin E; α-SMA: α-smooth muscle actin.

that alcohol-induced liver injury was associated with formation of the l-hydroxyl ethyl radical and lipid radicals[28]. Oxidative stress also plays an important role in high-fat forage induced liver injury. Free radicals attack and covalently bind to microsomal lipids and proteins, leading to lipid peroxidation and initiating secondary biochemical processes, which are the ultimate cause for unfolding the pathological consequences of CCl4.

In the current study, CCl4, alcohol and high-fat forage resulted in prominent hepatotoxicity as evidenced by the increases in serum TBil, ALT and AST levels and histopathology (HE and VG staining), and CAPE at all the three doses reduced TBil, ALT and AST levels in blood in a dose-dependent manner, indicating reduced hepatocellular damage. Histopathological observations also substantiated biochemical data of the study. CAPE treatment at 6 and 12 mg/kg resulted in the improvement in liver histoarchitecture and better recovery than that in the Vit E treated positive group.

Exogenous and endogenous protective agents with antioxidant properties demonstrated some protective effects against hepatotoxicity induced by various etiological factors. CAPE exhibits a number of beneficial effects against various types of degenerative diseases in humans, largely because the major components of CAPE have potent antioxidant activities[29,30]. GSH is a low molecular weight thiol antioxidant and a co-substrate for a variety of antioxidants and anti-xenobiotic enzymes of phase Ⅱ pathway of the CYP450 cycle. It has been reported that the covalent binding of most toxicants to hepatic protein occurs only after depletion of GSH, and the
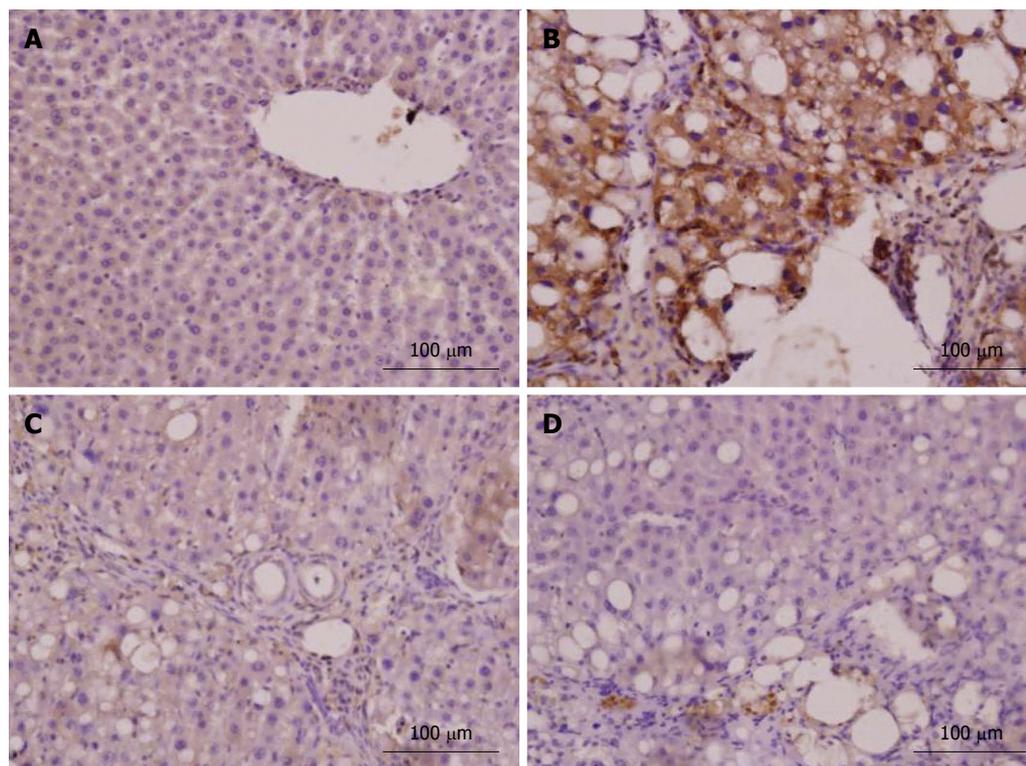
Figure 8 α-smooth muscle actin expression in liver tissue (immunohistochemistry, × 400). The α-smooth muscle actin (α-SMA) expression level in liver tissue was confirmed by immunohistochemistry (× 400). A: Liver sections of the normal group showed a small amount of α-SMA-positive cells only present in vascular wall; B: Liver sections of the model group showed that the α-SMA protein was predominantly present in the fibrous septa, inflamed area and portal area; C: Liver sections of the vitamin E group showed that α-SMA-positive cells were distributed in fibroplasia and inflamed area, but the number of positive cells was less than that in the model group; D: Liver sections of the CAPE (12 mg/kg) group showed that α-SMA-positive cells were sparse, located in inflamed area. The results of the other groups are not shown here.
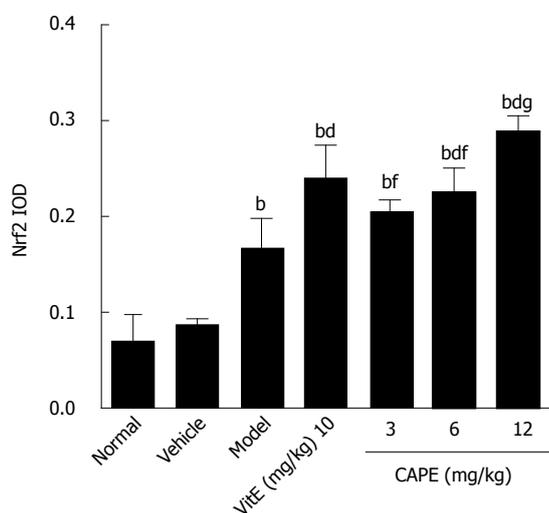
Figure 9 NF-E2-related factor 2 expression level in liver tissue. Bars and error bars represent mean ± SD. t = 6.412-14.54, [a]P < 0.05, [b]P < 0.01 *vs* normal group, respectively; t = 3.936-8.126, [c]P < 0.05, [d]P < 0.01 *vs* model group, respectively; t = 4.190-5.627, [e]P < 0.05, [f]P < 0.01 *vs* CAPE (12 mg/kg) group, respectively; t = 3.252, [g]P < 0.05 *vs* vit E group, respectively. CAPE: Caffeic acid phenethyl ester; Vit E: Vitamin E; Nrf2: NF-E2-related factor 2.

severity of hepatic necrosis is related to the degree of covalent binding[31]. In addition, antioxidant enzymes such as CAT and SOD are easily inactivated by lipid peroxides or reactive oxygen species, which results in decreased activities of these enzymes in CCl_4 toxicity. Thus, antioxidant enzymes also play an important role in the detoxification of xenobiotics, catalyzing their conjugation with reduced GSH[32]. Finally, it has been reported that antioxidants and flavonoids can act as inhibitors of LPO by scavenging peroxy radicals derived from polyunsaturated fatty acids and interrupt the chain reactions[33]. Therefore, LPO was monitored by measuring MDA - an end product of LPO in membrane components of cells[34].

In the present study, the model group showed significantly decreased GSH levels, CAT and SOD activities, and increased MDA levels compared with the normal group, indicating increased oxidative stress due to free-radical induced liver damage. CAPE treatment significantly reduced the elevated MDA levels and also increased tissue GSH levels, CAT and SOD activities. These results suggest that suppression of oxidative stress by CAPE in rats is an important aspect of the hepatoprotective effect of CAPE against hepatocytoxicity induced with CCl_4, alcohol and rich-fat forage.

Transcription factor Nrf2 is activated in response to electrophiles and reactive oxygen species. During oxidative stress, Nrf2 is released from sequestration in the cytoplasm and translocates to the nucleus[35]. It
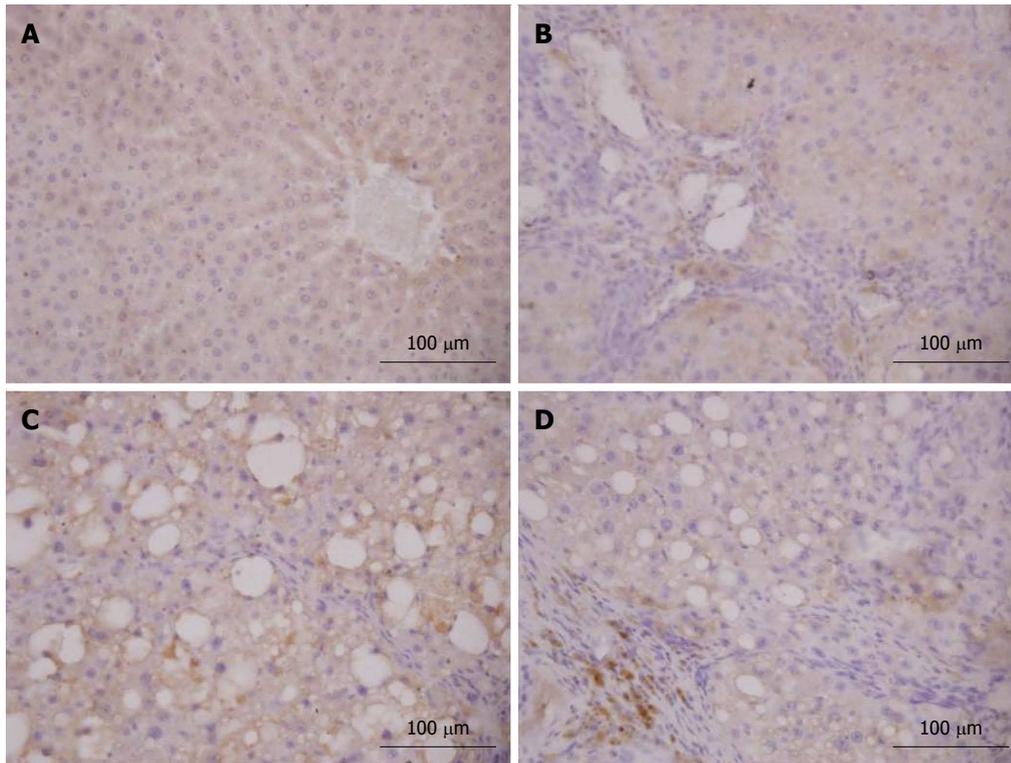
Figure 10 NF-E2-related factor 2 expression in liver tissue (immunohistochemistry). The NF-E2-related factor 2 (Nrf2) expression in liver tissue was confirmed by immunohistochemistry (× 400). A: Liver sections of the normal group showed a small amount of Nrf2-positive cells only present in the cytoplasm; B: Liver sections of the model group showed only a few Nrf2-positive cells present in the fibrous septa and inflamed area; C: Liver sections of the vitamin E group showed that Nrf2-positive cells were distributed in fibroplasia and inflamed area, but the number of positive cells was more than that in the model group; D: Liver sections of the caffeic acid phenethyl ester (12 mg/kg) group showed that Nrf2-positive cells were located mainly in fibrosis and inflamed area and were strongly stained. The results of the other groups are not shown here.

has been demonstrated that Nrf2 is a key transcription factor for both the inducible and constitutive expression of phase II enzymes. Previous research has shown that Nrf2 knockout mice are more susceptible to acetaminophen-induced hepatocellular injury and that Nrf2 may also be important in protecting against liver fibrosis[36]. In liver fibrosis, HSCs serve as the major source of extracellular matrices in liver fibrosis. During the activation process, HSCs undergo phenotypic changes in cellular morphology to a myofibroblast-like cell type with expression of α-SMA[12]. In the current study, immunohistochemistry results showed that treatment with CAPE at 6 mg/kg and 12 mg/kg significantly activated Nrf2, and inhibited α-SMA. These data indicate that CAPE may be able to protect liver cells from oxidative stress induced damage and inhibit activation of HSCs through up-regulated Nrf2 expression.

It should be considered that the protective effect of CAPE against hepatotoxicity might not be solely dependent on the antioxidant effect of CAPE, which may exert its hepatoprotective effect by other means. For example, it was shown that CCl4 induces liver cell apoptosis by cytochrome C release and caspase 3 activation, and causes hepatoxicity by increasing mRNA expression of NF-κB dependent cyclooxygenase-2 and iNOS. Alternatively, CAPE shows an anti-inflammatory action as lipoxygenase and cyclooxygenase inhibitor[37,38]. Furthermore, CAPE is a potent mitochondrial protective agent that blocks Ca^{2+}-induced cytochrome release and is a potent suppressor of pro-inflammatory cytokines by reducing caspase 1 activation and depressing NF-κB[39].

In conclusion, CAPE exhibits significant partial protection against hepatotoxicity as evidenced from the reversal of various biochemical indices and histopathology, which were altered due to CCl4, alcohol and high-fat forage induced toxicity. The protective effects against liver damage could be due to the antioxidant effect of CAPE. However, the protective effect of CAPE may not always be associated with its antioxidant effects. Owing to the hepatoprotective potential, CAPE has clinical importance and could be used to develop a safe hepatoprotective medicine.

COMMENTS

Background

Liver fibrosis results from chronic damage to the liver in conjunction with the accumulation of extracellular matrix proteins. Oxidative stress has been recognized as a fundamental factor in the pathological processes of liver fibrosis. Caffeic acid phenethyl ester (CAPE) has been widely accepted as a hepatoprotective folk medicine for many years. However, the hepatoprotective

effects of CAPE on liver fibrosis and the underlying mechanism are unclear.

Research frontiers

CAPE is one of the major components of honeybee propolis and has several biological and pharmacological properties, including cytoprotective, anti-oxidant, anti-proliferative, and anti-inflammatory effects. Moreover, it was reported that many CAPE activities were related to transcription factor inhibition of nuclear factor-kappa B.

Innovations and breakthroughs

Oxidative stress can cause excessive damage to hepatocytes through lipid peroxidation and protein alkylation. NF-E2-related factor 2 (Nrf2) is a key transcription factor that regulates the induction of genes encoding antioxidant proteins and phase 2 detoxifying enzymes, which is involved in drug metabolism, detoxification and antioxidant defenses. CAPE may inhibit liver fibrosis and oxidative stress by activating Nrf2 expression.

Applications

These findings show that the protective effects of CAPE against CCl_4, alcohol and high-fat forage induced liver fibrosis may be due to its ability to suppress activation of HSCs by inhibiting oxidative stress.

Terminology

CAPE is a flavonoid-like compound and is one of the major components of honeybee propolis.

Peer-review

This is an interesting study about CAPE in liver fibrosis. CAPE is a flavonoid-like compound and is one of the major components of honeybee propolis. In this study, the authors evaluated the hepatoprotective effects and antioxidant activity of CAPE in rats with liver fibrosis. The study design is good, and the results are interesting.

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ORIGINAL ARTICLE

Case Control Study

Interferon-λ3 polymorphisms in pegylated-interferon-α plus ribavirin therapy for genotype-2 chronic hepatitis C

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Abstract

AIM: To evaluate interferon-λ3 (*IFNL3*) polymorphisms in response-guided pegylated interferon-α plus ribavirin (Peg-IFNα/RBV) therapy for genotype 2 (G2) chronic hepatitis C.

METHODS: Between January 2006 and June 2012, a total of 180 patients with chronic infections of G2 hepatitis C virus (HCV) were treated with response-guided Peg-IFNα/RBV therapy. The treatment duration was 24 wk for patients who achieved rapid virologic response (RVR), and 36 or 48 wk for patients who did not. Then, the impact of the *IFNL3* single nucleotide polymorphism genotype (TT/non-TT at rs8099917) on treatment outcomes was evaluated in the 180 patients, and between patients infected with either HCV subgenotype 2a or 2b.

RESULTS: Of the 180 patients evaluated, 111 achieved RVR, while the remaining 69 patients did not. In RVR patients, the sustained virologic response (SVR) rate was 96.4%, and the *IFNL3* genotype did not influence the SVR rate (96.6% *vs* 95.8% in *IFNL3* genotype TT *vs* non-TT). However, in non-RVR patients, the SVR rate decreased to 72.5% ($P < 0.0001$), and this rate was significantly different between the *IFNL3* genotype TT and non-TT groups (80.0% *vs* 42.9%, $P = 0.0146$). Multivariate regression analysis in non-RVR patients identified the *IFNL3* genotype TT as the only baseline-

significant factor associated with SVR (OR = 5.39, 95%CI: 1.29-22.62; *P* = 0.0189). In analysis according to HCV sub-genotype, no significant difference in the SVR rate was found between HCV sub-genotypes 2a and 2b.

CONCLUSION: In response-guided Peg-IFNα/RBV combination therapy for chronically HCV G2-infected patients, the impact of the *IFNL3* genotype on SVR was limited to non-RVR patients.

Key words: Hepatitis C virus genotype 2; Interferon-λ3 single nucleotide polymorphism; Pegylated-interferon plus ribavirin response-guided therapy; Rapid virologic response; Sustained virologic response

Core tip: Interferon-λ3 (*IFNL3*) single nucleotide polymorphisms (SNPs), such as rs8099917 and rs12979860, affect the virologic responses of chronically hepatitis C virus genotype 1-infected patients to response-guided pegylated interferon-α plus ribavirin therapy. However, the significance of these SNPs in therapy for hepatitis C virus genotype 2 (G2)-infected patients is unclear. We show that rs8099917 significantly influences sustained virologic response (SVR) achievement only in patients who do not attain rapid virologic response. Therefore, *IFNL3* SNP genotyping is valuable for predicting SVR only in non-rapid virologic response patients, irrespective of the G2 subtype, even when therapy is extended up to 48 wk.

INTRODUCTION

Hepatitis C virus (HCV) genotype 2 (G2) is the second-most frequent HCV genotype and accounts for approximately 30% of chronic HCV infections in Japan[1,2]. However, the prevalence of HCV G2 is decreasing due to successful treatment with standard 24-wk regimens of pegylated interferon-α plus ribavirin (Peg-IFNα/RBV) combination therapy; with > 80% of patients achieving sustained virologic response (SVR)[3,4]. However, a fraction of patients who do not achieve a rapid virologic response (RVR) may remain uncured, even when therapy is extended for 36 or 48 wk[5,6].

The impact of single nucleotide polymorphisms (SNPs) near the interferon-λ3 (*IFNL3*)/interleukin-28B

gene on Peg-IFNα/RBV combination therapy for HCV genotype 1[7-9] has been firmly established. However, it remains controversial whether the *IFNL3* genotype is useful in predicting virologic responses to peg-IFNα/RBV therapy in HCV G2 patients[10-13].

Previously, we demonstrated the value of response-guided therapy for HCV G2 patients who were treated for 24 wk with Peg-IFNα/RBV combination therapy if they achieved RVR, and for 36 or 48 wk if they did not achieve RVR[6]. In the present study, we assessed the impact of *IFNL3* SNP (rs8099917) genotypes on virologic responses and outcomes of HCV G2 (subtype of G2a or G2b) patients who received response-guided Peg-IFNα/RBV combination therapy.

MATERIALS AND METHODS

Patients

Between January 2006 and June 2012, 180 chronically HCV G2-infected patients were treated with response-guided Peg-IFNα/RBV combination therapy at the Jikei University Katsushika Medical Center, the Jikei University Kashiwa Hospital, and the Shinmatsudo Central General Hospital. The treatment duration was 24 wk for patients who achieved RVR (RVR group) and 36 or 48 wk for patients who did not (non-RVR group). Patients received weekly subcutaneous injections of Peg-IFNα-2b (PegIntron; MSD K.K.; Tokyo, Japan) at a dose of 1.5 μg/kg, plus RBV (Rebetol; MSD K.K.) at a dose of 600-1000 mg/d according to body weight (< 60 kg: 600 mg/d; 60-80 kg: 800 mg/d; and > 80 kg: 1000 mg/d). Doses of Peg-IFNα-2b and/or RBV were appropriately adjusted if side effects were observed.

All the patients studied satisfied the following inclusion criteria: (1) serum HCV RNA levels ≥ 10000 copies/mL (Amplicor HCV Monitor Test, version 2.0; Roche Diagnostics, Basel, Switzerland; quantification limit: 50 IU/mL) or ≥ 5 $\log_{10}$ IU/mL (COBAS AmpliPrep/COBAS TaqMan HCV Test; Roche Diagnostics; quantification limit: 1.2 $\log_{10}$ IU/mL); (2) white blood cell counts ≥ 2000/mm^3; (3) neutrophil counts ≥ 1500/mm^3; (4) hemoglobin levels ≥ 11 g/dL; (5) platelet counts ≥ 60000/mm^3; and (6) serotype 2 or genotype 2a or 2b (G2a or G2b) determined by serologic and conventional PCR-based methods, as reported previously[14,15]. Patients were excluded from this study if they were positive for hepatitis B surface antigen or anti-human immunodeficiency virus antibody, consumed > 20 g of alcohol/d, had psychiatric disorders or hepatocellular carcinoma, or were diagnosed with other liver diseases. Patients with established liver cirrhosis that was easily diagnosed by image inspection or for whom laboratory tests did not indicate the need for liver biopsy (*e.g.*, low platelet count or prolonged prothrombin time) were not included in the present study. One hundred and sixty-five patients (91.7%) were treatment-naïve and the remaining 15 had previously been treated with 24-wk

Peg-IFNα/RBV combination therapy.

This study complied with the standards of the Declaration of Helsinki (revised edition 2008) and current ethical guidelines, and was approved by the human ethics review committees of each institution. Written informed consent was obtained from all patients.

Histology, HCV sub-genotyping, and detection of HCV RNA

Liver biopsies and HCV G2 sub-genotyping were performed in 152/180 and 159/180 patients, respectively. Histologic grades of liver fibrosis were classified as F1-F4, according to the METAVIR scoring system[16]. HCV G2 sub-genotyping was performed by the conventional PCR-based method[14]. HCV serotypes were determined by enzyme-linked immune assay[15]. The presence or absence of serum HCV RNA was evaluated after 4 wk of therapy, at the end of therapy, and at 24 wk after the completion of therapy. Serum HCV RNA levels were evaluated with the qualitative Amplicor HCV Monitor Test between January 2006 and November 2007, and the COBAS AmpliPrep/COBAS TaqMan HCV test was used thereafter. To evaluate potential discrepancies due to the use of different tests, 21 samples that were originally analyzed using the Amplicor HCV Monitor Test were re-tested with the COBAS AmpliPrep/COBAS TaqMan HCV test, using serum stocks stored at -30 ℃. Patients in whom serum HCV RNA levels were undetectable with the COBAS AmpliPrep/COBAS TaqMan HCV test at 4 wk after initiating therapy were designated as RVR patients, while the remaining patients were designated as non-RVR patients. The end point in this study was SVR (undetectable serum HCV RNA at 24 wk post-treatment).

Analysis of SNPs near IFNL3

Genomic DNA was extracted and isolated from whole blood using a MagNA Pure LC Instrument and the DNA Isolation Kit (Roche Diagnostics). Alleles of the rs8099917 SNP near *IFNL3* were determined using TaqMan SNP genotyping assays (Applied Biosystems of Thermo Fisher Scientific, Waltham, MA, United States), as described previously[9]. The rs8099917 genotypes were classified into TT (major homozygous genotype) and non-TT genotypes (heterozygous genotype TG or minor homozygous genotype GG). The rs8099917 genotype of all patients was determined at the Research Center for Medical Science at the Jikei University School of Medicine.

Statistical analysis

The Mann-Whitney *U*-test was used to analyze differences in continuous variables. Fisher's exact tests were used to analyze differences in categorical data. All tests of significance were two-tailed. $P < 0.05$ and < 0.1 were considered statistically significant and

marginal, respectively. To determine which factors were associated with SVR, variables that were significant or marginal in univariate analyses were analyzed by multiple logistic regression analysis. All statistical analyses were performed using Statistica for Windows version 6 (StatSoft; Tulsa, OK, United States).

RESULTS

Treatment responses

Of the 180 patients evaluated, 111 (61.7%) achieved RVR and received a 24-wk treatment course (RVR group). The remaining 69/180 (38.3%) patients failed to achieve RVR and the treatment duration was extended to 36 or 48 wk (non-RVR group; Figure 1). HCV G2a was more frequently detected in the RVR group than in the non-RVR group ($P = 0.0005$; Table 1). With respect to the HCV sub-genotype, 69/98 (70.4%) G2a patients had RVR, whereas only 23/57 (40.4%) G2b patients had RVR. The baseline level of HCV RNA was significantly lower in the RVR group than in the non-RVR group ($P < 0.0001$). Serum albumin levels were significantly higher in the RVR group than in the non-RVR group ($P = 0.0029$). Multivariate analysis identified the baseline levels of HCV RNA and serum albumin as significant factors associated with RVR (OR = 4.40, 95%CI: 2.25-8.63, $P < 0.0001$; and OR = 0.13, 95%CI: 0.04-0.42, $P = 0.0006$; respectively). However, no difference was observed in the distribution of *IFNL3* SNP genotypes between the RVR and non-RVR groups (Figure 1 and Table 1). The percentages of TT genotype patients were 78.4% (87/111) *vs* 79.7% (55/69) in the RVR and non-RVR groups, respectively.

SVR in RVR patients

Of the 111 RVR patients, 107 (96.4%) achieved SVR with the 24-wk treatment. Regarding the *IFNL3* SNP genotype, 84/87 (96.6%) TT patients and 23/24 (95.8%) non-TT patients achieved SVR (Figure 2A). As for HCV G2 subtype, 66/69 (95.7%) patients with G2a and 23/23 (100%) patients with G2b achieved SVR. There were also no significant differences in other variables between patients with SVR and non-SVR. Although only four patients failed to achieve SVR, no characteristics distinguishing them from SVR patients were identified. All four of the non-SVR patients (1 male and 3 female; age: 34-65 years) were treatment-naïve and completed treatment as scheduled. They had a mild degree of liver fibrosis and baseline HCV RNA levels of 5.0 log IU/mL to 6.5 log IU/mL.

SVR in non-RVR patients

Of the 69 non-RVR patients, 50 (72.5%) achieved SVR with the extended treatment to 36 or 48 wk. The SVR rate in the non-RVR group was significantly lower than in the RVR group (72.5% *vs* 96.4%, $P < 0.0001$). Thirty-eight patients (55.1%) received a 48-wk treatment course and 31/69 (44.9%) received a
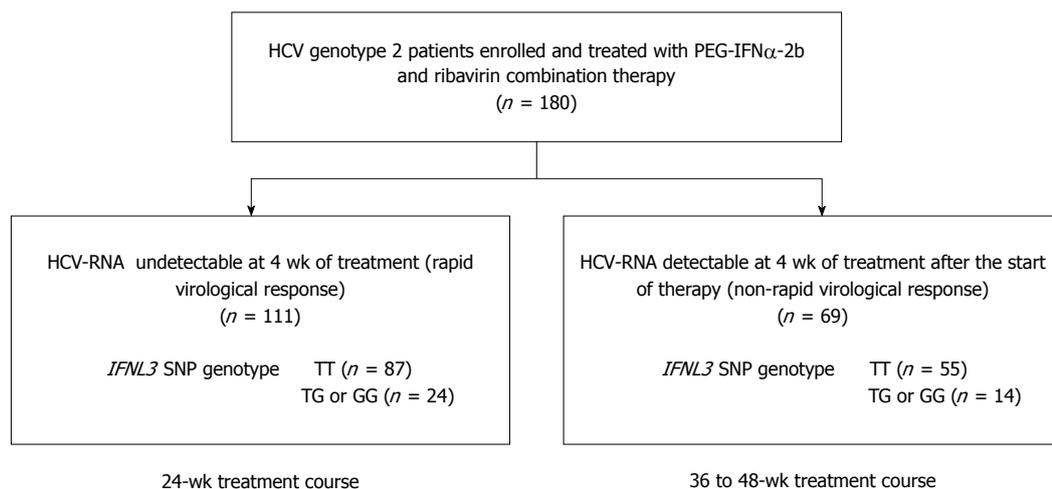
Figure 1 **Study flow chart.** PEG-IFNα: Pegylated interferon-α; HCV: Hepatitis C virus; SNP: Single nucleotide polymorphism.
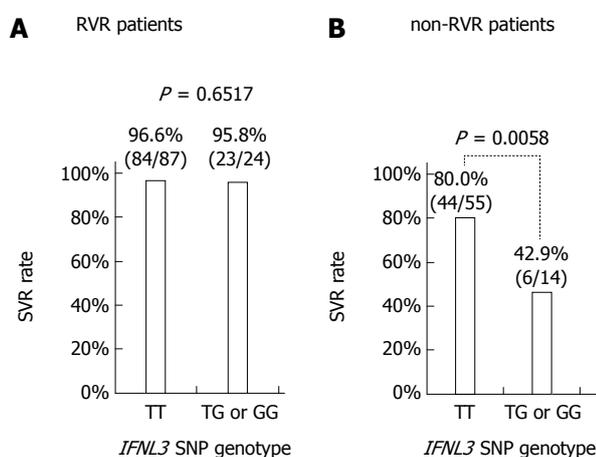
Figure 2 **Sustained virologic response rates according to interferon-λ3 single nucleotide polymorphism genotype.** A: Rapid virologic response (RVR); and B: Non-RVR patient groups. SVR: Sustained virologic response; SNP: Single nucleotide polymorphism; IFNL3: Interferon-λ3.

36-wk treatment course. SVR rates were higher in the 36-week treatment patient group than in the 48-wk group (80.6% *vs* 65.8%), but the difference was not significant. Regarding the *IFNL3* SNP genotype, 44/55 (80.0%) patients with the TT genotype and 6/14 (42.9%) patients with the non-TT genotype achieved SVR (*P* = 0.0058; Figure 2B). Among patients with the *IFNL3* TT genotype, the SVR rates were significantly different between RVR and non-RVR patient groups (96.6% *vs* 80.0%; *P* = 0.0033; Figure 2A and B). Similarly, among patients with non-TT genotypes, the SVR rates were significantly different between RVR and non-RVR patient groups (95.8% *vs* 42.9%, *P* = 0.0009). In HCV G2 sub-genotype patients, 21/29 (72.4%) patients with G2a and 24/34 (70.6%) patients with G2b achieved SVR.

Factors contributing to SVR in non-RVR patients
In the non-RVR patient group, the *IFNL3* TT genotype

was the only baseline factor that significantly related to SVR in univariate analysis (*P* = 0.0146). Among the other baseline factors, aspartate aminotransferase was marginal (*P* = 0.0751). The histologic stage of fibrosis and HCV G2 sub-genotypes were not significant factors for SVR. In multiple logistic regression analysis, only the *IFNL3* TT genotype was identified as an independent factor that was significantly associated with SVR (OR = 5.87, 95%CI: 1.62-21.22; *P* = 0.0058; Table 2).

Among the on-treatment factors, adherence to RBV was significantly higher in non-SVR patients than in SVR patients (*P* = 0.0457) and adherence to Peg-IFN was only marginally higher in non-SVR than in SVR patients (*P* = 0.0936), indicating that these adherence factors did not influence SVR. The duration of Peg-IFNα/RBV combination therapy (36 wk or 48 wk) did not affect the outcome of treatment (Table 2).

SVR rates according to RVR, G2 subtype, and IFNL3 SNPs
The SVR rates were not statistically different between patients with HCV G2a and G2b in either the *IFNL3* genotype TT patient group (90.7% *vs* 88.6%) or the non-TT group (82.6% *vs* 61.5%). The remaining 21 patients (11.7%) were not found to have G2a or G2b. Twenty of 21 patients achieved SVR and the remaining patient (who did not achieve RVR) showed relapse. In the RVR patient group, the SVR rates in HCV G2a patients were comparable to those observed with HCV G2b patients, regardless of the *IFNL3* genotype (Figure 3A).

Among non-RVR patients, the SVR rate in HCV G2b patients with the *IFNL3* TT genotype was significantly higher than in those with the non-TT genotype (81.5% *vs* 28.6%, *P* = 0.0231; Figure 3B). The SVR rate in G2a patients with the TT genotype was higher than in those with the non-TT genotype (78.3% *vs* 50.0%), though the difference was not statistically significant.

Table 1 Analysis of factors affecting rapid virologic response to pegylated interferon-α plus ribavirin combination therapy in patients infected with hepatitis C virus genotype 2

| | RVR | non-RVR | RVR *vs* non-RVR (1: non-RVR/2: RVR) | | | |
| | | | Univariate analysis | Multivariate analysis | | |
			P value	OR	95%CI	*P* value
Baseline factors						
Demographic data						
Number of patients	111	69				
Gender (1:male/2:female)	54/57	31/38	0.8705			
Age (yr)	60 (18-76)	60 (18-80)	0.9623			
Body weight (kg)	59.4 (35.6-90.9)	56.0 (37.0-104)	0.5154			
Body mass index (kg/m^2)	23.4 (17.1-31.2)	22.8 (15.2-34.8)	0.9645			
Histological fibrosis of liver (F0/1/2/3/4/ND)[1]	5/45/18/13/13/17	2/24/9/6/17/11				
(F0-3/F4)	81/13	41/17	0.0202			
Prior interferon and ribavirin treatment response						
Naïve/Relapse/Non response	101/10/0	64/3/2	0.8577			
Laboratory data						
Genetic variation at rs8099917 (TT/TG or GG)	87/24	55/14	0.9800			
HCV-RNA (log$_{10}$IU/mL)	5.5 (5.0-7.0)	6.4 (5.0-7.3)	< 0.0001	4.40	2.24-8.63 (per 1.0 log$_{10}$IU/mL)	< 0.0001
HCV Genotype (2a/2b/2a + 2b/ND)	69/23/2/17	29/34/2/4	0.0005			
White blood cells (/μL)	4800 (2400-10300)	4700 (2000-10200)	0.5170			
Hemoglobin (g/dL)	14.1(9.5-18.2)	13.8 (9.3-16.9)	0.1899			
Platelets (× 10^4/μL)	17.1 (4.8-34.1)	16.6 (5.5-34.1)	0.7993			
Aspartate aminotransferase (IU/L)	48 (16-317)	48 (13-455)	0.6753			
Alanine aminotransferase (IU/L)	57 (10-361)	56 (9-356)	0.9699			
Gamma-glutamyl-transpeptitase (IU/L)	43 (7-719)	48 (9-331)	0.7526			
Albumin (g/dL)	4.1 (3.2-5.0)	4.0 (2.9-4.8)	0.0029	0.13	0.04-0.42 (per 1.0 g/dL)	0.0006
Fasting total cholesterol (mg/dL)	170 (118-265)	173 (106-270)	0.8155			
Fasting low density lipoprotein-cholesterol (mg/dL)	99 (45-158)	103 (40-181)	0.4186			
Fasting plasma glucose (mg/dL)	99 (76-221)	100 (76-320)	0.2002			
Homeostasis model assessment-Insulin Resistance	1.59 (0.56-10.83)	1.38 (0.79-6.88)	0.4722			
Alpha-fetoprotein (ng/mL)	4.7 (1.6-193.6)	5.0 (1.0-130)	0.4101			

[1]Classified by METAVIR score. Data are expressed as number of patients or median (range); ND: Not done; HCV: Hepatitis C virus; RVR: Rapid virologic response.
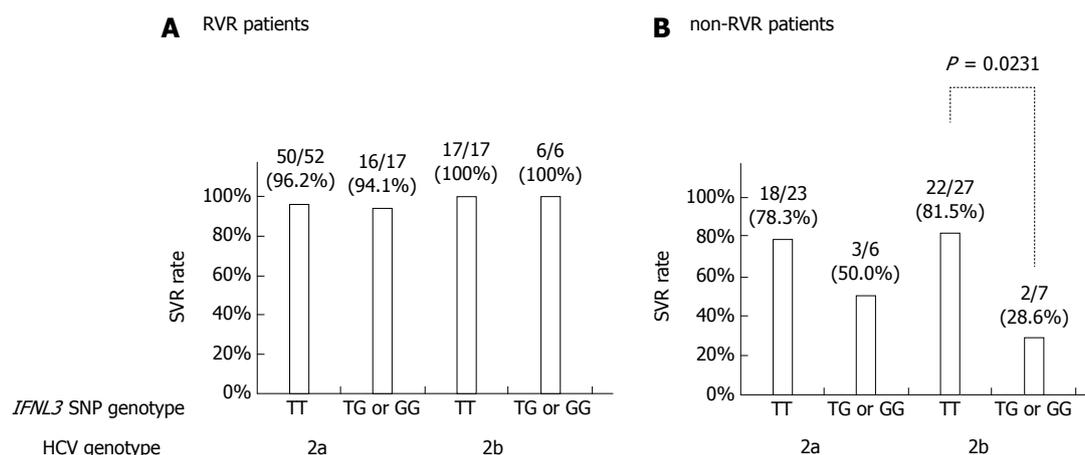
Figure 3 Sustained virologic response rates according to interferon-λ3 single nucleotide polymorphism genotype and hepatitis C virus sub-genotype. A: Rapid virologic response (RVR); and B: Non-RVR patient groups. HCV: Hepatitis C virus; RVR: Rapid virologic response; SNP: Single nucleotide polymorphism; IFNL3: Interferon-λ3.

DISCUSSION

IFNL3 SNPs, such as rs8099917 and rs12979860, have a strong impact on virologic responses in chronically HCV G1-infected patients to Peg-IFNα/RBV combination therapy[7-9]. However, more potent antiviral treatments, including direct-acting antiviral agents (DAAs), would attenuate the value of *IFNL3* SNPs as a predictor of treatment outcome, because they could markedly improve the SVR rate. In countries/

Table 2 Analysis of factors affecting sustained virologic response in non-rapid virologic response hepatitis C virus genotype 2 patients treated with 36 or 48-wk pegylated interferon-α plus ribavirin combination therapy

| | SVR | non-SVR | SVR *vs* non-SVR (1: non-SVR/2: SVR) | | | |
| | | | Univariate analysis | Multivariate analysis | | |
			P value	OR	95%CI	*P* value
Baseline factors						
Demographic data						
Number of patients	50	19				
Gender (1:male/2:female)	20/30	11/8	0.2873			
Age (yr)	60.5 (18-80)	53 (39-73)	0.2341			
Body weight (kg)	55.0 (37.0-101.0)	64.0 (44.0-104.0)	0.1563			
Body mass index (kg/m^2)	22.8 (15.2-32.8)	22.8 (18.8-34.8)	0.2426			
Histological fibrosis of liver (F0/1/2/3/4/ND)[1]	1/18/6/6/10/9	1/6/3/0/7/2				
(F0-3/F4)	31/10	10/7	0.2050			
Prior interferon and ribavirin treatment response						
Naïve/Relapse/Non response	46/3/1	18/0/1	0.8933			
Laboratory data						
Genetic variation at rs8099917 (TG or GG/TT)	6/44	8/11	0.0146	5.87 (1; TG or GG/ 2; TT)	1.62-21.22	0.0059
HCV-RNA (Log10IU/mL)	6.3 (5.0-7.3)	6.4 (5.0-7.2)	0.6064			
HCV Genotype (2a/2b/2a + 2b/ND)	21/24/2/3	8/10/0/1	0.8156			
White blood cells (/μL)	4700 (2800-7800)	5550 (2000-10200)	0.3001			
Hemoglobin (g/dL)	13.8 (11.0-16.9)	13.75 (11.0-16.8)	0.9279			
Platelets (× 10^4/μL)	16.6 (6.0-34.1)	16.55 (6.1-29.7)	0.9834			
Aspartate aminotransferase (IU/L)	54 (15-455)	32 (13-264)	0.0751			
Alanine aminotransferase (IU/L)	59 (12-356)	36.5 (9-231)	0.1866			
Gamma-glutamyl-transpeptitase (IU/L)	49 (9-292)	47 (11-331)	0.9237			
Albumin (g/dL)	4.0 (2.9-4.8)	3.9 (3.1-4.7)	0.5738			
Fasting total cholesterol (mg/dL)	172 (108-256)	178 (106-270)	0.6894			
Fasting low density lipoprotein-cholesterol (mg/dL)	102 (54-177)	110 (40-181)	0.9647			
Fasting plasma glucose (mg/dL)	98.5 (76-320)	103.5 (93-158)	0.1554			
Homeostasis model assessment-Insulin Resistance	1.36 (0.78-6.78)	1.44 (1.19-5.39)	0.3151			
Alpha-fetoprotein (ng/mL)	5.0 (1.0-96.0)	5.0 (2.0-130)	0.8593			
On-treatment factors						
Treatment						
Adherence of Peg-IFN (%)[2]	77.1 (62.5-100.0)	91.4 (66.0-104.6)	0.0936			
Adherence of RBV (%)[2]	75.0 (56.8-100.0)	86.1 (86.1-94.4)	0.0457			
Duration of treatment (wk)	37 (36-48)	48 (36-48)	0.1449			

[1]Classified by METAVIR score; [2]Calculated on the basis of 48-wk treatment. Note: Data are expressed as number of patients or median (range); ND: Not done; SVR: Sustained virologic response; HCV: Hepatitis C virus; Peg-IFN: Pegylated interferon; RBV: Ribavirin.

areas where DAAs are not available, Peg-IFNα/RBV combination therapy is still the standard of care for HCV G2 patients. Therefore, *IFNL3* SNPs still have prognostic value in such settings[10-13].

In a previous study from Japan, *IFNL3* SNPs were found to be an independent predictive factor for SVR (but not RVR) in patients infected with HCV subtype G2b, but not G2a[11]. However, the study analysis included both RVR patients and non-RVR patients in whom the treatment duration was limited to 24 wk and not extended to 36 or 48 wk. Another study conducted in the United States showed that the *IFNL3* rs12979860 genotype was associated with SVR to 24-wk Peg-IFNα/RBV combination therapy in HCV-2/3 patients who did not achieve RVR[12]. Our findings were in partial concordance with these results; the *IFNL3* SNP significantly influenced the achievement of SVR in patients who did not attain RVR, but did not affect SVR in RVR patients. Therefore, *IFNL3* SNP genotyping is valuable for predicting SVR only in non-RVR patients, irrespective of G2 subtype, even if Peg-

IFNα/RBV combination therapy is extended to 36 or 48 wk. Conversely, neither *IFNL3* SNPs nor G2 subtypes are associated with SVR in RVR patients. However, the relatively small number of patients in our study may limit the conclusions that can be drawn, and these results should be verified in a larger study cohort.

The SVR rate for HCV G2 patients in our study was similar to those reported in previous studies[17,18]. As the SVR rate was very high (96.4%) in patients who achieved RVR and were treated with standard 24-wk Peg-IFNα/RBV combination therapy, the treatment period of 24 wk is sufficient and could be abbreviated without reducing the SVR rate. Conversely, the SVR rates following 24-wk Peg-IFNα/RBV combination therapy were reported to be fairly low in non-RVR patients[6,19], and response-guided extension to 36 or 48 wk has been used to improve treatment efficacy[5,6,20]. However, our previous study[6] and the present study reveal that there are no distinct differences in the SVR rates of non-RVR patients who receive either 36 or 48 wk of therapy, and that the

SVR rate is significantly lower in non-RVR patients (treated for 36 wk or 48 wk) than in RVR patients (treated for 24 wk). These findings suggest that there are limitations to prolonged treatment duration in non-RVR patients. Specifically, this study highlights the low SVR rates in non-RVR patients with unfavorable *IFNL3* genotypes.

In the near future, DAA-based combination therapy will be used worldwide as the first-line therapy for treating chronic HCV G2 infection because extremely high SVR rates can be attained with shorter treatment durations and without distinctive side effects[21-23]. In many countries/areas, however, Peg-IFNα/RBV combination therapy will still be the standard of care before DAAs are approved and available. Until then, response-guided therapy based on RVR to Peg-IFNα/RBV combination therapy is useful in yielding high SVR rates for RVR patients and reducing economic and physical burdens by prematurely discontinuing unnecessary treatment for non-RVR patients. Alternatively, to make a decision to continue treatment in non-RVR patients, *IFNL3* genotyping may be valuable in predicting the probability of achieving SVR.

In conclusion, neither the *IFNL3* SNP genotype nor the G2 subtype influenced the probability of achieving SVR in RVR patients treated with response-guided Peg-IFNα/RBV combination therapy. However, the SVR rate in non-RVR patients was higher in those with the *IFNL3* TT genotype compared to those with the non-TT genotype, irrespective of G2 subtype, even if therapy was extended to 36 or 48 wk, indicating that the *IFNL3* SNP has a significant impact only on the achievement of SVR in non-RVR patients.

ACKNOWLEDGMENTS

COMMENTS

Background

Genotype 2 (G2) hepatitis C virus (HCV) is the second-most frequent HCV genotype and accounts for approximately 30% of chronic HCV infections in Japan. Most HCV G2 patients who achieve rapid virologic response (RVR) in 24-wk response-guided pegylated interferon-α plus ribavirin (Peg-IFNα/RBV) combination therapy achieve sustained virologic response, whereas a fraction of patients who do not achieve RVR may remain uncured even when therapy is extended for 36 or 48 wk. The impact of interferon-λ3 (*IFNL3*) single nucleotide polymorphisms (SNPs) on Peg-IFNα/RBV combination therapy for HCV G1 has been firmly established. However, it remains controversial whether the *IFNL3* genotype is useful in predicting virologic responses of HCV G2 patients to Peg-IFNα/RBV therapy.

Research frontiers

IFNL3 genotyping is advantageous in clinical practice for patients who do not achieve RVR. The results of this study provide a strong rationale for the use of *IFNL3* SNPs testing to personalize antiviral therapy.

Innovations and breakthroughs

This work aims at emphasizing the role of *IFNL3* SNPs in HCV G2 patients who received Peg-IFNα/RBV combination therapy. In non-RVR patients, the evaluation of the *IFNL3* SNPs still holds significance to establish the therapeutic schedule.

Applications

In patients with *IFNL3* non-TT genotypes and non-RVR, clinicians should not extend treatment with combination therapy. The relevance of this approach is cost-effective at the time of DAA therapy.

Terminology

IFNL3, located 8 kb upstream of the interleukin-28B gene, is a cytokine that plays a role in HCV clearance.

Peer-review

The authors describe associations of *IFNL3* genotypes with Peg-IFNα/RBV treatment outcome in HCV G2 patients who do not achieve RVR. The data are interesting, in that a role for *IFNL3* genotype in treatment outcome for HCV G2 patients is demonstrated only in patients not achieving RVR. These data contribute to the *IFNL3* literature and thus merit reporting.

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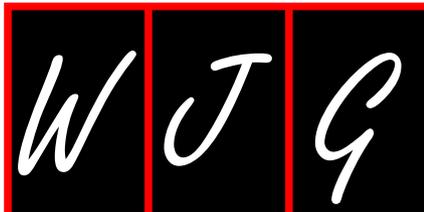
Retrospective Cohort Study

Hepatitis C virus recurrence after liver transplantation: A 10-year evaluation

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Abstract

AIM: To evaluate the predictors of 10-year survival of patients with hepatitis C recurrence.

METHODS: Data from 358 patients transplanted between 1989 and 2010 in two Italian transplant centers and with evidence of hepatitis C recurrence were analyzed. A χ^2, Fisher's exact test and Kruskal Wallis' test were used for categorical and continuous variables, respectively. Survival analysis was performed at 10 years after transplant using the Kaplan-Meier method, and a log-rank test was used to compare groups. A *P* level less than 0.05 was considered significant for all tests. Multivariate analysis of the predictive role of different variables on 10-year survival was performed by a stepwise Cox logistic regression.

RESULTS: The ten-year survival of the entire popu-

lation was 61.2%. Five groups of patients were identified according to the virological response or lack of a response to antiviral treatment and, among those who were not treated, according to the clinical status (mild hepatitis C recurrence, "too sick to be treated" and patients with comorbidities contraindicating the treatment). While the 10-year survival of treated and untreated patients was not different (59.1% *vs* 64.7%, *P* = 0.192), patients with a sustained virological response had a higher 10-year survival rate than both the "non-responders" (84.7% *vs* 39.8%, *P* < 0.0001) and too sick to be treated (84.7% *vs* 0%, *P* < 0.0001). Sustained virological responders had a survival rate comparable to patients untreated with mild recurrence (84.7% *vs* 89.3%). A sustained virological response and young donor age were independent predictors of 10-year survival.

CONCLUSION: Sustained virological response significantly increased long-term survival. Awaiting the interferon-free regimen global availability, antiviral treatment might be questionable in selected subjects with mild hepatitis C recurrence.

Key words: Hepatitis C; Liver transplantation; Hepatitis C virus recurrence; Antiviral treatment; Ten-year survival

Core tip: The recurrence of hepatitis C virus (HCV) infection after liver transplantation is still a great clinical challenge. Currently, the treatment opportunities are growing with the development of new antivirals; however, in several countries, their availability will not be immediate. The decision to start treatment for HCV recurrence might be difficult in some cases, and the data on the long-term impact are extremely useful in this setting. This study reports the results of 10-year survival analysis on an Italian cohort of liver transplant cases focusing on the differences in outcomes, not only between the treated and not-treated subjects but also in specific subgroups of patients with mild recurrence and those considered too sick to be treated.

INTRODUCTION

End stage liver disease (ESLD) due to hepatitis C virus (HCV) infection is the leading indication for liver transplantation (LT) in Europe and in the United States[1]. The lack of an effective prophylaxis makes the recurrence of the graft virtually universal and chronic hepatitis is found at liver biopsy in the vast majority of cases within one year after LT[2]. Recurrent liver disease is much more aggressive after a LT with respect to the pre-LT period, as progression to cirrhosis at 5 years occurs in 10%-50% of the recipients[2-4]. The efficacy of antiviral therapy with interferon-α (IFN-α), either pegylated (Peg) or not, and ribavirin (RBV) is lower in comparison to non-transplant patients. Moreover, significant concerns remain about potential serious adverse events in the post-LT period, including the risk of rejection[5,6]. Notably, indications for antiviral therapy have changed over the years. In the past, when only non-pegylated interferon was available and the data regarding its efficacy were very limited, antiviral therapy was started when there was evidence of disease progression (increasing fibrosis) at repeated liver biopsies[7]. More recently, after the introduction of pegylated interferon, antiviral therapy was initiated at an earlier stage, when active hepatitis was found at first year liver biopsy[7]. Although widely accepted guidelines for antiviral therapy in a LT setting do not exist[6], in every day practice, the vast majority of patients with HCV-recurrence experience at least one attempt of antiviral therapy. This purposeful approach is likely to become increasingly adopted by currently practicing clinicians according to the availability of shorter and better tolerated antiviral regimens.

By transposing the clinical end points of the antiviral treatment in the immunocompetent patient, published studies[8-10] have focused their attention on the identification of predictors of a virological response in the post-LT phase. However, very interesting studies[7,11-16] attempted to go deeper into the problem and to understand whether antiviral treatment could really change the long-term survival of recipients with HCV-recurrence. In particular, Bizollon *et al*[14] and Picciotto *et al*[15] reported the positive role of a sustained virological response (SVR) achievement in the post-LT period while Veldt *et al*[16] demonstrated that antiviral treatment itself was able to improve overall graft survival of treated patients in respect to the untreated.

In this complex context, direct acting antivirals (DAAs) represent a new era in HCV infection considering the excellent virological response rates. Triple regimens including PegIFN/RBV combined with first generation NS3/4A protease inhibitors (PIs) boceprevir (BOC) and telaprevir (TVR) were proposed, with an increased virological response rate but also with a high rate of adverse events. The second and third wave DAAs comprise new NS3/4A PIs, NS5A inhibitors, and nucleotide and non-nucleotide NS5B polymerase-inhibitors[17]. Among the latter, studies on the treatment of HCV recurrence are producing excellent results[18,19]. Nevertheless, the broad availability of these potent

Table 1 Study population characteristics *n* (%)

n	358
Gender (M/F)	281 (78.5)/77 (21.5)
Age at LT (yr, mean ± SD)	52 ± 9
Donor age (yr, mean ± SD)	53 ± 17
HCV Genotype	
1	212 (59.2)
2	48 (13.4)
3	57 (15.9)
4	41 (11.5)
Immunosuppression	
Cyclosporine	269 (75.1)
Tacrolimus	83 (23.2)
Other regimens	6 (1.7)
Antiviral therapy after LT	
Treated	150 (41.9)
Untreated	208 (58.1)

HCV: Hepatitis C virus; LT: Liver transplantation.

antiviral agents will raise important cost issues, especially, but not only, in developing countries. Thus, the careful identification of predictive factors of long-term overall efficacy is currently required.

In this study, we analyzed the clinical records of transplanted patients with HCV-recurrence followed up by two major Italian Tertiary Hospitals. The aim of this study was to evaluate the determinants of 10-year survival for patients with HCV recurrence and the differences between treated and untreated patients.

MATERIALS AND METHODS

The study population comprised 358 LT patients with established HCV-recurrence followed at the Hepatology Outpatient Clinic of Semeiotica Medica Unit, S. Orsola-Malpighi Hospital, University of Bologna, Italy and at the Department of Hepatology and Gastroenterology, Niguarda Hospital, Milan, Italy.

Patients were transplanted between January 1989 and December 2010, and most (73.5%) were at the Liver Transplant Centre in Milan. Study population characteristics are shown in Table 1.

HCV-recurrence after LT was defined in all cases by the positivity (> 50 IU/mL) of serum HCV-RNA and histological evidence of hepatitis at liver biopsy. Liver biopsies were evaluated by experienced pathologists using Ishak's scoring system[20].

For patients who underwent antiviral therapy, treatment consisted of IFN-α (either Peg- or not) in association with RBV in all cases with an intended duration of 48 wk of treatment. The type of virological response to treatment was established as a SVR or non-response (NR). SVR was defined as undetectable serum HCV-RNA 24 wk after discontinuation of treatment. The NR group included all non-SVR patients.

Statistical analysis

All sample data were encoded by a physician trained in statistics and data were included in a dedicated database.

Full descriptive statistical analysis was carried out on all evaluated parameters. Data are expressed as the mean ± SD or as median and range, where more appropriate and as indicated. Confidence intervals (CI) are presented whenever appropriate. The significance of differences between variables was calculated with nonparametric tests. A χ^2/Fisher's exact test was used for categorical variables. A Kruskal Wallis' test was used for continuous variables.

Survival analysis was performed at 10 years after LT using the Kaplan-Meier method, and a log-rank test was used to compare groups. A *P* level less than 0.05 was considered significant for all tests. Multivariate analysis of the predictive role of different variables on 10-year survival was performed by stepwise Cox logistic regression (variables were entered if *P* < 0.1 and were removed if *P* > 0.05). SPSS® software version 17.0 (MJ Norusis, Chicago, United States) was used to perform all statistics.

RESULTS

The 10-year cumulative survival of all patients included in the study was 61.2% (Figure 1A). In the post-LT period, 150 patients (41.9%) were treated with antiviral therapy while 208 were not. Treated and untreated patients had a similar mean age (52 ± 8 years *vs* 52 ± 9 years, *P* = NS) and were also comparable for gender, donor age and viral genotype. Regarding the type of immunosuppression, cyclosporine was administered more frequently among untreated patients (Table 2).

Sixty-three of 150 treated patients (42%) achieved a SVR.

NR patients had to reduce more frequently the dosage of antiviral therapy in comparison to patients achieving a SVR (72.4% *vs* 38.1%), neutron/thrombocytopenia being the main cause of a decrease in both the NR and SVR groups (74.6% *vs* 83.3%, respectively).

In the intention to treat analysis, the 10-year cumulative survival of treated and untreated patients was not significantly different (59.1% *vs* 64.7%, *P* = 0.192; Figure 1B). However, when analyzing the survival functions (Figure 1B), it can be noticed that curves were somehow irregular, especially those representing the survival of untreated patients. This latter curve clearly shows a rapid slope in its first section because some patients were deceased quite early after LT. Indeed, we stratified the study population according to the type of response to antiviral therapy (SVR *vs* NR) and, for patients who were not treated, according to their clinical status. Thus, we identified 5 different groups of patients: Group A: patients receiving antiviral therapy who achieved a SVR (*n* = 63); Group B: patients receiving antiviral therapy who were NR (*n* = 87); Group C: patients untreated with mild recurrence (*n* = 73);
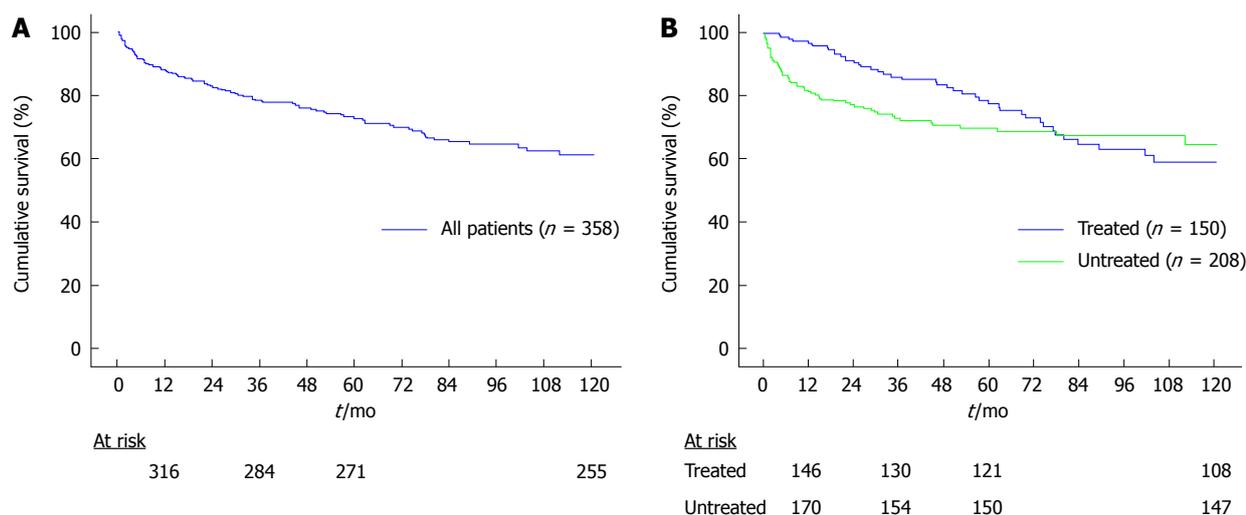
Figure 1 Kaplan-Meyer survival analysis of the entire population (A) and the treated and untreated patients (B).

Table 2 Characteristics of treated and untreated patients n (%)

	Treated	Untreated	χ^2/Mann Whitney-*U*
n	150	208	
Gender (M/F)	120/30	161/47	*P* = NS
Age at LT (yr, mean ± SD)	52 ± 8	52 ± 9	*P* = NS
Donor age (yr, mean ± SD)	53 ± 17	53 ± 17	*P* = NS
HCV genotype			*P* = NS
1	97 (64.7)	115 (55.3)	
2	16 (10.7)	35 (16.8)	
3	25 (16.6)	35 (16.8)	
4	12 (8.0)	23 (11.1)	
Immunosuppression			*P* = 0.011
Cyclosporine	102 (68.0)	165 (79.3)	
Tacrolimus	46 (30.7)	39 (18.8)	
Other regimens	2 (1.3)	4 (1.9)	

HCV: Hepatitis C virus; NS: Not significant.

Group D: patients too sick to be treated (*n* = 35); Group E: patients with clinically relevant comorbidities that contraindicated antiviral therapy (*n* = 100).

Patients were considered to have a mild recurrence (group C) in the case of a mild transaminase increase (alanine amino transferase < 3x the upper normal limit) and mild fibrosis (Ishak stage < 3) at the first post-LT liver biopsy performed within three years after LT. By definition, these subjects did not show graft malfunction or early complications after LT.

Patients were included in the too sick to be treated group when they were not suitable for antiviral treatment because of graft malfunction and/or early complications after LT.

Patients achieving a SVR showed the best survival time (mean 73 ± 35 mo, median 70, range: 13-120 mo), followed by subjects with mild recurrence (mean: 71 ± 37 mo, median 64, range: 7-120 mo), patients who were NR (mean 57 ± 34 mo, median 52, range: 4-120 mo), patients not treated for comorbidities
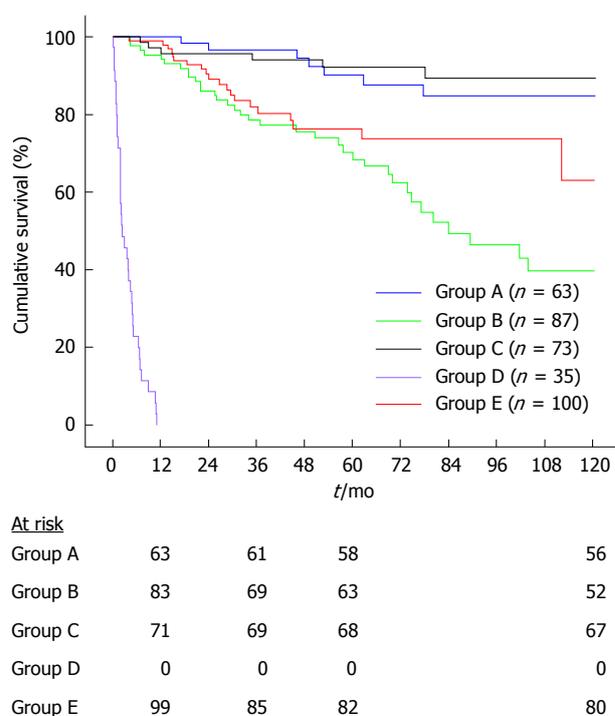
Figure 2 Kaplan-Meyer survival analysis by groups. Group A: patients receiving antiviral therapy who achieved a SVR; Group B: patients receiving antiviral therapy who were NR; Group C: patients untreated with mild recurrence; Group D: patients too sick to be treated; Group E: patients with clinically relevant comorbidities that contraindicated antiviral therapy. SVR: Sustained virological response; NR: Non-response.

(mean 48 ± 32 mo, median 36, range: 4-120 mo) and finally, as expected, patients too sick to be treated (mean 4 ± 3 mo, median 2, range: 0-11 mo).

The survival curves of the five groups are reported in Figure 2. Patients who achieved a SVR (group A) had a significantly higher 10-year survival than the NR group (group B; 84.7% *vs* 39.8%, respectively, *P* < 0.0001). Conversely, SVR patients (group A) had a 10-year survival rate comparable to that observed

Table 3 Characteristics of patients divided by groups

Group	A	B	C	D	E	χ^2/Kruskall Wallis
	SVR	NR	Mild recurrence	Too sick	Comorbidity	
n	63	87	73	35	100	
Gender (M/F)	47/16	73/14	63/10	20/15	78/22	*P* = 0.007
Age at LT (yr, mean ± SD)	52 ± 8	52 ± 9	53 ± 8	52 ± 9	51 ± 9	*P* = NS
Donor age (yr, mean ± SD)	49 ± 17	56 ± 15	48 ± 17	59 ± 15	55 ± 17	*P* = 0.001
HCV genotype						*P* = NS
1	37	60	45	16	44	
2	7	6	9	3	13	
3	9	13	6	5	14	
4	8	5	7	1	10	
Immunosuppression	48	55	54	24	83	*P* = NS
Cyclosporine	14	31	16	10	13	
Tacrolimus	1	1	3	1	4	
Other regimens						
Time between treatment start and LT [mo, median (min-max)]	20 (1-148)	16 (1-117)				*P* = NS
Treatment Length [mo, median (min-max)]	11 (1-61)	6 (1-63)				*P* = 0.010

SVR: Sustained virological response; NR: Non-response; HCV: Hepatitis C virus; NS: Not significant; LT: Liver transplantation.

Table 4 Causes of death

Group	A	B	C	D	E
	SVR	NR	Mild recurrence	Too sick	Comorbidity
n	63	87	73	35	100
No. of deaths at 10 yr after LT, *n* (%)	7 (11.1)	35 (40.2)	6 (8.2)	35 (100)	20 (20)
Liver related, *n* (%)	2 (28.6)	22 (62.9)	3 (50)	11 (31.4)	10 (50)
HCV recurrence	1	17	2	3	1
HCC	1	4	1	1	8
Primary non function	0	0	0	6	0
Other liver-related	0	1	0	1	1
Non liver-related, *n* (%)	5 (71.4)	13 (37.1)	3 (50)	24 (68.6)	10 (50)
Cardiac	1	0	0	0	0
Renal	0	2	0	0	0
Cancer	0	1	0	3	6
Infection	0	6	1	10	3
Vascular	0	0	1	0	0
Other comorbidity	4	4	1	11	1

SVR: Sustained virological response; HCC: Hepatocellular carcinoma; NR: Non-response; LT: Liver transplantation.

in untreated patients with mild recurrence (group C; 84.7% *vs* 89.3%, respectively, *P* = 0.639). As expected, patients with the worst prognosis were those included in group D (too sick to be treated).

The most relevant clinical features of the five proposed groups are reported in Table 3.

The groups were comparable for mean age at the time of LT and HCV-genotype. Patients who obtained a SVR (group A) and those who were not treated because of mild recurrence (group C) had a significantly younger donor age when compared to patients who were not treated because they were too sick (group D). In keeping with the available literature showing that cyclosporine may favor a response to anti-viral therapy[21], our patients achieving a SVR were more frequently on cyclosporine (76.19%) than those who did not achieve a SVR (63.21%) although there was no statistically significant difference. As expected,

our SVR patients (group A) were treated longer than patients who were NR (group B).

Going deeper into the analysis of patients with a mild recurrence (group C), we further identified a subgroup of 44 patients with mild recurrence and without any relevant comorbidity. Indeed, the results in terms of 10-year survival of this subgroup were the best among the considered groups (95.1%).

Causes of death

Main causes of death are reported in Table 4. Among the 63 patients who achieved a SVR (group A), 7 (11.1%) died within 10 years after LT, but only 2 (28.6%) died because of a liver-related cause. Among the 87 NR patients (group B), 35 (40.2%) died within 10 years after LT and, notably, 22 (62.9%) for a liver-related cause, which was mainly HCV-recurrence (17/22, 77.3%). Of the 73 patients with a mild

recurrence (group C), only 6 (8.2%) died within 10 years after LT, but only 3 (50.0%) for liver-related reasons, with 2 of these 3 (66.7%) dying because of HCV-recurrence. All 35 patients who were not treated because they were too sick to be treated (group D) died within 1 year after LT, with the main causes not being liver-related (24/35, 68.6%) and but linked to infections (10/24, 41.7%). Finally, of the 100 patients with relevant comorbidities (group E), 20 (20%) died within 10 years after LT, with half dying because of liver-related causes (10/20, 50%), mainly because of hepatocellular carcinoma recurrence (8/10, 80%).

Multivariate analysis
All the following variables were tested in a univariate analysis: gender, HCV genotype (1 and 4 *vs* others), type of immunosuppression (cyclosporine *vs* tacrolimus), donor age (< 53 years *vs* $\geq$ 53 years), recipient age (< 52 years *vs* $\geq$ 52 years), treatment *vs* no-treatment and SVR *vs* NR. The cut-off values of 53 years and 52 years were chosen as the means of both donor and recipient ages, respectively. Only having a SVR and a younger donor age showed a prognostic value ($P < 0.1$) in the univariate analysis.

In the multivariate analysis, a SVR and donor age < 53 years were confirmed to be independent predictors of 10-year survival (OR = 4.05, 95%CI: 1.81-9.05, P = 0.001 and OR = 3.05, 95%CI: 1.47-6.30, P = 0.001, respectively).

DISCUSSION

Because LT is agreed to be a life-saving therapy for end-stage liver disease, HCV has become one of its leading indications. Re-infection of the graft is virtually universal and HCV recurrence after LT is a "new disease" whose clinical, virological and immunological characteristics are determined by complex, and almost unknown, interactions between patient, graft and the virus. However, it is well known that fibrosis progression is accelerated in HCV recurrence compared with the pre-LT period and a small proportion of patients (approximately 5%) show a fast and aggressive disease after LT[22]. In this context, it can be very complex to decide whether and when it is appropriate to treat the HCV recurrence.

Berenguer *et al*[7] compared 89 treated patients with 75 matched untreated controls and, among the treated cases, patients who achieved a SVR with subjects who did not. The authors first demonstrated that all the main measures of outcome (mortality, development of cirrhosis and decompensation) were worse for untreated patients compared to patients who were treated and for NR compared to SVR. Our study confirms the findings of Berenguer *et al*[7] regarding the positive role of SVR. Moreover, it offers an additional analysis focused on the long-term outcome of the broad and heterogeneous population of

untreated patients, which has never been thoroughly investigated.

Our data indicate that the treatment itself was not associated with a better long-term outcome. In fact, we showed that treated and untreated patients had a comparable 10-year survival rate. However, the untreated group comprised a very inhomogeneous population. In particular, the reason for the choice not to start antiviral therapy could also be related to conditions different from a mild disease all having a distinct impact on survival, such as a too severe hepatic condition, a comorbidity (of varying severity) or the lack of compliance.

It should be acknowledged that the design of the study from Berenguer *et al*[7] was more appropriate as the results emerged from the comparison with a population of matched controls. Indeed, we analyzed a heterogeneous group of untreated patients, which comprised three different subgroups: (1) patients with mild HCV recurrence; (2) patients too sick to be treated; and (3) patients with comorbidities. Interestingly, untreated patients with mild HCV recurrence showed a 10-year survival rate that was comparable to treated patients achieving a SVR (89.3% *vs* 84.7%, respectively). Notably, patients included in the mild recurrence subgroup had no clinically relevant comorbidities nor graft malfunction and/or complications after LT. In fact, these conditions a priori can affect long-term survival.

Our data confirm that a SVR is an independent predictor of 10-year survival as only two SVR patients of 63 died of an HCV-related cause in the follow-up period. Together with achieving a SVR, our study once again confirms the impact of donor age on the severity of HCV-recurrence, the response to antiviral therapy and survival. In fact, it has been largely reported that older donor age negatively affects the progression of fibrosis in transplanted patients[23-27].

Regarding the treatment of HCV recurrence, it is important to consider the oncoming availability of DAAs. Currently, several reports on first wave PI treatment in the post-LT period are available. Recent studies reporting data on the efficacy and safety of BOC/TVR and PegIFN/RBV[28,29] showed encouraging efficacy results but also a complex side effects profile including infections, hematological and dermatological toxicity, renal failure, diabetes, drug-drug interactions with immunosuppressants and severe forms of plasma-cell hepatitis with occasional fatal outcomes[28-32]. The data on triple BOC/TVR based regimens are of great importance considering that the second/third wave DAA cost issue will continue to produce controversy for a certain amount of time in the future. Sofosbuvir, a pan-genotypic inhibitor of polymerase activity, is reported to have a good virological outcome and favorable safety profile when associated with Daclatasvir[33] or PegIFN ± RBV[18,19,34], and also in the treatment of severe HCV recurrence.

The main limitation of our study is the retrospective design. Nonetheless, this approach is a feasible one to analyze the long-term outcomes of transplanted patients. The most important issue emerging from our data is that the antiviral treatment should be undertaken in patients with moderate-severe disease because of the high risk of progression to recurrent cirrhosis, decompensation and death in the time of a few years[2,3]. Evidence has emerged from our study and many others that clearly indicate that achievement of a SVR may avoid this unfortunate course. On the other hand, our data suggest that patients with mild HCV recurrence have a very favorable long-term outcome even if untreated. Interestingly, a prospective randomized trial conducted in Italy confirms, at least partially, the observations emerging from our retrospective cohort[35]. Notably, it appears mandatory to have, in the first three years after LT, a correct histological classification for patients with HCV recurrence.

Our results can be useful in the complex decision-making process regarding whether and when to start antiviral treatment in LT recipients with HCV recurrence. This is even more important today with the availability of new DAAs, whose inappropriate use can dramatically increase costs, adverse event rates, drug-drug interactions and the risk of a virological resistance outbreak. Finally, the results from our cohort reveal that a certain rate of mortality in post-LT HCV recurrence concerns patients with comorbidities that are often considered as contraindications to antiviral treatment. Along with the availability of antiviral agents with a low-toxicity profile, the limitations related to the patients' eligibility for the treatment are expected to be reassessed. Furthermore, according to recent compassionate program derived results, the antiviral treatment will become increasingly applicable in the so called too sick to be treated population[18]. However, in those cases in whom a severe HCV recurrence is diagnosed, due to the latter's rapidly progressive nature, the treatment should be started as soon as possible.

Thus, more information from predictive analyses are necessary at this moment because data focused on the long-term effectiveness of antiviral therapy would help with a more feasible guidelines conception, correct clinical approach and rational cost-effectiveness treatment management in the LT population.

In conclusion, awaiting the consolidation of new interferon-free regimens, we suggest that, in carefully selected patients with predictors of long-term favorable outcomes, antiviral treatment might be delayed. Most likely, the development of interferon-free regimens will completely change the approach to HCV in both pre- and post-LT settings. Nevertheless, studies focusing on the mechanisms and factors leading to a mild HCV recurrence will still be extremely useful.

COMMENTS

Background
End stage liver disease due to hepatitis C is a leading indication for liver transplantation, however, infection recurrence is virtually universal. Accepted guidelines on the best timing for starting antiviral treatment do not exist. New direct antivirals represent a new era in hepatitis C virus (HCV) infection considering both the excellent virological response rates and the problems related to side effects and costs. In this complex context, the correct identification of predictive factors of long-term overall efficacy is required.

Research frontiers
It has already been demonstrated that to treat LT patients with hepatitis C recurrence with antiviral therapy leads to a better survival rate, decreasing the probability to develop cirrhosis and decompensation. Moreover, among treated patients, a sustained virological response (SVR) is a strong predictor of a good outcome. On the other hand, among the positive predictors of survival in transplanted subjects, young donor age is certainly the most consolidated predictor. Few data are available on predictors of long-term survival and outcomes of untreated patients.

Innovations and breakthroughs
This study firstly included a large number of patients evaluated with a ten-year analysis. In addition, this study did not only consider treated patients but also analyzed the outcomes of subjects who, for different reasons, did not receive antiviral therapy.

Applications
The most important conclusion emerging from our results is that antiviral treatment should be given to patients with moderate or severe recurrence because of the high risk of progression to recurrent cirrhosis, decompensation and death in a few years. Authors' data also showed that patients with mild recurrence have a good long-term outcome even if untreated. These results may be helpful in the decision-making process regarding whether and when to start antiviral therapy.

Terminology
Hepatitis C recurrence after liver transplantation was defined by positivity of serum HCV-RNA (> 50 IU/mL) and histological evidence of hepatitis at liver biopsy. A SVR was defined as undetectable serum HCV-RNA 24 wk after discontinuation of antiviral treatment.

Peer-review
The paper is interesting and has a surprising result. To identify a transplanted patient group who will fare even better without treatment than those with a sustained virological response is an important finding and helps to sharpen the discussion about treatment resources with the advent of efficient but expensive direct-acting antivirals.

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ORIGINAL ARTICLE

Retrospective Cohort Study

Clinical and computed tomography findings of appendiceal diverticulitis *vs* acute appendicitis

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Abstract

AIM: To study the clinical features and computed tomography (CT) findings of appendiceal diverticulitis *vs* acute appendicitis.

METHODS: We retrospectively reviewed the records of 451 patients who had undergone appendectomy in our institution from January 2007 to September 2012. Patient demographics, clinical features, pathological findings, and surgical outcomes were analyzed. We also compared preoperative CT images of 25 patients with appendiceal diverticulitis with those of 25 patients with acute appendicitis.

RESULTS: Among 451 patients, 44 (9.7%) were diagnosed to have appendiceal diverticulitis and 398 (86.9%) to have acute appendicitis. Patients with appendiceal diverticulitis were older (59 *vs* 37 years, P < 0.001) and had a longer duration of the illness (4.0 d *vs* 1.0 d, P < 0.001). Perforation rates in patients with appendiceal diverticulitis were higher (68% *vs* 27%, P < 0.001). The appendix could be visualized in only 13 patients (52%) among the appendiceal diverticulitis cases, but in all acute appendicitis cases. CT findings suggestive of appendiceal diverticulitis included the absence of fluid collection in the appendix (84% *vs* 12%, P < 0.001), absence of appendicolith (92% *vs* 52%, P = 0.005), and formation of abscess (68% *vs* 16%, P < 0.001). Appendiceal diverticula were identified in 6 patients (24%).

CONCLUSION: Among patients who had undergone appendectomy, 9.7% had appendiceal diverticulitis. Patients with appendiceal diverticulitis had different clinical features and CT findings from patients with acute appendicitis.

Key words: Appendiceal diverticulitis; Acute appendicitis; Computed tomography; Appendectomy; Diverticulosis

Core tip: To study the clinical features and computed tomography (CT) findings of appendiceal diverticulitis, we retrospectively reviewed 451 patients who had undergone appendectomy in our institution. Among 451 patients, 44 (9.7%) were diagnosed with appendiceal

diverticulitis. Patients with appendiceal diverticulitis were older and had a longer duration of illness. Perforation rates in patients with appendiceal diverticulitis were higher. CT findings suggestive of appendiceal diverticulitis included the absence of a fluid collection in the appendix, absence of an appendicolith (92% *vs* 52%, *P* = 0.004), and abscess formation. These findings make it possible to clinically differentiate appendiceal diverticulitis from acute appendicitis.

INTRODUCTION

Diverticula are small, bulging pouches within the bowel wall that typically form within the large intestine, including the appendix. Appendiceal diverticula are relatively rare[1]. Appendiceal diverticulitis is the result of inflammation of the appendiceal diverticulum.

This disease was first described by Kelynack[2] in 1893. The incidence of diverticulitis of the appendix in Europe[3] and the United States[4] is 0.2% and 1.7%, respectively. Appendiceal diverticulitis is considered a relatively rare disease, and has most often been reported in case reports[5].

Acute appendicitis occurs when the appendiceal lumen is obstructed, leading to fluid accumulation, luminal distention, inflammation, and ultimately perforation. Distension of the appendiceal lumen causes dull, periumbilical abdominal pain[6].

Since right lower quadrant pain is the main clinical symptom of both acute appendicitis and appendiceal diverticulitis, appendiceal diverticulitis has been commonly dismissed as a variant of acute appendicitis. Therefore, preoperative diagnosis has been rarely made, and pathologists may fail to differentiate between acute appendicitis and appendiceal diverticulitis.

Several studies have investigated the preoperative diagnosis of appendiceal diverticulitis by identifying inflammation in the appendiceal diverticula using ultrasonography[7] or CT[8]. Recently, two retrospective studies were reported from Japan[9] and Korea[10]. These studies considered appendiceal diverticulitis as a separate clinical entity apart from typical acute appendicitis. We retrospectively reviewed the pathological specimens and clinical charts of patients undergoing appendectomy with a preoperative diagnosis of acute appendicitis. We carefully examined the resected specimens in order to distinguish appendiceal diverticulitis from acute appendicitis.

The aim of this study was to clarify the incidence of appendiceal diverticulitis among patients who underwent appendectomy and to evaluate the clinical features and CT findings of appendiceal diverticulitis.

MATERIALS AND METHODS

In this study, we included 451 patients who had undergone appendectomy in our institution from January 2007 to September 2012. We retrospectively reviewed clinical records and re-examined the histopathological specimens to analyze the patients' pathological findings, clinical characteristics, laboratory findings, operative findings, operative procedures, and postoperative course.

In addition to our review of the pathology reports, the pathologist initially prepared and thoroughly examined the specimens microscopically to detect diverticula. Appendiceal diverticulitis was diagnosed as inflammation of one of the diverticula, with no or slight inflammation of the appendiceal wall[11].

In order to assess the feasibility of making a preoperative CT diagnosis, we compared preoperative CT findings of 25 patients pathologically diagnosed with appendiceal diverticulitis with those of 25 randomly selected patients diagnosed with acute appendicitis. For the retrospective data analysis, patient identification was blocked, and a research identification number was given to each patient.

Statistical analysis

Statistical analysis was performed for the comparison of cases of appendiceal diverticulitis to acute appendicitis using the Wilcoxon signed-rank test and Fisher's exact test. All statistical analyses were performed with JMP 5.0.1J, and all *P* values that were two-sided at a value of < 0.05 were considered statistically significant.

RESULTS

Among 451 patients who underwent appendectomy, 44 (9.7%) were pathologically diagnosed to have appendiceal diverticulitis, while 392 (86.9%), were confirmed to have acute appendicitis. No patients were diagnosed with appendiceal diverticulitis preoperatively. The preoperative clinical characteristics of both appendiceal diverticulitis and acute appendicitis are summarized in Table 1. Operative findings, operative procedures, and postoperative courses of the patients are summarized in Table 2.

Patients with appendiceal diverticulitis were older than those with acute appendicitis (59 *vs* 37 years, *P* < 0.001). Patients with appendiceal diverticulitis had lower white blood cell (WBC) count (13100 *vs* 14000 WBC/μL, *P* = 0.02) and higher C-reactive protein (CRP) level than patients with acute appendicitis (13.6 *vs* 6.8 mg/dL, *P* < 0.001). The median duration of preoperative symptoms (right lower quadrant abdominal pain and/or abdominal distension) was 4.0 d in the diverticulitis group and 1.0 d in the appendicitis group (*P* < 0.001). There was no significant difference

Table 1 Comparison of the clinical features between appendiceal diverticulitis and acute appendicitis

Variable	Appendiceal diverticulitis (*n* = 44)		Acute appendicitis (*n* = 398)		
	Median	Range	Median	Range	*P* value
Age (yr)	59	17-89	35	7-87	< 0.001[2]
Duration of symptoms (d)	4.0	10-30	1.0	0-20	< 0.001[2]
Preoperative CRP (mg/dL)	13.6	0.5-29.7	6.8	0.1-37.2	< 0.001[2]
Preoperative WBC (/μL)	13100	3580-20000	14000	2071-30100	0.02[2]
	Number	Rate	Number	Rate	
Sex (male/total)	32	72%	223	56%	0.06[1]
Comorbidity (yes/total)	26	60%	278	70%	0.20[1]

[1]Fisher's exact test; [2]Wilcoxon signed-rank test.

Table 2 Comparison of intra- and post-operative factor between appendiceal diverticulitis and acute appendicitis

Variable	Appendiceal diverticulitis (*n* = 44)		Acute appendicitis (*n* =398)		
	Median	Range	Median	Range	*P* value
Operating times (min)	77	33-165	55	15-172	< 0.001[2]
Inoperative blood loss (mL)	60	10-970	20	10-650	< 0.001[2]
Postoperative hospital stay (d)	8	4-38	5	2-48	< 0.001[2]
	Number	Rate	Number	Rate	
Ileocecal resection (yes/total)	6	13%	10	2%	0.008[1]
Skin incision (pararectal incision/total)	35	79%	143	36%	0.12[1]
Formation of localized abscess (yes/total)	26	60%	60	15%	< 0.001[1]
Perforation (yes/total)	32	72%	123	31%	< 0.001[1]
Postoperative wound infection (yes/total)	7	15%	32	8%	0.12[1]
Postoperative ileus (yes/total)	3	8%	16	4%	0.30[1]

[1]Fisher's exact test; [2]Wilcoxon signed-rank test.

Table 3 Comparison of intra- and post-operative factor between perforated appendiceal diverticulitis and perforated acute appendicitis

Variable	Perforated appendiceal diverticulitis (*n* = 30)		Perforated acute appendicitis (*n* = 111)		
	Median	Range	Median	Range	*P* value
Operating times (min)	65	45-165	67	25-172	0.98[2]
Inoperative blood loss (mL)	60	10-970	40	10-650	0.05[2]
Postoperative hospital stay (d)	9	5-38	9	3-48	0.77[2]
	Number	Rate	Number	Rate	
Ileocecal resection (yes/total)	4	13%	7	6%	0.20[1]
Skin incision (pararectal incision/total)	27	89%	82	74%	0.11[1]
Formation of localized abscess (yes/total)	22	72%	39	35%	< 0.001[1]
Postoperative wound infection (yes/total)	5	17%	19	17%	0.96[1]
Postoperative ileus (yes/total)	3	11%	12	11%	0.92[1]

[1]Fisher's exact test; [2]Wilcoxon signed-rank test.

in the ratio of comorbidities between the two groups.

All patients were treated with open appendectomy. The rate of perforation was significantly higher in the diverticulitis group than in the appendicitis group. Thirty-two patients (72%) in the diverticulitis group and 123 patients (31%) in the appendicitis group had perforated appendices. The number of patients with localized abscess was significantly higher in the diverticulitis group than the appendicitis group (60% *vs* 15%, *P* < 0.001). The median operative time was longer in the diverticulitis group (77 min *vs* 55 min, *P* < 0.001), and the average amount of operative blood loss was higher in the diverticulitis group than the appendicitis group (60 mL *vs* 20 mL, *P* < 0.001).

In the diverticulitis group, pathologic findings showed that all diverticula were of the false type, lacking a proper muscle layer. Multiple diverticula were found in 55% patients.

The differences in clinical characteristics between the patients with perforated appendiceal diverticulitis and perforated acute appendicitis are summarized in Table 3. In patients with perforations, mean blood loss and the ratio of localized abscess were significantly higher in the diverticulitis group than in the appendicitis group. However, the mean duration of postoperative hospital stay, postoperative morbidity rates, and mortality rates were not significantly different between the two groups.
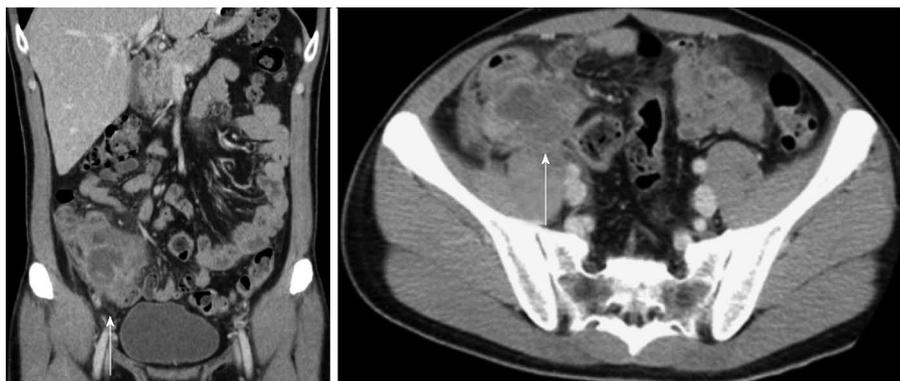
Figure 1 Representative computed tomography images of appendiceal diverticulitis. An oblique coronal reformation of a contrast-enhanced CT (5-mm thick) (arrow) showing localized periappendiceal abscess. However, the appendix itself is not visualized clearly.
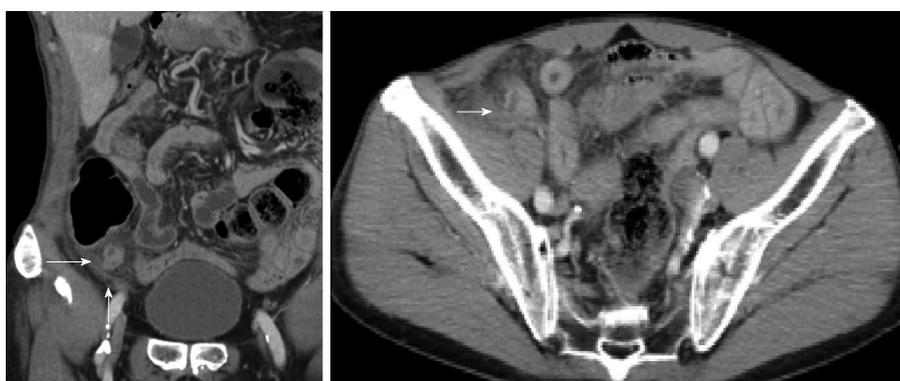
Figure 2 Computed tomography scan showing appendiceal diverticulitis. An oblique coronal reformation of contrast-enhanced CT scan (5-mm thick) showing an inflamed diverticulum (arrow) and stranded surrounding fat. The inflamed diverticulum is visualized as a small, round cyst with an enhancing wall attached to the distal segment of the appendix. However, this patient's appendix was not filled with fluid or enlarged.

The preoperative CT of patients with acute appendicitis showed several typical findings such as enlarged appendixes, appendiceal wall thickening and enhancement, and periappendiceal fat stranding (Figures 1 and 2). On the other hand, in the appendiceal diverticulitis group, the appendixes could not be clearly visualized on preoperative CT scans. In some cases, it was difficult to identify the appendix, even though a large abscess cavity was visible (Figure 3).

CT findings of appendiceal diverticulitis and acute appendicitis are described in Table 4. The appendix was detected in only 13 of 25 scans (52%) in the appendiceal diverticulitis group but was detected in all scans (100%) in the acute appendicitis group. Only 16% of the scans in the appendiceal diverticulitis group showed fluid in the appendix lumen compared to 88% of the scans in the acute appendicitis group. An appendicolith was visualized in 5 of 25 scans (20%) in the appendiceal diverticulitis group and 13 of 25 scans (52%) in the acute appendicitis group. Localized abscess was visualized in 17 scans (68%) with appendiceal diverticulitis, whereas an abscess was identified in only 4 (16%) scans of patients with acute appendicitis. The number of scans showing appendiceal diameter, appendiceal wall thickening, periappendiceal fat stranding, and appendiceal or abscess wall enhancements were not significantly different between the two groups. Appendiceal diverticula were identified in 6 (24%) of the 25 scans in the appendiceal diverticulitis group.

DISCUSSION

The three major findings of this study are as follows: First, among patients who underwent appendectomy, the incidence of appendiceal diverticulitis was 9.7%, which is much higher than that previously reported. Second, our results revealed several clinical characteristics that may assist in the diagnosis of appendiceal diverticulitis, such as older age, longer duration of symptoms, higher rate of perforation, and higher incidence of localized abscess. These findings are consistent with previous reports. Third, several CT findings suggestive of appendiceal diverticulitis were recognized with a comparison of preoperative CT scans of acute appendicitis with those of appendiceal diverticulitis.

Most appendiceal diverticula were false diverticula, formed by herniation of the mucosa and submucosa through a defect in the muscular layer. This is similar to the anatomical derangement seen in diverticula
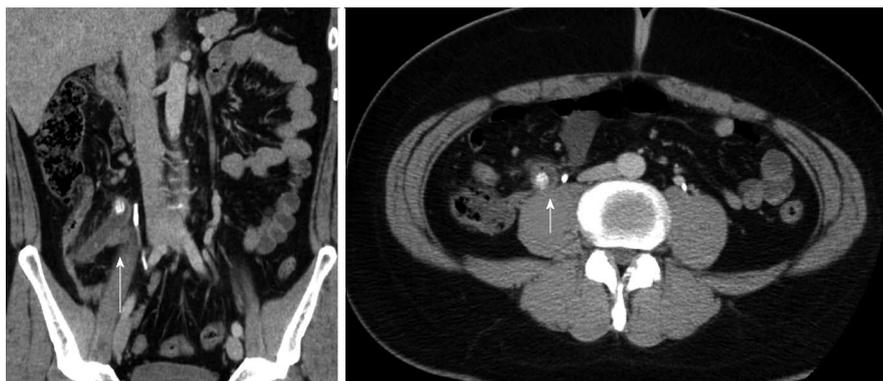
Figure 3 Representative computed tomography scan showing acute appendicitis. An oblique coronal reformation of contrast-enhanced computed tomography scan (5-mm thick) (arrow) is showing an enlarged appendix with fluid collection in the appendiceal lumen and appendicolith.

Table 4 Results of the retrospective computed tomography review

Variable	Appendiceal diverticulitis (*n* = 25)		Acute appendicitis (*n* = 25)		*P* value
	Median	**Range**	**Median**	**Range**	
Appendiceal diameter (mm)	13.2	4.3-23.4	12.4	8.1-18.9	0.25^2
Appendiceal wall thickening (mm)	3.1	1.6-5.3	3.7	2.0-6.2	0.77^2
	Number	**Rate**	**Number**	**Rate**	
Visualized appendix (yes/total)	13	52%	25	100%	$< 0.001^1$
Fluid collection in the appendix lumen (yes/total)	4	16%	22	88%	$< 0.001^1$
Formation of localized abscess (yes/total)	17	68%	4	16%	$< 0.001^1$
Periappendiceal fat stranding(yes/total)	16	66%	11	44%	0.17^1
Appendiceal or abscess wall enhancement (yes /total)	10	58%	9	41%	0.27^1
Apendicolith (yes/total)	2	8%	12	48%	0.0047^1
Visualized appendiceal diverticulum (yes /total)	6	24%	0	0%	0.025^1

[1]Fisher's exact test; [2]Wilcoxon signed-rank test.

of the colon. Since the incidence of appendiceal diverticulitis among patients undergoing appendectomy has been reported to vary from 0.004% to 2.1%[1], appendiceal diverticulitis has been considered very rare. However, in our study, appendiceal diverticulitis was found to be a relatively common condition. We believe that the higher incidence in our study is due to the careful resection and pathological processing of the resected appendixes, allowing visualization of the inflamed diverticula. Inflammation of the appendiceal wall is usually very slight in patients with appendiceal diverticulitis in contrast to the intense inflammation observed in patients with acute appendicitis. If only slight inflammation of the appendiceal wall was noted even if the area around the appendix had abscess, we suspected appendiceal diverticulitis and carefully manipulated the specimens, processing multiple thin sections to detect the diverticulum. Usually, appendiceal diverticulitis is diagnosed only when the diverticulum is clearly visualized on a pathologic section. We speculate that appendiceal diverticulitis is overlooked and frequently misdiagnosed as acute appendicitis because the resected tissue is not adequately examined for diverticula. It is very important for both pathologists and surgeons to consider the possibility of appendiceal diverticulitis and to examine the specimen carefully.

Second, several studies have reported that the clinical features of appendiceal diverticulitis are different from those of acute appendicitis[10,11], and that appendiceal diverticulitis should be classified as a separate and unique diagnostic entity. Indeed, when one considers the mechanisms of onset of both diseases, we may gain insight into the differences in clinical characteristics.

Acute appendicitis occurs when the appendix lumen is obstructed[6,12]. Appendiceal obstruction may be caused by fecaliths, calculi, lymphoid hyperplasia, infectious processes, and benign or malignant tumors. The appendix subsequently becomes filled with mucus and swells, increasing pressure within the lumen and causing dull central or periumbilical abdominal pain[13]. Finally, the appendix becomes ischemic and necrotic, resulting in perforation. Well-localized pain occurs later when inflammation spreads to the adjacent parietal peritoneum.

On the other hand, appendiceal diverticulitis usually occurs in acquired diverticula[14], which contain only the mucosal and submucosal layers without a muscular layer. Because of these anatomical characteristics, diverticula can be easily perforated. Since only slight inflammation may be noted initially, symptoms such as dull central or periumbilical abdominal pain may be very slight. The development

of localized abscess without well-localized pain occurs after several days, prolonging the duration of preoperative symptoms and increasing the rates of perforation and of intraperitoneal abscess in patients with appendiceal diverticulitis. The WBC level was decreased and the CRP level was increased in patients with appendiceal diverticulitis compared to those with acute appendicitis, suggesting that patients with appendiceal diverticulitis had a longer duration of inflammation at admission. These characteristics have also been reported by Yamana et al[10]. Compared to acute appendicitis, appendiceal diverticula occur mostly in older patients[11]. Appendiceal diverticulitis is often associated with localized abscess formation and perforation, often making surgery more difficult with increased surgical time and intraoperative blood loss compared to appendicitis surgery. The clinical features of appendiceal diverticulitis in our study are quite similar to those in previous reports, providing further evidence that the incidence of appendiceal diverticulitis is higher than previously reported.

Third, Lee et al[15] reported CT findings for 20 patients with appendiceal diverticulitis and commented on the diagnostic potential of CT in differentiating appendiceal diverticulitis from typical acute appendicitis. In 80% of patients with inflamed diverticula, CT revealed a round, cystic pouch with wall enhancement. An appendicolith was rarely present in patients with appendiceal diverticulitis compared to patients with acute appendicitis. Osada et al[16] reviewed the CT images of seven patients with pathologically diagnosed appendiceal diverticulitis. On CT scans, a total of 8 inflamed diverticula were observed as small fluid-filled luminal structures with thick, enhanced walls, or as solid, enhanced masses protruding from the appendix. Previous studies regarding the CT findings of appendiceal diverticulitis focused specifically on visualizing the inflamed diverticula. In this study, we compared the differences in CT findings between patients with acute appendicitis and those with appendiceal diverticulitis. In almost half of the cases with diverticulitis, the appendix was not visualized. We noted inflamed diverticula in only 24% of scans of patients with appendiceal diverticulitis. Fluid collection in the appendix was observed in 88% of patients with acute appendicitis, while seen in only 16% of patients with appendiceal diverticulitis. Therefore, while it is difficult to visualize the inflamed diverticula, CT findings showing an absence of a fluid level, absence of appendicolith, and the presence of localized abscess formation may indicate a possible inflamed appendiceal diverticulum.

Most of the patients who underwent appendectomy were diagnosed as either acute appendicitis or appendiceal diverticulitis. In patients presenting with possible appendicitis, but with atypical clinical features and the above-noted CT findings, a diagnosis of appendiceal diverticulitis should be considered. If CT shows that the appendix is not swollen, even though a localized abscess is noted around the appendix, this should be considered an indirect sign of appendiceal diverticulitis.

This study has several limitations, because it was a retrospective study. there may be a selection bias between the groups who performed CT scans and who did not.

To confirm the exact incidence of appendiceal diverticulitis and differeneces of CT findings between acute appendicitis and appendiceal diverticulitis, a prospective evaluation is necessary.

Despite these limitations, we believe that appendiceal diverticulitis can be diagnosed preoperatively with a combination of clinical features and suggestive findings on CT. If we are able to diagnose appendiceal diverticulitis accurately, we will be able to further investigate whether conservative antibiotic therapy or surgery is desirable for appendiceal diverticultis in the future.

In conclusion, the incidence of appendiceal diverticulitis was 9.7% in patients who underwent appendectomy. The CT findings and clinical features of patients with appendiceal diverticulitis were different from those of patients with acute appendicitis, suggesting it is possible to differentiate appendiceal diverticulitis from acute appendicitis on this basis.

COMMENTS

Background
Appendiceal diverticulitis is considered a relatively rare disease, and is usually the subject of case reports. Since right lower quadrant pain is the main clinical symptom of both acute appendicitis and appendiceal diverticulitis, appendiceal diverticulitis has been commonly dismissed as a variant of acute appendicitis. Therefore, a preoperative diagnosis is rarely made, and pathologists may fail to differentiate between acute appendicitis and appendiceal diverticulitis. However, recent studies have considered appendiceal diverticulitis as a separate clinical entity apart from typical acute appendicitis.

Research frontiers
With regard to appendiceal diverticulitis, the current research hotspot is how to distinguish appendiceal diverticulitis from acute appendicitis and to accurately determine the incidence of appendiceal diverticulitis.

Innovations and breakthroughs
Appendiceal diverticulitis has been considered very rare. Previous reports have found that the incidence of appendiceal diverticulitis among patients undergoing appendectomy has been from 0.004%-2.1%, However, in this study of 451 patients who underwent appendectomy, 44 (9.7%) were pathologically diagnosed with appendiceal diverticulitis. The authors' believe that the higher incidence in our study is due to the careful resection and pathological processing of the resected appendixes, allowing visualization of the inflamed diverticula. Moreover, the patients with appendiceal diverticulitis in this study had the same clinical features as in previous studies, even though the incidence of appendiceal diverticulitis was much higher in this study. This similar clinical presentation is further evidence suggesting a higher incidence of appendiceal diverticulitis. Computed tomography findings suggestive of appendiceal diverticulitis included the absence of a fluid collection in the appendix, absence of an appendicolith (92% vs 52%, P = 0.004), and abscess formation.

Applications
The study results suggest that it may be possible to clinically differentiate appendiceal diverticulitis from acute appendicitis.

Terminology
Diverticula are small bulging pouches within the bowel wall that typically form within the large intestine, including the appendix. Appendiceal diverticula are relatively rare. Appendiceal diverticulitis is the result of inflammation of the

appendiceal diverticulum.

Peer-review

This interesting study is focused on the clinical, pathological, and radiological features of appendiceal diverticulitis, an uncommon, but more common than previously believed condition commonly confused with acute appendicitis.

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ORIGINAL ARTICLE

Retrospective Study

Diagnostic value of PIVKA-II and alpha-fetoprotein in hepatitis B virus-associated hepatocellular carcinoma

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Abstract

AIM: To determine the cutoff values and to compare the diagnostic role of alpha-fetoprotein (AFP) and prothrombin induced by vitamin K absence-II (PIVKA-II) in chronic hepatitis B (CHB).

METHODS: A total of 1255 patients with CHB, including 157 patients with hepatocellular carcinoma (HCC), 879 with non-cirrhotic CHB and 219 with cirrhosis without HCC, were retrospectively enrolled. The areas under the receiver operating characteristic (AUROC) curves of PIVKA-II, AFP and their combination were calculated and compared.

RESULTS: The optimal cutoff values for PIVKA-II and AFP were 40 mAU/mL and 10 ng/mL, respectively, for the differentiation of HCC from nonmalignant CHB. The sensitivity and specificity were 73.9% and 89.7%, respectively, for PIVKA-II and 67.5% and 90.3% for AFP, respectively. The AUROC curves of both PIVKA-II and AFP were not significantly different (0.854 *vs* 0.853, *P* = 0.965) for the differentiation of HCC from nonmalignant CHB, whereas the AUROC of PIVKA-II was significantly better than that of AFP in patients with cirrhosis (0.870 *vs* 0.812, *P* = 0.042). When PIVKA-II and AFP were combined, the diagnostic power improved significantly compared to either AFP or PIVKA-II alone for the differentiation of HCC from nonmalignant CHB (*P* < 0.05), especially when cirrhosis was present (*P* < 0.05).

CONCLUSION: Serum PIVKA-II might be a better tumor marker than AFP, and its combination with AFP may enhance the early detection of HCC in patients with CHB.

Key words: Hepatitis B virus; Hepatocellular carcinoma;

Alpha-fetoprotein; Prothrombin induced by vitamin K absence-Ⅱ

Core tip: Hepatocellular carcinoma (HCC) surveillance is crucial for patients with chronic hepatitis B (CHB). There have been few studies that have compared the levels of prothrombin induced by vitamin K absence-Ⅱ (PIVKA-Ⅱ) and AFP in hepatitis B virus-associated HCC. Serum PIVKA-Ⅱ, at a level of 40 mAU/mL, is a useful tumor marker to distinguish patients with HCC from those with nonmalignant CHB, especially liver cirrhosis (LC). A combination of AFP and PIVKA-Ⅱ could enhance early detection of HCC in patients with CHB. Therefore, serum PIVKA-Ⅱ levels should be measured in combination with serum AFP levels during the follow-up of patients with CHB and particularly those with LC.

INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common cancers and is the leading cause of cancer-related deaths worldwide. HCC appears to be increasing in incidence[1-4] and still has a dismal prognosis in spite of recent advancements in therapeutic intervention because of its diagnosis at advanced stages. Previous studies have reported the benefits of HCC surveillance on survival thanks to the detection of HCC at earlier stages[5-7]. Thus, many guidelines recommend HCC surveillance for at-risk populations[8-10].

Of the known biomarkers, alpha-fetoprotein (AFP) has been the most widely used as a tumor marker for diagnosis and surveillance of HCC. The sensitivity and specificity of AFP, however, have been reported to vary from 39% to 64% and from 76% to 91%, respectively[11-13]. Furthermore, AFP levels may be elevated in a number of nonspecific conditions in patients with cirrhosis or when cases of chronic hepatitis are exacerbated[14].

Due to such limitations, ultrasonography (US) alone without concurrent detection of AFP levels has been recommended for the surveillance of HCC, according to the representative guidelines in the United States and Europe[8,10]. US is the primary surveillance tool that is used in patients with chronic liver diseases that can detect the development of HCC. The sensitivity

and specificity have been reported to be 65% to 80% and 90% to 93%, respectively[8]. However, the interpretation is not only highly operator-dependent but is also insufficiently sensitive in the patients who are obese or who have underlying liver cirrhosis (LC). Additionally, the combined use of AFP and US not only increases the detection rates but also increases the false-positive rates[8]. Therefore, reliable biomarkers to complement the pitfalls of US are needed, especially in patients with cirrhotic patients.

Prothrombin induced by vitamin K absence-Ⅱ (PIVKA-Ⅱ) is an abnormal prothrombin protein that is elevated in HCC. The overall sensitivity and specificity of serum PIVKA-Ⅱ in the detection of HCC has been reported to be 48%-62% and 81%-98%, respectively[15]. Unlike AFP, the serum levels of PIVKA-Ⅱ are not elevated in patients with chronic liver disease such as exacerbations of chronic hepatitis and cirrhosis. That PIVKA-Ⅱ is more specific that AFP represents a highly specific feature of this protein[16,17].

To date, many studies have been conducted to determine the role of PIVKA-Ⅱ in patients with HCC, but most included only small numbers of patients or patients with heterogeneous etiologies, with a predominance of individuals with hepatitis C virus (HCV) infection[15,18-23]. Considering that the clinical features and mechanisms of hepatocarcinogenesis vary according to the etiologies[24-26], the roles of PIVKA-Ⅱ in hepatitis B virus (HBV)-associated HCC might be different from those of HCV-related HCC.

To the best of our knowledge, there have been few reports that have evaluated the role of PIVKA-Ⅱ in HBV-associated HCC. Therefore, additional studies are warranted to determine the role of PIVKA-Ⅱ in the diagnosis of HBV-associated HCC. The aim of this study was to compare the diagnostic role of PIVKA and AFP and to determine the best cutoff values of both tumor markers in patients with chronic hepatitis B (CHB).

MATERIALS AND METHODS

Patients and study design

A total of 1255 patients with CHB were retrospectively included at Hallym University Medical Center, Seoul, Korea, from January 2005 to December 2012. All patients who enrolled in this study demonstrated positivity for hepatitis B surface antigen for at least 6 mo.

Demographic and clinical information was collected from the medical records of the subjects. LC was diagnosed by histology and/or ultrasonographic/CT imaging features and was supplemented by clinically relevant portal hypertension (*e.g.,* esophageal and/or gastric varices, ascites, splenomegaly with a platelet count of < 100000/mm^3) or hepatic encephalopathy. All patients with HCC were newly diagnosed, and the diagnosis of HCC was based on liver histology

or appropriate imaging characteristics as defined by accepted guidelines[8]. The HCC stage was determined according to the TNM staging system by the Liver Cancer Study Group of Japan[27]. Early stage HCC was defined as a single tumor nodule < 3 cm in diameter with no evidence of vascular invasion or metastasis.

All patients were divided into three groups: (1) non-cirrhotic CHB (G1); (2) cirrhosis without HCC (G2); and (3) HCC (G3). In the non-HCC groups, laboratory tests were performed on the most recent clinic visit, and the following parameters were assessed: albumin, total bilirubin, alanine aminotransferase (ALT), international normalized ratio of prothrombin time, model for end-stage liver disease score, and the levels of AFP and PIVKA-Ⅱ. The same laboratory data were obtained at the time of diagnosis of HCC in the HCC group.

The exclusion criteria were as follows: the patients who (1) were positive for other markers of hepatitis such as hepatitis C virus or human immunodeficiency virus; (2) were heavy alcoholics (more than 80 g of ethanol daily); and (3) were taking warfarin or antibiotics that might influence the metabolism of vitamin K.

This study was approved by the Investigation and Ethics Committee for Human Research at Hallym University Medical Center, Seoul, Korea.

Assay of serum levels of AFP and PIVKA-Ⅱ
The serum AFP concentrations were determined with a commercially available electrochemiluminescence immunoassay kit (Elecsys AFP immunoassay, Roche, Mannheim, Germany). The serum PIVKA-Ⅱ level was measured by a revised enzyme immunoassay (Eitest PIVKA-II; Eisai, Tokyo, Japan).

Statistical analysis
A one-way analysis of variance (ANOVA) test was used for continuous variables and a χ^2 test was used for categorical variables in order to compare variables between the three groups. Four groups were compared with Kruskal-Wallis tests and a χ^2 test. Log transformation was used for the AFP and PIVKA-Ⅱ values to account for the large range of values among the groups for both markers. We used a new variable, the combination of AFP and PIVKA-Ⅱ levels (logAFP + 4.6*logPIVKA-Ⅱ), which was conceived in a previous study[15]. To find the optimal cutoff value of AFP and PIVKA-Ⅱ in the diagnosis of HCC, the receiver operating characteristic (ROC) curves were plotted using all possible cutoff values for each assay. The areas under the ROC (AUROC) curves of PIVKA-Ⅱ, AFP and the combination of the two were calculated and compared. Youden's index was calculated as an index of sensitivity and specificity. A *P* value < 0.05 was considered significant. Statistical analyses were performed using SPSS, version 16 and Medcalc, version 12.3.

RESULTS

Comparison of the clinical features between the HCC and non-HCC groups
A total of 1255 patients were divided into three subgroups: (1) non-cirrhotic CHB (G1, *n* = 879); (2) cirrhosis without HCC (G2, *n* = 219); and (3) HCC (G3, *n* =157). The median levels (range) of both PIVKA-Ⅱ and AFP were significantly higher in the HCC group compared to the non-cirrhotic CHB group and the cirrhosis without HCC group [PIVKA-Ⅱ; 202 (10-2000) mAU/mL *vs* 23 (6-162) mAU/mL *vs* 19 (4-312) mAU/mL, AFP; 55.9 (0.6-121000.0) ng/mL *vs* 2.5 (0.6-602.8) ng/mL *vs* 3.3 (0.6-233.6) ng/mL, *P* < 0.001]. Additionally, patients in the HCC group (G3) showed advanced liver dysfunction compared with patients in the non-HCC group (G1 and G2). The characteristics of these patients are summarized in Table 1.

Ninety-two (10.5%), 21 (9.6%), and 116 (73.9%) patients in G1, G2, and G3, respectively, had PIVKA-Ⅱ values above 40 mAU/mL, which was previously reported as the upper limit of normal. Elevated AFP (current clinical cutoff level, > 20 ng/mL,) was observed in 40 (4.6%), 22 (10.0%), and 94 (61.0%) patients of G1, G2, and G3, respectively. Of the patients with HCC, 26 patients (16.6%) had an AFP level < 20 ng/mL and a PIVKA-Ⅱ level < 40 mAU/mL, 37 (23.6%) had isolated PIVKA-Ⅱ elevation, and 15 (9.6%) had isolated AFP elevation.

Diagnostic values for PIVKA-Ⅱ and AFP in the differentiation of HCC from nonmalignant CHB
To determine the optimal cutoff values in the differentiation of HCC (G3) from nonmalignant CHB (G1 and G2), ROC curves were drawn (Figure 1). The best cutoff values for PIVKA-Ⅱ and AFP were 40 mAU/mL and 10 ng/mL, respectively. The sensitivity and specificity at these cutoff values were 73.9% and 89.7%, respectively, for PIVKA-Ⅱ and 67.5% and 90.3%, respectively, for AFP.

The AUROC curves of PIVKA-Ⅱ and AFP were not significantly different for the differentiation of HCC from nonmalignant CHB (0.854 *vs* 0.853, *P* = 0.965). After the combined levels of PIVKA-Ⅱ and AFP were considered, a significant improvement was observed in the diagnostic power compared with either PIVKA-Ⅱ or AFP alone (AUROC = 0.898; *vs* PIVKA-Ⅱ, *P* < 0.001; *vs* AFP, *P* = 0.03, respectively). The combination of these tumor markers yielded a sensitivity and specificity of 75.2% and 95.4%, respectively.

We compared the baseline characteristics of all of the patients according to the cutoff levels that we calculated from the ROC curve. A comparison of these baseline characteristics is represented in Table 2.

The optimal cutoff values of PIVKA-II and AFP for the differentiation of HCC (G3) from LC (G2) were 40 mAU/mL and 25 ng/mL, respectively (Figure 2). These

Table 1 Baseline characteristics of the study population (*n* = 1255)

	Non-cirrhotic CHB (*n* = 879)	Cirrhosis without HCC (*n* = 219)	HCC (*n* = 157)	*P* value
Age (yr)	45 (17-97)	54 (26-92)	57 (34-89)	0.0001
Gender (M:F)	549:330	151:68	124:33	0.0001
AFP (ng/mL)	2.5 (0.6-602.8)	3.3 (0.6-233.6)	55.9 (0.6-121000.0)	0.0001
PIVKA-II (mAU/mL)	23 (6-162)	19 (4-312)	202 (10-2000)	0.0001
INR	1.00 (0.84-3.24)	1.11 (0.92-2.38)	1.12 (0.79-8.93)	0.0001
Albumin (g/dL)	4.5 (2.3-5.3)	4.2 (0.5-5.2)	3.6 (2.0-5.2)	0.0001
Total bilirubin (mg/dL)	0.7 (0.2-19.2)	0.9 (0.2-18.9)	1.0 (0.3-14.7)	0.0001
ALT (IU/L)	26.5 (2-3112)	31 (8-886)	39 (6-1155)	0.1220
MELD score	7.1	10.1	9.9	0.0001
TNM stage *n* (%)	NA	NA		
I			22 (14.0)	
II			58 (36.9)	
III			32 (20.4)	
IV			45 (28.7)	
Early HCC *n* (%)	NA	NA	46 (29.3)	

Data are expressed as number or median (range). CHB: Chronic hepatitis B; HCC: Hepatocellular carcinoma; M: Male; F: Female; AFP: Alpha-fetoprotein; PIVKA-II: Prothrombin induced by vitamin K absence-II; INR: International normalized ratio; ALT: Alanine aminotransferase; MELD: Model for end-stage liver disease; TNM: Tumor-nodes-metastasis; NA: Not applicable.
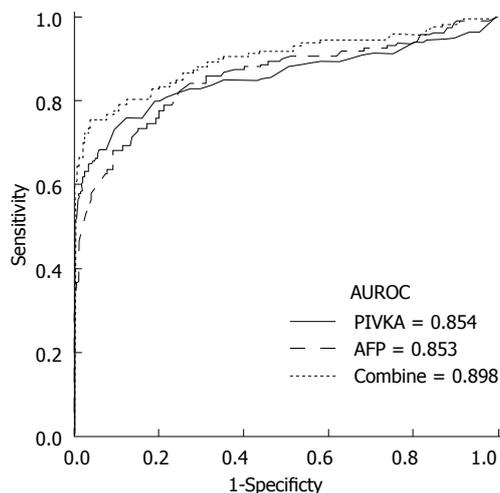
Figure 1 Receiver operating characteristic curves comparing prothrombin induced by vitamin K absence-II, alpha-fetoprotein and a combination of alpha-fetoprotein and prothrombin induced by vitamin K absence-II in patients with hepatocellular carcinoma *vs* those with nonmalignant chronic hepatitis B. The AUROC curves for PIVKA-II, AFP, and Combination are indicated in the inset. *P* = 0.965 for AFP *vs* PIVKA-II, *P* < 0.001 for combination *vs* PIVKA-II and *P* = 0.037 for combination *vs* AFP. PIVKA-II: Prothrombin induced by vitamin K absence-II; HCC: Hepatocellular carcinoma; AFP: Alpha-fetoprotein.
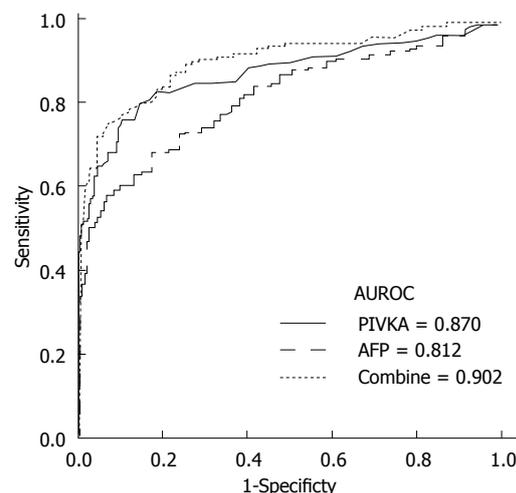
Figure 2 Receiver operating characteristic curves comparing prothrombin induced by vitamin K absence-II, alpha-fetoprotein and a combination of alpha-fetoprotein and prothrombin induced by vitamin K absence-II in patients with hepatocellular carcinoma *vs* those with liver cirrhosis. The AUROC curves for PIVKA-II, AFP, and Combination are indicated in the inset. *P* = 0.042 for AFP *vs* PIVKA-II, *P* = 0.001 for combination *vs* PIVKA-II and *P* < 0.001 for combination *vs* AFP. PIVKA-II: Prothrombin induced by vitamin K absence-II; HCC: Hepatocellular carcinoma; AFP: Alpha-fetoprotein.

values gave a sensitivity and specificity of 73.9% and 90.4%, respectively, for PIVKA-II and 58.6% and 92.7%, respectively, for AFP. Interestingly, the AUROC curve indicated a better sensitivity and specificity for PIVKA-II than for AFP in the differentiation of HCC from LC (0.870 *vs* 0.812, *P* = 0.042). When PIVKA-II and AFP were combined, the diagnostic power was significantly enhanced compared with that of either marker alone (AUROC = 0.902; *vs* PIVKA-II, *P* = 0.001; *vs* AFP, *P* < 0.001). The combination of these

tumor markers yielded a sensitivity and specificity of 75.2% and 92.7%, respectively.

After an analysis of the diagnostic power of PIVKA-II and AFP for the differentiation of early HCC from LC, the AUROC curve of PIVKA-II tended to be better than that of AFP (AUROC = 0.752 *vs* 0.712, *P* = 0.512). Additionally, the combination of these two markers demonstrated a significant improvement in the diagnostic power compared with each marker alone (Combination *vs* PIVKA-II, *P* = 0.033; Combination *vs* AFP, *P* = 0.019, respectively).

Table 2 Comparision of baseline characteristics according to cutoff values of prothrombin induced by vitamin K absence-Ⅱ (40 mAU/mL) and AFP (10 ng/mL)

	PIVKA-Ⅱ < 40 AFP < 10	PIVKA-Ⅱ ⩾ 40 AFP < 10	PIVKA-Ⅱ < 40 AFP < 10	PIVKA-Ⅱ ⩾ 40 AFP ⩾10	*P* value
Number	918	125	108	104	
Age (yr)	47.9 (17-97)	48.4 (18-83)	51.4 (23-92)	55 (25-88)	0.0001
Gender (M:F)	578:340	97:28	67:41	82:22	0.0001
INR	1.0 (0.8-3.2)	1.0 (0.8-2.3)	1.1 (0.9-2.1)	1.13 (0.7-8.9)	0.0001
Albumin (g/dL)	4.5 (0.5-5.3)	2.9 (0.6-9.6)	3.8 (2-5.2)	3.5 (1.7-4.9)	0.0001
TB (mg/dL)	0.7 (0.2-18.9)	0.7 (0.2-19.2)	1.1 (0.2-17.3)	1.1 (0.3-14.7)	0.0001
ALT (IU/L)	25.4 (2-803)	30.1 (5-1664)	50 (9-1531)	43.4 (9-3112)	0.0001
MELD score	7.4	8.2	9.9	10.5	0.0001
LC, *n* (%)	188 (20.5)	42 (33.6)	50 (46.3)	89 (85.6)	0.0001
HCC, *n* (%)	21 (2.3)	30 (24)	20 (18.5)	86 (82.7)	0.0001

Data are expressed as number or median (range). PIVKA-Ⅱ: Prothrombin induced by vitamin K absence-Ⅱ; AFP: Alpha-fetoprotein; M: Male; F: Female; INR: International normalized ratio; TB: Total bilirubin; ALT: Alanine aminotransferase; MELD: Model for end-stage liver disease; LC: Liver cirrhosis; HCC: Hepatocellular carcinoma.

Table 3 Sensitivity and specificity of alpha-fetoprotein and prothrombin induced by vitamin K absence-Ⅱ in differentiating hepatocellular carcinoma from nonmalignant chronic hepatitis B

	Sensitivity	Specificity	PPV	NPV
PIVKA-Ⅱ (mAU/mL)				
> 40				
HCC *vs* CHB	73.9%	89.7%	50.7%	96.0%
HCC *vs* LC	73.9%	90.4%	84.7%	82.8%
> 100				
HCC *vs* CHB	57.3%	98.8%	87.4%	94.2%
HCC *vs* LC	57.3%	96.8%	82.8%	76.0%
> 125				
HCC *vs* CHB	56.1%	99.2%	90.7%	94.0%
HCC *vs* LC	56.1%	97.3%	93.6%	75.5%
> 150				
HCC *vs* CHB	52.9%	99.4%	92.2%	93.6%
HCC *vs* LC	52.9%	97.3%	93.3%	74.2%
AFP (ng/mL)				
> 10				
HCC *vs* CHB	67.5%	90.3%	50.0%	95.1%
HCC *vs* LC	67.5%	82.6%	73.6%	78.0%
> 20				
HCC *vs* CHB	59.9%	94.4%	60.3%	94.3%
HCC *vs* LC	59.9%	90.0%	81.0%	75.8%
> 200				
HCC *vs* CHB	36.3%	99.3%	87.7%	91.6%
HCC *vs* LC	36.3%	99.1%	96.6%	68.5%
> 400				
HCC *vs* CHB	29.9%	99.7%	94.0%	90.9%
HCC *vs* LC	29.9%	100.0%	100.0%	66.6%
Combination[1]				
HCC *vs* CHB	75.2%	95.4%	69.8%	96.4%
HCC *vs* LC	75.2%	92.7%	88.1%	83.9%

[1]Combination obtained from variable $\log$AFP + 4.61$\log$PIVKA-Ⅱ. PPV: Positive predictive value; NPV: Negative predictive value; PIVKA-Ⅱ: Prothrombin induced by vitamin K absence-Ⅱ; HCC: Hepatocellular carcinoma; CHB: Chronic hepatitis B; LC: Liver cirrhosis; AFP: Alpha-fetoprotein.

Diagnostic performance of PIVKA-Ⅱ and AFP in order to distinguish patients with HCC from those with CHB or LC

Table 3 shows the sensitivity and specificity of PIVKA-Ⅱ alone, AFP alone, and the combination of both markers in the differentiation of HCC from nonmalignant CHB at the various cutoff values based on our study and on previously published studies: 10, 20, 200, 400 ng/mL for AFP[11-13,15,23,28,29] and 40, 100, 125, 150 mAU/mL for PIVKA-Ⅱ [15,20,21,30]. PIVKA-Ⅱ values > 40 mAU/mL had better sensitivity, specificity, positive predictive value, and negative predictive value than PIVKA-Ⅱ values of 100, 125 and 150 mAU/mL that were accepted in other studies. In addition, PIVKA-Ⅱ values > 40 mAU/mL demonstrated a better diagnostic performance than AFP, regardless of the cutoff value that was chosen.

When the two markers were combined, the sensitivity increased from 73.9% for PIVKA-Ⅱ alone and 67.5% for AFP alone to 75.2%; similarly, the specificity also increased from 89.7% for PIVKA-Ⅱ alone and 90.3% for AFP alone to 95.4% in the differentiation of patients with HCC from those without HCC.

DISCUSSION

PIVKA-Ⅱ is an abnormal prothrombin molecule, known as des-gamma-carboxy prothrombin, which is generated as a result of an acquired defect in the posttranslational carboxylation of the prothrombin precursor in malignant cells. Since the first report by Liebman *et al*[31] in 1984, many studies have demonstrated the usefulness of PIVKA-Ⅱ for the diagnosis of HCC[17-22,31-33]. Earlier studies showed that PIVKA-Ⅱ had a low sensitivity compared with AFP[30,34]. However, after the introduction of a revised enzyme immunoassay kit, more recent studies revealed that PIVKA-Ⅱ is comparable or more sensitive than AFP for the differentiation of HCC from nonmalignant chronic liver disease; moreover, most studies have shown that PIVKA-II is more specific than AFP[15,17-22,32,33].

Most studies that have been concerned with the role of PIVKA-Ⅱ were conducted in Japan and in Western countries where the etiology of liver disease

varied greatly, primarily associated with HCV infection. Therefore, we focused on the role of PIVKA-Ⅱ in HBV-related HCC after a consideration of the differences in hepatocarcinogenesis and in the clinical features between HBV-related HCC and HCV-related HCC. Actually, HBV-related HCC typically presents more frequently as an aggressive tumor compared with HCV-related HCC, and the levels of AFP are higher in HBV-related HCC than in HCV-related HCC[24-26].

In this study, we showed that PIVKA-Ⅱ is more accurate than AFP in the ability to distinguish patients with HCC from those with nonmalignant CHB. The sensitivity and specificity at the cutoff values which were identified by the ROC curve were 73.9% and 89.7%, respectively, for PIVKA-Ⅱ and 67.5% and 90.3%, respectively, for AFP. Although the AUROC curves of PIVKA-Ⅱ and AFP showed a similar diagnostic efficacy for the differentiation of HCC from nonmalignant CHB, when the analysis was limited to patients with cirrhosis, the AUROC curve indicated a significantly better sensitivity and specificity for PIVKA-Ⅱ than for AFP for differentiation of HCC from LC; this was also the case for the differentiation of early HCC from LC. These data are in contrast to those of previous studies that showed that the sensitivity of PIVKA-Ⅱ was inferior to AFP in the detection of small HCC[33,34], and this result suggests that PIVKA-Ⅱ is a more reliable tumor marker than AFP for the detection of early HCC in patients with CHB.

This difference might be due to the difference in the etiology of liver diseases of the patients. Previous studies have included patients with heterogeneous etiologies of liver diseases (mainly HCV infection or alcohol) and were case-controlled studies that were conducted in relatively small numbers of selected patients of the population. In contrast, our study included patients with only HBV as an etiology of liver disease and nearly all patients with CHB who visited our institute. Therefore, our study represents a real clinical situation, and indeed, advanced liver diseases such as LC or HCC account for approximately 30% in this study. This is in accordance with the disease progression that occurs as part of the natural history of CHB. To our knowledge, our study is the first large-scale study that has demonstrated the diagnostic role of PIVKA-Ⅱ in HBV-associated HCC.

The ROC curve identified the optimal cutoff value of PIVKA-Ⅱ as 40 mAU/mL for the differentiation of patients with HCC from those with nonmalignant CHB in our study. This value is comparable with the result of a Japanese study, but is lower than that of a previous American study. It is possible that the cutoff value of PIVKA-Ⅱ may vary among different ethnic groups. Indeed, American patients typically have PIVKA-Ⅱ values up to 63 mAU/mL, whereas studies in Japan have used 40 mAU/mL as the upper limit of normal[15,18-22].

We found that the optimal cutoff value for the level of AFP for the diagnosis of HCC was 10 ng/mL. This result was substantially lower than the current clinical cutoff level (20 ng/mL), but was in accordance with the results of recent studies with cutoff values of 10.9 ng/mL and 11 ng/mL[15,23]. In addition, the optimal cutoff value of AFP for the differentiation of HCC from LC was 25 ng/mL. These results are in agreement with those of previous prospective studies that have included patients with LC[11].

Because AFP and PIVKA-Ⅱ levels do not correlate in patients with HCC and are complementary tumor markers, it would be reasonable to determine that using both markers might improve the accuracy of a diagnosis of HCC. Indeed, a few studies have demonstrated an increased sensitivity and specificity when these tumor markers are combined[35-37]. We also demonstrated that the combination of these two markers showed a significant improvement with respect to the diagnostic power compared with each marker alone for the differentiation of HCC from nonmalignant CHB, especially in patients with cirrhosis. Clinically, this is very important because US is the primary surveillance tool for LC, but this method is not sensitive enough to detect HCC in many patients with cirrhosis. Although AFP is no longer considered in surveillance tests for HCC in American and European practice guidelines, our study suggests that the combination of AFP and PIVKA-Ⅱ could enhance the diagnostic accuracy. A prospective study of patients with LC is warranted to further validate the utility of this combination for the detection of early HCC.

Our results are interesting, but there are some potential limitations to our study. First, this is a single-center study with a retrospective design. Second, the number of patients with HCC (n = 157) is relatively small compared to those with nonmalignant CHB (n = 1098); nonetheless, it reflects the natural course of CHB progression. Finally, our study cannot be generalized to patients with liver diseases that are not caused by HBV or to patients who are not Asian.

In conclusion, serum PIVKA-Ⅱ, at the level of 40 mAU/mL, is a useful tumor marker to distinguish patients with HCC from those with nonmalignant CHB, especially LC. A combination of AFP and PIVKA-Ⅱ could enhance the early detection of HCC in patients with CHB. Therefore, the measurement of the serum levels of PIVKA-Ⅱ should be applied in combination with the measurement of the AFP levels in the follow-up of patients with CHB, particularly those with LC. Further large-scale prospective studies are needed to verify the utility of PIVKA-Ⅱ for the detection of early HCC.

COMMENTS

Background

Hepatocellular carcinoma (HCC) is one of the most common cancers, and therefore, the early detection of HCC is crucial for patients with chronic liver disease. Ultrasonography is the primary tool that is used for surveillance, but there are limitations associated with this method.

Research frontiers
Alpha-fetoprotein (AFP) has been most widely used as a tumor marker for the diagnosis and surveillance of HCC; however, the sensitivities and specificities that have been reported have been varied. Prothrombin induced by vitamin K absence-Ⅱ (PIVKA-Ⅱ) can be more specific than AFP, and therefore, additional studies are needed in order to determine the role of PIVKA-Ⅱ in the diagnosis of hepatitis B virus-associated HCC.

Innovations and breakthroughs
Previous studies regarding the role of PIVKA-Ⅱ in patients with HCC were conducted in small numbers of patients or in patients with heterogeneous etiologies of liver disease (primarily those with hepatitis C virus). Hence, the authors compared the diagnostic roles of PIVKA-Ⅱ and AFP and determined the best cutoff value of both tumor markers in patients with chronic hepatitis B (CHB).

Applications
The study results suggest that serum PIVKA-Ⅱ levels might be a useful tumor marker to distinguish patients with HCC from those with nonmalignant CHB, especially liver cirrhosis. The combination of AFP and PIVKA-Ⅱ may also enhance the early detection of HCC in patients with CHB.

Terminology
PIVKA-II is an abnormal prothrombin molecule, known as des-gamma-carboxy prothrombin, which is generated as a result of an acquired defect in the posttranslational carboxylation of the prothrombin precursor in malignant cells.

Peer-review
The research is important and the research findings are significant. The novelty and innovative nature of the research is acceptable because the other similar reports but this one depicts an interesting number of patients.

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ORIGINAL ARTICLE

Retrospective Study

Eradication rate and histological changes after *Helicobacter pylori* eradication treatment in gastric cancer patients following subtotal gastrectomy

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Abstract

AIM: To investigate the eradication rate and histological changes after *Helicobacter pylori* (*H. pylori*) eradication treatment following subtotal gastrectomy for gastric cancer.

METHODS: A total of 610 patients with *H. pylori* infection who had undergone surgery for either early or advanced gastric adenocarcinoma between May 2004 and December 2010 were retrospectively studied. A total of 584 patients with proven *H. pylori* infection after surgery for gastric cancer were enrolled in this study. Patients received a seven day standard triple regimen as first-line therapy and a 10 d bismuth-containing quadruple regimen as second-line therapy in cases of eradication failure. The patients underwent an esophagogastroduodenoscopy (EGD) between six and 12 mo after surgery, followed by annual EGDs. A further EGD was conducted 12 mo after confirming the result of the eradication and the histological changes. A gastric biopsy specimen for histological examination and *Campylobacter*-like organism testing was obtained from the lesser and greater curvature of the corpus of the remnant stomach. Histological changes in the gastric mucosa were assessed using the updated Sydney system before eradication therapy and at follow-up after 12 mo.

RESULTS: Eradication rates with the first-line and second-line therapies were 78.4% (458/584) and 90% (36/40), respectively, by intention-to-treat analysis and 85.3% (458/530) and 92.3% (36/39), respectively, by per-protocol analysis. The univariate and multivariate analyses revealed that Billroth II surgery was an independent factor predictive of eradication success in the eradication success group (OR = 1.53, 95%CI: 1.41-1.65, *P* = 0.021). The atrophy and intestinal

metaplasia (IM) scores 12 mo after eradication were significantly lower in the eradication success group than in the eradication failure group (0.25 ± 0.04 *vs* 0.47 ± 0.12, *P* = 0.023; 0.27 ± 0.04 *vs* 0.51 ± 0.12, *P* = 0.015, respectively). The atrophy and IM scores 12 mo after successful eradication were significantly lower in the Billroth II group than in the Billroth I group (0.13 ± 0.09 *vs* 0.31 ± 0.12, *P* = 0.029; 0.32 ± 0.24 *vs* 0.37 ± 0.13, *P* = 0.034, respectively).

CONCLUSION: Patients with *H. pylori* following subtotal gastrectomy had a similar eradication rate to patients with an intact stomach. *H. pylori* eradication is recommended after subtotal gastrectomy.

Key words: *Helicobacter pylori*; Eradication; Atrophy; Intestinal metaplasia; Esophagogastroduodenoscopy; Gastrectomy

Core tip: This is the first study to investigate the eradication rate and histological changes after *Helicobacter pylori* (*H. pylori*) eradication treatment in patients following subtotal gastrectomy for gastric cancer. The patients with *H. pylori* infection who had undergone a subtotal gastrectomy for gastric cancer had a similar eradication rate to patients with an intact stomach. *H. pylori* eradication in gastric cancer patients following subtotal gastrectomy resulted in histological improvement, especially in the Billroth II group.

INTRODUCTION

Helicobacter pylori (*H. pylori*) infection is a leading cause of gastric cancer[1,2] and *H. pylori* eradication therapy is thought to convey beneficial effects in preventing metachronous cancer after endoscopic resection of early gastric cancer[3]. As *H. pylori* infection remains a risk factor for malignancy after subtotal gastrectomy, several guidelines recommend *H. pylori* eradication therapy in patients who have undergone surgery for gastric cancer[4,5].

Several guidelines recommend *H. pylori* eradication in patients following surgery for gastric cancer[4,5] but its beneficial effects have not been established. In general, glandular atrophy from *H. pylori* infection is reversible

after eradication but intestinal metaplasia (IM) cannot be improved in patients who have not undergone surgery[6,7]. Onoda *et al*[8] reported significant changes in glandular atrophy after eradication in the remnant stomach. However, Matsukura *et al*[9] determined no significant improvements in glandular atrophy or IM. Cho *et al*[10] reported that *H. pylori* eradication following subtotal gastrectomy significantly reduced both glandular atrophy and IM scores, 36 mo after eradication.

In this retrospective study, we investigated whether *H. pylori* eradication resulted in histological improvement in patients who underwent a subtotal gastrectomy for gastric cancer. We also compared the *H. pylori* eradication rates in patients who underwent subtotal gastrectomy with the recently reported eradication rates in a group that did not undergo surgery.

MATERIALS AND METHODS

Study population

A total of 610 patients with *H. pylori* infection who had undergone surgery for either early or advanced gastric adenocarcinoma at Seoul National University Bundang Hospital between May 2004 and December 2010 were retrospectively studied. The patients underwent either open or laparoscopically-assisted distal gastrectomy and either Billroth I (gastroduodenostomy) or II (gastrojejunostomy) surgery was used for reconstruction. The exclusion criteria were as follows: (1) age below 18 years; (2) previous *H. pylori* eradication before diagnosis of malignancy; (3) previous gastric surgery or endoscopic resection for gastric cancer; (4) severe concurrent disease (hepatic, renal, respiratory or cardiovascular); (5) pregnancy; (6) palliative therapy; and (7) any condition probably associated with poor compliance (*e.g.,* alcoholism or drug addiction).

All patients gave written informed consent and the study was performed according to the directions of the Declaration of Helsinki. The study protocol was approved by the Ethics Committee at Seoul National University Bundang Hospital (Institutional Review Board number: B-1306/206-109).

H. pylori infection and histological evaluation

The patients underwent an esophagogastroduodenoscopy (EGD) between six and 12 mo after surgery, followed by annual EGDs. Of the 610 patients who were offered eradication therapy, all consented to the treatment. A further EGD was conducted 12 mo after confirming the result of the eradication and the histological changes. *H. pylori* infection and eradication failure were defined on the basis of at least one of the following three tests: first, a positive ^{13}C-urea breath test (^{13}C-UBT); second, histological evidence of *H. pylori* by modified Giemsa staining

in the lesser and greater curvature of the corpus of the remnant stomach; and third, a positive rapid urease test (CLOtest; Delta West, Bentley, Australia) in gastric mucosa biopsy samples from the lesser and greater curvatures of the corpus of the remnant stomach. An endoscopic specialist performed the biopsies and described the endoscopic findings (Dong Ho, Lee). A gastric biopsy specimen for histological examination and *Campylobacter*-like organism testing was obtained from the lesser and greater curvature of the corpus of the remnant stomach and immediately fixed in formalin. The tumor location was determined with reference to the Japanese Classification of Gastric Cancer[11]. The degree of atrophy and IM, polymorphonuclear neutrophil activity and mononuclear cell count were graded according to the updated Sydney system (0 = none, 1 = mild, 2 = moderate and 3 = marked)[12].

H. pylori eradication regimen

All the patients received seven day standard triple therapy [amoxicillin 1000 mg twice a day (b.i.d.), clarithromycin 500 mg b.i.d. and esomeprazole 20 mg b.i.d.] as first-line therapy. Patients who failed to respond to first-line therapy received a 10 d bismuth-containing quadruple regimen [tripotassium dicitratobismuthate 300 mg four times a day (q.i.d.), tetracycline 500 mg q.i.d., metronidazole 500 mg three times a day and esomeprazole 20 mg b.i.d.] as second-line therapy, with a subsequent ^{13}C-UBT for evaluation of eradication. Patients who took < 85% of the prescribed medication were considered to have low compliance.

Statistical analysis

H. pylori eradication rates were determined using intention-to-treat (ITT) and per-protocol (PP) analyses. ITT analysis compared treatment groups, including all the patients as originally allocated. PP analysis compared treatment groups, including only those patients who completed the treatment as originally allocated. The mean ± SD was calculated for quantitative variables. The Student's *t* test was used to evaluate continuous variables and the χ^2 test and Fisher's exact test were used to assess non-continuous variables. Additionally, univariate and multivariate analyses were conducted to assess the effects of factors on the eradication rate. Updated Sydney system scores were compared using the Wilcoxon signed-rank test and Mann-Whitney *U* test for unpaired data. All of the statistical analyses were performed using Predictive Analytics Software (PASW) version 20.0 for Windows (SPSS Inc., IBM, Chicago, IL, United States). A *P* value of less than 0.05 was defined as carrying statistical significance. The statistical methods of this study were reviewed by Medical Research Collaborating Center at Seoul National University Bundang Hospital.

RESULTS

Characteristics of patients

A schematic diagram of the study is provided in Figure 1. Of the 610 patients, 26 were excluded from the study because of previous *H. pylori* eradication before diagnosis of malignancy (nine patients), endoscopic submucosal dissection before surgery (nine patients), palliative surgery (four patients), liver cirrhosis (three patients) and chronic renal failure (one patient). A total of 584 patients underwent first-line eradication treatment. Forty-four were lost to follow-up and 10 had low treatment compliance. Eradication was surveyed using a ^{13}C-UBT in all the patients after treatment but histological changes were surveyed in only 326 patients after 12 mo. The remaining 204 patients were not examined for histological changes after eradication because they were lost to follow-up. Finally, 326 patients had histological changes of their gastric mucosa analyzed after eradication of *H. pylori*. Of these, 290 patients in whom *H. pylori* was successfully eradicated with first-line therapy were assigned to the eradication success group and 36 patients in whom first-line therapy failed were assigned to the eradication failure group. In patients in the eradication failure group, second-line therapy was used to eradicate *H. pylori* infection. However, this was not successful in most patients because of either poor compliance or adverse events. The mean ages of the eradication success and failure groups were 56.7 ± 10.4 and 56.9 ± 10.7 years (*P* = 0.124), respectively. The enrolled patients' baseline demographic and clinical characteristics are provided in Table 1. The eradication rate of patients who underwent Billroth Ⅱ surgery (96.7%, 89/92) was significantly higher than that of patients who underwent Billroth Ⅰ surgery in the eradication success group (85.8%, 201/234, *P* = 0.012). There were no statistically significant differences in gender distribution, smoking status, alcohol use, underlying disease, early gastric cancer/advanced gastric cancer or tumor location between the two groups (*P* > 0.05).

H. pylori eradication rates of first- and second-line therapy

Table 2 shows the rates of eradication of *H. pylori* infection according to the ITT and PP analyses. The eradication rates of first-line therapy by ITT and PP analyses were 78.4% (95%CI: 74.9-81.6%) and 85.3% (95%CI: 82.1-88.1%), respectively. Forty of the 72 patients who failed first-line therapy underwent second-line treatment and their eradication rates by ITT and PP analyses were 90% (95%CI: 77.0-96.0%) and 92.3% (95%CI: 79.7-97.4%), respectively.

Clinical factors influencing H. pylori eradication

To evaluate the clinical factors influencing the efficacy of *H. pylori* eradication, univariate analyses were
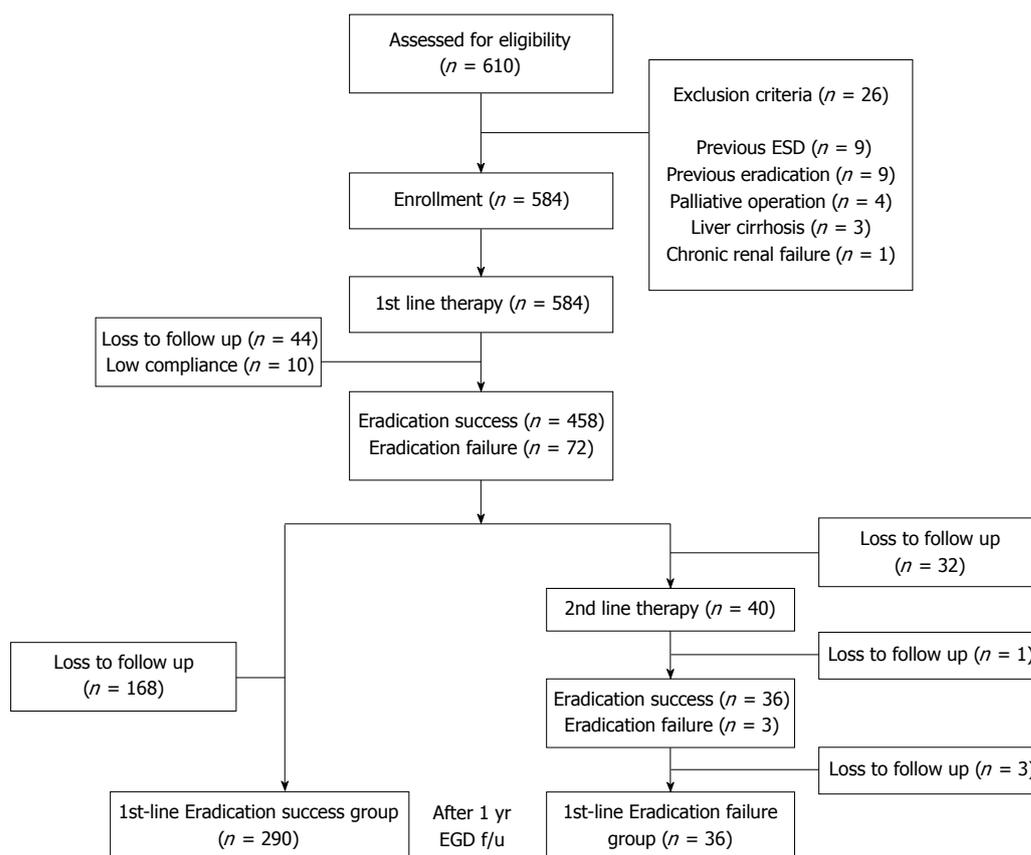
Figure 1 Schematic diagram of the study. ESD: Endoscopic submucosal dissection.

performed, which are listed in Table 1. The eradication rate of patients who underwent Billroth Ⅱ surgery was significantly higher than that of patients who underwent Billroth Ⅰ surgery (*P* = 0.012). The other factors did not affect the eradication response. The multivariate analysis revealed that Billroth Ⅱ surgery (OR = 1.53, 95%CI: 1.41-1.65, *P* = 0.021) was an independent factor predictive of eradication success in the eradication success group (Table 3).

Sequential histological changes after eradication
Table 4 shows the histological changes of the gastric mucosa according to eradication. Histological changes were compared before and after eradication and scored according to the updated Sydney system. The atrophy scores were significantly lower than the baseline after eradication in the eradication success group (0.25 ± 0.04 *vs* 0.46 ± 0.04, *P* < 0.001). The atrophy scores were not significantly different compared to the baseline after eradication in the eradication failure group (0.47 ± 0.12 *vs* 0.50 ± 0.11, *P* = 0.698). The atrophy scores after eradication were significantly lower in the eradication success group than in the eradication failure group (0.25 ± 0.04 *vs* 0.47 ± 0.12, *P* = 0.023). The IM scores were significantly lower than the baseline after eradication in the eradication success group (0.27 ± 0.04 *vs* 0.41 ± 0.05, *P* < 0.001). The IM scores were not significantly different compared to

the baseline after eradication in the eradication failure group (0.51 ± 0.12 *vs* 0.56 ± 0.14, *P* = 0.226). The IM scores after eradication were significantly lower in the eradication success group than in the eradication failure group (0.27 ± 0.04 *vs* 0.51 ± 0.12, *P* = 0.015). Activity and chronic inflammation scores decreased in all the groups. In metachronous cancer patients (*n* = 7), the atrophy and IM scores were lower than the baseline after eradication but the differences were not statistically significant (*P* > 0.05; Table 5).

Sequential histological changes according to reconstructive surgery method
Table 6 shows the histological changes of the gastric mucosa according to reconstructive surgery method after successful eradication. Thirty-six eradication failure patients were excluded to analyze the histological changes of the gastric mucosa after successful eradication between the Billroth Ⅰ and Billroth Ⅱ groups. The atrophy scores were significantly lower than the baseline after successful eradication in the Billroth Ⅱ group (0.13 ± 0.09 *vs* 0.53 ± 0.19, *P* < 0.001). The atrophy scores were not significantly different compared to the baseline after successful eradication in the Billroth Ⅰ group (0.31 ± 0.12 *vs* 0.33 ± 0.10, *P* = 0.831). The atrophy scores after successful eradication were significantly lower in the Billroth Ⅱ group than in the Billroth Ⅰ group (0.13 ± 0.09 *vs* 0.31

Table 1 Baseline demographic and clinical characteristics of patients and univariate analysis of clinical factors influencing the eradication rate n (%)

	Eradication success (n = 290)	Eradication failure (n = 36)	P value	Univariate analysis
Age	56.7 ± 10.4	56.9 ± 10.7	0.124	0.127
Gender			0.229	0.233
Male	183 (63.1)	19 (52.7)		
Female	107 (36.9)	17 (47.3)		
Smoking			0.789	0.792
Non-smoker	228 (78.6)	29 (80.5)		
Smoker	62 (21.4)	7 (19.5)		
Alcohol			0.213	0.219
Non-drinker	224 (77.2)	23 (63.8)		
Drinker	66 (22.8)	13 (36.2)		
Underlying disease				
Hypertension	65 (22.4)	6 (16.6)	0.431	0.437
Diabetes mellitus	24 (8.2)	4 (11.1)	0.531	0.539
EGC/AGC			0.451	0.463
EGC	217 (74.8)	29 (80.5)		
AGC	73 (25.2)	7 (19.5)		
Tumor location			0.695	0.707
Lower (Antrum)	147 (50.6)	17 (47.2)		
Middle (Body)	143 (49.3)	19 (52.7)		
Surgery			0.012	0.016
Billroth-I	201 (69.4)	33 (91.7)		
Billroth-II	89 (30.6)	3 (8.3)		

EGC: Early gastric cancer; AGC: Advanced gastric cancer; CI: Confidence interval.

Table 2 *H. pylori* eradication rates of first- and second-line therapy

	First-line therapy	Second-line therapy	P value
ITT analysis			
%, Eradication(ratio)	78.4 (458/584)	90 (36/40)	0.027
95%CI	74.9-81.6	77.0-96.0	
PP analysis			
%, Eradication(ratio)	85.3 (458/530)	92.3 (36/39)	0.034
95%CI	82.1-88.1	79.7-97.4	

ITT: Intention-to-treat; PP: Per-protocol; CI: Confidence interval.

Table 3 Multivariate analysis for the clinical factors related to the eradication success in the eradication success group

	Odds ratio	95%CI	P value
Billroth-II *vs* Billroth-I	1.53	1.41-1.65	0.021
Male *vs* female	1.01	0.87-1.15	0.125
Smoking (+) *vs* (-)	1.07	0.89-1.25	0.163
Alcohol (+) *vs* (-)	0.98	0.94-1.08	0.187

CI: Confidence interval.

± 0.12, *P* = 0.029). The IM scores were significantly lower than the baseline after successful eradication in the Billroth II group (0.32 ± 0.24 *vs* 0.67 ± 0.21, *P* < 0.001). The IM scores were not significantly different compared to the baseline after successful eradication in the Billroth I group (0.37 ± 0.13 *vs* 0.39 ± 0.13, *P* = 0.572). The IM scores after successful eradication were significantly lower in the Billroth II group than in the Billroth I group (0.32 ± 0.24 *vs* 0.37 ± 0.13, *P* = 0.034). Activity and chronic inflammation scores were decreased in all the groups.

DISCUSSION

Anatomical and physiological changes after surgery are inevitable and may affect the *H. pylori* eradication rate and histological findings. The effects on *H. pylori* eradication of bacterial overgrowth due to low acidity, impairment of absorption as a result of intestinal hurry, changes in blood flow and bile reflux caused by interruption of the pyloric sphincter are well-known[13-19]. However, some questions remain about the effects of *H. pylori* eradication therapy after gastric surgery.

First, it is uncertain whether differences in histological improvements between patients undergoing and patients not undergoing gastric surgery are associated with *H. pylori* eradication therapy. One study reported no significant changes in either glandular atrophy or IM scores after eradication in the remnant stomach[9], whereas another study showed significant improvements in glandular atrophy compared with normal mucosa[8]. Second, it is unknown whether the eradication rate differs between patients who have undergone and those who have not undergone gastric surgery and whether the eradication rate in patients after surgery decreases, as in the current study[20], because histological improvement was significantly higher in the eradicated group[8,9].

In this study, we evaluated histological changes, particularly glandular atrophy and IM, and the eradication rate of *H. pylori* infection in patients who underwent a subtotal gastrectomy for gastric cancer. Our data indicated that only the eradication success group had significantly improved glandular atrophy and IM scores 12 mo after treatment, suggesting the importance of *H. pylori* eradication rate. The eradication rate in patients who underwent Billroth II surgery was significantly higher than that of patients who underwent Billroth I surgery. The multivariate analysis revealed that Billroth II surgery was an independent factor predictive of eradication success in the eradication success group. The atrophy and IM scores were significantly lower than the baseline after eradication in the eradication success group. The atrophy and IM scores after eradication were significantly lower in the eradication success group than in the eradication failure group. Moreover, the atrophy and IM scores were significantly lower than the baseline after successful eradication in the Billroth II group. These results suggest that *H. pylori* eradication would result in histological improvement in patients who underwent surgery for gastric cancer,

Table 4 Histological changes of gastric mucosa according to eradication

	Eradication success (*n* = 290)			Eradication failure (*n* = 36)			*P* value
	Baseline	12 mo	*P* value	Baseline	12 mo	*P* value	
Atrophy	0.46 ± 0.04	0.25 ± 0.04	< 0.001	0.50 ± 0.11	0.47 ± 0.12	0.698	0.023
IM	0.41 ± 0.05	0.27 ± 0.04	< 0.001	0.56 ± 0.14	0.51 ± 0.12	0.226	0.015
Neutrophil count	2.12 ± 0.04	0.40 ± 0.04	< 0.001	2.08 ± 0.12	1.11 ± 0.18	< 0.001	< 0.001
Mononuclear cells	2.10 ± 0.03	1.45 ± 0.03	< 0.001	2.08 ± 0.08	1.64 ± 0.11	0.004	0.085

0 = none, 1 = mild, 2 = moderate, and 3 = marked. IM: Intestinal metaplasia.

Table 5 Comparison of histological changes of gastric mucosa in metachronous cancer patients (*n* = 7)

	Baseline	12 mo	*P* value
Atrophy	1.29 ± 0.42	0.71 ± 0.36	0.436
Intestinal metaplasia	1.34 ± 0.41	1.00 ± 0.38	0.457
Neutrophil count	2.00 ± 0.22	0.86 ± 0.34	0.071
Mononuclear cell	2.00 ± 0.31	1.85 ± 0.20	0.289

0 = none, 1 = mild, 2 = moderate, and 3 = marked.

especially Billroth Ⅱ surgery.

At least 50% of the Korean population has a *H. pylori* infection and its eradication rate with standard triple therapy is reported to be between 72.5% and 83.7%[21]. A 14 d bismuth-containing quadruple therapy as the second-line treatment resulted in an eradication rate of 82.6%-93.6% in Korea[22]. After surgery, the efficacy of eradication therapy varies from 70% to 90%[9,23]. In our results, the eradication rates of the seven day standard triple therapy as first-line therapy and the 10 d bismuth-containing quadruple therapy as second-line therapy were 78.4% and 90%, respectively, using ITT analysis and 85.3% and 92.3%, respectively, using PP analysis. Our data suggest a slightly higher result than previous studies. As histological improvement was seen only in the *H. pylori* negative group, the eradication rate is important[8,10]. In our results, there were also significant histological improvements in the eradication success group. Therefore, *H. pylori* eradication following subtotal gastrectomy might lead to histological improvement.

Billroth Ⅱ surgery has been reported to result in a higher reflux rate than Billroth Ⅰ surgery[24] and bile reflux plays a role in the eradication of *H. pylori* after subtotal gastrectomy. Rino *et al*[25] reported that the overall rate of *H. pylori* infection was 37.1%: 39.6% in Billroth Ⅰ reconstruction, 0% in Billroth Ⅱ reconstruction and 55.6% in pylorus-preserving gastrectomy. We hypothesized that the reconstructive surgery method would influence eradication and histological changes. In our study, atrophy and IM scores were significantly lower than the baseline after successful eradication in the Billroth Ⅱ group. The atrophy and IM scores after successful eradication were also significantly lower in the Billroth Ⅱ group than in the Billroth Ⅰ group, suggesting that the reconstructive surgery method

influences eradication and histological changes. Previous studies have shown that the *H. pylori* infection rate was significantly lower in Billroth Ⅱ patients than Billroth Ⅰ due to the role of bile reflux which interferes with *H. pylori* colonization. If *H. pylori* is still left after Billroth Ⅱ surgery, the gastric carcinogenesis process is promoted because of the synergistic effect of bile reflux and *H. pylori* infection[24-26]. Therefore, *H. pylori* eradication should be strongly recommended following subtotal gastrectomy, especially in the Billroth Ⅱ group.

Metachronous cancer developed in seven patients in this study and all of these were patients in which *H. pylori* eradication had been successful. The atrophy and IM scores were lower than the baseline after eradication but the differences were not statistically significant. However, the atrophy and IM scores in this group were higher than the mean for all the patients and increased after eradication. These results indirectly indicate that *H. pylori* eradication alone does not ensure prevention of metachronous cancer after surgery.

This study had several limitations. First, due to its retrospective nature at a single center, only two thirds of the patient population underwent histological examination after eradication and only 40 of the 72 patients who failed first-line treatment went on to second-line eradication. Second, the follow-up time to evaluate the changes in atrophy and IM was relatively short.

Although our study was limited by its retrospective nature, we enrolled a large number of patients and evaluated the eradication rate of second-line treatment and histological changes according to eradication and reconstructive surgery method. The patients with *H. pylori* infection who underwent subtotal gastrectomy for gastric cancer had a similar eradication rate when compared with the patients with an intact stomach. The eradication success group showed histological improvement in glandular atrophy and IM. After successful eradication, the Billroth Ⅱ group showed a significant decrease in atrophy and IM scores over the Billroth Ⅰ group. Therefore, *H. pylori* eradication is needed for these patients and more active treatment is required in the Billroth Ⅱ group. Our study may support the recommendation that *H. pylori* should be treated even after gastrectomy for gastric cancer, especially after Billroth Ⅱ reconstruction.

Table 6 Histological changes of gastric mucosa according to reconstruction surgery after successful eradication

	Billroth-I (*n* = 201)			Billroth-II (*n* = 89)			*P* value
	Baseline	12 mo	*P* value	Baseline	12 mo	*P* value	
Atrophy	0.33 ± 0.10	0.31 ± 0.12	0.831	0.53 ± 0.19	0.13 ± 0.09	< 0.001	0.029
IM	0.39 ± 0.13	0.37 ± 0.13	0.572	0.67 ± 0.21	0.32 ± 0.24	< 0.001	0.034
Neutrophil count	2.24 ± 0.10	0.21 ± 0.08	< 0.001	2.33 ± 0.16	0.33 ± 0.16	< 0.001	< 0.001
Mononuclear cells	2.21 ± 0.10	1.33 ± 0.12	0.831	1.93 ± 0.19	1.47 ± 0.09	0.540	0.085

0 = none, 1 = mild, 2 = moderate, and 3 = marked. IM: Intestinal metaplasia.

COMMENTS

Background

It is well established that *Helicobacter pylori* (*H. pylori*) infection is a strong risk factor for gastric cancer. Several guidelines recommend *H. pylori* eradication in patients after surgery for gastric cancer but its beneficial effects have not been established.

Research frontiers

There is controversy as to whether *H. pylori* eradication results in histological improvement in patients following subtotal gastrectomy for gastric cancer.

Innovations and breakthroughs

This is the first study to investigate the eradication rate and histological changes after *H. pylori* eradication treatment in patients following subtotal gastrectomy for gastric cancer. The patients with *H. pylori* infection who had undergone a subtotal gastrectomy for gastric cancer had a similar eradication rate when compared with the patients with an intact stomach. *H. pylori* eradication in gastric cancer patients following a subtotal gastrectomy resulted in histological improvement, especially in the Billroth II group.

Applications

This study urges clinicians to confirm *H. pylori* infection and to start eradication therapy to prevent gastric cancer recurrence or metachronous cancer in patients following subtotal gastrectomy.

Terminology

H. pylori is a bacterium found in the stomach. It is linked to the development of gastritis, peptic ulcers and stomach cancer. To prevent recurrence in patients following subtotal gastrectomy, it is necessary to eradicate *H. pylori* infections.

Peer-review

H. pylori eradication alone does not ensure prevention of metachronous cancer after surgery but after successful eradication, the Billroth II group showed a significant decrease in atrophy and IM scores compared with the Billroth I group. So, they should make *H. pylori* eradication an important factor in the treatment of patients following Billroth II reconstruction.

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ORIGINAL ARTICLE

Retrospective Study

Is endoscopic submucosal dissection safe for papillary adenocarcinoma of the stomach?

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Abstract

AIM: To identify the clinicopathological predictors of lymph node (LN) metastasis and evaluate the outcomes of endoscopic submucosal dissection (ESD) in papillary adenocarcinoma-type early gastric cancers (EGCs).

METHODS: From January 2005 to May 2013, 49 patients who underwent surgical operation and 24 patients who underwent ESD for papillary adenocarcinoma-type EGC were enrolled to identify clinicopathological characteristics and predictive factors of LN metastasis and to evaluate the outcomes of ESD for papillary adenocarcinoma-type EGC.

RESULTS: Most papillary adenocarcinoma-type EGCs were located in the lower third of the stomach and had an elevated macroscopic shape. The overall prevalence of LN metastasis was 18.3% (9/49). The presence of lymphovascular invasion was found to be a predictor of LN metastasis ($P = 0.016$). According to current indication criteria of ESD, 6 and 11 of the 49 patients had absolute and expanded indications for ESD, respectively. Two patients (11.8%) with expanded indication for ESD had LN metastasis. Of the 24 patients who underwent ESD, 13 (54%) achieved out-of-ESD indication, with 9 of those 13 patients undergoing surgical operation due to non-curative resection.

CONCLUSION: The use of ESD should be carefully considered for papillary adenocarcinoma-type EGC with suspected ESD indication after pre-treatment work-up because of the higher frequency of LN metastasis and additional surgeries.

Key words: Gastric cancer; Papillary adenocarcinoma; Endoscopic submucosal dissection; Metastasis; Lymph node

Core tip: Papillary adenocarcinoma-type early gastric cancers (EGCs) are classified as differentiated-

type adenocarcinoma and, therefore, treated with endoscopic submucosal dissection (ESD) according to the same indication criteria as other differentiated-type adenocarcinoma, such as tubular adenocarcinoma. However, the rate of lymph node metastasis under the current ESD indication criteria was somewhat high, and more than half of the patients who underwent ESD as a primary treatment for papillary carcinoma-type EGC ultimately achieved out-of-ESD indication. Therefore, the use of ESD should be more carefully considered for papillary adenocarcinoma-type EGCs with suspected ESD indication after pre-treatment work-up.

INTRODUCTION

Recent developments in endoscopic submucosal dissection (ESD) have enabled *en bloc* resection of early gastric cancer (EGC) with a negligible risk of lymph node (LN) metastasis irrespective of tumor size or the presence of submucosal fibrosis. ESD has many advantages compared with surgical treatments, such as more accurate histological diagnosis, minimally invasive procedure, high curative resection rate, and low local recurrence rate. Therefore, ESD is widely accepted as a standard treatment strategy for EGC[1-4]. The indications of ESD for EGC have been gradually expanded[1,4]. ESD of absolute and expanded indications is recognized as a safe treatment modality for EGC based on recent studies of the long-term outcomes of ESD[2,4,5].

The World Health Organization classification of gastric carcinomas recognized four main histological patterns based on the predominant histological pattern of the carcinoma, which often co-exists with less dominant elements of other histological patterns: tubular, papillary, mucinous, and poorly cohesive (including signet ring cell type)[6]. Among these entities, papillary adenocarcinoma is a rare histologic variant of gastric adenocarcinoma that is characterized histologically by epithelial projections scaffolded by a central fibrovascular core[7]. Its biological behavior and prognostic significance is still unclear because of its rarity. Currently, papillary adenocarcinoma is classified into differentiated-type adenocarcinoma (based on the Japanese classification of gastric carcinoma)[8] and intestinal type (based on the Lauren classification)[6]. However, papillary adenocarcinoma has been reported to have a higher rate of liver metastasis and LN

involvement, as well as a lower overall 5-year survival rate compared with non-papillary gastric carcinomas such as tubular adenocarcinoma[7,9].

Given the more aggressive features of papillary adenocarcinoma, an inevitable question is whether papillary and tubular adenocarcinomas should be treated according to the same ESD indication criteria. It is doubtful that the same ESD indication criteria can be rationally applied to gastric carcinomas with or without considerable papillary adenocarcinoma components[10-12]. To date, many studies have reported on the safety and outcomes of ESD for differentiated-type adenocarcinoma of the stomach[1-4]. However, the number of papillary adenocarcinomas included in these studies was small. To our knowledge, studies investigating the safety and outcomes of ESD for papillary adenocarcinoma-type EGC alone have not been conducted. Therefore, this study aimed to investigate the clinicopathological predictors of LN metastasis and evaluate the outcomes of ESD in papillary adenocarcinoma-type EGCs.

MATERIALS AND METHODS

Study population

From January 2005 to May 2013, a total of 1,510 patients underwent surgical operation or endoscopic resection for EGC at the Pusan National University Hospital. Of these 1510 patients, 64 were histologically diagnosed with papillary adenocarcinoma-type EGC (Figure 1). All patients underwent abdominal computed tomography (CT) to determine the presence of LN or distant metastases before ESD. Endoscopic ultrasonography was also performed as needed in order to rule out submucosal invasion. We divided the 64 patients into 2 groups: group 1 consisted of 49 patients who underwent surgical operation for papillary adenocarcinoma-type EGC and group 2 consisted of 24 patients who underwent ESD as a primary treatment for papillary adenocarcinoma-type EGC. Group 1 patients were enrolled to identify clinicopathological characteristics and predictive factors of LN metastasis in papillary adenocarcinoma-type EGC. Group 2 patients were enrolled to evaluate the outcomes of ESD for papillary adenocarcinoma-type EGC. All patients provided written informed consent before ESD or surgical operation. The study protocol was reviewed and approved by the Institutional Review Board of the Pusan National University Hospital.

Surgical procedures

Laparoscopy-assisted or open gastrectomy was performed with lymphadenectomy, resection, and reconstruction. In laparoscopy-assisted gastrectomy, a laparoscope and trocars were inserted through small incisions in the abdominal wall under general anesthesia. The decision of range for surgical operation
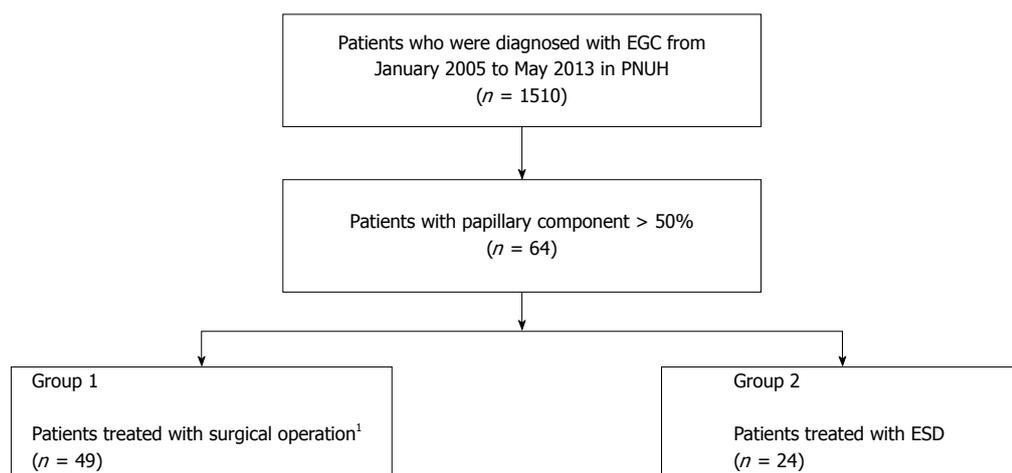
Figure 1 Flowcharts showing the patients enrolled in this study. [1]Nine patients: Additional gastrectomy after endoscopic submucosal dissection. EGC: Early gastric cancer; ESD: Endoscopic submucosal dissection.

was based on the degree of cancer progression, histopathological diagnosis of the biopsy specimen, tumor location, and risk of LN metastasis, morbidity, and mortality. In cases with proximal tumor location, near-total or total gastrectomy was done with Billroth-Ⅰ or Billroth-Ⅱ and Roux-en-Y reconstruction methods. Subtotal gastrectomy was done for tumors located in the middle third or lower third of the stomach. D1 or D2 lymphadenectomies were performed on a case-by-case basis.

ESD procedures

ESD procedures were performed by two experienced endoscopists (GH Kim and GA Song) using a single-channel endoscope (GIF-H260 or GIF-Q260; Olympus Co., Ltd., Tokyo, Japan). ESD was performed under conscious sedation with cardiorespiratory monitoring. For sedation, 5-10 mg of midazolam and 25 mg of meperidine were administered intravenously; propofol was administered as needed during the procedure. First, argon plasma coagulation was used to mark the borders of the lesion, which had been identified by conventional endoscopy or chromoendoscopy with the application of an indigo carmine solution. After marking, a saline solution (0.9% saline with a small amount of epinephrine and indigo carmine) was injected submucosally around the lesion to lift it off the muscular layer. Next, a circumferential mucosal incision was made around the marking dots with an IT knife (Olympus) and/or Flex knife (Olympus). The lesion was completely removed by submucosal dissection with these knives. If necessary, the submucosal injection was repeated and endoscopic hemostasis was achieved. A high-frequency electrosurgical current generator (Erbotom VIO 300D; ERBE, Tübingen, Germany) was used during marking, mucosal incision, submucosal dissection, and hemostasis.

Histopathological evaluation

The macroscopic shapes of lesions were categorized as either protruding (Ⅰ), non-protruding and non-excavated (Ⅱ), or excavated (Ⅲ). Type Ⅱ lesions were subclassified as slightly elevated (Ⅱa), flat (Ⅱb), or slightly depressed (Ⅱc). Lesions were classified into 3 groups: elevated (Ⅰ and Ⅱa), flat (Ⅱb), and depressed (Ⅱc and Ⅲ) types. Resected specimens were fixed in a 10% formalin solution and serially sectioned at 2-mm intervals to assess tumor involvement in the lateral and vertical margins. Tumor size, depth of invasion, presence of ulceration, lymphovascular invasion (LVI), and LN metastasis were evaluated microscopically.

The tumors were classified histopathologically according to the Japanese classification of gastric carcinoma[8]. Papillary adenocarcinoma was defined as a tumor in which more than 50% of the tumor area contained papillary structures composed of epithelial projections scaffolded by a central fibrovascular core. Histological types were classified as pure papillary (PP) and mixed papillary (MP) types. The MP type was subclassified as MP type with differentiated component (well and moderately differentiated tubular adenocarcinoma) and MP type with undifferentiated component (poorly differentiated adenocarcinoma and signet-ring cell carcinoma). The depth of mucosal invasion was classified as m1 (confined to the epithelial layer), m2 (invasion of the lamina propria), and m3 (invasion of the muscularis mucosa). The depth of submucosal invasion was classified as sm1 (submucosal invasion of $\leq$ 500 μm from the muscularis mucosa) and sm2 (submucosal invasion of > 500 μm from the muscularis mucosa).

In ESD cases, *en bloc* resection was defined as resection in a single piece as opposed to piecemeal resection (in multiple segments). Resection was regarded to be curative when *en bloc* resection

Table 1 Clinicopathological characteristics of patients who underwent surgical operation for papillary adenocarcinoma-type early gastric cancers *n* (%)

Characteristic	
No. of patients	49
Median age, yr (range)	66 (48-80)
Gender	
Male	35 (71)
Female	14 (29)
Location	
Upper third	5 (10)
Middle third	2 (4)
Lower third	42 (86)
Macroscopic shape	
Elevated	36 (73)
Flat	0 (0)
Depressed	13 (27)
Ulceration	12 (24)
Histologic type	
PP type	28 (57)
MP with diff.	14 (29)
MP with undiff.	7 (14)
Median size, mm (range)	30 (9-105)
≤ 30	24 (49)
> 30	25 (51)
Depth of invasion	
Mucosa (m1:m2:m3)	14 (0:0:14) (29)
Submucosa (sm1:sm2)	35 (8:27) (71)
Lymphovascular invasion	15 (31)
Lymph node metastasis	9 (18)

PP type: Pure papillary type; MP with diff.: Mixed papillary type with differentiated component; MP with undiff.: Mixed papillary type with undifferentiated component.

was achieved with tumor-free lateral and vertical margins, with histopathological examinations revealing differentiated-type adenocarcinoma, submucosal invasion of ≤ 500 μm from the muscularis mucosa, and the absence of LVI. Resection was regarded to be non-curative if the above criteria were not met despite complete endoscopic removal of the lesion. For surgical operation, curative resection was defined as the compete removal of macroscopic tumor tissue and tumor-free resection margin on histological examination.

Follow-up
All patients who were treated with ESD underwent post-procedural chest and abdominal radiography and second-look endoscopy on the following day to detect any perforation or bleeding. Proton pump inhibitors and sucralfate were administered to relieve pain, prevent procedure-related bleeding, and promote ulcer healing. Patients without serious symptoms or adverse events were permitted to start food intake the day after the procedure and were discharged within 3-4 d.

In cases of curative resection, follow-up endoscopy was conducted 6 mo after ESD and annually thereafter. Abdominal CT, chest radiography, and laboratory measurements of tumor markers were performed 6 mo after ESD and annually thereafter. In cases of non-

curative resection such as those with LVI, a positive vertical margin, or deep submucosal invasion, surgical operation was first recommended to all patients for curative resection. However, for patients who refused surgical operation, follow-up endoscopy with biopsies and abdominal CT were conducted 1-2 mo and 4-6 mo after ESD.

Statistical analysis
Variables were expressed as median (range) values and simple proportions. Fisher's exact test or χ^2 test was used to identify predictive factors of LN metastasis in papillary adenocarcinoma-type EGCs. Statistical calculations were performed using SPSS version 18.0 for Windows software (SPSS Inc., Chicago, IL, United States). A *P* value of < 0.05 was considered statistically significant.

RESULTS

Clinicopathological characteristics of patients with papillary adenocarcinoma-type EGC
Clinicopathological characteristics of the 49 patients who underwent surgical operation for papillary adenocarcinoma-type EGC are summarized in Table 1. Of the 49 patients, 35 were male and 14 were female, with a median age of 66 years (range: 48-80 years). Forty patients underwent primary surgical operation, with the remaining 9 undergoing an additional surgical operation for non-curative resection after ESD. Laparoscopy-assisted gastrectomy with D1 lymphadenectomy was performed in 9 patients, laparoscopy-assisted gastrectomy with D2 lymphadenectomy in 17 patients, and open gastrectomy with D2 lymphadenectomy in 23 patients. Most papillary adenocarcinomas (86%) were located in the lower third of the stomach. Macroscopically, elevated shape was more frequent than depressed shape (73% *vs* 27%). Regarding histological type, 28 patients (57%) had PP type and 21 patients (43%) had MP type. Median tumor size was 30 mm (range: 9-105 mm). Fourteen patients (29%) had mucosal cancer, and the other 35 patients (71%) had submucosal cancer. The frequencies of LVI and LN metastasis were 31% (15/49) and 18% (9/49), respectively.

Predictive factors of LN metastasis in papillary adenocarcinoma-type EGCs
Clinicopathological characteristics of patients with LN metastasis are described in Table 2. Location, macroscopic shape, ulceration, histological type, and tumor size were not associated with LN metastasis (Table 3). The incidence of LN metastases was higher in submucosal cancers than in mucosal cancers; however, this difference did not reach statistical significance [22.9% (8/35) *vs* 7.1% (1/14), *P* = 0.195]. LVI was the only clinicopathological factor significantly

Table 2 Clinicopathological characteristics of patients with lymph node metastasis

Patient No.	Gender/ age	Tumor location	Macroscopic type	Ulceration	Histologic type	Tumor size (mm)	Depth of invasion	Lymphovascular invasion	Pre-treatment ESD indication	Primary treatment
1	F/63	Lower third	Ⅱa	-	PP	35	sm2	+	Out	Op
2	M/58	Lower third	Ⅱa	-	MP + diff.	48	m3	-	In (expanded)	Op
3	F/75	Lower third	Ⅰ + Ⅱa	-	MP + undiff.	105	sm2	+	Out	Op
4	F/71	Lower third	Ⅱc	+	PP	25	sm2	-	Out	Op
5	M/67	Lower third	Ⅰ	-	MP + undiff.	23	sm2	-	Out	Op
6	M/64	Lower third	Ⅱc	+	PP	35	sm2	+	Out	Op
7	M/58	Lower third	Ⅱc + Ⅲ	+	MP + undiff.	55	sm2	+	Out	Op
8	M/68	Lower third	Ⅱa + Ⅱb	-	PP	27	sm2	+	Out	Op
9	F/63	Lower third	Ⅱa	-	MP + diff.	30	sm1	+	In (expanded)	ESD

PP type: Pure papillary type; MP + diff.: Mixed papillary type with differentiated component; MP + undiff.: Mixed papillary type with undifferentiated component; ESD: Endoscopic submucosal dissection; Out: Out-of-ESD indication; In: In ESD indication; Op: Operation.

Table 3 Predictive factors of lymph node metastasis in papillary adenocarcinoma-type early gastric cancers *n* (%)

Factors	Lymph node metastasis		*P* value
	Present (*n* = 9)	Absent (*n* = 40)	
Location			0.712
Upper third	0 (0)	5 (100)	
Middle third	0 (0)	2 (100)	
Lower third	9 (21.4)	33 (78.6)	
Macroscopic type			0.683
Elevated	6 (16.7)	30 (83.3)	
Depressed	3 (23.1)	10 (76.9)	
Ulceration			0.498
Absent	6 (16.2)	31 (83.8)	
Present	3 (25.0)	9 (75.0)	
Histologic type			0.470
PP type	4 (14.3)	24 (85.7)	
MP with diff.	2 (16.7)	10 (83.3)	
MP with undiff.	3 (33.3)	6 (66.7)	
Tumor size			0.253
≤ 30 mm	3 (12.5)	21 (87.5)	
> 30 mm	6 (24.0)	19 (76.0)	
Depth of invasion			0.195
Mucosa	1 (7.1)	13 (92.9)	
Submucosa	8 (22.9)	27 (77.1)	
Lymphovascular invasion			0.016
Absent	3 (8.6)	32 (91.4)	
Present	6 (42.9)	8 (57.1)	

PP type: Pure papillary type; MP + diff.: Mixed papillary type with differentiated component; MP + undiff.: Mixed papillary type with undifferentiated component.

associated with LN metastasis (*P* = 0.016).

Application of current criteria of ESD

Table 4 shows the prevalence of LN metastasis in 49 patients with papillary adenocarcinoma-type EGC according to the current indication criteria of ESD. Among the 49 patients, 6 had absolute indication for ESD (intramucosal cancer ≤ 20 mm in size without ulceration) and 11 had expanded indication for ESD (4 patients, intramucosal cancer > 20 mm in size without ulceration; 2 patients, intramucosal cancer ≤ 30 mm with ulceration; and 5 patients, sm1 cancer ≤ 30 mm). The other 32 patients were in out-of-ESD

indication because of tumor size, ulceration, or deep submucosal invasion.

Among the 17 patients with EGC indicated for ESD, 2 patients (11.8%) had LN metastasis. One patient had intramucosal cancer (m3) of histological MP type (with differentiated component) 48 mm in size without ulceration, and the other patient had a submucosal cancer (sm1) of histological MP type (with differentiated component) 30 mm in size with LVI.

Outcomes of patients who underwent ESD for papillary adenocarcinoma-type EGC

During the study period, 24 patients with papillary adenocarcinoma-type EGC underwent ESD as a primary treatment (Figure 2). The clinicopathological characteristics of these 24 patients are summarized in Table 5. In the pre-treatment work-up, 10 patients had absolute indication for ESD and 14 patients had expanded indication for ESD. In all cases, *en bloc* resection was obtained without treatment-related adverse events. In the final histopathology, 13 patients (54%) had out-of-ESD indication; 9 patients had LVI or deep submucosal invasion, 3 patients had intramucosal cancer > 30 mm in size with ulceration, and 1 patient had sm1 cancer > 30 mm in size (Table 6). Of these patients, 9 underwent additional surgical operation because of non-curative resection, and the other 4 patients were followed-up without operation. Recurrence was not observed during the median follow-up period of 19 mo (range: 6-51 mo) in the 15 patients who only underwent ESD.

DISCUSSION

In the present study, the overall incidence of LN metastasis in the 49 patients who underwent surgery for papillary adenocarcinoma-type EGC was 18.3%. Of the 17 patients who met the current indication criteria of ESD (6 patients, absolute indication; 11 patients, expanded indication), 2 patients with expanded indication of ESD had LN metastasis. Therefore, the rate of LN metastasis in patients with EGC indicated

Table 4 Lymph node metastasis in papillary adenocarcinoma-type early gastric cancers according to the current endoscopic submucosal dissection indication criteria *n* (%)

| | Mucosal cancer (*n* = 14) | | | | Submucosal cancer (*n* = 35) | | |
| | Ulcer (-) (*n* = 10) | | Ulcer (+) (*n* = 4) | | sm1 (*n* = 8) | | sm2 (*n* = 27) |
	⩽ 20 mm (*n* = 6)	> 20 mm (*n* = 4)	⩽ 30 mm (*n* = 2)	> 30 mm (*n* = 2)	⩽ 30 mm (*n* = 5)	> 30 mm (*n* = 3)	Any size (*n* = 27)
Lymph node metastasis	0 (0)	1 (25)	0 (0)	0 (0)	1 (20)	0 (0)	7 (26)
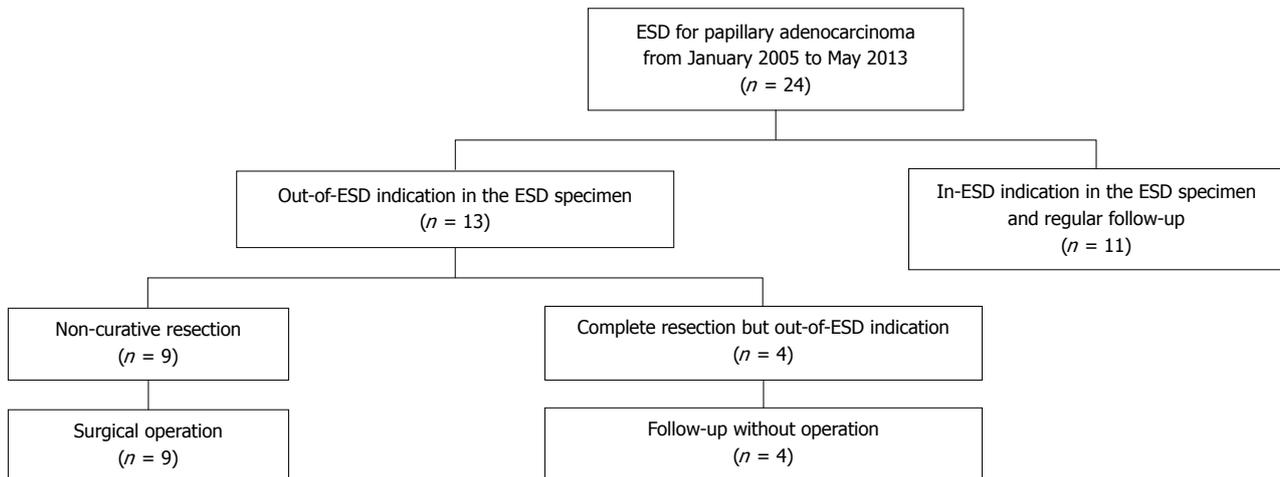
Figure 2 Outcomes of patients who underwent endoscopic submucosal dissection for papillary adenocarcinoma-type early gastric cancer. ESD: Endoscopic submucosal dissection.

Table 5 Clinicopathological characteristics of patients who underwent endoscopic submucosal dissection for papillary adenocarcinoma-type early gastric cancer *n* (%)

Characteristic	
No. of patients	24
Median age, yr (range)	68 (56-80)
Gender	
Male	19 (79)
Female	5 (21)
Location	
Upper third	4 (17)
Middle third	0 (0)
Lower third	20 (83)
Macroscopic shape	
Elevated	23 (96)
Flat	1 (4)
Depressed	0 (0)
Ulceration	5 (21)
Histologic type	
PP type	13 (54)
MP with diff.	9 (38)
MP with undiff.	2 (8)
Median size, mm (range)	22 (6-59)
⩽ 30	18 (75)
> 30	6 (25)
Depth of invasion	
Mucosa (m1:m2:m3)	14 (0:4:10) (58)
Submucosa (sm1:sm2)	10 (4:6) (42)
Lymphovascular invasion	5 (21)
Lymph node metastasis	1 (4)

PP type: Pure papillary type; MP with diff.: Mixed papillary type with differentiated component; MP with undiff.: Mixed papillary type with undifferentiated component.

for ESD was somewhat high (11.8%). Furthermore, of the 24 patients who underwent ESD as a primary treatment for papillary carcinoma-type EGC, more than half (13 patients) achieved an out-of-ESD indication. Among the 13 patients in out-of-ESD indication, 9 underwent surgical operation because of LVI or deep submucosal invasion.

Papillary adenocarcinoma accounts for approximately 6%-11% of gastric carcinomas and 1% of EGCs[7,9,13,14]. There have been few reports on the clinicopathological characteristics of papillary adenocarcinoma because of its rarity[7,9]. Papillary adenocarcinomas tend to occur in older patients and have a proximal tumor location, elevated shape in the early stage, frequent liver metastasis, higher rate of LN involvement, and lower overall 5-year survival rate compared with non-papillary gastric carcinomas. In the present study, the frequency of papillary adenocarcinoma-type EGC was 4.2% (64/1510). Most papillary adenocarcinoma-type EGCs had an elevated shape (73%) and were located in the lower third of the stomach (86%).

Despite the more aggressive features of papillary adenocarcinoma, it is currently classified as a differentiated-type adenocarcinoma and, therefore, papillary adenocarcinoma-type and tubular adenocarcinoma-type EGCs have been treated according to the same ESD indication criteria. Many recent studies have reported a high curative resection rate (84%-95%) and excellent long-term outcomes (a 5-year survival rate of

Table 6 Clinicopathologic characteristics of patients in out-of-endoscopic submucosal dissection indication

Patient No.	Gender/age	Ulceration	Histologic type	Tumor size (mm)	Pre-treatment ESD indication	Depth of invasion	LVI	Additional surgery	Follow-up period (mo)	Recurrence
1	M/74	-	PP	18	Absolute	m3	+	Yes	3	No
2	M/80	-	MP + undiff.	18	Absolute	sm2	+	Yes	7	No
3	M/68	-	PP	18	Absolute	sm2	-	Yes	18	No
4	M/66	-	PP	13	Absolute	sm2	-	Yes	20	No
5	M/56	+	PP	32	Expanded	m2	-	No	34	No
6	M/78	+	MP + diff.	34	Expanded	m3	-	No	3	No
7	M/74	+	PP	59	Expanded	m3	-	No	26	No
8	M/56	-	PP	24	Expanded	sm1	+	Yes	3	No
9	F/63	-	MP + diff.	30	Expanded	sm1	+	Yes	51	No
10	F/71	+	MP + diff.	32	Expanded	sm1	-	No	41	No
11	M/65	-	PP	22	Expanded	sm2	-	Yes	8	No
12	M/66	-	MP + diff.	24	Expanded	sm2	+	Yes	26	No
13	F/73	-	MP + undiff.	31	Expanded	sm2	-	Yes	24	No

ESD: Endoscopic submucosal dissection; LVI: Lymphovascular invasion; PP type: Pure papillary type; MP + diff.: Mixed papillary type with differentiated component; MP + undiff.: Mixed papillary type with undifferentiated component.

92%-100%) for ESD in EGC based on the absolute and expanded indications[1-4,11]. However, the number of patients with papillary adenocarcinoma included in these studies was small. Therefore, it is possible that the safety risks of ESD for papillary adenocarcinoma-type EGC have been overlooked.

Known predictive factors of LN metastasis in EGC include tumor size, histological type, depth of invasion, and LVI[15]. The overall incidence of LN metastasis in EGC is known to exceed 10% (2%-5% for mucosal cancer *vs* > 20% for submucosal cancer; 0.4% for differentiated-type intramucosal cancer *vs* 4.2%-7.3% for undifferentiated-type intramucosal cancer)[5,15,16]. In the present study, LVI was a predictive factor of LN metastasis in papillary adenocarcinoma-type EGC. The incidence of LN metastasis was 7.1% in mucosal cancer and 22.9% in submucosal cancer. Therefore, the rate of LN metastasis in papillary adenocarcinoma type-EGCs was higher in the present study than in previous reports.

The criteria of surgical operation after non-curative endoscopic resection of EGC have yet to be clearly defined. To date, the criteria of surgical operation after ESD for EGC include positive lateral or vertical margins, deep submucosal invasion (> 500 μm), LVI, or undifferentiated-type histology[10,11,17,18]. The frequency of surgical operation after ESD is 2.1%-14.6%, according to previous reports[19,20]. The rate of surgical operation for papillary adenocarcinoma-type EGC because of LVI or deep submucosal invasion was higher (37.5%) in our study than in previous reports. It is impossible to directly compare our results with those of previous studies because of the relatively small number of patients and the exclusion of other carcinoma types in our study. However, papillary adenocarcinoma-type EGCs tended to have a higher frequency of LN metastasis and additional surgical operation after ESD in the present study compared with other differentiated-

type EGCs in the previous reports, even for tumors that met the current indication criteria of ESD[5,15,16,19,20].

Our study demonstrated that ESD for papillary adenocarcinoma-type EGC should be approached more carefully because of the higher frequency of LN metastasis, LVI, and deep invasion. To our knowledge, this is the first study to demonstrate the outcomes of ESD for papillary adenocarcinoma-type EGC alone. However, our study has some limitations. Firstly, potential selection biases may have been present because of the retrospective nature of the study. Treatment options were selected on a case-by-case basis according to clinical judgment and patient factors. Secondly, our study had a relatively small number of patients and a short follow-up period. Further large-scale prospective studies with longer follow-up periods are needed to clarify the clinicopathological characteristics and outcomes of ESD in papillary adenocarcinoma-type EGC. Lastly, because a tumor's histopathological type is typically defined as the predominant type in cases with mixed histopathological components, we defined papillary adenocarcinoma as a tumor in which more than 50% of the tumor area contained papillary structure. However, it is possible that other histopathological components of papillary adenocarcinomas could confer different clinicopathological characteristics and ESD outcomes. Further investigation is required to determine the contribution of papillary and non-papillary components of EGC to clinicopathological characteristics and ESD outcomes.

In conclusion, papillary adenocarcinoma-type EGCs are classified as differentiated-type carcinoma and, therefore, treated with ESD according to the same indication criteria as other differentiated-type carcinomas such as tubular adenocarcinoma. However, our study demonstrated that the rate of LN metastasis under the current ESD indication criteria was somewhat high (11.8%). In addition, more than half of the

patients who underwent ESD as a primary treatment for papillary carcinoma-type EGC ultimately achieved out-of-ESD indication. Therefore, the use of ESD should be more carefully considered for papillary adenocarcinoma-type EGCs with suspected ESD indication after pre-treatment work-up compared with other differentiated-type adenocarcinomas because of their higher frequency of LN metastasis and additional surgical operation.

COMMENTS

Background

Papillary adenocarcinoma of the stomach is associated with a higher frequency of lymph node (LN) and liver metastasis, as well as poor surgical outcome, compared with tubular adenocarcinoma. Given the more aggressive features of papillary adenocarcinoma, an inevitable question is whether papillary and tubular adenocarcinomas should be treated according to the same endoscopic submucosal dissection (ESD) indication criteria.

Research frontiers

Studies investigating the safety and outcomes of ESD for papillary adenocarcinoma-type EGC alone are currently few. In this study, the authors investigated the clinicopathological predictors of LN metastasis and evaluated the outcomes of ESD in papillary adenocarcinoma-type early gastric cancers (EGCs).

Innovations and breakthroughs

The overall prevalence of LN metastasis in papillary adenocarcinoma-type EGCs was 18.3%. Of patients with papillary adenocarcinoma-type EGCs who met the current indication criteria of ESD, the rate of LN metastasis somewhat high (11.8%). Furthermore, of patients who underwent ESD as a primary treatment for papillary carcinoma-type EGC, more than half achieved an out-of-ESD indication.

Applications

The use of ESD should be more carefully considered for papillary adeno-carcinoma-type EGCs with suspected ESD indication after pre-treatment work-up compared with other differentiated-type adenocarcinomas because of their higher frequency of LN metastasis and additional surgical operation.

Terminology

Papillary adenocarcinoma is a rare histologic variant of gastric adenocarcinoma that is characterized histologically by epithelial projections scaffolded by a central fibrovascular core. Currently, papillary adenocarcinoma is classified as a differentiated-type adenocarcinoma.

Peer-review

The authors present a large retrospective study with 49 patients with papillary adenocarcinoma of the stomach treated with either surgical operation or endoscopic submucosal dissection. The study is the largest study to date to compare the outcome of surgical interventions and ESD for this rare histopathologic entity.

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ORIGINAL ARTICLE

Retrospective Study

Differentiation of acute and chronic hepatitis B in IgM anti-HBc positive patients

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Abstract

AIM: To identify the factors that differentiate acute hepatitis B (AHB) from chronic hepatitis B with acute exacerbation (CHB-AE).

METHODS: From 2004 to 2013, a total of 82 patients (male n = 52, 63.4%; female n = 30, 36.6%) with clinical features of acute hepatitis with immunoglobulin M antibodies to the hepatitis B core antigen (IgM anti-HBc) were retrospectively enrolled and divided into two groups; AHB (n = 53) and CHB-AE (n = 29). The AHB group was defined as patients without a history of hepatitis B virus (HBV) infection before the episode and with loss of hepatitis B surface antigen within 6 mo after onset of acute hepatitis. Biochemical and virological profiles and the sample/cutoff (S/CO) ratio of IgM anti-HBc were compared to determine the differential diagnostic factors.

RESULTS: The multivariate analysis demonstrated that, the S/CO ratio of IgM anti-HBc and HBV DNA levels were meaningful factors. The S/CO ratio of IgM anti-HBc was significantly higher in the AHB group, while the HBV DNA level was significantly higher in the CHB-AE group. The optimal cutoff values of IgM anti-HBc and HBV DNA levels for differentiating the two conditions were 8 S/CO ratio and 5.5 $\log_{10}$ IU/mL, respectively. The sensitivity and specificity were 96.2% and 89.7% for the S/CO ratio of IgM anti-HBc and 81.1% and 72.4% for HBV DNA levels, respectively. The area under receiver operating characteristic curves of both the S/CO ratio of IgM anti-HBc and HBV DNA levels were not significantly different (0.933 *vs* 0.844, *P* = 0.105). When combining IgM anti-HBc and HBV DNA, the diagnostic power significantly improved compared to HBV DNA alone (*P* = 0.0056). The combination of these factors yielded a sensitivity and specificity of

98.1% and 86.2%, respectively.

CONCLUSION: The combination of the S/CO ratio of IgM anti-HBc and HBV DNA levels was a useful tool for differentiating AHB from CHB-AE in patients with positive IgM anti-HBc.

Key words: Acute hepatitis; Differential diagnosis; Chronic hepatitis; Hepatitis B virus

Core tip: Distinguishing between acute hepatitis B (AHB) and chronic hepatitis B with acute exacerbation (CHB-AE) is important because of the different prognosis and treatment strategy. However, distinguishing AHB and CHB-AE is difficult due to their similar clinical features and serologic profiles, especially in patients with IgM anti-HBc positivity. This is the first study to differentiate between AHB and CHB-AE in a distinct group of subjects with positive IgM anti-HBc. The quantitative determination of IgM anti-HBc was useful for differentiating AHB from CHB-AE in patients IgM anti-HBc positive. The combination of serum IgM anti-HBc $\geq$ 8 S/CO ratio with HBV-DNA levels < 5.5 $\log_{10}$ IU/mL could effectively distinguish AHB from CHB-AE.

INTRODUCTION

Chronic Hepatitis B virus (CHB) infection has been an important global health problem. Cirrhosis related complications and/or hepatocellular carcinoma (HCC) are found in 25%-40% of the patients with CHB infection[1]. Thus antiviral therapy is indicated for patients with CHB with acute exacerbation (CHB-AE). When adults with an intact immune system become infected with hepatitis B virus (HBV), they usually recover without antiviral therapy[2], and the risk of developing a CHB infection after acute hepatitis B (AHB) is less than 5% in adults[3]. Nonetheless, AHB is worthy of our attention due to recent public health management, such as HBV outbreak[4]. Considering the different prognoses, treatment strategies, and prevention and public health management strategies for these conditions, correct diagnoses are extremely important. However, it is difficult due to the similar clinical features and serologic profiles[2,5]. Little is known about designing a simple assay to distinguish between these conditions.

Immunoglobulin M antibody to the hepatitis B core antigen (IgM anti-HBc) has been considered as a valuable diagnostic marker of AHB[6-8]. However, 20%-27.5% of CHB-AE patients have an IgM anti-HBc positive result with the fully automated, quantitative analysis method, and these patients could be misclassified as AHB[9]. For this reason, the cut-off index value of IgM anti-HBc was suggested to be set at a higher level for the differentiation of AHB and CHB-AE in Taiwanese patients[10]. Some authors suggested measuring IgM anti-HBc by semi-quantitative assays as a meaningful serological marker to monitor the disease activity of CHB and also to reflect the host's active immune response[11-13].

In Korea, the prevalence of HBV infection is estimated to be 3.7%[14], it is not rare to encounter CHB-AE patients with positive IgM anti-HBc. This study determined the optimal sample/cutoff (S/CO) ratio of IgM anti-HBc by chemiluminescent immunoassay which is recently available semi-quantitative assay that can identify characteristics to discriminate AHB from IgM anti-HBc positive CHB-AE.

MATERIALS AND METHODS

Patients and study design

A total of 82 patients with positive result for IgM anti-HBc upon clinical presentation of acute hepatitis or AE in CHB were retrospectively analyzed at Hallym University Medical Center, Seoul, Korea, from December 2004 to December 2013. All patients were divided into two groups according to their clinical diagnosis, AHB and CHB-AE.

The AHB group was defined patients with the clinical signs or symptoms suggestive of acute hepatitis without a history of HBV infection prior to this episode and with the loss of hepatitis B surface antigen (HBsAg) within 6 mo after onset of acute hepatitis. AE was defined as serum elevation of alanine aminotransferase (ALT) levels more than 10 times the upper limit of normal. Identified CHB patients with AE and positive IgM anti-HBc were recruited as the control group. Patients with other viral infections (hepatitis A virus, hepatitis C virus, human immunodeficiency virus and hepatitis D virus) or other concomitant liver diseases (alcoholic liver disease and autoimmune liver disease) or a recent history of hepatotoxic drugs including herbal medications or HCC were excluded.

The biochemical and virological profiles were compared between the AHB and CHB-AE groups. Then, the parameters with the greatest differences were selected to assess the diagnostic power for differentiating between AHB and CHB-AE. This study was approved by the Investigation and Ethics Committee for Human Research at the Hallym University Medical Center, Seoul, Korea.

Serum assay methodology

Routine biochemical tests were performed using standard laboratory procedures. HBsAg, antibody

Table 1 Comparison clinical features between acute hepatitis B and chronic hepatitis B with acute exacerbation				
Variables	Total (*n* = 82)	AHB (*n* = 53)	CHB-AE (*n* = 29)	*P* value
Age (yr)[1]	41.9 ± 12.7	40.2 ± 13.4	45.0 ± 11.0	0.023
Sex (male, %)	52 (63.4%)	30 (56.6%)	22 (75.9%)	0.098
WBC (× 10^3/μL)[1]	6.0 ± 1.9	6.1 ± 2.1	5.9 ± 1.6	0.808
Hb (g/dL)[1]	14.0 ± 1.7	13.9 ± 1.8	14.2 ± 1.7	0.782
Platelet (× 10^3/μL)[1]	212.1 ± 84.3	232.8 ± 85.9	174.3 ± 67.4	0.001
ALT (IU/L)[1]	1864.2 ± 1397.2	2273.2 ± 1373.1	1116.8 ± 1118.3	< 0.001
Total bilirubin (mg/dL)[1]	6.2 ± 5.6	7.0 ± 4.6	4.7 ± 7.0	< 0.001
Albumin (g/dL)[1]	3.5 ± 0.5	3.6 ± 0.5	3.4 ± 0.5	0.151
INR[1]	1.4 ± 0.9	1.5 ± 1.1	1.3 ± 0.5	0.861
HBeAg positivity (+, %)[2]	53 (64.6%)	31 (60.8%)	22 (75.9%)	0.226
HBeAg titer (S/CO)[1]	133.5 ± 261.5	49.2 ± 60.9	415.7 ± 367.8	0.001
IgM anti-HBc titer (S/CO)[1]	16.4 ± 10.7	22.3 ± 7.1	6.0 ± 6.9	< 0.001
HBV DNA (log$_{10}$ IU/mL)[1]	5.3 ± 1.8	4.5 ± 1.6	6.7 ± 1.4	< 0.001

[1]Mean ± SD; [2]HBeAg was not checked in two patients of AHB group. AHB: Acute hepatitis B; CHB-AE: Chronic hepatitis B with acute exacerbation; WBC: White blood cell; Hb: Hemoglobin; ALT: Alanine aminotransferase; INR: International normalized ratio; HBeAg: hepatitis B e antigen; IgM anti-HBc: Immunoglobulin M antibody to hepatitis B core antigen; S/CO: Sample/cutoff value; HBV: Hepatitis B virus.

to HBsAg (anti-HBs), hepatitis B e antigen (HBeAg), and antibody to HBeAg (anti-HBe) were measured using a microparticle enzyme immunoassay (Abbott Laboratories, North Chicago, IL, United States). Serum HBV DNA levels were measured by the VERANT 3.0 assay (Bayer Healthcare, Tarrytown, NY, United States; lower limit of detection 2000 copies/mL) or COBAS TaqMan PCR assay (Roche, Branchburg, NJ, United States; lower limit of detection 60 copies/mL). IgM anti-HBc was performed using the chemiluminescent immunoassay on the Abbott Architect (Abbot GmbH, Wiesbaden Delkenheim, Germany). There is a direct relationship between the amount of IgM anti-HBc in the sample and chemiluminescent reaction measured as relative light units. The presence or absence of IgM anti-HBc in the sample is determined by comparing the chemiluminescent signal in the reaction to the cutoff signal determined from a previous manufacturer's IgM anti-HBc calibration. According to the product reference, positivity was defined by an S/CO ratio ⩾ 1, and an S/CO ratio between 0.5 and 0.999 was categorized as a "gray zone". In this study, all patients had an S/CO ratio for IgM anti-HBc ⩾ 1.

Statistical analysis

The Mann-Whitney *U*-test for continuous variables and χ^2 test for categorical variables were used for the analyses as appropriate. HBV DNA levels were logarithmically transformed for analysis. Multiple logistic regression analysis was used to identify the independent factors that were significantly associated with AHB. Candidate variables with a *P*-value of less than 0.05 on a univariate analysis were entered into the regression analysis. A *P*-value of less than 0.05 was considered significant. The Youden's index was calculated as an index of sensitivity and specificity. To determine the optimal cutoff value of the variables differentiating AHB from CHB-AE, the receiver operating characteristic (ROC) curves were plotted using all possible cutoff values. The area under ROC (AUROC) curves of identified factors

were calculated and compared. The Mann-Whitney *U*-test, χ^2 test and multiple logistic regression analysis were performed with SPSS version 16 (IBM corporation, New York, United States). STATA version 11 (StataCorp, Texas, United States) was used to evaluate if the combination of identified factors could be better than either of these factors alone[15,16].

RESULTS

Comparison of clinical features between AHB and CHB-AE groups

A total 82 patients were enrolled and divided into two groups: AHB (*n* = 53, 64.6%) and CHB-AE (*n* = 29, 35.4%). The baseline characteristics of both groups are shown in Table 1. Compared to patients in the CHB-AE group, AHB patients had more severe necroinflammation of the liver, which was characterized by higher levels of serum bilirubin and ALT. The S/CO ratio of IgM anti-HBc were significantly higher in AHB group, while the HBV DNA level was significantly higher in the CHB-AE group. The HBeAg status was measured in 80 patients (51 patients in the AHB group; 29 patients in the CHB-AE group). Although the proportion of HBeAg positive patients was not significantly different between the two groups, the HBeAg titers, as reflected by the S/CO ratio, were significantly higher in the CHB-AE group than in the AHB group (415.7 ± 367.8 *vs* 49.2 ± 60.9, *P* = 0.001). The alpha fetoprotein (AFP) test was performed in only 54 patients (Thirty-two patients in the AHB group; 22 patients in the CHB-AE group). The CHB-AE group had higher AFP than the AHB group (133.5 ± 395.7 *vs* 6.7 ± 6.3, *P* < 0.001).

Independent predictor for differentiating between AHB and CHB-AE

A multivariate logistic regression analysis was performed to determine the independent predictors for the discrimination of AHB from CHB-AE using variables

Table 2 Multivariate analysis for predicting acute hepatitis B			
Variables	RR	95%CI	*P* value
Age (yr)	0.957	0.895-1.023	0.197
Platelet (× 10^3/μL)	1.001	0.988-1.014	0.934
Total bilirubin (mg/dL)	1.071	0.927-1.237	0.350
ALT (IU/L)	1.001	1.000-1.001	0.055
HBV DNA (log_{10} IU/mL)	0.323	0.135-0.773	0.011
IgM anti-HBc titer (S/CO)	1.293	1.125-1.486	< 0.001

AHB: Acute hepatitis B; RR: Relative risk; ALT: Alanine aminotransferase; HBV: Hepatitis B virus; IgM anti-HBc: Immunoglobulin M antibody to hepatitis B core antigen; S/CO: Sample/cutoff value.
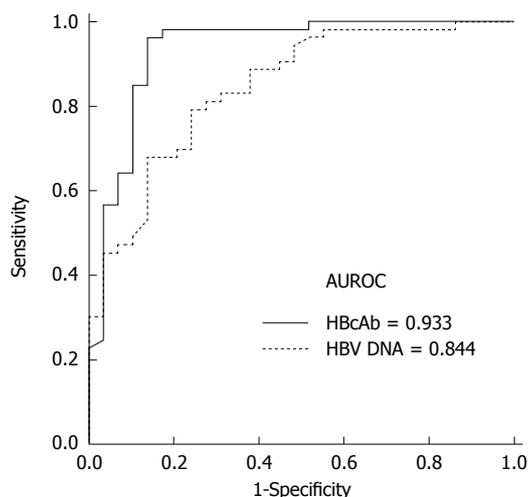
Figure 1 Receiver operating characteristic curves of sample/cutoff ratio of I immunoglobulin M antibodies to the hepatitis B core antigen and hepatitis B virus DNA levels. The area under receiver operating characteristic (AUROC) of IgM anti-HBc and hepatitis B virus DNA levels for diagnosing acute hepatitis B (AHB) were 0.933 (95%CI: 0.869-0.998, *P* < 0.001) and 0.844 (95%CI: 0.757-0.931, *P* < 0.001), respectively.

that were significant in the univariate analyses. With the multivariate analysis, high IgM anti-HBc titers and low serum HBV DNA levels were identified as independent prediction factors for AHB (Table 2).

Diagnostic values for IgM anti-HBc and HBV DNA for the differentiation of AHB from CHB-AE

To determine the optimal cutoff values for the differentiation of AHB from CHB-AE, ROC curves were plotted (Figure 1). Figure 1 shows that the AUROC of IgM anti-HBc and hepatitis B virus DNA levels for diagnosing AHB were 0.933 (95%CI: 0.869-0.998, *P* < 0.001) and 0.844 (95%CI: 0.757-0.931, *P* < 0.001), respectively. The best cutoff values for IgM anti-HBc and HBV DNA were 8 S/CO and 5.5 log_{10} IU/mL, respectively. The sensitivity and specificity at these cutoff values were 96.2% and 89.7% for IgM anti-HBc and 81.1% and 72.4% for HBV DNA, respectively. The AUROC curves of IgM anti-HBc and HBV DNA were not significantly different for differentiating AHB from CHB-AE (0.933 *vs* 0.844, *P* = 0.105). To determine if the combination of IgM anti-HBc S/CO ratio and HBV-DNA
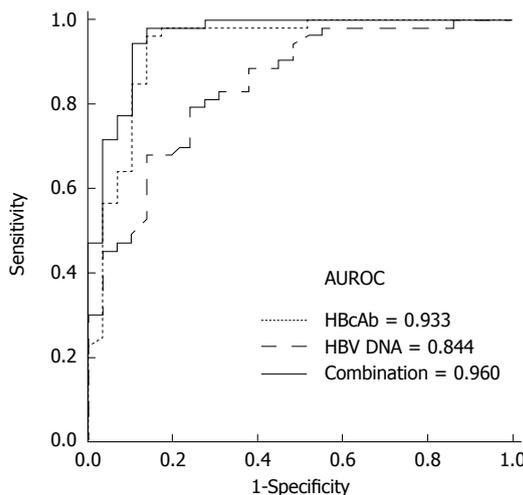
Figure 2 Receiver operating characteristic curves comparing immunoglobulin M antibodies to the hepatitis B core antigen, hepatitis B virus DNA and a combination of immunoglobulin M antibodies to the hepatitis B core antigen and hepatitis B virus DNA in patients with acute hepatitis B *vs* those with chronic hepatitis B with acute exacerbation. The area under receiver operating characteristic (AUROC) curves for IgM anti-HBc, HBV-DNA and combination are indicated in the inset. *P* = 0.0056 for HBV DNA *vs* combination, *P* = 0.22 for combination vs immunoglobulin M antibodies to the hepatitis B core antigen (IgM anti-HBc).

level was better than either of these markers alone, we created a new variable combining the IgM anti-HBc S/CO ratio and HBV-DNA level (0.2303*IgM anti-HBc - 1.0694*logHBV-DNA), which was made by a logistic regression using the "lroc" function in STATA[15]. The AUROC curve of the combination is shown in Figure 2. The AUROC curves of the combination and HBV-DNA were significantly different for the differentiation of AHB from CHB-AE (combination; 0.960 *vs* HBV DNA; 0.844, *P* = 0.0056). There was no significant difference between the combination and IgM anti-HBc (combination; 0.960 *vs* IgM anti-HBc; 0.933, *P* = 0.22). When combining the IgM anti-HBc and HBV DNA factors, there was a significant improvement in the diagnostic power compared to HBV DNA alone. The combination of these factors yielded a sensitivity and specificity of 98.11% and 86.2%, respectively.

Diagnostic performance of IgM anti HBc and HBV DNA for differentiating AHB from CHB-AE

Table 3 shows the sensitivity and specificity of IgM anti-HBc alone, HBV-DNA alone and the combination of both factors in differentiating AHB from CHB-AE at various cutoff values. The S/CO ratio of IgM anti-HBc ≥ 8 had better sensitivity, specificity, positive predictive value, and negative predictive values than the other values 7 and 9. Furthermore, the S/CO ratio of IgM anti-HBc ≥ 8 had a better diagnostic performance than HBV-DNA, regardless of the cutoff value chosen.

When the two factors were combined, the sensitivity and specificity increased from 81.1% and 72.4% for HBV-DNA alone to 98.1% and 86.2%, respectively,

Table 3 Diagnostic performance of laboratory tests for acute hepatitis B

	Sensitivity	Specificity	PPV	NPV
IgM anti-HBc (S/Co)				
≥ 7	98.1	82.8	91.2	96.0
≥ 8	96.2	89.7	94.4	92.9
≥ 9	94.3	86.2	92.6	89.3
HBV DNA (log10 IU/mL)				
< 5	56.6	86.2	88.2	52.1
< 5.5	81.1	72.4	84.3	67.7
< 6	83.0	65.5	81.5	67.9
Combination[1]	98.1	86.2	92.7	92.6

[1]Combination obtained from variable 0.2303*IgM anti-HBc-1.0694*Log HBV-DNA. The values are expressed in percent. PPV: Positive predictive value; NPV: Negative predictive value; IgM anti-HBc: Immunoglobulin M antibody to hepatitis B core antigen; S/CO: Sample to the cutoff value; HBV: Hepatitis B virus.

but when comparing IgM anti-HBc, only the sensitivity increased from 96.2% to 98.1%.

DISCUSSION

In Korea, it is not rare to encounter HBV infected patients with clinical and biochemical features resembling acute hepatitis. Historical information of chronicity or recent HBV exposure would facilitate the differential diagnosis between AHB and CHB-AE. However, this information is not always provided. Discriminating between the two conditions is difficult, especially in patients who are IgM anti-HBc positive.

In this study, an IgM anti-HBc ≥ 8 S/CO ratio and an HBV-DNA level < 5.5 $\log_{10}$ IU/mL aided the differentiation of AHB from CHB-AE in IgM anti-HBc positive patients. The sensitivity and specificity at these cutoff values were 96.2% and 89.7% for IgM anti-HBc, 81.1% and 72.4% for HBV DNA, respectively. These results are consistent with the data from previous studies comparing AHB and CHB-AE patients. AHB patients demonstrate a high titer of IgM anti-HBc and a low level of HBV DNA[17-19]. However, our study recruited only IgM anti-HBc positive CHB-AE patients as a control, unlike previous studies. This prevented the misclassification of the patient groups and enhanced the discrimination between the two groups. Furthermore, the AUROC curves of the combination of the S/CO ratio of IgM anti-HBc and HBV-DNA level was better than each factor alone, a significant difference was shown only with the AUROC curve of the combination and HBV-DNA level alone. In addition to these factors, Han *et al*[17] suggested that the S/CO ratio of HBeAg could be a diagnostic parameter for distinguishing AHB from CHB-AE. In the present study, similar results were observed between the two groups. Although the proportion of patients with positive HBeAg in the two groups were not significantly different (60.8% *vs* 75.9%), the HBeAg positive patients from the CHB-AE group had significantly

higher S/CO HBeAg ratios than the AHB group (380.8 *vs* 34.2 S/CO, *P* = 0.001). A high S/CO IgM anti-HBc ratio, low HBV DNA level and HBeAg titer in the AHB group suggests a more robust immune response. This finding is underpinned by the previous data, the HBcAg effectively activates antigen presenting cells, leading to a strong proliferative T-cell response and triggering the humoral immune response for the production of IgM and IgG anti-HBc. Consequently, the cellular and humoral immune systems suppress HBV replications and clear HBsAg[20].

The level of AFP was higher in the CHB-AE group than the AHB group (24.9 ng/mL *vs* 5.0 ng/mL, *P* < 0.001). AFP, a tumor marker for HCC diagnosis and surveillance, can be elevated in a number of nonspecific conditions in patients with cirrhosis or hepatitis[21,22]. Increased serum AFP levels in patients with non-malignant liver disease was suggested to be associated with ongoing liver cell regeneration in response to liver injury[23,24]. Other possible mechanisms include viral derepression of the genome controlling AFP synthesis or an acute-phase reaction to liver injury[25]. However, these mechanisms cannot sufficiently explain why the AFP levels of the CHB-AE group are higher than the AHB group. In CHB-AE, the additive hepatic necrosis in pre-existent altered hepatocyte architecture, such as fibrosis, cirrhosis or liver cell dysplasia, may lead to more severe parenchymal damage leading to greater stimulation and regeneration than with AHB. Further study is necessary to answer this question.

In this study, we defined AHB using the clinical criteria and taking the patient's history into account. It is very challenging to correctly classify AHB and the first presentation of CHB-AE patients in the clinical settings. Therefore, the classification of patients according to our strict criteria might be the best practical approach to determine chronicity. A small percentage of CHB patients may have been classified as AHB in our study. Considering the natural course of CHB, the possibility of HBsAg loss within 6 months after CHB-AE would be extremely rare and the differences in IgM anti-HBc titer and serum HBV DNA levels between the two groups would decrease if a subset of the CHB-AE patients had indeed been misallocated into the AHB group. Nevertheless, the IgM anti-HBc titers and serum HBV DNA levels remained significant factors for the differentiation of AHB from CHB-AE with a multivariate analysis. This suggests that IgM anti-HBc titers and serum HBV DNA levels are indeed discriminating factors between AHB and CHB-AE, although there is a limitation in the classification of chronicity based on the patient's medical history.

This is the first study to differentiate between AHB and CHB-AE in a distinct group of subjects with positive IgM anti-HBc. There are some limitations. First, it was retrospectively designed, and some patients were misclassified as mentioned above. Second, the number

of patients with CHB-AE (n = 29) was relatively small compared to the patients with AHB (n = 53). Finally, the IgM anti-HBc assay by Abbot Architect is actually a semi-quantitative method, but it is accommodated for the quantitation in this study, and it is unknown whether the formula for IgM anti-HBc S/CO by the Abbot Architect is useful with other commercial IgM anti-HBc kits. Nevertheless, the distinct difference of IgM anti-HBc titers between the two groups was similar to previous studies[10,19].

In conclusion, the quantitative determination of IgM anti-HBc could be a useful, simple tool for differentiating AHB from CHB-AE in IgM anti-HBc positive patients. The combination of a serum IgM anti-HBc $\geq$ 8 S/CO ratio with an HBV-DNA level < 5.5 $\log_{10}$ IU/mL can effectively distinguish AHB from CHB-AE.

COMMENTS

Background
Distinguishing between acute hepatitis B (AHB) and chronic hepatitis B with acute exacerbation (CHB-AE) is extremely important because of the patients' different prognoses and treatment strategies as well as different prevention and public health management strategies.

Research frontiers
Immunoglobulin M antibody to hepatitis B core antigen (IgM anti-HBc) is a valuable diagnostic marker of AHB. However, 20%-27.5% of CHB-AE patients are IgM anti-HBc positive according to the fully automated, quantitative analysis method. Thus, these patients could be misclassified as AHB. In this study, the authors demonstrated that the combination of the IgM anti-HBc sample/cutoff (S/CO) value and hepatitis B virus (HBV) DNA level had a good sensitivity and specificity when distinguishing AHB and CHB-AE in patients positive for IgM anti-HBc.

Innovations and breakthroughs
Recent reports comparing AHB and CHB-AE patients indicated that a high titer of IgM anti-HBc and a low level of HBV DNA were the characteristics of AHB patients. However, our study recruited only IgM anti-HBc positive CHB-AE patients as a control, unlike previous studies. Furthermore, the authors created formula to prove that the combination of IgM anti-HBc and HBV DNA levels is a better diagnostic parameter than either of them alone.

Applications
By providing a cutoff value of IgM anti-HBc S/CO values and HBV DNA levels, this study may help physicians differentiate between AHB and CHB-AE in patients who are positive for IgM anti-HBc.

Terminology
HBV infection is one of the causes responsible for acute or chronic hepatitis. Chronic hepatitis is inflammation of the liver that lasts more than six months. IgM anti-HBc is the first antibody observed in the serum of patients with AHB. Therefore, it has been considered to be a diagnostic marker.

Peer-review
The authors compared different cutoff values of the S/CO ratio of IgM anti-HBc and HBV-DNA, and concluded that the S/CO ratio of IgM anti-HBc $\geq$ 8 with an HBV-DNA level < 5.5 $\log_{10}$ IU/mL can discriminate AHB and CHB-AE. Although this manuscript presents a retrospectively study, the statistical analysis is good.

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ORIGINAL ARTICLE

Retrospective Study

Methylation of *IRAK3* is a novel prognostic marker in hepatocellular carcinoma

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Abstract

AIM: To examine the methylation levels of interleukin-1 receptor-associated kinase 3 (*IRAK3*) and *GLOXD1* and their potential clinical applications in hepatocellular carcinoma (HCC).

METHODS: mRNA expression and promoter methylation of *IRAK3* and *GLOXD1* in HCC cells were analyzed by reverse transcription-polymerase chain reaction (RT-PCR) and methylation-specific PCR (MSP), respectively. Using pyrosequencing results, we further established a quantitative MSP (Q-MSP) system for the evaluation of *IRAK3* and *GLOXD1* methylation in 29 normal controls and 160 paired HCC tissues and their adjacent nontumor tissues. We also calculated Kaplan-Meier survival curves to determine the applications of gene

methylation in the prognosis of HCC.

RESULTS: *IRAK3* and *GLOXD1* expression was partially restored in several HCC cell lines after treatment with 5-aza-2'-deoxycytidine (DNA methyltransferase inhibitor; 5DAC). A partial decrease in the methylated band was also observed in the HCC cell lines after 5DAC treatment. Using *GLOXD1* as an example, we found a significant correlation between the data obtained from the methylation array and from pyrosequencing. The methylation frequency of *IRAK3* and *GLOXD1* in HCC tissues was 46.9% and 63.8%, respectively. Methylation of *IRAK3* was statistically associated with tumor stage. Moreover, HCC patients with *IRAK3* methylation had a trend toward poor 3-year disease-free survival ($P < 0.05$).

CONCLUSION: *IRAK3* and *GLOXD1* were frequently methylated in HCC tissues compared to normal controls and nontumor tissues. *IRAK3* methylation was associated with tumor stage and poor prognosis of patients. These data suggest that *IRAK3* methylation is a novel prognostic marker in HCC.

Key words: *IRAK3*; *GLOXD1*; Hepatocellular carcinoma; DNA methylation biomarker; Quantitative methylation-specific polymerase chain reaction; Pyrosequencing

Core tip: The methylation biomarker is relatively stable in tissue samples and body fluids, suggesting that it is a good tool for the detection, diagnosis, prognosis, and even therapy of hepatocellular carcinoma (HCC). Our study not only demonstrated frequent methylation of interleukin-1 receptor-associated kinase 3 (*IRAK3*) and *GLOXD1* in HCC but also found that *IRAK3* methylation was positively associated with poor 3-year disease-free survival of patients. This indicates that *IRAK3* methylation could be used as a potential biomarker for prediction of prognosis in HCC.

INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common causes of cancer deaths in the world[1]. HCC is a serious disease because it is difficult to detect in its early stages; this leads to a very poor prognosis and high mortality. It is believed that studying the molecular mechanisms of HCC development can help

us to design better strategies for disease detection or prognosis prediction[2].

Aberrant changes in DNA methylation patterns, which alter gene expression and subsequently drive malignant transformation, are recognized as a common event during carcinogenesis[3] and are also found during the development of HCC[4,5]. Identification of these events not only allows for a detailed understanding of the hepatocarcinogenesis but also provides potential clinical applications in the diagnosis or prognosis of HCC[6]. Recently, technical advances in array systems have led to the development of higher-resolution genome-wide methods for DNA methylation analysis, such as Infinium assay[7]. It has also been successfully used in the study of HCC[8-17]. By using array-based platforms, researchers can simultaneously profile the DNA methylation of a large number of genes or the entire genome. Furthermore, by validating the results from the high-throughput screening approach, researchers can effectively discover more novel genes that may have potential applications in clinical practice.

In our recent study[18], we found several aberrantly methylated genes in HCC by using the Infinium HumanMethylation27 BeadChip and then verified 34 genes by methylation-specific PCR (MSP). Of these genes, we further showed that frequent methylation of homeobox A9 (*HOXA9*) in HCC tissues and plasma samples from patients could be a helpful biomarker to assist in HCC detection. However, several novel genes in our array data were not further validated by quantitative MSP (QMSP), such as interleukin-1 receptor-associated kinase 3 (*IRAK3*) and 4-hydroxyphenylpyruvate dioxygenase-like (*HPDL*, also known as *GLOXD1*). IRAK3 plays an important role in alcohol-induced liver injury[19], and HPDL is an important enzyme in the catabolic pathway of tyrosine in the liver[20]. Moreover, there are no quantitative data about the methylation levels of *IRAK3* and *GLOXD1* in HCC. In this study, we aimed to examine the methylation levels of *IRAK3* and *GLOXD1* in HCC by QMSP and to further test whether these two genes have potential clinical applications in the diagnosis or prognosis of HCC.

MATERIALS AND METHODS

Cell lines and samples for methylation analysis

A normal liver cell line (THLE-3) and 6 HCC cell lines (HepG2, SK-HEP1, TONG, Mahlavu, PLC/PRF/5, and HuH6) were used in this study. THLE-3, HepG2, and SK-HEP1 cells were purchased from American Type Culture Collection. TONG, Mahlavu, PLC/PRF/5, and HuH6 cells were provided by Professor K.H. Lin (Chuang-Gung University, Taiwan). For 5-aza-2'-deoxycytidine (5DAC) treatment, HCC cells were prepared as previously described and harvested directly for reverse transcription-polymerase chain reaction (RT-PCR) and MSP[18]. THLE-3, 3 HCC cell lines (PLC/PRF/5, HepG2,

Table 1 Primer and probe sequences for reverse-transcription polymerase chain reaction, methylation-specific polymerase chain reaction, pyrosequencing, and quantitative methylation-specific polymerase chain reaction

Primer	Sequence (5'→3')	Amplicon (bp)
RT-PCR		
IRAK3-Forward	ATGCAGTGTAAGAAGCATTGGA	247
IRAK3-Reverse	GCAGGTAGTGAATGGCTTTGG	
GLOXD1-Forward	CCCTTCCTACCCGGCTTCA	122
GLOXD1-Reverse	TGGAACCAGCGCAAAAGTGT	
Pyrosequencing		
GLOXD1-Forward	GAAGGGAGGTTTAGTGTTTAAGGA	242
GLOXD1-Reverse	AGCTGGACATCACCTCCCACAACGCCACCCCAACCAAAAACA	
Universal primer	AGCTGGACATCACCTCCCACAACG-Biotin	
Sequencing primer	AGGTTTAGTGTTTAAGGAT	
MSP/Q-MSP		
IRAK3-Forward	AGGAGATCGTTTAGTCGTGGGGTAC	110
IRAK3-Reverse	ACCTCTACGATAAAAACGAAACTACCG	
IRAK3-Probe	CTACCGAAACAAACAAAATA	
GLOXD1-Forward	AGGATGTGATTAGGCGTGAGGTTC	122
GLOXD1-Reverse	AAAAAAACGAAACCCGTAACTCCG	
GLOXD1-Probe	FAM-CGCTACTCTTTCCCC	

Allele-specific primer sequences for MSP and Q-MSP are the same. RT-PCR: Reverse transcription-polymerase chain reaction; MSP: Methylation-specific polymerase chain reaction; Q-MSP: Quantitative MSP.

Table 2 Clinicopathological characteristics of hepatocellular carcinoma patients

Characteristic	Cases
Age, yr	59 ± 14
Mean ± SD	
Gender	
Female	94
Male	66
Hepatitis	
HBV-positive	68
HCV-positive	62
Double-negative	30
Cirrhosis	
No	77
Yes	80
Unknown	3
Tumor size, cm	
≤ 3	52
> 3	108
Nodule	
Solitary	98
Multiple	62
AFP level, ng/mL	
≤ 10	45
> 10	113
Unknown	2
Stage	
I	60
II	46
III	47
IV	7
Invasion	
No	85
Yes	75
Recurrence	
No	58
Yes	36
Unknown	66
Survival	
No	71
Yes	27
Unknown	62

HBV: Hepatitis B virus; HCV: Hepatitis C virus; AFP: Alpha-fetoprotein.

and HuH6), and 7 types of pooled tissues (each type was independently pooled with an equal amount of DNA from 5 tissues) were used as the samples for methylation analysis by pyrosequencing. The primer sequences of RT-PCR and MSP are summarized in Table 1.

Patients

The Taiwan Liver Cancer Network (TLCN) is funded by the National Science Council to provide researchers in Taiwan with primary liver cancer tissues and their associated clinical information. With the approval by the TLCN User Committee and the Institutional Review Board of the Tri-Service General Hospital (TSGH), 29 normal parts of liver hemangiomas (as normal controls) and a total of 160 HCC tissues and their paired adjacent nontumor tissues were used in this study. Among these samples, 40 HCC tissues and their paired adjacent nontumor tissues were obtained from TSGH; the others were obtained from TLCN. These specimens were obtained during surgery, frozen immediately in liquid nitrogen and preserved at -80 ℃ until DNA extraction. The diagnosis of HCC samples was confirmed by histology. The clinicopathological characteristics of the patients are summarized in Table 2.

Sodium bisulfite treatment, pyrosequencing, and Q-MSP

Genomic DNA from tissue samples was extracted and prepared for sodium bisulfite treatment and methylation analysis as previously described[21]. Pyrosequencing for the methylation levels of 11 CpG sites in a *GLOXD1* promoter was carried out using PCR and sequencing primers, as previously described[22]. The primers for pyrosequencing were designed with PyroMark Assay Design 2.0 software (Qiagen, Hilden, Germany) to amplify and sequence bisulfite-treated DNA. PCR was carried out in a 20

A

B
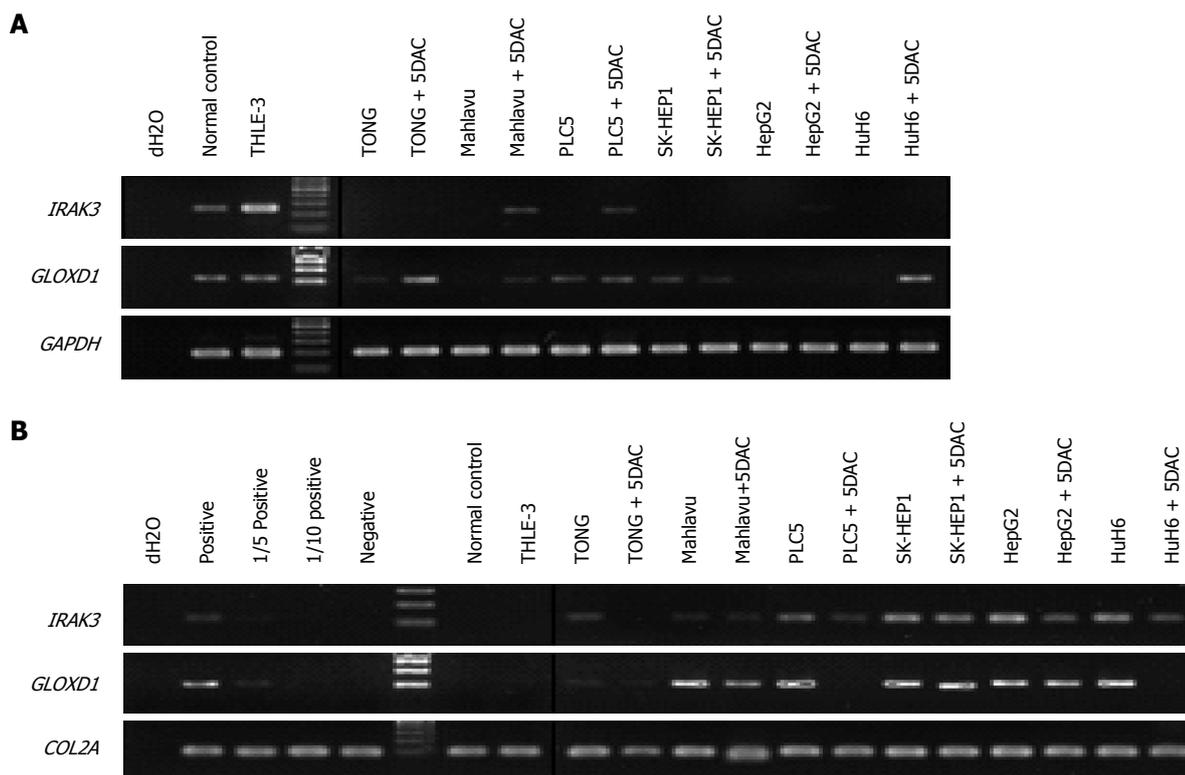
Figure 1 Gene expression and methylation analyses of *IRAK3* and *GLOXD1*. A: Gene expression levels of *IRAK3*, *GLOXD1*, and *GAPDH* (an internal reference gene) were analyzed by RT-PCR in normal controls, THLE-3 cells, 6 HCC cell lines, and HCC cell lines treated with 5DAC; B: Methylation status of *IRAK3*, *GLOXD1*, and *COL2A* (an internal reference gene) was analyzed by MS-PCR with methylated primers in normal controls, THLE-3 cells, 6 HCC cell lines, and HCC cell lines treated with 5DAC. Positive and negative are peripheral *blood* lymphocyte (*PBL*) DNA *in vitro* treated with or without CpG methyltransferase (*M.SssI*). 1/5 positive and 1/10 positive indicate 1:5 and 1:10 dilution of the positive control.

μL reaction mix containing 1 μL bisulfite-converted DNA, 2 × RBC SensiZyme HotStart Taq Mastermix (RBC Bioscience Corp., Taipei, Taiwan), and primers using the following program: 95 ℃ for 15 min, then 49 cycles of 95 ℃ for 30 s, 62 ℃ for 30 s and 72 ℃ for 30 s, with a final extension at 72 ℃ for 10 min. The biotinylated PCR product was purified by binding to streptavidin-sepharose beads, washed, and denatured. The sequencing primer was then added to the PCR products, and pyrosequencing was performed using the PyroMark Q24 system (Qiagen). Q-MSP was performed in the TaqMan probe system using the LightCycler 480 system (Roche Applied Science, Mannheim, Germany) and prepared as previously described[18]. The *COL2A* gene was used as an internal reference by amplifying non-CpG sequences. Results with cycle threshold values (Cq values) of *COL2A* > 38 were defined as detection failures. The DNA methylation level was determined as a methylation index using the following formula: 100 × 2 [(Cq of COL2A) - (Cq of target genes)][23]. Each set of amplifications included a positive control, a negative control, and a non-template control. The primer and probe sequences of pyrosequencing and Q-MSP are summarized in Table 1.

Statistical analysis

The prism software (version 4.03; Graphpad Software Inc, La Jolla, CA) was used for statistical analyses. The unpaired *t*-test and paired *t*-test were used to determine the difference of the methylation index between tissues with different disease status. Fisher's exact test, χ^2 test, and χ^2 test for trend were used to evaluate the association between gene methylation and clinical parameters. Pearson correlation was used to compare the consistency of different techniques. Receiver operating characteristic (ROC) curves were generated to determine the optimal cut-off point of gene methylation for discriminating tumors and normal controls. Kaplan-Meier curves were used to estimate survival fraction of patients for 3 years after treatment. Log-rank tests were used to *compare* the *survival* of patients with or without gene methylation.

RESULTS

Correlation between gene expression and promoter methylation of IRAK3 and GLOXD1 in cell lines

To confirm the results from the methylation array, we first analyzed the correlation between gene expression and promoter methylation of *IRAK3* and *GLOXD1* in cell
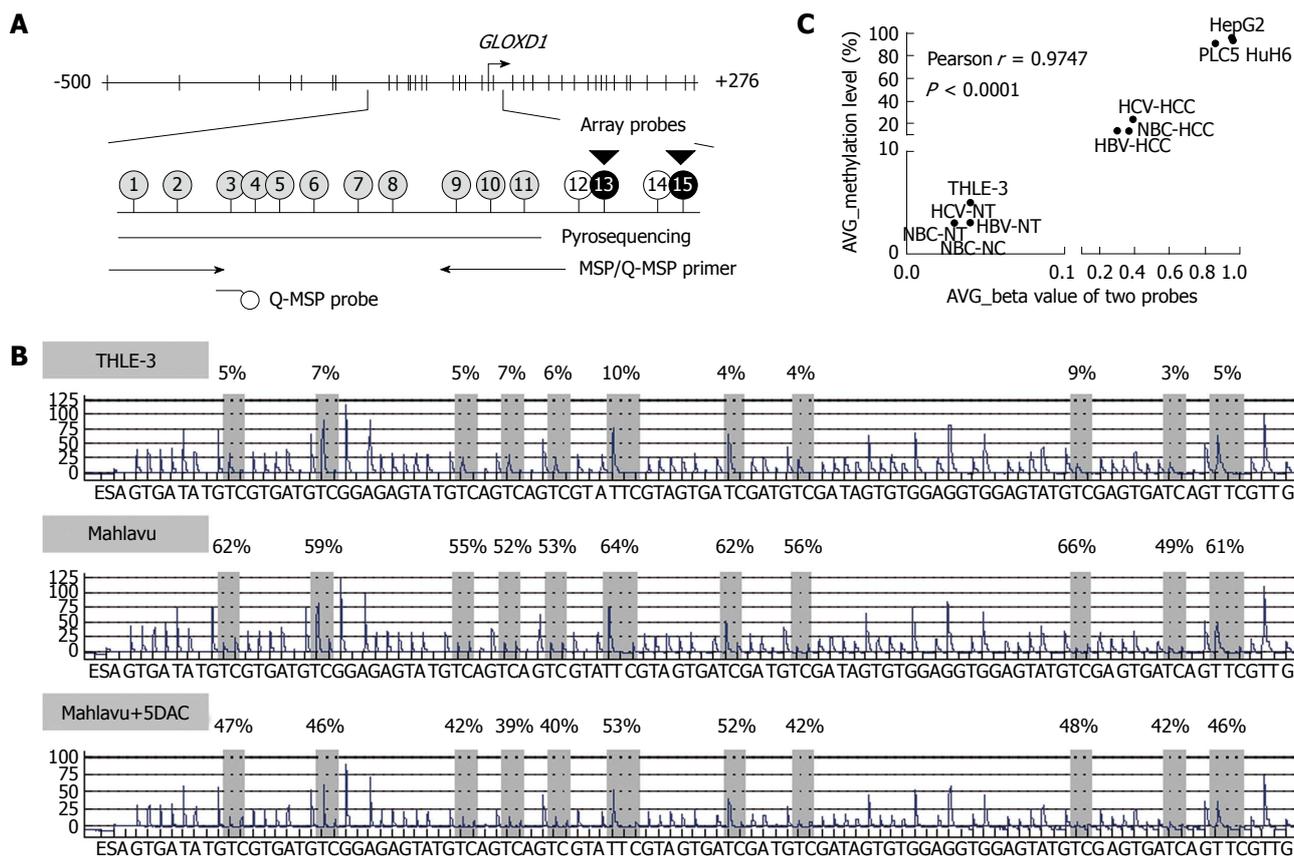
Figure 2 Map of the *GLOXD1* promoter region and representative methylation pattern determined by pyrosequencing. A: The 15 CpG sites within the *GLOXD1* promoter (-500/+276) were addressed using different techniques. Two black circles indicate the 2 CpG sites recognized by probes of the methylation array chip, respectively. Eleven gray circles indicate the 11 CpG sites addressed by pyrosequencing and the 6 CpG sites that MSP/Q-MSP primer set covered (5 CpG sites for allele-specific*, one CpG site for probe*); B: Methylation level of 11 CpG sites addressed by pyrosequencing in THLE-3 cells, Mahlavu cells, and Mahlavu cells treated with 5DAC; C: Pearson correlation was analyzed between the average β value of two array probes and average methylation levels of the 11 CpG sites assessed by pyrosequencing in samples for methylation array, including 4 cell lines and 7 types of liver tissues.

lines by RT-PCR and MSP (Figure 1). Expression analysis showed that *IRAK3* and *GLOXD1* were expressed in normal control and THLE-3 cells but down-regulated in several HCC cell lines (Figure 1A). In addition, the expression of *IRAK3* and *GLOXD1* was partially restored after treatment with 5DAC (a DNA methyltransferase inhibitor). Methylation analysis revealed that *IRAK3* and *GLOXD1* methylation was detected mainly in HCC cell lines, and a partial decrease in the methylated band was also observed in the HCC cell lines after 5DAC treatment (Figure 1B). These results implied that *IRAK3* and *GLOXD1* were down-regulated in HCC cell lines through promoter methylation.

Verification of gene methylation in cell lines and pooled samples by pyrosequencing
We then confirmed the methylation levels of *IRAK3* and *GLOXD1* in cell lines and pooled samples by pyrosequencing (Figure 2; *GLOXD1* as an example). Methylation levels of 11 CpG sites in *GLOXD1* promoter that is close to the two probe sites on array were examined (Figure 2A). It revealed that the *GLOXD1* methylation level was 3%-10% in THLE-3 cells and 49%-66% in Mahlavu cells (Figure 2B). Consistent

with MSP, a partial decrease in the methylation level of the *GLOXD1* promoter was observed in Mahlavu cells after 5DAC treatment (39%-53%). Furthermore, the average β value for different array probes was significantly correlated to the average methylation level of the 11 CpG sites in the samples used in the methylation array ($r = 0.9747$, Figure 2C). In addition, *GLOXD1* methylation was much lower in THLE-3 cells, the pooled normal controls, and each type of pooled nontumor tissues compared to HCC cell lines and all types of pooled tumor tissues. Finally, we designed a primer and probe set based on the CpG methylation results of pyrosequencing to carry out Q-MSP analysis in larger clinical samples.

Methylation analysis of IRAK3 and GLOXD1 in HCC tissues by Q-MSP
To examine the methylation levels of *IRAK3* and *GLOXD1* in HCC, we analyzed 29 normal controls, 160 paired HCC tissues, and their adjacent nontumor tissues using Q-MSP (Figure 3). Promoter methylation of *IRAK3* and *GLOXD1* was both significantly increased in HCC tissues compared to normal controls and nontumor tissues (Figure 3A). Furthermore, to find
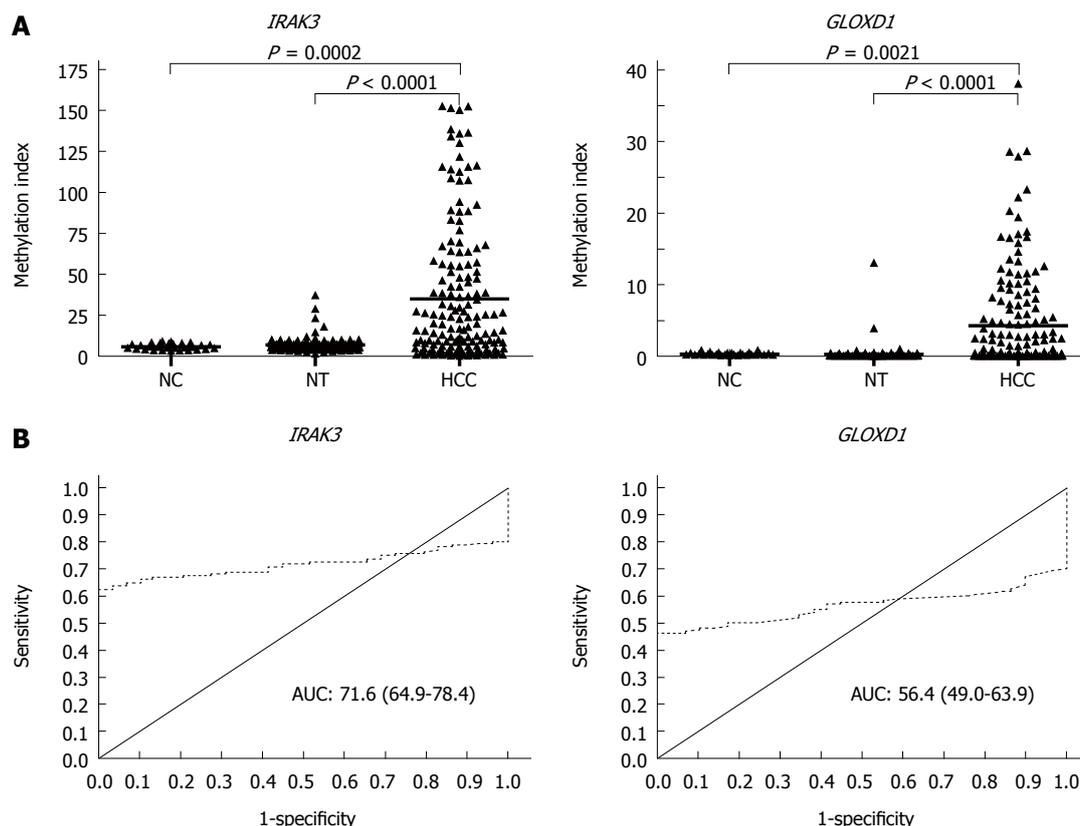
Figure 3 Methylation levels and receiver operating characteristic curve analysis of *IRAK3* and *GLOXD1* in liver tissues. A: Gene methylation was determined in 29 normal controls (NC) and 160 paired hepatocellular carcinoma (HCC) tissues and their adjacent notumor tissues (NT) by quantitative methylation-specific polymerase chain reaction. The results are represented as the difference in the methylation index. The black lines indicate the mean of the methylation index. (NC *vs* HCC, unpaired *t*-test; NT *vs* HCC, paired *t*-test); B: The area under the receiver operating characteristic curve (AUC) for each gene was calculated to discriminate 29 normal individuals and 160 HCC cases.

Table 3 Methylation frequency of *IRAK3* and *GLOXD1* in liver tissues

Symbol	M-Index[1] cut-off value	No. of methylated cases/total			P value[2]
		Normal controls	Nontumor tissues	HCC tissues	
IRAK3	8.90	1/29 (3.4%)	23/160 (14.4%)	102/160 (63.8%)	< 0.0001
GLOXD1	0.60	2/29 (6.9%)	4/160 (2.5%)	75/160 (46.9%)	< 0.0001

[1]Methylation-Index: the best cut-off value to discriminate 29 normal controls and 160 hepatocellular carcinoma (HCC) tissues; [2]χ^2 test for trend.

a best cut-off value for defining methylated cases, ROC curve analysis of each gene was performed to discriminate normal controls and HCC tissues (Figure 3B). As summarized in Table 3, *IRAK3* and *GLOXD1* methylated cases were mainly present in HCC tissues (102/160, 63.8%; 75/160, 46.9%) compared to normal controls (1/29, 3.4%; 2/29, 6.9%).

Association between clinicopathologic parameters and gene methylation

To evaluate the association of gene methylation with clinicopathological characteristics, we analyzed a total of 160 HCC patients (Table 4 and Figure 4). As shown in Table 4, there was a statistically significant correlation between *IRAK3* methylation and tumor stage (*P* = 0.03), but no significant association was shown

between *GLOXD1* methylation and clinicopathological parameters. As shown in Figure 4, HCC patients with *IRAK3* methylation was found to have a trend toward poor 3-year disease-free survival (*P* = 0.0386, log-rank test) but not in patients with or without *GLOXD1* methylation.

DISCUSSION

Recently, several high-resolution methods for genome-wide methylation analysis have been used in the study of HCC, such as methylated CpG island amplification microarray, bacterial artificial chromosome array-based methylated CpG island amplification, GlodenGate assay, and Infinium assay[8-17]. These results provide evidence that HCC tumors with specific DNA methylation patterns
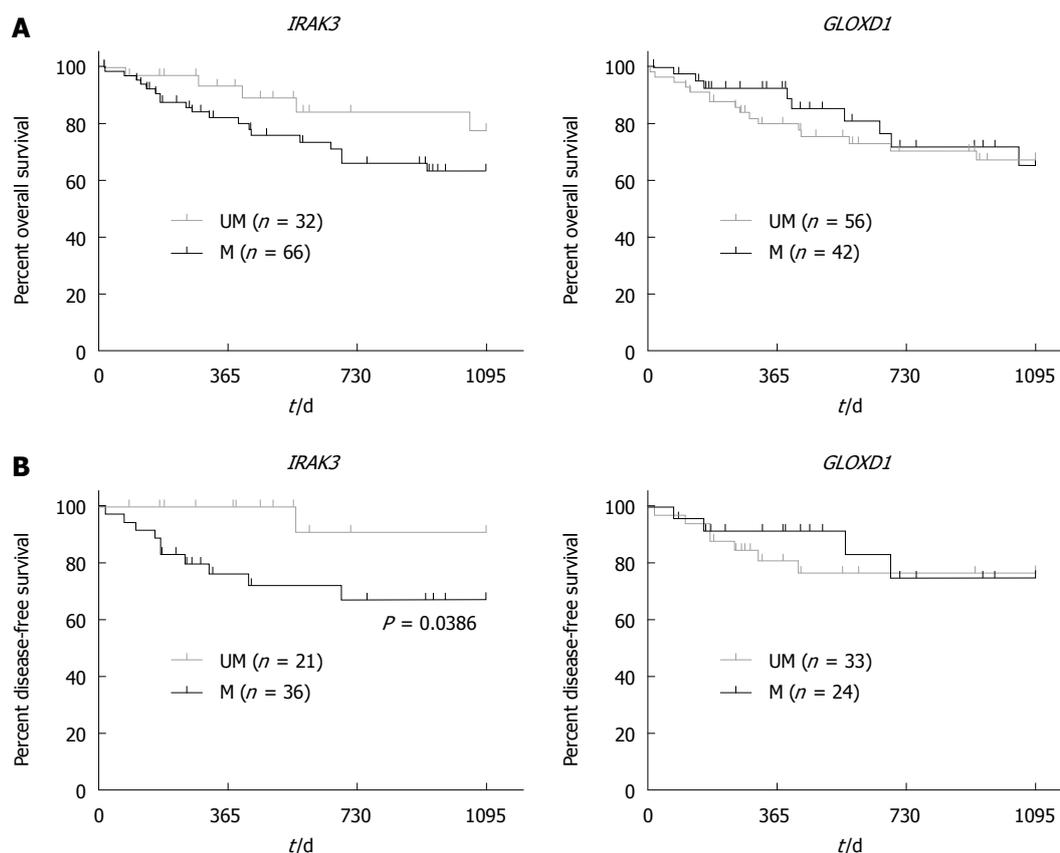
Figure 4 Correlation analyses between gene methylation and the survival of hepatocellular carcinoma patients. A: Survival was analyzed using Kaplan-Meier curves. The plots were made according to the patients with *IRAK3* and *GLOXD1* methylation and 3-year overall survival in 98 hepatocellular carcinoma (HCC) patients, respectively; B: Kaplan-Meier survival curves were made according to the cases with *GLOXD1* and *IRAK3* methylation and 3-year disease-free survival in 57 HCC patients (*P = 0.0386, log-rank test,* UM, unmethylated cases *vs* M, methylated cases).

associated with risk factors or progression of HCC have important clinical applications. In our recent study, we also used the Infinium HumanMethylation27 BeadChip to analyze DNA methylation signatures of HCC and found 1968 genes that were hypermethylated in non-tumor tissue and/or tumor tissue with different viral etiologies. Among 34 genes selected for verification, we further identified that methylation of the *HOXA9* gene could be a helpful biomarker to assist in HCC detection. In this study, we further identified that two novel genes, *IRAK3* and *GLOXD1,* were frequently methylated in HCC. However, both of these two genes were undetectable in plasma. Moreover, *IRAK3* methylation was statistically associated with tumor stage and poor 3-year disease-free survival of HCC patients.

IRAK3 encodes a member of the interleukin-1 receptor-associated kinase protein family that is an essential component of the Toll/IL-R immune signal transduction pathways. This gene is primarily expressed in monocytes and macrophages, and it is also detected in various adult human tissues including the liver[24]. It has been known that IRAK3 functions as a negative regulator in Toll-like receptor signaling and plays an important role in alcohol-induced liver

injury[19,25]. In this study, we demonstrated that *IRAK3* was mainly methylated in HCC, and its methylation was positively associated with tumor stage and poor 3-year disease-free survival of patients. Furthermore, the inverse correlation between *IRAK3* expression and methylation status in HCC cell lines was also observed. Overall, our study indicates that *IRAK3* methylation is associated with tumor stage and *poor prognosis* of patients and also implies that IRAK3 might play an important role in the development of HCC. Confirmation of this hypothesis requires further investigation.

GLOXD1 (the official gene symbol is *HPDL*) encodes a protein that may function like 4-hydroxyphenylpyruvate dioxygenase. Although the function of GLOXD1 is still unclear, 4-hydroxyphenylpyruvate dioxygenase is known as an important enzyme in the catabolic pathway of tyrosine in the liver, and defects in this gene will cause diseases such as tyrosinemia type 3[20]. Till now, there are no data regarding the *GLOXD1* methylation in any cancer, even in HCC. We showed that *GLOXD1* expression was down-regulated in HCC cell lines, which was inversely correlated with its methylation status, and *GLOXD1* was frequently

Table 4 Association between gene methylation and clinicopathological characteristics of 160 hepatocellular carcinoma patients

Methylation status Characteristic	*IRAK3*, M-Index		*P* value	*GLOXD1*, M-Index		*P* value
	≤ 8.90	> 8.90		≤ 0.60	> 0.60	
Cases	58	102		85	75	
Age, yr						
≤ 59	27	47	1.00	45	29	0.08
> 59	31	55		40	46	
Gender						
Female	25	41	0.74	39	27	0.26
Male	33	61		46	48	
Hepatitis						
HBV-positive	21	47	0.32	38	30	0.22
HCV-positive	23	39		28	34	
Double-negative	14	16		19	11	
Cirrhosis						
No	33	44	0.07	46	31	0.15
Yes	23	57		38	42	
Unknown	2	1		1	2	
Tumor size, cm						
≤ 3	16	36	0.38	25	27	0.40
> 3	42	66		60	48	
Nodule						
Solitary	37	61	0.74	53	45	0.87
Multiple	21	41		32	30	
AFP level, ng/mL						
≤ 10	14	31	0.58	20	25	0.16
> 10	42	71		65	48	
Unknown	2	0		0	2	
Stage						
I	22	38	0.03	28	32	0.66
II	23	23		26	20	
III	13	34		27	20	
IV	0	7		4	3	
Invasion						
No	30	55	0.87	39	46	0.06
Yes	28	47		46	29	
Recurrence						
No	22	36	0.38	34	24	0.83
Yes	10	25		19	16	
Unknown	26	41		32	35	
Survival						
No	25	46	0.47	38	33	0.26
Yes	7	20		18	9	
Unknown	26	36		29	33	

HBV: Hepatitis B virus; HCV: Hepatitis C virus; AFP: Alpha-fetoprotein.

methylated in HCC tissues. All these results suggest that *GLOXD1* expression might be down-regulated in HCC through the promoter methylation. However, the role of GLOXD1 in the development of HCC requires further investigation.

In this study, we used pyrosequencing to verify the actual methylation pattern of CpG sites within the promoter of the target genes, similar to previous studies. Then, we used the results of pyrosequencing to design a Q-MSP system for validation in a large clinical cohort. Therefore, we easily determined the methylation frequency of the target genes in 349 tissue samples, including 29 normal controls and 160 HCC tissues and their paired adjacent nontumor tissues. According to

these results, our data indicate that this quantitative methylation analysis workflow is an efficient and economical approach to verify initially and validate further the data from high-throughput screening.

In summary, our data demonstrated that *IRAK3* and *GLOXD1* were frequently methylated in HCC tissues. Furthermore, *IRAK3* methylation was statistically associated with tumor stage and a poor 3-year disease-free survival rate of HCC patients. This indicated that detection of IRAK3 methylation would be helpful in the prediction of patients' survival as well as the follow-up of patients. Taken together, these findings reveal that methylation of *IRAK3* and *GLOXD1* has a potential clinical application.

COMMENTS

Background

Hepatocellular carcinoma (HCC) is a serious disease because it is difficult to detect and therefore leads to a very poor prognosis and high mortality rates. Studying the molecular *mechanisms* of HCC development can help us to design better strategies for disease detection or prognosis prediction.

Research frontiers

Aberrant DNA methylation is associated with the development of HCC, suggesting that gene methylation could provide potential clinical applications in the diagnosis or prognosis of HCC. The authors' previously identified that *IRAK3* and *GLOXD1* were frequently methylated in HCC using a methylation array. However, there are no quantitative data about the methylation level of two novel genes in HCC.

Innovations and breakthroughs

This study demonstrated frequent methylation of two novel genes [interleukin-1 receptor-associated kinase 3 (*IRAK3*) and *GLOXD1*] in HCC and further showed the potential value of *IRAK3* methylation as a biomarker in the prognosis of HCC.

Applications

IRAK3 methylation would be helpful in prediction of patients' survival as well as the follow-up of patients.

Terminology

DNA methylation is a common epigenetic event that alters gene expression. Identification of DNA methylation pattern not only allows for a detailed understanding of the hepatocarcinogenesis but also provides potential clinical applications in the diagnosis or prognosis of HCC.

Peer-review

In this study, the authors demonstrated that *IRAK3* and *GLOXD1* gene expression was down-regulated in HCC cell lines and that it was partially restored after treatment with 5DAC. Importantly, they also found that *IRAK3* methylation was statistically associated with tumor stage and with a trend of poor 3-year disease-free survival in HCC samples. Data are very interesting.

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ORIGINAL ARTICLE
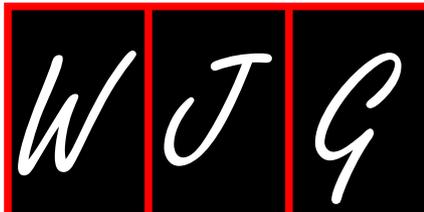
Retrospective Study

Oxaliplatin and 5-fluorouracil hepatic infusion with lipiodolized chemoembolization in large hepatocellular carcinoma

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Abstract

AIM: To investigate transarterial chemoembolization (TACE) with hepatic infusion of oxaliplatin and 5-fluorouracil and Lipiodol chemoembolization in large hepatocellular carcinoma (HCC).

METHODS: In this retrospective study, 132 patients with unresectable HCCs larger than 10 cm were treated with hepatic infusion of oxaliplatin and 5-fluorouracil followed by Lipiodol chemoembolization. The primary endpoint was overall survival (OS). Sixteen-week disease-control rate, time to progression (TTP), and major complications were also studied. Univariate and multivariate analyses were performed to identify prognostic factors affecting OS and TTP.

RESULTS: A total of 319 procedures were performed in the 132 patients. Eleven (8.3%) patients received radical resection following TACE treatment (median time to initial TACE 4.3 ± 2.3 mo). The median OS and TTP were 10.3 and 3.0 mo respectively, with a 50.0% 16-wk disease-control rate. Major complications were encountered in 6.0% (8/132) of patients following TACE and included serious jaundice in 1.5% (2/132) patients, aleukia in 1.5% (2/132), and hepatic failure in 3.0% (4/132). One patient died within one month due to serious hepatic failure and severe sepsis after receiving the second TACE. The risk factor associated with TTP was baseline alpha-fetoprotein level, and vascular invasion was an independent factor related to OS.

CONCLUSION: Hepatic infusion of oxaliplatin and

5-fluorouracil followed by lipiodolized-chemoembolization is a safe and promising treatment for patients with HCCs larger than 10 cm in diameter.

Key words: Hepatic infusion; Large hepatocellular carcinoma; Oxaliplatin; Transarterial chemoembolization; 5-fluorouracil

Core tip: Treatment of large unresectable hepatocellular carcinomas (HCCs) with diameters exceeding 10 cm is clinically challenging due to the low response rate and high rate of major complications. In this study, the safety and efficacy of a transarterial chemoembolization modality that included a combination of oxaliplatin and 5-fluorourcil infusion followed by embolization with a mixture of mitomycin and Lipiodol was tested in patients with large HCCs. The results indicate that this modality is a promising treatment for certain patients with large HCCs.

INTRODUCTION

Hepatocellular carcinoma (HCC) is the fifth most common cancer worldwide based on incidence[1]. Although patients at high risk of developing HCC can be monitored, only a small portion (20%-30%) of patients are eligible for curative treatments, such as resection, liver transplantation, and local ablation. Others may only receive palliative treatments, including transarterial chemoembolization (TACE)[2].

TACE is the main treatment option for unresectable HCC and is used in 58% of recurrent patients[2], and achieves partial responses in 15%-55% of patients with HCC who are not candidates for curative treatments[3]. Also, according to a recent prospective cohort study, TACE is safe and effective for elderly patients $\geq$ 75 years of age[4]. However, not all patients with unresectable HCC derive similar benefits from TACE. A systematic review in the Cochrane database showed that TACE or transarterial embolization (TAE) did not significantly increase survival for unresectable HCCs, particularly those with poor compensate liver function[5]. This may due in part to tumor heterogeneity, tumor burden, liver function, and the general condition of the individual patient. The heterogeneity for treatment modalities and for patients with different tumor stage or Child-Pugh classification of HCC may explain why some randomized, controlled trials of TACE failed to demonstrate prolonged survival in the patients[6].

Currently, the most widely used TACE modalities for unresectable HCC are conventional TACE, drug-eluting TACE beads (DB-TACE), and radioembolization[7]. There is increasing evidence supporting the safety and efficacy of DB-TACE, especially doxorubicin-eluting beads, and radioembolization, including iodine-131-labeled Lipiodol or microspheres containing yttrium-90[8-11]. However, conventional TACE remains the standard first-line choice. In addition, oxaliplatin shows promising efficiency and safety profiles in the treatment of HCC[12,13]. Thus, we evaluated the efficacy and safety of hepatic infusion of oxaliplatin in combination with Lipiodol embolization in patients with HCC.

Tumor size, especially a large tumor size, influences survival following TACE[14,15]. The difficulties related to treating a larger tumor include inadequate embolization of the tumor blood supply and major embolization-related complications, such as tumor lysis syndrome, tumor rupture, and hemolytic uremic syndrome. Several studies show that a tumor size larger than 10 cm is associated with poor prognosis following TACE[14-16]. However, one limitation of past studies was the relatively small number of patients with HCCs larger than 10 cm. Therefore, the safety and efficacy of TACE could not be analyzed. We performed this study to evaluate safety and efficiency of the protocol with local regional infusion of oxaliplatin and 5-fluorouracil (FU) followed with chemoembolization to treat large HCCs ($\geq$ 10 cm), which is generally not considered as a good indication for TACE, because of the lack of complete tumor eradication[17].

MATERIALS AND METHODS

Patients

This study was conducted in accordance with the principles of the Declaration of Helsinki and approved by the institutional review board. All patients provided written informed consent for the treatment procedure.

This retrospective study review included 597 patients who were diagnosed with large HCCs (single nodule $\geq$ 10 cm or the largest nodule $\geq$ 10 cm for multiple nodules) and were consecutively treated with TACE at the Liver Cancer Institute, Zhongshan Hospital, Fudan University, from January 2008 to December 2012. Diagnosis was based on either histologic confirmation or non-invasive AASLD criteria[18,19]. The main inclusion criteria were the following: (1) patients received the defined TACE procedure in accordance with the treatment protocol described below; (2) liver function was maintained with Child-Pugh A or B; (3) performance was ECOG 0 to 2; (4) renal function was normal; and (5) there was adequate bone marrow

function with a peripheral white blood cell count > 3.0 × 10^9/L and platelet count > 50 × 10^6/L. The patients were excluded in the cases of arterial-venous shunting identified by arterial angiography and lost follow-up after the first session of TACE. Thus, 132 patients were included in the study. Among these patients, 103 patients had well-documented follow-up imaging data [CT or magnetic resonance imaging (MRI)] to evaluate the objective response.

Hepatic infusion and chemoembolization procedure

Angiography and chemoembolization were performed according to the standard operating protocol of the hospital. Briefly, a 5 F RH catheter was inserted into the common hepatic artery using the Seldinger method. Hepatic angiography was performed to evaluate the tumor-feeding artery. Doses of 100-150 mg oxaliplatin and 1000 mg 5-FU were diluted with 5% dextrose and normal saline, respectively, and slowly infused *via* the common hepatic artery. The catheter was selected and inserted into the arterial branch as close as possible to the tumor. The chemoembolization was performed with 10 mg mitomycin C mixed with 10-30 mL Lipiodol, which was slowly injected into tumor vessels. Gelatin-sponge particles were used in some of the patients (*n* = 50; 37.88%) with significant hypervascularization. The procedure was repeated with an interval of approximately 6 wk until complete necrosis of the tumor was shown with enhanced CT or MRI, tumor shrinkage was achieved and thus eligible for surgical resection, or treatment failure due to tumor progression. However, the procedure would be postponed or terminated in the case of impaired liver function or bone marrow function.

Follow-up

Patients received follow-up CT or MRI evaluation of tumor response and tumor markers [alpha-fetoprotein (AFP), carcinoembryonic antigen, and carbohydrate antigen 19-9] and routine biochemical and blood tests approximately one month after completing the first session of TACE. If a tumor showed enhancement by CT or MRI indicating viable tumor tissue, a repeat procedure was conducted with an interval of six weeks. This repeat procedure was postponed if impaired liver function had not recovered to an acceptable level compared to baseline. Patients received follow-up examinations for AFP, CT, or MRI every two or three months after the termination of TACE, while complete tumor necrosis was indicated by absence of artery enhancement by CT or MRI.

Tumor response and survival assessments

The primary endpoint was overall survival (OS) after the first TACE procedure. Other endpoints included 16-wk disease-control rate (16w-DCR) and time to progression (TTP). Target lesions were evaluated by measuring the longest diameter and using Response Evaluation Criteria in Solid Tumor (RECIST)[20].

TTP was calculated from the date of TACE to the date of radiologic disease progression or the last date on which imaging showed stable disease; data for patients who died were censored. The 16w-DCR was defined as the percentage of patients with complete response, partial response, or stable disease lasting until 16 wk after treatment. The best tumor response according to RECIST was evaluated. AFP response was also evaluated. AFP levels were measured before and after the first TACE (4 ± 2 wk), and the change in AFP levels was calculated. Patients were separated into three groups based on the change in serum AFP from that at baseline: > 25% AFP decline, > 25% AFP increase, and < 25% change in either direction. An AFP decrease > 25% was defined as an AFP response, whereas the other two were defined as a lack of AFP response. OS was measured from the first TACE treatment until death from any cause or until the last date of follow up. Data were censored for patients who remained alive at the end of the study.

Major complications were evaluated after treatment, defined as life-threatening events and events with medical importance requiring inpatient hospitalization or prolongation of existing hospitalization within 30 d after TACE. Criteria specific for the diagnosis of post-TACE syndrome include pain, fever, nausea, and vomiting.

Statistical analysis

Continuous variances are indicated as mean ± standard deviation. TTP and OS were analyzed by the Kaplan-Meier method, and survival curves were compared with the log-rank test. Multivariate analysis was performed with the Cox proportional hazards model. All analyses were performed with SPSS version 19.0 (IBM Corp., Armonk, NY, United States). A $P < 0.05$ was defined as statistically significant.

RESULTS

Baseline characteristics of patients

Detailed characteristics for the 132 patients are shown in Table 1. The median age was 53 ± 12.7 year (range: 20-83 year), and the female to male ratio was 1:11. Almost all patients (97.7%) had chronic HBV disease. Among these, there were 50 patients with HBV DNA > 10^4 copies/mL, but none of them were positive for HCV antibodies. The performance status was ECOG 0 for most patients. The majority of patients had intact liver function, with 97.7% of patients recorded as Child-Pugh A. The mean tumor diameter was 11.5 ± 2.2 cm (range: 10-20 cm), and 110 patients had a solitary lesion. Vascular invasion was detected in 56.8% of patients and included the portal and hepatic veins. Extrahepatic metastases were found in 30.3% of patients, affecting the lymph nodes in 34 patients,

Table 1 Baseline characteristics of all 132 patients *n* (%)

Baseline characteristics	Value
Age, yr	
> 65	20 (15.2)
≤ 65	112 (84.8)
Gender	
Male	121 (91.7)
Female	11 (8.3)
CLIP score	
0 or 1	0 (0)
2	38 (28.8)
3	47 (35.6)
4	47 (35.6)
BCLC	
A	0 (0)
B	53 (40.2)
C	79 (59.8)
D	0 (0)
ECOG Performance Status	
0	87 (65.9)
1	35 (26.5)
2	10 (7.6)
AFP, ng/mL (mean ± SD)	15038.8 ± 22661.5
< 20	27 (20.5)
20-400	23 (17.4)
> 400	82 (62.1)
Tumor nodules	
Solitary	110 (83.3)
Multiple	22 (16.7)
Vascular invasion	
With	75 (56.8)
Without	57 (43.2)
Extrahepatic metastasis	
With	40 (30.3)
Without	92 (69.7)
Child-Pugh stage	
A	129 (97.7)
B	3 (2.3)
HBV infection[1]	
Yes	129 (97.7)
No	3 (2.3)
HBV DNA level > 10^4 copies/mL	50 (37.9)
HCV antibody	
Positive	0
Negative	132 (100)
Cirrhosis	
With	124 (93.9)
Without	8 (6.1)

[1]Patients with chronic hepatitis B history, HBsAg, or anti-HBcAg-positive. AFP: Alpha-fetoprotein; BCLC: Barcelona Clinic Liver Cancer; ECOG: Eastern Cooperative Group; HBV: Hepatitis B virus; HCV: Hepatitis C virus; SD: Standard deviation.

Table 2 Univariate and multivariate analysis of predictors of tumor progression of all 132 patients

Characteristic	Univariate	Multivariate	HR	95%CI
Age (≤ 65 yr >)	0.84			
Gender	0.88			
Clip score	0.05			
BCLC (B, C)	0.02			
ECOG (0, 1, 2)	0.59			
Cirrhosis	0.79			
Solitary or multiple nodules	0.50			
Vascular invasion	0.01	0.30	0.75	0.44-1.29
Extrahepatic spread	0.04	0.26	0.73	0.42-1.27
AFP-pre	0.02			
< 20 ng/mL		0.05		
20-400 ng/mL		0.04	0.55	0.30-0.99
> 400 ng/mL		0.50	1.23	0.67-2.28
HBV DNA > 10^4 copies/mL	0.66			
Gelatin-sponge application	0.26			

AFP-pre: Alpha-fetoprotein at baseline; BCLC: Barcelona Clinic Liver Cancer; CI: Confidence interval; ECOG: Eastern Cooperative Group; HBV: Hepatitis B virus; HR: Hazard ratio.

Table 3 Univariate and multivariate analysis of predictors of survival of all 132 patients

Characteristic	Univariate	Multivariate	HR	95%CI
Age (≤ 65 yr >)	0.53			
Gender	0.46			
Clip score	0.29			
BCLC (B, C)	0.03			
ECOG (0, 1, 2)	0.57			
Cirrhosis	0.73			
Solitary or multiple nodules	0.74			
Vascular invasion	0.00	0.00	2.36	1.41-2.94
Extrahepatic spread	0.04	0.34	1.27	0.78-2.10
AFP-pre	0.02			
< 20 ng/mL		0.09		
20-400 ng/mL		0.19	0.71	0.43-1.20
> 400 ng/mL		0.22	1.41	0.81-2.49
HBV DNA > 10^4 copies/mL	0.21			
Gelatin-sponge application	0.47			

AFP-pre: Alpha-fetoprotein at baseline; BCLC: Barcelona Clinic Liver Cancer; CI: Confidence interval; ECOG: Eastern Cooperative Group; HBV: Hepatitis B virus; HR: Hazard ratio.

the lungs in 4 patients, and bone in 2 patients.

Survival

The median follow-up period was 12.0 mo (range: 1.0-48.0 mo). There were 98 deaths recorded by April of 2013. Among them, 83/98 (84.7%) patients died of tumor progression, 14/98 (14.3%) died of uncontrollable gastrointestinal bleeding, and 1/98 (1.0%) died of lung infection with subsequent respiratory failure. The median OS of all 132 patients was 10.3 ± 1.2 mo (range: 1.0-45.7) (Figure 1). The one-, two-, and three-year OS rates were 48, 15, and 5%, respectively. Among those who received TACE only, the median OS was 9.4 ± 1.4 mo (range: 1.0-45.7 mo), and the one-, two-, and three-year OS rates were 44, 14, and 6%, respectively. The median OS of those with BCLC stage B or BCLC stage C HCC was 14.2 and 7.4 mo, respectively.

Four potential prognostic variables were identified by univariate analysis (Table 2). The Clip score and BCLC staging of large HCCs were determined by baseline AFP, the presence of ascites, and extrahepatic metastasis. Thus the Clip score and BCLC staging were excluded from multivariate analysis. Only one factor significantly increased the hazard of reduced OS: the presence of vascular invasion before the TACE procedure (Table 3).

TTP was 3.00 ± 0.45 mo (95%CI: 2.11-3.89) in all
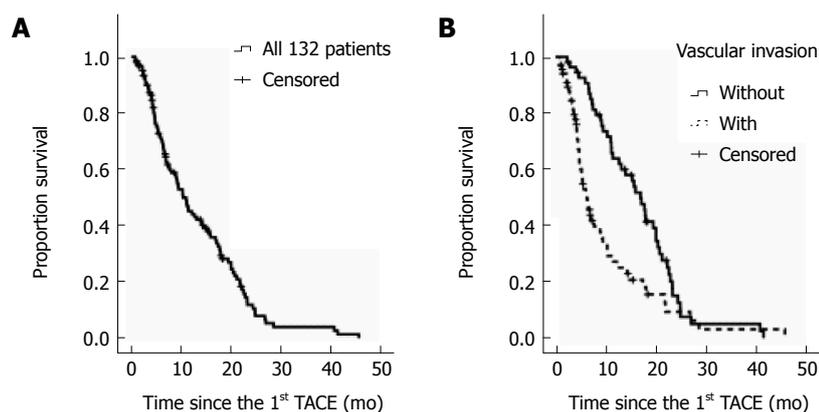
Figure 1 Overall survival curves. A: The Kaplan-Meier overall survival curve for all 132 patients in this study; B: The factor that significantly influenced survival was revealed by multivariate analysis. *P* value stratified log-rank test; Hazard ratio was obtained with the Cox model; TACE: Transarterial chemoembolization.
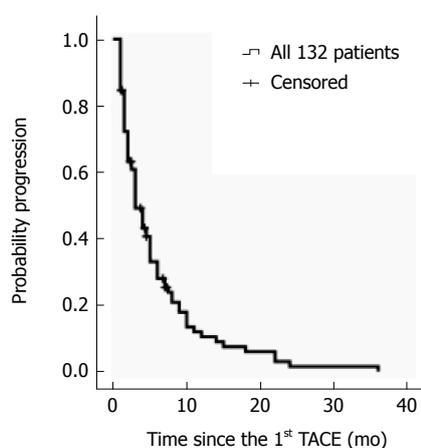
Figure 2 Time to progression curve. The Kaplan-Meier time to progression curve for all 132 patients in this study. TACE: Transarterial chemoembolization.

132 patients, and 3.00 ± 0.22 mo (95%CI: 2.57-3.43) in the 121 patients who received TACE only (Figure 2). Further analyses focused specifically on the 121 patients who received TACE only. Univariate analysis identified five potential factors related to TTP. Multivariate analysis revealed the AFP values at baseline were associated with TTP (Table 2).

Tumor objective response

Three hundred and nineteen sessions of TACE were performed in 132 patients with a mean of 2.4 sessions per patient (range: 1-8). Among the 132 patients, 11 (8.3%) received a secondary radical resection due to successful shrinking of the tumor. The median interval between the first TACE and resection was 4.3 ± 2.3 mo (range: 1.0-7.2 mo).

Excluding 29 patients who had no following-up image data and five patients who already received surgical resection within 16 wk after the first TACE, the 16w-DCR was 50.0% in the 98 patients. And among all 103 patients with well-documented follow-up imaging data (CT or MRI), partial response was achieved in 21.4%, while no complete response was observed.

Table 4 Tumor responses after treatment of 103 patients

Parameter	*n* (%)
16-wk disease-control rate	50%
Best response	
Complete response	0 (0.0)
Partial response	22 (21.4)
Stable disease	58 (56.3)
Progressive disease	23 (22.3)
AFP response rate (decrease > 25%)	39 (37.9)
No AFP response rate	64 (62.1)
Change < 25%	39 (37.9)
Increase > 25%	25 (24.3)

AFP: Alpha-fetoprotein.

Around one month after the first TACE, 64.5% patients showed a decrease in AFP value, but the defined AFP response was achieved in 37.9% of patients (Table 4).

The median OS of those with or without tumor response was 21.7 ± 3.1 and 9.4 ± 0.8 mo, respectively ($P = 0.000$). The median OS of those with or without an AFP response was 17.7 ± 1.6 and 10.2 ± 1.6 mo, respectively ($P = 0.14$). The difference was not statistically significant.

Safety

Among the 319 TACE procedures in 132 patients, only one patient died within the 30-d period following the procedure.

Post-TACE syndrome was the most common treatment-related adverse event, but was usually reversible and some patients received pain relievers or antipyretics. Increased enzyme levels were also common after TACE, but most cases were reversible and needed no specific treatment.

Major complications were encountered in 6.0% (8/132) of patients, including jaundice ($n = 2$), grade 3/4 liver dysfunction ($n = 4$; 1 patient died of hepatic failure and sepsis), and grade 3/4 aleukia ($n = 2$). One of the four patients who developed hepatic failure died on the 28th d after the second TACE, resulting in a procedure-related mortality rate of 0.76%.

DISCUSSION

The survival benefit of TACE has been explored in several randomized, controlled trials and meta-analyses, which are generally considered to be robust[21]. However, an optimal regimen has not yet been defined for conventional TACE, particularly for target lesions that are larger than 10 cm. These large HCCs cannot be completely embolized, and a high risk of complications accompanies the TACE procedure due to necrosis of the large tumor.

We tested the safety of a TACE protocol in patients with large HCCs that included a combination of oxaliplatin and 5-FU infusion followed by embolization with a mixture of mitomycin and Lipiodol. The toxicities of similar chemotherapeutic schedules have been shown to be tolerable during TACE of liver lesions in patients with advanced colorectal cancer[22,23] or intrahepatic cholangiocarcinoma[24,25]. Similarly, our study shows that the TACE protocol is safe and tolerable for patients with large HCCs. The most common adverse effects included tolerable post-embolism syndrome and temporarily elevated total bilirubin and alanine aminotransferase, which are generally reversible. Decreased platelet count and albumin were also common changes after therapy, but most grade 3/4 reductions were restricted to those who had low baseline values. One patient died of hepatic failure accompanied by suspicious infectious shock, giving a 0.75% 30-d mortality rate, which is acceptable as compared with other studies[26,27].

Considering the heavy tumor burden of the patients in our study, in which 59.8% belonged to BCLC-C, 56.8% had vascular invasion, and 30.3% had extrahepatic metastasis, the efficacy of the TACE protocol was acceptable to a certain extent. One recent study showed the median survival time was 6.6 months in patients with HCC lesions > 7 cm that were treated with TAE or TACE based on doxorubicin or cisplatin. These patients with large HCC lesions had double the risk of death and 60% reduction in median survival compared with those with tumors ⩽ 7 cm[28]. In addition, similar overall (5.2 mo) and two-year (19.2%) survival data were reported previously in a study that recruited HCC patients with vascular invasion[29]. In that study, cisplatin and doxorubicin were used as chemoembolization drugs. Although one study focused on patients with BCLC-C HCC showed that DB-TACE achieved a better median OS (13.3 mo, 95%CI: 10.0-19.8)[30], most patients in that study were classified as BCLC-C based on ECOG score, rather than vascular invasion or metastasis.

Although vascular invasion was found to be a risk factor associated with OS, it may not be an absolute contraindication to conventional TACE. Based on the most recent EASL-EORTC guidelines, TACE is recommended for those with BCLC-B, which

lacks vascular invasion of extrahepatic spread[18]. However, in a phase III trial of sorafenib in the Asia-Pacific[31], patients with HCC and vascular invasion or extrahepatic spread showed a similar median TTP (2.7 mo) and shorter median OS (5.6 mo) compared to the present study. These findings support the efficacy of our TACE protocol. Moreover, sorafenib does not induce tumor shrinkage[32], and our TACE protocol achieved cytoreduction in some patients. Radical resection was performed in 8.3% of patients following TACE. Thus, patients with BCLC-C HCC, including those with vascular invasion or extrahepatic spread, might also benefit from TACE if liver function and the general condition of the patient are intact. In fact, clinical practice guidelines proposed by some Asian countries noted that TACE was frequently performed in patients with minimal portal invasion[33,34].

AFP status was associated with TTP in patients with large HCCs. Previous studies demonstrated that a change in AFP during treatment might serve as a predictor of clinical outcome in advanced HCC[35,36]. However, the specific amount of change varied among studies. In our study, no significant associations were found between AFP response and survival proportion. Changes in other biochemical or hematologic results, such as total bilirubin and alanine aminotransferase, were also analyzed, but no links to treatment outcome were found.

Extrahepatic spread is not an independent predictor in our study. Intrahepatic tumor progression or liver failure was the main causes of death from HCC, rather than metastasis. In fact, two-thirds of patients with HCC die without metastasis. Thus, it is necessary to control intrahepatic tumors with loco-regional therapies[37].

Other factors that have been associated with outcome from TACE were also analyzed in our study. However, no significant relationships were found for ECOG score, HBV DNA copy number, or ascites. These may be due to differences in the patient population of each study.

Our study had several limitations. It was a retro-spective study from a single institution. A multicenter, prospective study is desirable to validate our results. Combined TACE and sorafenib is also a promising strategy for patients with advanced HCC. In addition, most patients were Child-Pugh A, which may have led to more favorable outcomes as compared to the general population. Finally, ECOG status remains a highly subjective measurement with clinical variability.

In conclusion, TACE with hepatic infusion of oxa-liplatin and 5-FU and Lipiodol embolization may be considered as a safe and promising treatment for patients with HCCs larger than 10 cm. Although systemic chemotherapy is usually recommended for advanced-stage patients, certain TACE regimens may be considered as adjuvant or sole therapies in a select group of patients.

COMMENTS

Background

The prognosis of hepatocellular carcinoma (HCC) is poor due, and only a small portion (20%-30%) of patients are eligible for curative treatments, such as resection, liver transplantation, and local ablation. Patients with unresectable HCC are typically treated with transarterial chemoembolization (TACE) or systemic therapy, and the long-term survival is far more unsatisfactory.

Research frontiers

TACE is the most widely used standard treatment for unresectable HCC. However, treatment of large HCCs (> 10 cm in diameter) with TACE is clinically challenging due to the low response rate and high rate of major complications.

Innovations and breakthroughs

Treatment of large unresectable HCCs is clinically challenging, thus we tested the safety and efficacy of a certain TACE modality that included a combination of oxaliplatin and 5-fluorourcil infusion followed by embolization with a mixture of mitomycin and Lipiodol for these patients. The results show that this modality is a promising treatment for certain patients with large HCCs.

Applications

TACE with hepatic infusion of oxaliplatin and 5-fluorouracil and Lipiodol embolization may be a safe and promising treatment for patients with HCCs larger than 10 cm in diameter. Although systemic chemotherapy is usually recommended for advanced-stage patients, certain TACE regimens may be considered as adjuvant or sole therapies in a select group of patients.

Terminology

Chemoembolization is a local regional therapy for unresectable HCC based on the principle of exposure of the HCC to a high concentration of chemotherapeutic agents *via* tumor feeding artery infusion followed by embolization of this artery, and the therapeutic benefit is achieved due to tumor necrosis.

Peer-review

There are many studies assessing the role of TACE for unresected HCC. However, this study focuses on HCC with a size larger than 10 cm. This subgroup of HCC is not common in Western countries and would be less in China due to the early diagnosis *via* regular screening. Thus the data provided by the manuscript is valuable.

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ORIGINAL ARTICLE

Retrospective Study

Endoscopic transpancreatic septotomy as a precutting technique for difficult bile duct cannulation

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Abstract

AIM: To evaluate the technique of transpancreatic septotomy (TS) for cannulating inaccessible common bile ducts in endoscopic retrograde cholangiopancreatography (ERCP).

METHODS: Between May 2012 and April 2013, 1074 patients were referred to our department for ERCP. We excluded 15 patients with previous Billroth Ⅱ gastrectomy, Roux-en-Y anastomosis, duodenal stenosis, or duodenal papilla tumor. Among 1059 patients who underwent ERCP, there were 163 patients with difficult bile duct cannulation. Pancreatic guidewire or pancreatic duct plastic stent assistance allowed for successful ERCP completion in 94 patients. We retrospectively analyzed clinical data from 69 failed patients (36 transpancreatic septotomies and 33 needle-knife sphincterotomies).

RESULTS: Of the 69 patients who underwent precut papillotomy, common bile duct cannulation was successfully achieved in 67. The success rates in the TS and needle knife sphincterotomy (NKS) groups were 97.2% (35/36) and 96.9% (32/33), respectively, which were not significantly different ($P > 0.05$). Complications occurred in 11 cases, including acute pancreatitis ($n = 6$), bleeding ($n = 2$), and cholangitis ($n = 3$). The total frequency of complications in the TS group was lower than that in the NKS group (8.3% *vs* 24.2%, $P < 0.05$).

CONCLUSION: Pancreatic guidewire or pancreatic duct plastic stent assistance improves the success rate of selective bile duct cannulation in ERCP. TS and NKS markedly improve the success rate of selective bile duct cannulation in ERCP. TS precut is safer as compared with NKS.

Key words: Cholangiopancreatography; Endoscopic retrograde; Transpancreatic septotomy; Needle-knife precut; Complication

Core tip: In this study, we describe that pancreatic guidewire or pancreatic duct plastic stent assistance improves the success rate of selective bile duct cannulation in endoscopic retrograde cholangiopancreatography

(ERCP). Transpancreatic septotomy (TS) and needle knife sphincterotomy (NKS) markedly improve the success rate of selective bile duct cannulation in ERCP. TS precut is safer and effective than NKS.

Figure 1 Septum between the pancreatic duct and common bile duct.

INTRODUCTION

Endoscopic retrograde cholangiopancreatography (ERCP) is an important technique in the diagnosis and treatment of biliary and pancreatic diseases. Successful bile duct cannulation in ERCP is key to follow-up treatment. Biliary cannulation rates during ERCP exceed 90% in most large centers worldwide[1,2]. However, selective bile duct cannulation in ERCP is difficult to perform in patients with small duodenal papilla, papilla opening sclerosis, papilla looseness, peripapillary diverticulum or a history of Billroth Ⅱ gastrectomy. Although a variety of catheters and guidewires are used to guide the knife, the failure rate of bile duct cannulation ranges from 5%-20%[3]. Prolonged or repeated attempts at cannulation in these patients increase the risk of ERCP related complications[2,4].

Pancreatic guidewire or stent placement can improve the success rate of bile duct cannulation during ERCP[5,6]. Endoscopic needle-knife precut papillotomy can also significantly increase ERCP cannulation success rate, but the complication rate is 6%-20%[7]. A thin septum between the pancreatic duct and the common bile duct has been reported (Figure 1)[8]; therefore, we hypothesized that difficult bile duct cannulation and repeated insertion into the pancreatic duct may be due to bile duct blockage by the septum in some cases. In the present study, a bow knife was used to cut the septum through the pancreatic duct toward the bile duct. Transpancreatic septotomy (TS) was compared with needle-knife sphincterotomy (NKS) during the same period to explore its clinical value.

MATERIALS AND METHODS

Clinical data

From May 2012 to April 2013, 1074 patients were referred to our department for ERCP. Fifteen patients with a history of Billroth Ⅱ gastrectomy, Roux-en-Y anastomosis, duodenal stenosis, or duodenal papilla tumor were excluded, and ERCP was performed in 1059 patients. Among these, 163 patients experienced difficult bile duct cannulation in ERCP. With the assistance of a pancreatic guidewire or plastic stent, 94 of the 163 patients had successful ERCP procedures. Of the other 69 patients with failed cannulation, 36 underwent TS (referred to as TS group) and 33 had NKS (referred to as NKS group). The TS group included 19 males and 17 females, and they ranged in age from 17 to 89 years, with an average of 59.5 years. The NKS group included 16 males sand 15 females, and they ranged in age from 17 to 85 years, with an average of 57.9 years. There was no significant statistical difference in etiology distribution or duodenal papilla morphology between the two groups (Tables 1 and 2).
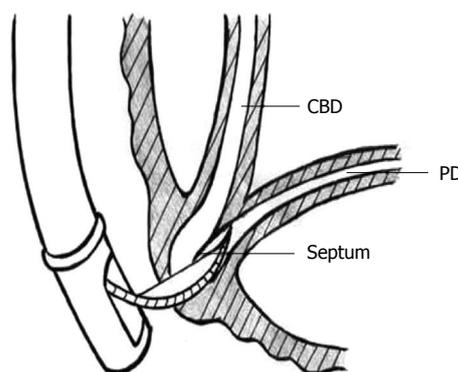
Apparatus

Side-view duodenoscope (Olympus TJF-260), needle knife and bow knife (Boston or COOK), and high-frequency surgical unit (ERBE CC80) were used.

Operation methods

A duodenoscope was inserted into the duodenal papilla. A catheter was then inserted *via* the papilla. In the case of failing to enter the bile duct but repeated (more three times) insertion of the catheter into the pancreatic duct, a pancreatic guidewire or plastic stent was placed, and bile duct cannulation was attempted again. In cases of failed bile duct cannulation, the following procedures were carried out randomly: (1) TS: If the guidewire repeatedly entered the pancreatic duct but failed to enter the bile duct, the guidewire was left in the pancreatic duct. A bow knife was then inserted into the pancreatic duct to make a small incision (< 5 mm) into the papilla toward the bile duct. The transpancreatic septum was cut, and the bile duct cannulation was then performed again (Figure 2). All patients underwent pancreatic stenting after TS; and (2) conventional NKS: A needle knife was inserted upward into the papilla opening or into the papilla uplift about 5-10 mm above the papilla opening. Electrical excision and electrocoagulation were then applied from the top down to cut the mucous membrane and papilla ampulla sphincter layer by layer in the 11-12 o'clock direction. All patients underwent pancreatic stenting

Table 1 Distribution of etiology in the transpancreatic septotomy and needle-knife sphincterotomy groups (case number)

Etiology	TS	NKS	Total
Benign	22	21	43
Common bile duct stones	9	8	17
Cystic dilatation of the common bile duct	1	1	2
Cholangitis	4	6	10
SOD	5	4	9
Others	3	2	5
Malignant	14	12	26
Cholangiocarcinoma	4	3	7
Pancreatic cancer	4	5	9
Duodenal papilla tumor	2	1	3
Gallbladder carcinoma	4	3	7

SOD: Sphincter of Oddi dysfunction; TS: Transpancreatic septotomy; NKS: Needle-knife sphincterotomy.

Table 2 Duodenal papilla morphology in the transpancreatic septotomy and needle-knife sphincterotomy groups (case number)

Papilla morphology	TS	NKS
Small papilla	5	6
Sclerosis of papilla opening	6	4
Looseness of papilla	11	9
Peripapillary diverticulum	7	8
Normal papilla	7	6

TS: Transpancreatic septotomy; NKS: Needle-knife sphincterotomy.

after NKS.

Diagnostic criteria for post-ERCP complications

ERCP complications included: (1) acute pancreatitis (AP): at 24 h after ERCP, the serum amylase value exceeded 3 times the upper limit of normal and was accompanied by persistent abdominal pain; (2) infection: The patient had right upper quadrant pain accompanied by fever > 38.5 ℃, as well as a white blood cell count > 10×10^9/L without infectious lesions within 24 h after ERCP; (3) gastrointestinal bleeding: The patient had vomiting or black stools after ERCP or hemoglobin < 95% of normal level within 24 h[9]; and (4) perforation: Subcutaneous emphysema, retroperitoneal air, or subphrenic free air was detected after ERCP.

Statistical analysis

The cannulation success rate and complications were compared by χ^2 tests using SPSS 15.0. A *P*-value < 0.05 was considered statistically significant.

RESULTS

Bile duct cannulation success rate

A total of 1059 patients underwent ERCP, and 896 patients achieved successful bile duct cannulation. Of the other 163 patients who experienced difficult

bile duct cannulation during ERCP, 94 completed ERCP successfully with the assistance of a pancreatic guidewire or plastic stent. The total success rate was 93.4% (990/1059). Of the remaining 69 patients, 67 underwent successful bile duct cannulation after precut papillotomy, with a success rate of 97.1%. Therefore, the total success rate of bile duct cannulation was 99.8% (1057/1059). The total success rate was 97.2% (35/36) in the TS group, and one unsuccessful case was managed by percutaneous transhepatic cholangial drainage. The total success rate was 96.9% (32/33) in the NKS group, and one unsuccessful case was treated by surgical intervention. The total success rates were not statistically different between the two groups (*P* > 0.05).

Postoperative complications

Complications occurred in 11 out of the 69 precut cases, including AP (*n* = 6), bleeding (*n* = 2), and cholangitis (*n* = 3). Two cases of AP and one case of cholangitis occurred in the TS group, and four cases of AP, two cases of bleeding and two cases of cholangitis occurred in the NKS group. The total complication rate was significantly lower in the TS group than in the NKS group (8.3% *vs* 24.2%, *P* < 0.05). The patients with AP were fasted and given anti-inflammatory treatment and inhibition of pancreatic secretion, and recovery was achieved one week later. Intravenous hemostatic agents were administrated for patients with bleeding, which was stopped 2 d later. Patients with cholangitis recovered one week after anti-inflammatory treatment.

Follow-up

The 69 patients in the TS and NKS groups were followed for four months. No recurrence of bile duct stones was seen, and SOD was relieved in both groups. One patient with cholangitis in the NKS group relapsed. One patient with cholangiocarcinoma in the TS group died of liver failure by tumor invasion. One patient with pancreatic cancer in the NKS group died of tumor cachexia.

DISCUSSION

Endoscopy plays an increasingly important role in treating biliary and pancreatic diseases. Successful deep bile duct cannulation in ERCP is key to follow-up treatment of biliary diseases. However, the failure rate of bile duct cannulation is as high as 5%-20%[3]. At present, the most common methods to manage difficult bile duct cannulation include placement of a pancreatic guidewire or plastic stent, NKS, and TS. In this study, the success rate of bile duct cannulation increased to 93.4% by placement of a pancreatic guidewire or plastic stent, which is supposed to relieve the blockage of the pancreatic duct opening and facilitate the insertion of the second guidewire into the bile duct[10]. However, we believe that the guidewire or
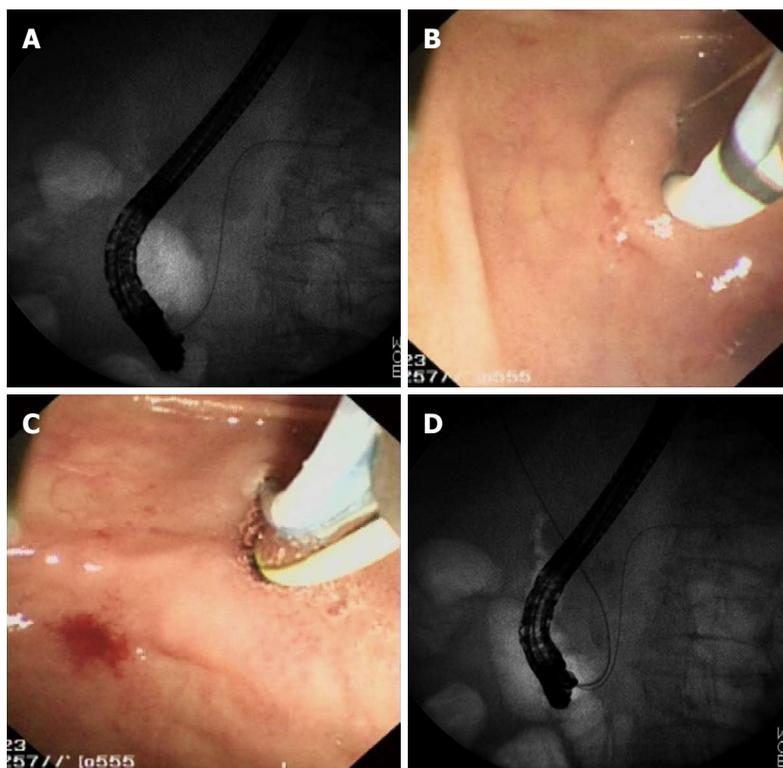
Figure 2 Transpancreatic septotomy procedure. A guidewire enters the pancreatic duct (A); the septum is cut from the pancreatic duct to the bile duct (B); the catheter is inserted toward the bile duct (C); and the guidewire enters the bile duct (D).

stent also changes the axis direction of the bile duct and straightens it.

Precut papillotomy is a complementary technique for conventional ERCP and endoscopic sphincterotomy (EST) that improves the success rate of ERCP and increases the success rate of papillotomy to 97%[11]. Needle-knife precut papillotomy is another commonly applied precut technique that has a high success rate of cannulation, as well as a high rate of complications (6%-20%), including perforation, bleeding, pancreatitis, and cholangitis[7]. Endoscopy researchers have been trying other precut techniques and hope to find a technique with a high success rate of cannulation and a low complication rate. Pancreatic duct cannulation has a relatively high success rate in clinical practice, but deep bile duct cannulation is relatively difficult, especially in patients with small papilla, peripapillary diverticulum, malposition of the papilla, or a history of Billroth Ⅱ gastrectomy. We hypothesized that difficult bile duct cannulation may be related to duct blockage by the ampulla septum in cases where the guidewire repeatedly enters the pancreatic duct. The septum is located between the pancreatic duct and the common bile duct[8]. Therefore, we investigated TS in this study. Both NKS and TS resulted in high success rates, which were 96.9% and 97.2%, respectively, which were not significantly different between the two groups. However, the incidence of complications was significantly lower for TS than for NKS (8.3% *vs* 24.2%, *P* < 0.05).

TS was first reported by Goff[12]. In clinical practice, it is easy to insert the guidewire into the pancreatic duct but difficult to insert it into the bile duct. If the guidewire repeatedly enters the pancreatic duct but is unable to access the bile duct, a bow knife is used to make a 5-mm incision along the guidewire from the pancreatic duct at the 11-12 o'clock direction. After removing the bow knife, and the catheter is re-inserted into the bile duct along the upper left of the guidewire. This method has the advantages of a small incision; manageable direction; and a low incidence of bleeding, perforation, and infection. Goff reported an overall success rate up to 97.5% in his long-term study of 51 patients[13]. Weber *et al*[14] performed transpancreatic precut sphincterotomy for cannulating inaccessible common bile ducts in 108 patients. The success rate of selective bile duct cannulation was 95.4%, and the incidence of complications reached 11.1%. They suggested that the technique could be applied to the unsuccessful cases of conventional EST or needle-knife precutting. Catalano *et al*[8] compared TS with standard precut papillotomy in a randomized study and found that the former resulted in a higher success rate of bile duct cannulation and a lower incidence of complications; pancreatitis occurred in 3.4% and 11.8% of cases, respectively. Kahaleh *et al*[15] reported an overall success rate of 85% and a complication rate of 12% for 116 TS cases. In the present study, both TS and NKS had high success rates, which is similar to those reported in the literature. The results indicate

that both techniques can improve bile duct surgery success rate, but TS is safer because of its lower complication rate. Of course, both techniques must be performed by skilled ERCP surgeons.

In summary, pancreatic guidewire or plastic stent assistance improves the success rate of selective bile duct cannulation in ERCP. TS and NKS further improve the success rate; however, TS is safer than NKS.

COMMENTS

Background
Pre-cut techniques, with the most commonly described being needle knife sphincterotomy (NKS), have been used to facilitate biliary access in failed standard biliary cannulation. Transpancreatic septotomy (TS) is a new pre-cut technique with limited outcome data.

Research frontiers
Precut papillotomy is a complementary technique for endoscopic sphincterotomy that improves the success rate of endoscopic retrograde cholangiopancreatography (ERCP) and increases the success rate of papillotomy. Endoscopy researchers have been trying other precut techniques and hope to find a technique with a high success rate of cannulation and a low complication rate.

Innovations and breakthroughs
In the present study, TS has the advantages of a small incision; manageable direction; and a low incidence of bleeding, perforation, and infection.

Applications
In clinical practice, it is easy to insert the guidewire into the pancreatic duct but difficult to insert it into the bile duct. If the guidewire repeatedly enters the pancreatic duct but is unable to access the bile duct, TS is a good choice.

Terminology
Transpancreatic septotomy is defined as a controlled incision into the common bile duct to achieve selective biliary cannulation. During ERCP, TS and NKS further improve the success rate; however, TS is safer than NKS.

Peer-review
In this manuscript the authors report their experience in the treatment of 163 patients with difficult bile duct cannulation who underwent a second attempt by either transpancreatic septotomy or needle knife sphincterotomy. Fortunately, they have two comparable groups of patients subjected to each of both procedures, the results are interesting and conclusions are correctly derived from these results.

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ORIGINAL ARTICLE

Clinical Trials Study

Decreased STAT4 indicates poor prognosis and enhanced cell proliferation in hepatocellular carcinoma

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Abstract

AIM: To investigate the role of signal transduction and activation of transcription 4 (STAT4) in the development and progression of human hepatocellular carcinoma (HCC).

METHODS: Recent genetic investigations have identified that a genetic variant of STAT4 is associated with hepatitis B virus (HBV)-related HCC. The level of STAT4 in 90 HCC patients was examined *via* Western blot and immunohistochemical analyses. The correlation between STAT4 expression and the clinicopathological characteristics of the patients was analyzed. The level of STAT4 expression in the HCC liver tissues was significantly lower than that in the non-HCC liver tissues and correlated with tumor size, histological grade of HCC and serum hepatitis B surface antigen level in HCC patients. The data were statistically analyzed using SPSS. Furthermore, siRNA oligos targeting STAT4 were employed to investigate the influence of STAT4 RNA interference on HCC cell physiology. Based on Cell Counting Kit-8 and flow cytometric assays, we found that depletion of STAT4 expression significantly enhanced the proliferation of L02 cells.

RESULTS: STAT4 protein expression was significantly lower in HCC tissues than in normal liver tissues. Immunohistochemistry followed by statistical analysis revealed that the expression of STAT4 negatively correlated with Ki67 expression (r = 0.851; $P < 0.05$) and positively correlated with maximal tumor size ($P < 0.05$), HBV ($P = 0.012$) and histological grade ($P < 0.05$). Kaplan-Meier analysis revealed significant differences in the survival curves between HCC patients expressing low and high levels of STAT4 and Ki67 ($P < 0.05$). Based on a multivariate Cox proportional hazard model, STAT4 expression was an independent prognostic indicator for HCC patients who underwent curative resection. *In vitro*, following the release of L02 cell lines from serum starvation, the expression of STAT4 was downregulated, and transfection of L02 cells with siRNA targeting STAT4 inhibited cell proliferation.

CONCLUSION: Our data indicate that STAT4 may inhibit HCC development by modulating HCC cell proliferation.

Key words: Signal transduction and activation of transcription 4; Prognosis; Proliferation; Hepatocellular carcinoma

Core tip: In this study, we assessed the role of signal transduction and activation of transcription 4 in hepatocellular carcinoma (HCC) and discussed the possible function of this protein in the development of HCC.

INTRODUCTION

Hepatocellular carcinoma (HCC) is among the most common cancers and is the third most common cause of cancer mortality worldwide[1]. Worldwide, more than 600000 new HCC cases are diagnosed annually, among which about 55% are in China[2]. Although intensive efforts have been made by clinical practitioners and basic researchers to identify prognostic markers of and therapeutic targets for HCC, the molecular mechanisms underlying HCC progression remain largely elusive[3,4]. Moreover, effective treatments for HCC are essentially absent. This deficiency emphasizes the urgency to develop new diagnostic and therapeutic strategies for HCC[5].

Signal transducers and activators of transcription (STATs) are members of a well-conserved family of transcription factors that play integral roles in various cellular processes[6,7]. STATs are latent cytoplasmic proteins that are promptly activated by tyrosine phosphorylation by receptor-associated JAK (Janus) kinases in response to cytokine or growth factor exposure. The resulting functional STATs are capable of entering the nucleus, where they directly bind to DNA and activate the transcription of a variety of target genes[8,9]. Generally, STAT proteins regulate cytokine-mediated cell proliferation by modulating the expression of crucial cell cycle regulators, such as cyclin D1, p21 and p27[6]. In addition to cell cycle regulation, STATs modulate various other cellular processes, such as apoptosis, differentiation and migration, *via* the transcription of various target genes, including Bcl-2 family members, cytokines, matrix metalloproteinases and miRNAs. Accordingly, dysregulated STAT proteins are closely associated with the pathogenesis of human cancers. The hyperactivation of STAT signaling has been widely documented in various cancer types, including ovarian cancer, breast cancer, brain tumors, gastric cancer and colon cancer[10-13]. Consistent with these findings, STATs have been considered as promising therapeutic targets in cancer drug discovery. However, much remains unclear with respect to the expression profiles and roles of STATs in HCC development[14].

Studies have indicated that several members of the STAT family play crucial roles in the pathology of liver diseases. STAT1 has been demonstrated to play a key role in antiviral defense, inflammation, and injury in the liver of STAT1 knockout mice[15-18], and STAT1 negatively regulates HCC cell proliferation[19]. STAT2-deficient mice exhibit an increased susceptibility to viral infections, and the loss of a type I IFN autocrine/paracrine loop indicates that STAT2 performs an antiviral defense function in the liver[20]. STAT3, which is activated by a variety of extracellular signals, has been shown to play key roles in the acute phase response, protection against liver injury, the promotion of liver regeneration, glucose homeostasis, and hepatic lipid metabolism[14]. STAT5 is primarily activated by growth hormone, which regulates the expression of a wide range of hepatic genes, including cytochrome P450, glutathione S-transferase, sulfotransferase enzyme, the growth hormone receptor, serine protease inhibitor Sp12.1, insulin-growth factor I, and hepatocyte growth factor[21,22], which suggests that STAT5 regulates HCC cell proliferation[23,24]. STAT6, which is primarily activated by interleukin (IL)-12, IL-4, and IL-13, plays an important role in Th2 differentiation[6]. The study of STAT4 in liver diseases, compared with other members of the STAT family, is limited. STAT4 was primarily regarded as a transducer of IL-12 signaling, affecting a broad range of immune cell physiology[25,26]. A very recent study by Jiang and colleagues reported that STAT4 might prevent HBV-related hepatocarcinogenesis[27]. However, the precise involvement of STAT4 in HCC development remains unclear.

Our study aimed to investigate the possible involvement of STAT4 in HCC pathology and to evaluate the prognostic value of STAT4 expression for HCC development. We found that STAT4 was significantly downregulated in HCC specimens compared with adjacent nontumorous specimens. Furthermore, we showed that the expression of STAT4 correlated with HBV, the maximal tumor size, the histological grade and Ki67 expression. These findings provide novel insight into the mechanisms underlying HCC development.

MATERIALS AND METHODS

Patients and tissue samples

Paired samples of tumor and adjacent nontumor tissues were obtained from 90 HCC patients who underwent curative surgery at the Affiliated Cancer Hospital of Nantong University. After surgical removal, a portion of the paired tissue samples was snap-frozen in liquid nitrogen and then maintained at -80 ℃ until use for protein extraction, and another portion of the paired tissue samples was immediately

fixed in formalin and embedded in wax for immuno-histochemistry.

The inclusion criteria for all patients in this study were: (1) HCC diagnosis as confirmed by experienced pathologists based on histological examination of HE-stained biopsy sections; (2) no anticancer treatment before surgery; (3) curative resection, defined as the macroscopically complete removal of the tumor and histologically demonstrated tumor-free margins; and (4) the availability of complete clinicopathologic and follow-up data. Informed consent was obtained from each patient, and the study protocols were approved by the Institutional Review Board of the Affiliated Cancer Hospital of Nantong University. The patients included 57 males and 33 females with a mean age of 47.3 years (range: 21-75 years). The primary clinicopathological characteristics of the subjects were recorded. The differentiated tumors were histologically classified as grade Ⅰ-Ⅱ (*n* = 49) or grade Ⅲ-Ⅳ (*n* = 41). The follow-up duration ranged from 1 to 96 mo.

Immunohistochemistry

Tissues were formalin-fixed and paraffin-embedded for immunohistochemical analysis. The sections were dewaxed in xylene and rehydrated in graded ethanol solutions. Then, the sections were processed in 10 mmol/L citrate buffer (pH 6.0) and heated in a microwave at high power (750 W) in 10 mm for three cycles of 5 min each for antigen retrieval. Then, endogenous peroxidase activity was blocked by soaking the sections in 0.3% hydrogen peroxide for 15 min after cooling at room temperature for 1 h. Goat serum was applied for 15 min to block nonspecific reactivity. The sections were incubated overnight at 4 ℃ in a rabbit anti-human STAT4 polyclonal antibody (diluted 1:100; Santa Cruz Biotechnology), and an anti-Ki-67 mouse monoclonal antibody (diluted 1:100; clone 7B11; Zymed Laboratories, San Francisco, CA, United States). Negative control samples were processed in parallel using a nonspecific immunoglobulin IgG (Sigma Chemical Co, St. Louis, MO) at the same concentration as the primary antibody. All sections were processed using the peroxidase-antiperoxidase method (Dako, Hamburg, Germany). After rinsing in water, the sections were counterstained with hematoxylin, dehydrated, and coverslipped. All of the immunostained sections were evaluated in a blinded manner with respect to the clinical and pathological characteristics of the patients. For the assessment of STAT4 expression, five high-power fields for each specimen were randomly selected, and nuclear (cytoplasmic) staining was examined. More than 500 cells were counted to determine the LI (labeling index) which represented the percentage of immunostained cells relative to the total number of cells. In half of the samples, the staining was repeated twice to avoid possible technical errors, but similar results were obtained for these samples. The results obtained were confirmed by another investigator (J.Y.X.) using a multihead microscope, and a consensus was achieved.

Cell culture and cell cycle analysis

One normal hepatocyte cell line (L02) and 3 HCC cell lines (HepG2, HuH7, and Hep1) were obtained from the Institute of Cell Biology of the Chinese Academy of Sciences and were cultured in RPMI 1640 and Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 μg/mL streptomycin (all media were from Invitrogen, Carlsbad, CA, United States) in 5% - at 37 ℃.

Two human STAT4 siRNA expression vectors and pSilencer siRNA were constructed. The siRNA sequences targeting the nucleotide residues AAATCCGGCATCT-GCTAGCTC and AATTGGATGAACAGTTGGGGC were termed siRNA-1 and -2, respectively. L02 cells were seeded the day before transfection using DMEM containing 10% FBS lacking antibiotics. Transfection was performed using the transfection reagent Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. The cells were incubated in pSilencer vector-Lipofectamine 2000 complexes for 4-6 h at 37 ℃, and FBS was added to the DMEM to a final concentration of 10%. The cells were used for subsequent experiments at 48 h after transfection.

Western blot analysis

Tissue and cellular protein samples were promptly homogenized in homogenization buffer containing 1 mol/L Tris HCl pH7.5, 1% Triton X-100, 1% Nonidet p-40 (NP-40), 10% sodium dodecyl sulfate (SDS), 0.5% sodium deoxycholate, 0.5 mol/L EDTA, 10 μg/mL leupeptin, 10 μg/mL aprotinin, and 1 m mol/L PMSF, followed by centrifugation at 10000 ×g for 30 min to collect the supernatants. The protein concentrations were determined *via* a Bio-Rad protein assay (Bio-Rad, Hercules, CA, United States). The total cellular protein extracts were separated *via* SDS-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. The membranes were blocked with 5% nonfat dry milk in PBS for approximately 2 h at room temperature and then incubated in antibodies against STAT4 (1:1000; Santa Cruz Biotechnology) and GAPDH (1:1000; Sigma) in PBS containing 5% milk for approximately 1 h at room temperature. The membranes were washed three times in PBS buffer, followed by incubation in the appropriate horseradish peroxidase-conjugated secondary antibodies (1:500; Santa Cruz Biotechnology). Specific protein bands in the membranes were visualized using an enhanced chemiluminescence reagent (NEN, Boston, MA). Three independent experiments were performed.

Cell proliferation assay and cell cycle analysis

Cell proliferation was determined *via* a Cell Counting Kit-8 (CCK)-8 assay according to the manufacturer's
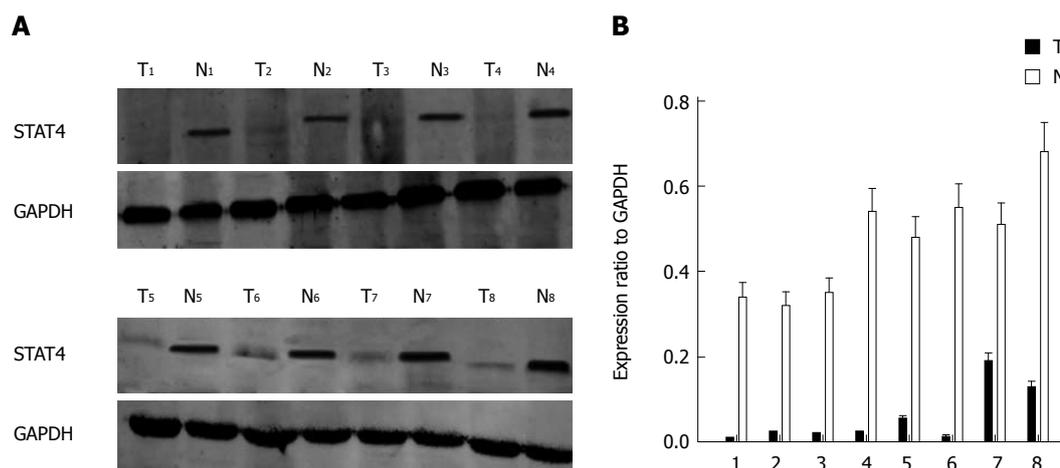
Figure 1 Expression of signal transducer and activator of transcription 4 in human hepatocellular carcinoma tissues. A, B: Expression of STAT4 in eight representative matched samples of HCC tissue (T) and adjacent noncancerous liver tissues (N) *via* Western blot and SigmaPlot. STAT4: Signal transducer and activator of transcription 4; HCC: Hepatocellular carcinoma.

instructions. In brief, cells which were transfected with siRNA were seeded at a density of 2×10^4 cells/well in a 96-well cell culture dish (Corning, Corning, NY, United States) in 100 μL of culture medium and incubated overnight. CCK-8 (Dojindo, Kumamoto, Japan) reagents were added to a subset of the wells, and the cells were incubated for 2 h at 37 ℃. The absorbance was recorded using an automated plate reader. Each experiment was performed in triplicate and repeated at least three times.

For cell cycle analysis, the cells were fixed in 70% ethanol for 1 h at 4 ℃ and then incubated in 1 mg/mL RNase A for 30 min at 37 ℃. Subsequently, the cells were stained with propidium iodide (PI; 50 μg/mL) (Becton-Dickinson, San Jose, CA, United States) in PBS containing 0.5% Tween-20, followed by flow cytometry using a Becton-Dickinson FACScan and Cell Quest acquisition and analysis software. Gating was applied to exclude cell debris, cell doublets, and cell clumps.

Statistical analysis
Statistical analysis was performed using the SPSS software package. The association between STAT4 expression and the clinicopathological characteristics was analyzed using the χ^2 test. The relationship between the STAT4 and Ki-67 expression levels was evaluated using the Spearman rank correlation test because the data were not normally distributed. Survival analysis was performed using the Kaplan-Meier method, and the difference in the survival rates was assessed using the generalized log-rank test. Multivariate analysis was performed using Cox proportional hazard models. The data were expressed as the means ± SE, and $P < 0.05$ was considered to be significant.

RESULTS

STAT4 expression in tumor and adjacent nontumor tissues from HCC patients
To determine the role of STAT4 in HCC development, we first investigated the expression profile of STAT4 in HCC and non-tumorous tissues *via* Western blot and immunohistochemical analyses. Western blot analysis of eight paired tumor and adjacent non-tumorous tissues confirmed that STAT4 expression was lower in tumor tissues than in adjacent non-tumorous tissues (Figure 1). Immunohistochemical analysis indicated that STAT4 expression was low or undetectable in most tumorous tissues and was highly expressed in most adjacent nontumor tissues (Figure 2). In addition, the proliferation index of Ki67 was used as a control to indicate the tumorous and non-tumorous tissues.

Correlation between STAT4 expression and the clinicopathological characteristics
Next, we analyzed the relationship between STAT4 expression and the clinicopathological characteristics of HCC patients. To this end, the patients were stratified into those exhibiting high and low STAT4 expression according to the immunostaining intensity score (high > 0.53, low ≤ 0.53; 0.53 was the mean proportion of STAT4-expressing cells) The clinicopathological and demographic characteristics of the HCC patients are listed in Table 1. Statistical analysis indicated that STAT4 expression significantly correlated with histological grade, HBV infection and the tumor size ($P < 0.05$) but not to other characteristics, including gender, age, metastasis, the tumor count, the serum AFP level, cirrhosis, and vascular invasion. Furthermore, the patients were stratified into those exhibiting high and low Ki67 expression depending
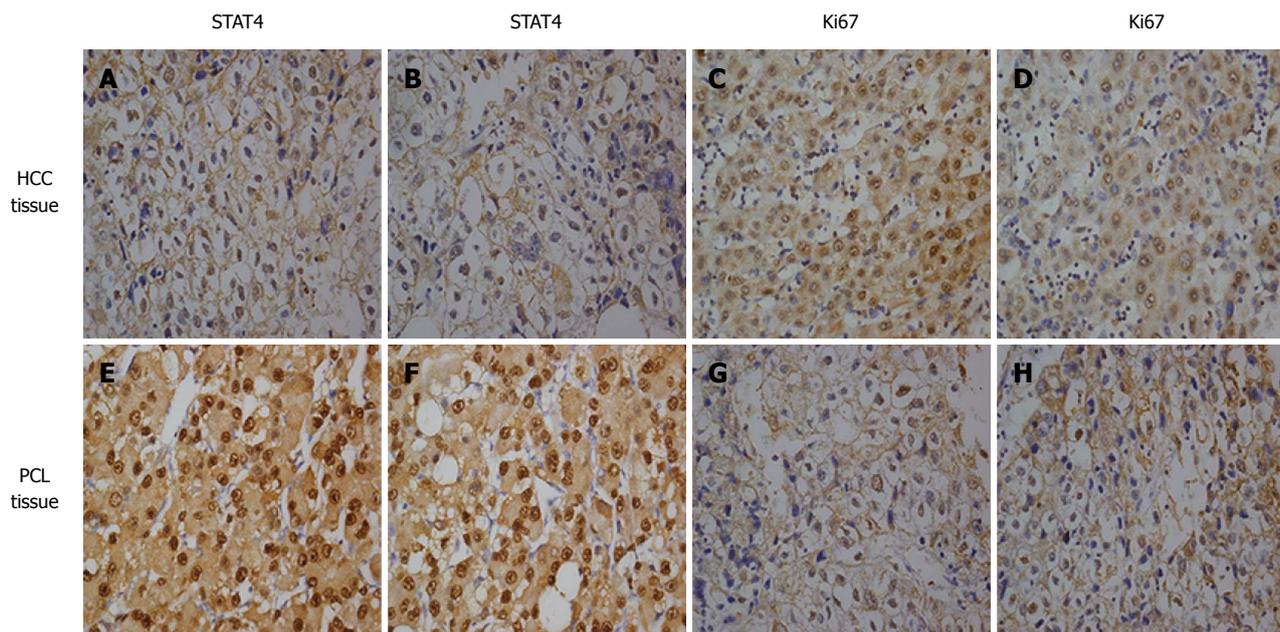
Figure 2 Immunohistochemical analysis of signal transducer and activator of transcription 4 and Ki67 expression in hepatocellular carcinoma and adjacent noncancerous liver tissues. Paraffin-embedded tissue sections were stained with antibodies against STAT4 and Ki67 and counterstained with hematoxylin. High expression of STAT4 (E, F) and Ki67 (C, D) was detected in the HCC tissues. Low expression of STAT4 (A, B) and high expression of Ki67 (G, H) were detected in adjacent noncancerous liver tissues (SP × 400) based on immunohistochemistry. STAT4: Signal transducer and activator of transcription 4; HCC: Hepatocellular carcinoma.

Table 1 Clinicopathological features of hepatocellular carcinoma in relation to the Stat4/Ki67 expression pattern in 90 patients

Clinicopathological features	Total	Stat4		P value	χ^2 value	Ki67		P value	χ^2 value
		Low < 0.53 n = 40	High > 0.53 n = 50			Low < 0.48 n = 49	High > 0.48 n = 41		
Age (yr)									
< 45	33	13	20	0.463	0.538	17	16	0.671	0.180
≥ 45	57	27	30			32	25		
Gender									
Male	57	26	31	0.769	0.086	31	26	0.988	0.000
Female	33	14	19			18	15		
Serum AFP level (ng/mL)									
< 25	34	19	15	0.089	2.895	18	16	0.823	0.050
≥ 25	56	21	35			31	25		
Liver cirrhosis									
No	26	13	13	0.499	0.457	13	13	0.589	0.291
Yes	64	27	37			36	28		
No. of tumor nodes									
Single	51	24	27	0.568	0.326	29	22	0.598	0.278
Multiple	39	16	23			20	19		
HBV									
Negative	43	25	18	0.012[1]	6.255	27	16	0.128	2.313
Positive	47	15	32			22	25		
Maximal tumor size (cm)									
< 4.5	40	30	9	0.000[1]	29.403	31	8	0.000[1]	17.402
≥ 4.5	50	10	41			18	33		
Tumor metastasis									
No	77	36	41	0.283	1.151	42	35	0.963	0.002
Yes	13	4	9			7	6		
Microvascular invasion									
No	66	30	36	0.749	0.102	36	30	0.975	0.001
Yes	24	10	14			13	11		
Histological grade									
I - II	49	36	13	0.000[1]	36.699	35	14	0.000	12.510
III-IV	41	4	37			14	27		
Ki67 expression									
Low	49	33	16	0.000[1]	22.849				
High	41	7	34						

[1]The *P* value was considered significant. Statistical analyses were carried out using pearson χ^2 test. HBV: Hepatitis B virus.
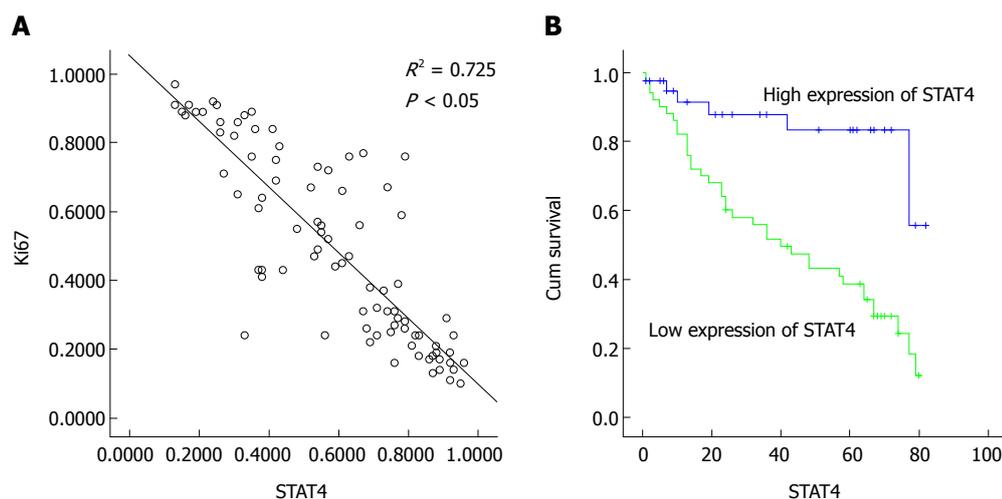
Figure 3 Relationship between signal transducer and activator of transcription 4 expression and the Ki67 proliferation index in hepatocellular carcinoma. A: Scatterplot of STAT4 expression *vs* Ki-67 expression; the regression line indicates a correlation based on the Spearman correlation coefficient; B: Kaplan-Meier survival curves of 90 HCC patients stratified into low and high STAT4 expression show a highly significant difference between these groups ($P < 0.05$, log-rank test using SPSS). STAT4: Signal transducer and activator of transcription 4; HCC: Hepatocellular carcinoma.

on the expression score for Ki67. We found that Ki67 expression significantly correlated with histological grade and tumor size ($P < 0.05$) but not with the other characteristics. Very importantly, there was a negative correlation between STAT4 and Ki67 expression ($R = 0.85$; Figure 3A). These findings suggested that STAT4 downregulation might contribute to HCC progression.

Prognostic significance of STAT4 expression to HCC prognosis

The above data indicated that STAT4 downregulation might commonly occur in HCC specimens, indicating the potential value of STAT4 expression for predicting the prognosis of HCC. Therefore, we analyzed whether the STAT4 expression level was indicative of HCC prognosis. Survival analysis was restricted to 90 cases for which complete follow-up data and STAT4 expression scores were available. Kaplan-Meier analysis of the overall survival of these HCC patients demonstrated that the patients displaying low STAT4 expression exhibited significantly worse overall survival than those displaying high STAT4 expression ($P < 0.05$; Figure 3B). Multivariate analysis using a Cox proportional hazard model showed that STAT4 was an independent prognostic indicator of the overall survival of HCC patients ($P < 0.05$; Table 2). As shown in Table 3, STAT4 was an independent prognostic indicator based on a multivariate Cox proportional hazard model. These findings convincingly demonstrated that STAT4 may serve as a valuable prognostic marker for HCC prognosis.

STAT4 was downregulated in HCC cell lines and was associated with the proliferation status of HCC cells

Next, HCC cell lines and L02 hepatocytes were employed to analyze the role of STAT4 in HCC cell physiology. The expression of STAT4 in these cell lines

was initially determined *via* Western blot analysis. L02 cells displayed the highest expression of STAT4 of all of the cell lines examined (Figure 4A). Given that STAT4 expression negatively correlated with histological grade and Ki67 expression in the HCC specimens, we hypothesized that STAT4 might play an inhibitory role in the proliferation of HCC cells. Thus, we analyzed whether the expression of STAT4 was altered in HCC cells during various proliferation statuses using a serum-starvation and refeeding experiment. After serum depletion for 72 h, HepG2 cells were arrested at the G1 phase (Figure 4B). Then, after serum refeeding, the cells progressively entered the S phase. Accordingly, we detected the progressive accumulation of cyclin D1 in HepG2 cells after serum refeeding, whereas the expression level of STAT4 decreased (Figure 4C). These data demonstrated that the expression of STAT4 was associated with HCC cell proliferation.

Effects of altered STAT4 expression on cell growth and the cell cycle in human L02 cell lines

To further examine the functional role of STAT4 in HCC proliferation, STAT4-siRNA oligos were employed to silence STAT4 expression in L02 cells. After transfection with two different STAT4-targeting siRNAs, L02 cells were subjected to Western blot analysis to determine the interference efficiency of each siRNA oligo. As shown in Figure 5A, STAT4 was significantly decreased after transfection with the STAT4-siRNAs. In addition, the expression level of cyclin D1 was elevated after STAT4 depletion, implying that STAT4 negatively regulates the expression of cyclin D1 in L02 cells.

Next, we analyzed the impact of STAT4 depletion on the proliferation of L02 cells. Flow cytometry was performed to determine the cell cycle distribution of L02 cells at 72 h after mock transfection or transfection

Table 2 Survival status and clinicopathological parameters in 90 hepatocellular carcinoma speciments

| Parameters | Total | Survival Status | | *P* value | χ^2 value |
		Dead $n = 43$	Alive $n = 47$		
Age (yr)					
< 45	33	16	17	0.919	0.010
⩾ 45	57	27	30		
Gender					
Male	57	26	31	0.589	0.292
Female	33	17	16		
Serum AFP level (ug/mL)					
< 25	34	15	19	0.588	0.293
⩾ 25	56	28	28		
Cirrhosis					
No	26	13	13	0.788	0.072
Yes	64	30	34		
No. of tumor nodes					
Single	51	23	28	0.561	0.339
Multiple	39	20	19		
HBV					
Negative	43	14	29	0.006	7.644
Positive	47	29	18		
Maximal tumor size (cm)					
< 4.5	39	7	32	< 0.051	24.543
⩾ 4.5	51	36	15		
Tumor metastasis					
No	77	36	41	0.636	0.224
Yes	13	7	6		
Microvascular invasion					
No	66	31	35	0.799	0.065
Yes	24	12	12		
Histological grade					
Ⅰ-Ⅱ	49	14	35	< 0.051	15.902
Ⅲ-Ⅳ	41	29	12		
Stat4 expression					
Low	40	6	34	< 0.051	31.003
High	50	37	13		
Ki67 expression					
Low	49	10	39	< 0.051	32.293
High	41	33	8		

[1]The *P* value was considered significant. Statistical analyses were performed using Pearson χ^2 test. HBV: Hepatitis B virus.

Table 3 Contribution of various potential prognostic factors to survival by Cox regression analysis in hepatocellular carcinoma specimens

Parameters	RR	95%CI	*P* value
Age (yr)	0.156	0.060-0.405	0.069
Gender	1.629	0.843-3.147	0.147
Serum AFP Level (ng/mL)	0.885	0.476-1.647	0.701
Cirrhosis	2.066	0.934-4.572	0.073
Tumor numbers	1.166	0.595-2.288	0.654
HBV	0.742	0.546-1.013	0.057
Maximal tumor size (cm)	0.448	0.460-0.951	0.014
Tumor metastasis	1.732	0.644-4.658	0.277
Microvascular Invasion	0.874	0.430-1.778	0.710
Histological grade	0.343	0.599-0.817	0.023[1]
STAT4 expression	0.411	0.222-0.758	< 0.001[1]
Ki67 expression	0.454	0.193-1.065	0.004[1]

[1]The *P* value was considered significant. Statistical analyses were performed using Long-rank test. HBV: Hepatitis B virus.

with control or STAT-targeted siRNA. As predicted, the STAT4-depleted L02 cells consisted of a markedly higher proportion of cells in the S phase (29.09% and 31.46%) than the control cells (20.29% and 20.28%), whereas the percentage of cells in the G1 phase (64.64% and 63.13%) after transfection with STAT4 siRNA-1 and 2 was significantly lower than that in the control cells (73.96% and 73.39%) (Figure 5B). Moreover, the impact of STAT4 depletion on HepG2 cell proliferation was determined using the CCK-8 assay. As shown in Figure 5C, the STAT4-depleted cells exhibited enhanced cell growth compared to the mock transfected and control siRNA-transfected cells. These data suggested that the downregulation of STAT4 might be associated with enhanced HCC cell proliferation.

DISCUSSION

HCC prognosis remains unsatisfactory because of its high recurrence and metastasis rates, despite significant improvements in surveillance and clinical treatment strategies[28]. The efficacy of traditional
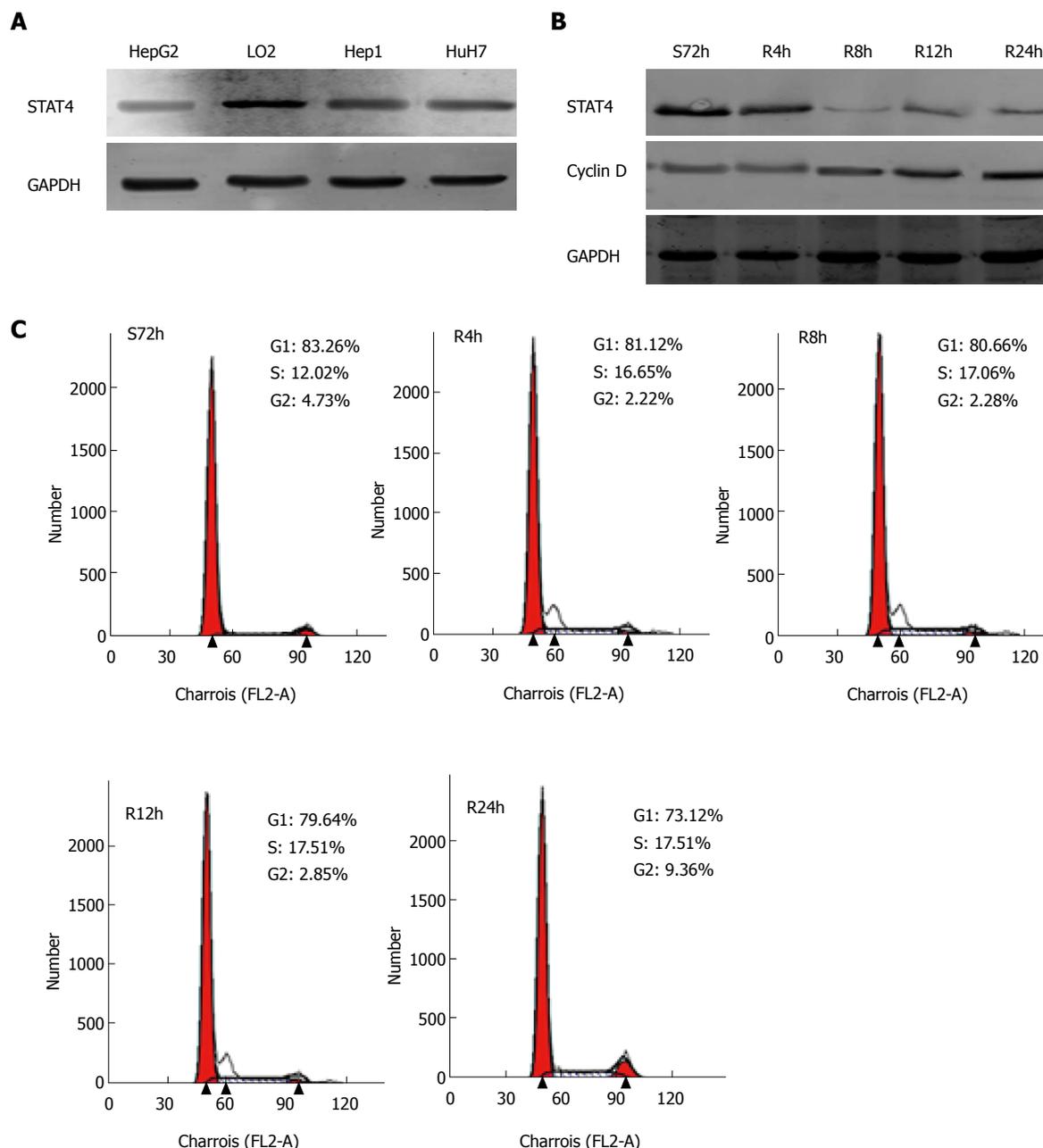
Figure 4 Western blot analysis of signal transducer and activator of transcription 4 protein expression in hepatocellular carcinoma cells compared to normal hepatocyte (L02) cells. A: GAPDH was used as a loading control. Each experiment was repeated at least 3 times; B: Expression of STAT4 and cell cycle-related molecules in proliferating HCC cells. HepG2 cells were synchronized *via* serum starvation for 72 h. Upon serum refeeding, cell lysates were prepared and analyzed *via* Western blot using antibodies directed against STAT4 and cyclin D1. GAPDH was used as a control for protein load and integrity. S: Serum starvation; R: Serum refeeding; C: Flow cytometric quantification of the cell cycle status in HepG2 cells. The cells were synchronized at G1 *via* serum starvation for 72 h; then, progression into the cell cycle was induced by adding medium containing 10% FBS for the indicated period (R4 h, R8 h, R12 h, or R24 h). STAT4: Signal transducer and activator of transcription 4; HCC: Hepatocellular carcinoma.

therapeutic methods, such as chemotherapy and surgical operation, remains limited. Therefore, it is critical to identify patients exhibiting poor prognosis for timely intervention and to develop novel targeted therapeutic strategies. Our current study showed that the decreased expression of STAT4 was significantly associated with a poor prognosis among HCC patients, which is partially attributed to uncontrolled HCC cell proliferation. Thus, our findings may promote the development of novel therapeutic strategies for HCC

patients based on STAT4.

The STAT family members were originally identified as cytokine-related signaling factors and have emerged as promising molecular targets for cancer therapy[29]. Among these proteins, STAT4 plays a critical role in the regulation of diverse biological actions, including anti-viral defense, the induction of cell death and growth arrest[24,30]. In the present study, we detected significantly lower levels of STAT4 expression in HCC tissues. Furthermore, we found that
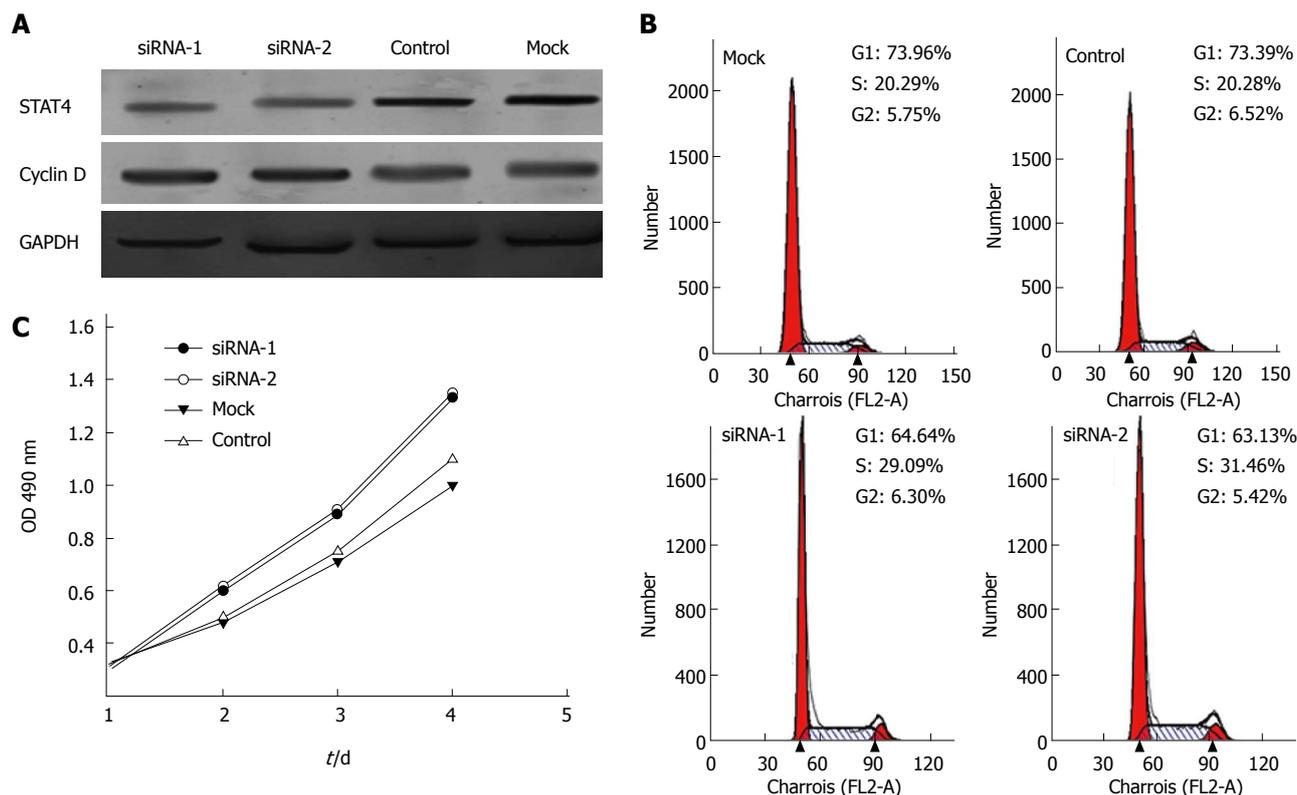
Figure 5 Signal transducer and activator of transcription 4 knockdown inhibited cell proliferation and the effects of altered signal transducer and activator of transcription 4 expression on the cell cycle status of L02 cells. A: Western blot analysis revealed that treatment with STAT4-siRNA markedly decreased the STAT4 and cyclin D1 levels at 48 h after siRNA transfection in L02 cells compared to transfection with negative control siRNA and mock transfection; B: siSTAT4-1 and -2 were used to knockdown STAT4 in HepG2 cells, resulting in the delay of the G1-S transition and significant arrest in the G1 phase after these cells were released from starvation. The data are presented as the mean ± SD of three experiments; C: A CCK-8 assay showed that STAT4 knockdown inhibited cell proliferation. CCK-8 reagents were added to the medium for 2 h. The absorbance was measured at each indicated time point (0, 1, 2, and 3 d). Each value was derived from three independent experiments. STAT4: Signal transducer and activator of transcription 4; HCC: Hepatocellular carcinoma.

the level of STAT4 in HCC tissues positively correlated with the degree of HCC differentiation and the serum hepatitis B surface antigen levels in HCC patients. It is well known that HBV infection is a risk factor for the development of HCC, contributing to the progression of HCC. Hence, our data suggest that STAT4 may serve as a negative regulator of HCC development and progression. Importantly, recent reports found that genetic alteration of STAT4 was a key risk factor for HBV-related HCC, which is consistent with our data showing that STAT4 downregulation was associated with HBV infection in HCC specimens[27]. However, another recent study reported that the mRNA level of STAT4 was not correlated with HBV infection in HCC patients[31]. Given that, STAT4 may be regulated at both the transcriptional and posttranscriptional levels. However, the detailed relevance of STAT4 to HBV-related hepatocarcinogenesis remains virtually unknown and requires clarification in future studies.

We showed that the expression of STAT4 correlated with the histological degree of HCC and the prognosis of HCC patients. Therefore, STAT4 may serve as an effective biomarker to evaluate the prognosis of HCC in Chinese patients. However, ubiquitous activation of JAK/STAT pathways is detected in human HCC

tissues[32]. This discrepancy may be due to the different genetic backgrounds and the various pathologic factors that contribute to the development of HCC. Although chronic viral hepatitis is the predominant factor for the development of HCC in the Chinese population, hyperlipidemia-related and alcoholic liver diseases are crucial for the development of HCC in Western countries. Indeed, the level of STAT4 expression varies for different types of cancers, and even for the same type of cancer in different genetic backgrounds and in patients from different geographic regions. We are interested in further investigating how these factors modulate STAT4 expression and activation, thereby contributing to the development and progression of HCC.

As a crucial member of the STAT family, STAT4 was widely considered to be primarily expressed in immune cells, including T helper cells, natural T killer cells, dendritic cells and macrophages, to mediate IL-12-dependent signaling. However, the function of STAT4 in non-immune cells remains poorly understood. We and other groups recently revealed a role of STAT4 in HCC development. In this regard, it was unexpectedly found that STAT4 was highly expressed in normal liver cells and was dramatically downregulated in HCC cells.

In addition, the low expression of STAT4 was verified in HCC cell lines and was found to be associated with the proliferation of HCC cells. Therefore, STAT4 in hepatocytes may exert a suppressive effect on the development of HCC tumors. Aside from HCC, STAT4 has been reported to be expressed in breast cancer cells and to play an important role in the regulation of breast cancer physiology[33]. Moreover, varying degrees of STAT4 activation have been detected in both prostate cancer and normal prostate tissues[34]. Evidence has also indicated that STAT4 might be expressed in several other cancer types, including gastric cancer and ovarian cancer[10,35]. These findings may provide novel insight into the role of STAT4 in the pathogenesis of various human cancers. However, a majority of recent studies inferred that the role of STAT4 in the prevention of liver disease was related to inflammatory pathways[31,36,37]. Therefore, it remains unclear to what extent an inflammation-independent role of STAT4 may contribute to HCC prevention. Further investigation should be performed to resolve this intriguing issue.

In summary, our findings suggest that STAT4 represents a novel and promising therapeutic target and prognostic biomarker for HCC. Our data may be of important clinical value for estimating prognosis and for determining the treatment of HCC patients. Technological development may lead to new treatments based on STAT4 that improve the therapies against HCC.

COMMENTS

Background
Signal transducers and activators of transcription (STATs) are members of a well-conserved family of transcription factors that play integral roles in various cellular processes. It is well known that STAT4 is expressed in many cancers, such as breast cancer. However, the expression of STAT4 in hepatocellular carcinoma (HCC) patients has yet to be reported.

Research frontiers
The STAT family includes many prominent members, such as STAT1, STAT3, which contribute to the early diagnosis of many cancers. Few studies of STAT4, which inhibits HCC, are available.

Innovation and breakthroughs
Tissue microarray was performed to analyze STAT4 and Ki67 expression and other clinical characteristics, revealing that low STAT4 expression indicates a poor clinical prognosis. Transfection and flow cytometry were used to analyze the proliferation of HCC cells after transfection of siRNA targeting STAT4, demonstrating that high expression of STAT4 inhibits HCC cell proliferation.

Applications
STAT4 expression may serve as an indicator of clinical prognosis.

Peer-review
This is a very interesting study of STAT4 and its possible effects on HCC. The expression of STAT4 in HCC with different methods is very good.

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ORIGINAL ARTICLE

Observational Study

Importance of reporting segmental bowel preparation scores during colonoscopy in clinical practice

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Abstract

AIM: To evaluate the impact of reporting bowel preparation using Boston Bowel Preparation Scale (BBPS) in clinical practice.

METHODS: The study was a prospective observational cohort study which enrolled subjects reporting for screening colonoscopy. All subjects received a gallon of polyethylene glycol as bowel preparation regimen. After colonoscopy the endoscopists determined quality of bowel preparation using BBPS. Segmental scores were combined to calculate composite BBPS. Site and size of the polyps detected was recorded. Pathology reports were reviewed to determine advanced adenoma detection rates (AADR). Segmental AADR's were calculated and categorized based on the segmental BBPS to determine the differential impact of bowel prep on AADR.

RESULTS: Three hundred and sixty subjects were enrolled in the study with a mean age of 59.2 years, 36.3% males and 63.8% females. Four subjects with incomplete colonoscopy due BBPS of 0 in any segment were excluded. Based on composite BBPS subjects were divided into 3 groups; Group-0 (poor bowel prep, BBPS 0-3) n = 26 (7.3%), Group-1 (Suboptimal bowel prep, BBPS 4-6) n = 121 (34%) and Group-2 (Adequate bowel prep, BBPS 7-9) n = 209 (58.7%). AADR showed a linear trend through Group-1 to 3; with an AADR of 3.8%, 14.8% and 16.7% respectively. Also seen was a linear increasing trend in segmental AADR with improvement in segmental BBPS. There was statistical significant difference between AADR among Group 0 and 2 (3.8% vs 16.7%, P < 0.05), Group 1 and 2 (14.8% vs 16.7%, P < 0.05) and Group 0 and 1 (3.8%

vs 14.8%, *P* < 0.05). χ^2 method was used to compute *P* value for determining statistical significance.

CONCLUSION: Segmental AADRs correlate with segmental BBPS. It is thus valuable to report segmental BBPS in colonoscopy reports in clinical practice.

Key words: Colorectal cancer screening; Adenomas; Polyps; Boston Bowel Preparation Score

Core tip: Bowel preparation quality determines the yield of colonoscopy. Most endoscopists continue to use the subjective systems of reporting bowel preparation. Boston Bowel Preparation Score (BBPS) helps to understand segment-specific risks for missed pathology based on the degree of bowel cleanliness. Our study showed that segmental Advanced Adenoma detection rate correlate with segmental BBPS. Segmental reporting will help in careful examination during repeat colonoscopy of segments with poor or sub-optimal BBPS on previous colonoscopy, in determining appropriate surveillance interval and the procedure for surveillance and in determining appropriate interventions to improve bowel preparation for colonoscopy in future.

INTRODUCTION

Colorectal cancer is the third most common cancer and the third leading cause of cancer related death in the United States[1]. It has been postulated that with increase in colorectal screening rates, risk reduction and availability of newer chemotherapeutic agents will likely reduce the colorectal cancer mortality rates in the United States by 50% by 2020[2]. Five year survival rates for colorectal cancer survival can be highly dependent upon stage of cancer at diagnosis, and can range from 90% for cancers detected at the localized stage; 70% for regional; to 10% for people with metastatic cancer[3,4].

Multiple risk reduction, prevention and early detection strategies have led to declining rates in colorectal cancer (CRC) incidence and mortality[5]. Colonoscopy is the only test which can target prevention through the detection and removal of adenomatous polyps. Removal of polyps during colonoscopy has been shown to have predominantly indirect, but convincing evidence in prevention of

CRC[6-8]. Bowel preparation is an important factor that determines the yield of colonoscopy and suboptimal preparation is associated with missed lesions[9]. Bowel preparation should be tolerable, effective without any side effects or changes in colonic mucosa[10-12]. Unfortunately, most of the currently available bowel preparations have some limitations[11-13].

Colonoscopies with suboptimal bowel prep quality are likely to have higher rates of missed lesions and there is a dire need for uniform and more efficient reporting of bowel preparation during colonoscopies. Interventions to increase bowel preparation quality utilizing visual aids (cartoons and photographs), simplified written materials and in-person and telephone counseling have resulted in mixed findings, but show promise in certain populations[14,15].

The Boston Bowel Preparation Score (BBPS) score was developed by Boston Medical Centre section of gastroenterology to provide a standardized score to rate the quality of bowel preparation during colonoscopy which can be used for clinical practice, quality assurance and outcome research in colonoscopy[16]. Three segments of colon are given a rating based on its cleanliness and the three section scores are added together for a BBPS score[16]. The scale is valid and demonstrates good inter and intra-rater reliability[16].

The efficiency of colonoscopy as a CRC screening method depends on the quality of bowel preparation. The interpretation of colonoscopy results depends on looking at the bowel preparation in addition to other findings. It is common for endoscopists to use the subjective systems of reporting bowel preparation which have high inter-observer variability. This study was designed to evaluate the impact of reporting bowel preparation using Boston Bowel Preparation Scale in clinical practice.

MATERIALS AND METHODS

Study objective
To determine advanced adenoma detection rate (AADR) in relation to segmental and composite BBPS's during colonoscopy.

Study design
The study was a prospective observational cohort study conducted at an urban teaching hospital. The study was approved by the Institutional Review Board.

Inclusion/exclusion criteria
Consecutive patients presenting for average risk screening colonoscopy were enrolled in the study. Subjects having colonoscopy for evaluation of symptoms and personal history of colon cancer, inflammatory bowel disease or colon surgery for any reason were excluded. Patients who were unable to comply with the preparation instructions were excluded.

Bowel preparation

All subjects received clear liquid diet the day before colonoscopy and a gallon of polyethylene glycol as bowel preparation the evening prior to colonoscopy.

Study method

Study was an observational study and no intervention or deviation from standard practice protocols for patients were done for the study purposes. All subjects were asked questions to determine that they met inclusion and exclusion criteria for participation in study. All study participants, or their legal guardian, provided informed verbal consent prior to study enrolment. All colonoscopies were performed by either board certified gastroenterology physicians or gastroenterology fellows under direct supervision of the board certified gastroenterology physicians. Before enrolling patients into the study all endoscopists involved were in serviced on boston bowel preparation score and scoring cards were made available in each endoscopy suite. BBPS was categorized as described by Lai *et al*[16]: 0: Unprepared colon segment with mucosa not seen due to solid stool that cannot be cleared; 1: Portion of mucosa of the colon segment seen, but other areas of the colon segment not well seen due to staining, residual stool and/or opaque liquid; 2: Minor amount of residual staining, small fragments of stool and/or opaque liquid, but mucosa of colon segment seen well; and 3: Entire mucosa of colon segment seen well with no residual staining, small fragments of stool or opaque liquid.

For the purpose of study, the colon was divided into three segments- Right (R) (Caecum and Ascending Colon), Transverse (T) (Hepatic Flexure, Transverse Colon and Splenic flexure) and Left (L) (Descending colon, Sigmoid colon and Rectum). A research associate was present during each procedure to record the BBPS reported by the endoscopist in each segment during the procedure (R-0/1/2/3, T-0/1/2/3, L-0/1/2/3). Segmental scores were combined to calculate the composite BBPS. Based on Composite BBPS, subjects were divided into three groups: Group 0- Composite BBPS 0-3, Poor bowel preparation; Group 1- Composite BBPS 4-6, Sub-optimal bowel preparation; and Group 2 - Composite BBPS 7-9, adequate bowel preparation.

As per national guidelines all procedures had a minimal withdrawal time of 6 minutes. Also the site, size and number of polyps were recorded during the procedure. High definition endoscopes were used for the colonoscopy of all enrolled subjects. Pathology report of each polyp was followed to determine segmental and combined AADR. The advanced adenoma bridges benign and malignant states and may be the most valid neoplastic surrogate marker for present and future colorectal cancer risk[17]. Advanced adenoma was defined as 3 or more adenomatous polyps, polyps greater than or equal to 10 mm or
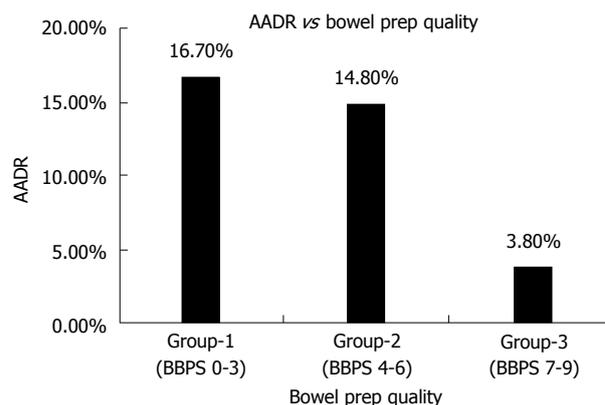
Figure 1 Advanced adenoma detection rates across different groups based on composite Boston Bowel Preparation Score. AADR: Advanced adenoma detection rates.

histologically having high-grade dysplasia or significant villous components.

Endpoint

To determine the association between AADR and quality of bowel preparation by using segmental and composite BBPS during colonoscopy.

Statistical analysis

Microsoft Excel software for Windows version 2010 was used. Cross tables with χ^2 test were used to compare differences among groups.

RESULTS

The statistical review of the study was done by one of the authors with biomedical research experience. Three hundred and sixty subjects were enrolled in the study. Mean age was 59.2 years, gender distribution was 36.3% males and 63.8% females. Four subjects with incomplete colonoscopy due BBPS of 0 in any segment were excluded. Based on composite BBPS subjects were divided into 3 groups; Group 0: *n* = 26 (7.3%), Group 1: *n* = 121 (34%) and Group 3: *n* = 209 (58.7%). AADR showed a linear trend through Group-0 to 2; with an AADR of 3.8%, 14.8% and 16.7% respectively (Figure 1).

Also seen was a linear increasing trend in segmental AADR with improvement in segmental BBPS (1 to 3); with an AADR of 3%, 6.8% and 8.1% for R-1, R-2, R-3 respectively; 0%, 4.4% and 7.4% for T-1, T-2, T-3 respectively; and 0%, 10% and 11.5% for L-1, L-2, L-3 respectively (Figure 2). There was statistical significant difference between AADR among Group 0 and 2 (3.8% *vs* 16.7%, *P* < 0.05), Group 1 and 2 (14.8% *vs* 16.7%, *P* < 0.05) and Group 0 and 1 (3.8% *vs* 14.8%, *P* < 0.05).

DISCUSSION

The bowel preparation process before a colonoscopy
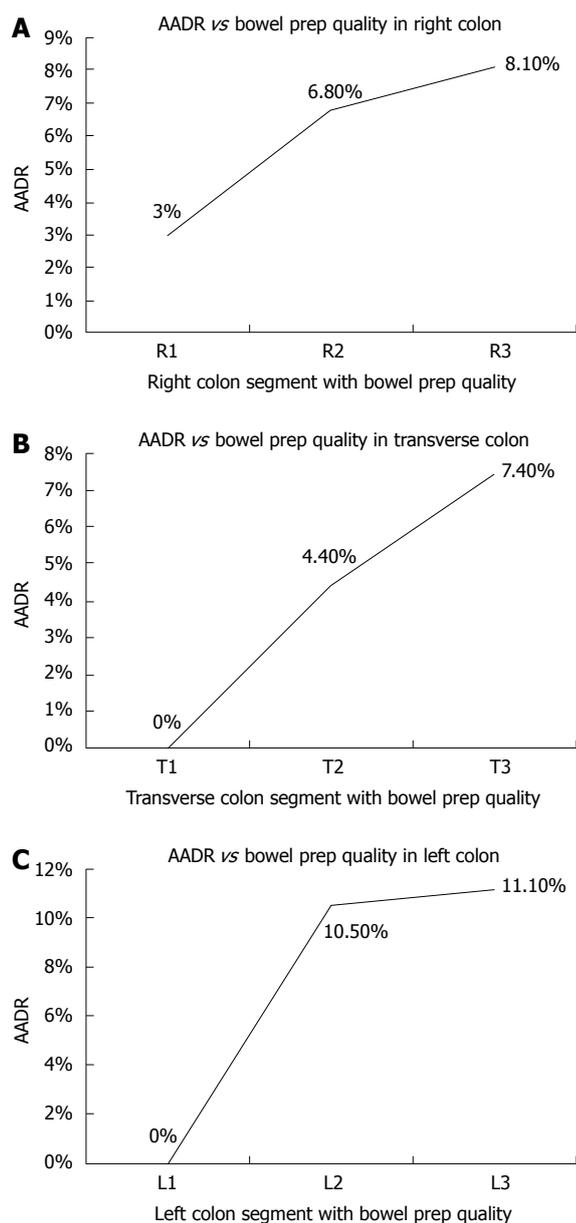
Figure 2 Advanced Adenoma detection rate across three segments (A, B, C) of colon and variation with segmental Boston Bowel Preparation Score 1-3. AADR: Advanced adenoma detection rate; R: Right; T: Transverse; L: Left.

is directed towards cleaning the colon of the faecal material for better visualisation of colonic mucosa and detection of abnormalities especially polyps present in the colon. Optimal bowel cleansing is pre-requisite for successful colonoscopy, indirectly having impact on both the performance and the effectiveness of the colonoscopy. Colonoscopies with suboptimal bowel preparation have significant adenoma miss rates, suggesting that suboptimal bowel preparation substantially decreases efficiency of colonoscopy as a CRC screening tool[9].The incidence of inadequate bowel preparation for colonoscopy has been reported to be as high as 25%[18]. The American Society for Gastrointestinal Endoscopy and American College of Gastroenterology Taskforce on Quality in Endoscopy

have recommended inclusion of assessment of the quality of bowel preparation in each colonoscopy report[19]. Terms such as excellent, good, fair, and poor were considered appropriate but the committee emphasized that the terms lack standardized definitions[19]. There are several other issues which are unclear such as whether the bowel preparation quality should be documented based on findings upon insertion of the colonoscope, or during withdrawal. Impact of cleansing maneuvers such as washing and suctioning of fluid is not accounted when using this terminology[16]. While former is an assessment of colonic preparation, and the latter is an assessment of the likelihood for missed lesions, a more clinically relevant measure, hence the distinction is important[16]. Furthermore, the variation of bowel preparation in different segments of colon is also not accounted.

Insufficient mucosal visualization during colonoscopy can result in lesions being missed[18,20]. Poor bowel preparation may also result in difficult progression, an increase risk of complications, prolonged procedure duration and an increase in the amount of sedatives and analgetics required[21]. Poor bowel preparation is also a frequent cause for incomplete procedures, resulting in the need for a repeat colonoscopy[21]. It has been suggested that the fact that colonoscopic surveillance does not prevent right-sided cancers is caused by the often worse quality of cleansing of the right side of the colon[22].

Because of these consequences, the quality of bowel preparation needs to be assessed and documented[23]. Suboptimal bowel preparation rates during colonoscopy can be as high as 1/3rd of total colonoscopies[24]. Therefore, knowledge of its risk factors can be very important. A model based on risk factors, such as male gender, inpatient status, and older age, correctly predicted inadequate bowel preparation in only 60% of patients[25].

In an effort to improve colonoscopy outcome, it is essential to report the quality of bowel preparation accurately. Most gastroenterologist continue to use the subjective systems of reporting bowel preparation. Many endoscopist find it difficult to report the bowel preparation quality accurately because of inter-segmental variation. BBPS score allows gastroenterologist to report the quality of bowel preparation for each colon segment in an objective manner. BBPS is sensitive to differences in bowel prep quality within different segments of colon, and therefore helps to identify segment-specific risks for missed pathology. It helps in identifying the potential colon segments which require more detailed examination in repeat colonoscopy. Total and individual segment BBPS scores have demonstrated strong inter- and intra-rater reliability over the full range of possible segment scores[16]. The BBPS is simple to learn and practice and can be seen as a useful tool in standardizing the reporting of bowel prep quality.

Our study showed that segmental AADR correlate with segmental BBPS. Also, AADR shows linear increasing trend with composite BBPS. It is thus valuable to report segmental BBPS in colonoscopy reports in clinical practice. Segmental BBPS can also aid gastroenterologists in deciding the surveillance method for colorectal screening. Patients with suboptimal scores only on the left side can have surveillance using a flexible sigmoidoscopy rather than having a complete colonoscopy. Similarly patients with suboptimal preparation on the right or transverse colon need to have complete colonoscopy. Reporting segmental bowel preparation will also help us identify patient related factors which are associated with suboptimal preparation on one particular segment and hence study interventions that can improve bowel preparation on that segment.

In conclusion, the BBPS is a valid and reliable scoring system for assessing adequacy of bowel preparation during colonoscopy regardless of degree of cleanliness. Documentation of BBPS in all colonoscopy reports will help in: (1) careful examination during repeat colonoscopy of segments which had poor or sub-optimal BBPS on previous colonoscopy; (2) determining appropriate surveillance interval and the procedure for surveillance (flexible sigmoidoscopy *vs* colonoscopy); (3) determining appropriate inter- ventions to improve bowel preparation for colonoscopy in future; and (4) quality improvement research in colonoscopy when we need to control for bowel preparation quality.

This practice will help in better documentation of the colonoscopy results in relation to the quality of bowel preparation and will be helpful in planning the appropriate course of future intervention for every subject.

COMMENTS

Background
The efficiency of colonoscopy as a colorectal cancer screening method depends on the quality of bowel preparation. The interpretation of colonoscopy results depends on looking at the bowel preparation in addition to other findings. Many endoscopist find it difficult to report the bowel preparation quality accurately because of inter-segmental variation. Colonoscopies with suboptimal bowel prep quality are likely to have higher rates of missed lesions and there is a dire need for uniform and more efficient reporting of bowel preparation during colonoscopies.

Research frontiers
The American Society for Gastrointestinal Endoscopy and American College of Gastroenterology Taskforce on Quality in Endoscopy have recommended inclusion of assessment of the quality of bowel preparation in each colonoscopy report. Terms such as excellent, good, fair, and poor were considered appropriate but the committee emphasized that the terms lack standardized definitions. Few bowel preparation scales have been validated till now and their clinical use is still not widely accepted.

Innovations and breakthroughs
Boston bowel preparation score was devised to address the need for reporting segmental bowel preparation scores. A recent study has demonstrated higher polyp detection rate in patients with higher Boston Bowel Preparation Score (BBPS) scores than in those with lower BBPS scores during a colonoscopic procedure, consistent with our study results.

Applications
Composite and segmental reporting of bowel preparation during colonoscopy will be helpful in following ways: (1) careful examination during repeat colonoscopy of segments which had poor or sub-optimal BBPS on previous colonoscopy; (2) determining appropriate surveillance interval and the procedure for surveillance (flexible sigmoidoscopy *vs* colonoscopy); (3) determining appropriate interventions to improve bowel preparation for colonoscopy in future; and (4) quality improvement research in colonoscopy when we need to control for bowel preparation quality.

Terminology
Advanced adenoma was defined as presence of 3 or more adenomatous polyps, polyps greater than or equal to 1 cm or having high-grade dysplasia or significant villous components. Advanced adenoma detection rate - percentage of patients who have one or more advanced adenoma detected. BBPS - Boston Bowel Preparation Score, a validated tool to report quality of bowel preparation

Peer-review
This article focused on an interesting issue: The standardization of preparation colonoscopy evaluation. The results are intuitive, the paper is well-written and easy to understand. The number of patients studied is good.

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ORIGINAL ARTICLE

Observational Study

Rectal tone and compliance affected in patients with fecal incontinence after fistulotomy

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Abstract

AIM: To investigate the anal sphincter and rectal factors that may be involved in fecal incontinence that develops following fistulotomy (FIAF).

METHODS: Eleven patients with FIAF were compared with 11 patients with idiopathic fecal incontinence and with 11 asymptomatic healthy subjects (HS). All of the study participants underwent anorectal manometry and a barostat study (rectal sensitivity, tone, compliance and capacity). The mean time since surgery was 28 ± 26 mo. The postoperative continence score was 14 ± 2.5 (95%CI: 12.4-15.5, St Mark's fecal incontinence grading system).

RESULTS: Compared with the HS, the FIAF patients showed increased rectal tone (42.63 ± 27.69 *vs* 103.5 ± 51.13, $P = 0.002$) and less rectal compliance (4.95 ± 3.43 *vs* 11.77 ± 6.9, $P = 0.009$). No significant differences were found between the FIAF patients and the HS with respect to the rectal capacity; thresholds for the non-noxious stimuli of first sensation, gas sensation and urge-to-defecate sensation or the noxious stimulus of pain; anal resting pressure or squeeze pressure; or the frequency or percentage of relaxation of the rectoanal inhibitory reflex. No significant differences were found between the FIAF patients and the patients with idiopathic fecal incontinence.

CONCLUSION: In patients with FIAF, normal motor anal sphincter function and rectal sensitivity are preserved, but rectal tone and compliance are impaired. The results suggest that FIAF is not due to alterations in rectal sensitivity and that the rectum is more involved than the anal sphincters in the genesis of FIAF.

Key words: Fecal incontinence; Anorectal surgery; Fistulotomy; Visceral sensitivity; Barostat

Core tip: Fistula and anorectal abscess are common, and 40% of such abscesses result in fistulas. Postoperative fecal incontinence (FI) is frequent. It has

been reported that rectal distensibility and thresholds for sensations decrease after hemorrhoidopexy, and that the perception of rectal distension is not always reduced in FI. In our patients with FI after fistulotomy (FIAF), anal sphincter function and rectal sensitivity are preserved, but rectal tone and compliance are impaired. The results suggest that FIAF is not due to alterations in rectal sensitivity and that the rectum is more involved than the anal sphincters in the genesis of FIAF.

INTRODUCTION

Fecal incontinence (FI) results in poor quality of life[1]. FI can appear spontaneously (idiopathic)[2], secondary to systemic diseases or traumatic events, as a result of congenital alteration[3], or after pelvic radiation or anorectal surgery. Fistula in ano is a common proctological disease[4]. Anorectal abscess is very frequent in adults[5]. About 40% of such abscesses result in fistulas[5]. Fistulas are usually treated surgically; postoperative anal incontinence is common, and the recurrence rate is high for complex fistulas. Of the various possible surgical approaches, fistulotomy is the treatment of choice, although all of the surgical treatments are associated with success rates ranging from 30%-80%[6]. The reported incidence of postsurgical incontinence varies according to the procedure performed[7-9]: 20% for fistulotomy, 11.6% for fistulotomy with end-to-end primary sphincteroplasty, 20.3% for fistulotomy treating intersphincteric fistula, 13% for complex fistulae undergoing sphincter division, 12% for the cutting seton fistulotomy, and 6% for the ligation of the intersphincteric fistula tract procedure. It has been reported that FI can present as a late complication of anal fissures[10] or other anorectal procedures[11]. It has also been demonstrated that rectal distensibility and the volume thresholds for sensations decrease after stapled hemorrhoidopexy[12], and the perception of rectal distension is not always reduced in FI[13]. The lack of a universal and effective treatment for FI highlights the need to investigate the underlying mechanisms and develop new therapeutic targets. These mechanisms can be assessed by an electronic barostat, measuring the visceral afferent sensation[14], and by anorectal manometry, measuring motor function in patients with FI after anorectal surgery. This work intended to recognize the rectal and anal sphincter factors that might contribute to the development of fecal incontinence after fistulotomy (FIAF).

MATERIALS AND METHODS

Subjects

The work was conducted at the Experimental Medicine and Motility Unit in the Mexico City General Hospital. The Ethical and Research board approved the protocol, and signed informed consent was obtained from all the subjects. The study was performed in accordance with the Declaration of Helsinki and its subsequent revisions. The epidemiological and clinical data obtained for all of the outpatients presenting with FIAF during a 12-mo period at our motility tertiary unit were saved in a file. The inclusion criteria were being older than 18, having FI for a minimum of 6 mo after anorectal surgery, and giving consent for the study. The exclusion criteria were previous non-anorectal and anorectal surgery, idiopathic or postpartum FI, pelvic radiation, treatments affecting the smooth muscles during the precedent month, a state of pregnancy or nursing, any concomitant disease, and psychiatric alterations. Eleven patients who suffered from anal fistulae (mean age 45 ± 8, range 34-67 years) were enrolled in the study. One patient presented with chronic anal pain. Demographic data and etiology of fistula are shown in Table 1. The laboratory test parameters (chemistry panel, coproparasitoscopic studies, and amoeba assessment) performed on all of the patients was normal. None of the patients had presented with FI before anorectal surgery. The severity of FI after anorectal surgery was graded using the St. Mark's fecal incontinence grading system (minimum score = 0 = perfect continence; maximum score = 24 = totally incontinent)[15].

Controls

The controls were 11 patients with idiopathic FI (mean age 48 ± 17, range 17-71 years) and healthy subjects (HS) who voluntarily agreed to participate in the study as controls. None of the healthy controls was a proctologic patient. Due to ethical restrictions against the performance of manometry and barostat procedures on the same group of volunteers, a different group of subjects was utilized as controls for the manometry (*n* = 11, mean age 22 ± 2 years, 10 females) and barostat (*n* = 10, mean age 25 ± 7 years, three females) procedures. However, all patients underwent clinical history, laboratory test, recto-sigmoidoscopy (Welch Allyn 32823 sigmoidoscope, Skaneateles Falls, NY, United States), anorectal manometry (MMS, AN Enschede, The Netherlands) and barostat rectal sensitivity studies (Distender Ⅱ; G. J. Electronics, Toronto, Canada).

Study and distension protocol

The methodology that we use regularly in our laboratory to evaluate visceral sensation has already

Table 1 Demographic data and etiology of fistula				
Case	Age	Gender	Etiology	Type
1	38	Male	Idiopathic (perianal abscess)	Simple
2	34	Male	Idiopathic (perianal abscess)	Simple
3	67	Female	Idiopathic (perianal abscess)	Simple
4	50	Male	Idiopathic (abscess)	Complex
5	39	Male	Idiopathic (abscess)	Simple
6	45	Female	Idiopathic (abscess)	Complex
7	42	Female	Iatrogenic (miotomy)	Simple
8	47	Female	Iatrogenic (surgery)	Simple
9	48	Male	Idiopathic (abscess)	Simple
10	47	Male	Idiopathic (inter-sphincteric abscess)	Complex
11	41	Female	Idiopathic (inter-sphincteric abscess)	Complex

been published in detail elsewhere[14]. In brief, we have reported[14] that rectal visceral sensitivity is evaluated with an electronic barostat that is a tool that keeps a constant pressure in an air-filled bag through a feedback mechanism. This cylindrical ultra-thin polyethylene bag (Mui Scientific, Ontario, Canada) has infinite compliance and reaches 600 mL. The individual operating pressure (IOP) is defined as the minimum pressure required to overcome passive resistance to bag inflation[16]. To obtain muscle tone, the bag is inflated to the IOP, and the volume recorded over a 15-min period. We[14] and others investigators[16,17] have also reported that the barostat using the ascending method of limits procedure performs rectal distension for one minute, with increase of 4 mmHg each time and separated by periods of a minute, in which the bag pressure returns to the IOP. The distensions continue until the subject reports pain, press the panic button or reach a pressure of 48 mmHg.

Statistical analysis

The outcome measures were the anorectal manometry and barostat results in patients with FIAF compared with that of the idiopathic FI and HS. A 95%CI was analyzed for all of the variables and used when was appropriate. The results are expressed as means ± SD. $P < 0.05$ and Student's two-tailed t tests were utilized. The statistical analyses were performed using 2000 GraphPad Software (San Diego, CA, United States).

RESULTS

Subjects

The mean body mass indices of the FIAF patients (29 ± 3 kg/m^2), the idiopathic FI patients (27 ± 3 kg/m^2) and the HS (26 ± 5 kg/m^2) in the barostat group were similar and not significant. The average time for the FIAF patients since surgery was over two years (28 ± 26 mo; 95%CI: 10.8-45.3). All of the patients suffered FI. The mean postoperative continence score was 14 ± 2.5 (95%CI: 12.4-15.5) based on St Mark's faecal incontinence grading system. The results of laboratory

tests and the recto-sigmoidoscopy were normal in all patients.

Rectal tone, compliance and capacity

As shown in Table 2, there was no difference in the IOP between FIAF patients (8.7 ± 1 mmHg; 95%CI: 7.7-9.6), idiopathic FI patients (7.9 ± 10 mmHg; 95%CI: 7.8-8; $P = 0.10$) and the HS (9.6 ± 2 mmHg; 95%CI: 8.3-10.8; $P = 0.28$). The rectal bag volume was lower in the FIAF patients compared with the HS ($P = 0.002$), indicating an increased smooth muscle tone in the rectum of the FIAF patients (Table 2). Rectal bag volume in the FIAF patients compared with the idiopathic FI patients was not different ($P = 0.12$; Table 2).

The FIAF patients showed less rectal compliance (mL per mmHg; $P = 0.009$; Table 2) than the HS. There was no significant difference in the rectal capacity (balloon volume at the maximum imposed pressure[18]) of the FIAF patients compared with the HS ($P = 0.73$; Table 2).

Sensory thresholds to rectal distension

As shown in Table 2, the FIAF patients reported their first sensations and noxious stimulus of pain at levels that were not significantly different from the idiopathic FI patients and HS.

Anorectal manometry

There was no significant difference in the anal resting or squeeze pressures or the frequency or percentage of relaxation of the spontaneous rectoanal inhibitory reflex (RAIR) of the FIAF patients and the idiopathic FI patients and the HS (Table 2).

DISCUSSION

FI is generally considered to be primarily related to the dysfunction of the anal sphincters[19,20]. Interestingly, the present study demonstrated that in patients with FIAF, the function of the internal anal sphincter is preserved. Normal sphincter function in FI was reported by Siproudhis *et al*[21] who found that one-third of their subjects who suffered from FI had anal manometry values within the normal range. This finding was more recently supported by Burgell *et al*[22], who reported that only one-third of 160 male patients with FI demonstrated sphincter dysfunction.

In our study, the anal squeeze pressure of the FIAF patients was not significantly different than that of the idiopathic FI patients and the HS. This finding is in contrast with that of Bharucha *et al*[19], who reported lower anal squeeze pressures in women with idiopathic FI compared with healthy controls. The etiology of this incontinence may explain this discrepancy. However, the fact that our study included both men and women may be significant because squeeze pressure has been reported to be higher on average in men than

Table 2 Physiological data						
	Fecal incontinence after Fistulotomy	Idiopathic fecal incontinence	Healthy subjects	*P* value		
	(*n* = 11)	(*n* = 11)	(*n* = 11)	FIAF *vs* IFI	FIAF *vs* HS	IFI *vs* HS
Barostat						
IOP (mmHg)	8.7 ± 1.6	7.9 ± 0.1[a]	9.6 ± 2.1	0.106	0.282	0.012
Tone (bag volume, mL)	42.6 ± 27.7[a]	63.6 ± 34.1[a]	103.5 ± 51.1	0.129	0.002	0.047
Compliance (v/p)	5 ± 3.4[a]	8.1 ± 4.3	11.8 ± 6.9	0.075	0.009	0.151
Rectal capacity (mL)	314.7 ± 76.1	332.6 ± 97.1	302.9 ± 82.3	0.636	0.735	0.460
First sensation (mmHg)	15.8 ± 3.1	16.6 ± 4.2	14.1 ± 5	0.627	0.336	0.220
Gas sensation (mmHg)	22 ± 3	20.5 ± 3.6	18 ± 6	0.381	0.107	0.292
Urge defecate (mmHg)	28.1 ± 6.6	30.5 ± 10.9	22.5 ± 7.2	0.600	0.107	0.069
Pain sensation (mmHg)	36.8 ± 7.2	34.1 ± 8.4	35.9 ± 8.8	0.480	0.818	0.656
Anorectal manometry data						
Anal resting pressure (mmHg)	41.7 ± 24.3	44.2 ± 18.2	34.9 ± 22.9	0.791	0.506	0.306
Anal squeeze pressure (mmHg)	94.1 ± 88.8	109.9 ± 88.9[a]	43.2 ± 24.9	0.680	0.082	0.026
Spontaneous RAIR frequency (#)	1.9 ± 2.8	1.5 ± 1.5	1.4 ± 1.6	0.641	0.583	0.893
Induced RAIR relaxation (%)	66.3 ± 20.5	63.4 ± 16.7	74.4 ± 31.6	0.721	0.483	0.319
Induced RAIR duration (s)	18.4 ± 6.7	22.3 ± 6.5	19.8 ± 5.9	0.186	0.610	0.367

[a]$P < 0.05$ *vs* healthy subjects. All data represent the mean ± SD. FIAF: Fecal incontinence after Fistulotomy; IFI: Idiopathic fecal incontinence; HS: Healthy subjects; RAIR: Rectoanal inhibitory reflex; IOP: Individual operating pressure.

in women[23]. In addition, our data partially agree with Lindsey *et al*[24] who reported that the maximum squeeze pressure was normal in 52% of 93 patients with FI after anal surgery.

In the current study, there was no difference in the RAIR parameters of the FIAF patients, the idiopathic FI patients and the HS. This finding is inconsistent with a recent study that reported differences in RAIR parameters between incontinent and normal cohorts[25] as well as with another study that reported a reduced frequency of RAIR in patients with idiopathic FI compared with normal controls[26].

Different methods and etiology of incontinence could explain this disagreement. The presence of RAIR may exclude neuropathy as a factor related to FIAF. It is therefore necessary to identify other potential participants in the continence mechanism, such as the rectum.

In the present study, the FIAF patients and the idiopathic FI patients demonstrated higher rectal tone than the HS. This finding is inconsistent with that of Worsøe *et al*[26], who reported that in patients with idiopathic FI, the rectal tone during fasting is similar to that of HS. Since we used a barostat technique and Worsøe *et al*[26] used rectal distensibility in 12 patients, differences in the methodology and etiology of incontinence may account for the different results.

Compared with the HS, the FIAF patients demonstrated lower rectal compliance (distensibility). This finding is consistent with that of Corsetti *et al*[12], who reported that rectal distensibility decreases after a stapled hemorrhoidopexy, and Krol *et al*[27], who reported that a different form of rectal damage from prostate radiotherapy can also lead to reduced rectal distensibility. Considering that fecal continence requires the relaxation of the rectal wall[28], post-surgery rectal wall stiffness may explain the observed alteration in the tone and rectal distensibility. Related to this fact

is a report that increased rectal wall stiffness after radiotherapy alters the rectal distensibility, most likely by fibrosis[27].

Furthermore, patients with FI and sphincter defects have been reported to have a reduced rectal capacity[20]. Our results do not agree with this report; our patients with FIAF and idiopathic FI demonstrated rectal capacities similar to those of the HS. Thus, not all FIAF patients present with a reduced rectal capacity after surgery. Our data are consistent with a report that only 25% of women with idiopathic FI have a reduced rectal capacity[29].

The thresholds for the first, gas and urge-to-defecate sensations as well as the noxious stimulus of pain sensation were similar in FIAF patients, idiopathic FI patients and the HS. Our results differ from those of Burgell *et al*[22], who reported that one-sixth of incontinent men have rectal hyposensitivity, Bharucha *et al*[29], who demonstrated rectal hypersensitivity in women with idiopathic FI, and Chan *et al*[30], who reported that approximately 50 percent of their patients with urge FI demonstrated rectal hypersensitivity. Because we utilized a barostat technique while others typically evaluated sensory threshold volume during balloon distension performed with a hand-held syringe, the etiology of incontinence through differences in methodology may account for these discrepancies. However, it should be noted that in a preliminary report, we have described rectal hyposensitivity in patients with FI after sphincterotomy[31], and we have also described rectal hyposensitivity in subjects with complete spinal cord injury with neurogenic bowel dysfunction[14]. Interestingly, irradiation for prostate cancer that reduced rectal distensibility did not affect the thresholds of rectal sensation[27]. Therefore, based on these findings, we can infer that FI appearing after fistulotomy is not due to alterations in rectal sensitivity.

We recognize that the small number of subjects

limits the power of this work and that a small study is at risk to a type-II error. Nevertheless, it is reported that in such cases, power is properly indicated by CI[32], which we used in this work. Another limitation could be that we did not perform rectal sensitivity studies before surgery. However, due to the large number of colorectal surgical procedures performed[33], it is not reasonable to perform barostat procedures on all of these patients before surgery. Because only a small percentage of patients develop incontinence after surgery[8], a comparison of data from FIAF patients with idiopathic FI patients and with HS constitutes a more reliable assessment of physiological and structural changes.

In summary, the finding that internal anal sphincter function is preserved in FIAF patients but rectal tone and compliance is impaired compared with HS suggests that the rectum is more involved than the internal anal sphincter in the genesis of FIAF. Furthermore, based on these results, we can infer that FI that appears after fistulotomy is not due to alterations in rectal sensitivity. Therefore, surgeons should be careful to preserve the rectal anatomy and neural networks as much as possible when performing anorectal surgery.

COMMENTS

Background

Fecal incontinence (FI) results in poor quality of life. Anorectal abscess is extremely common in adults. Approximately 40% of such abscesses result in fistulas. Fistulas are usually treated surgically; postoperative anal incontinence is common, and the recurrence rate is high for complex fistulas. This work intended to recognize the rectal and anal sphincter factors that might contribute to the development of fecal incontinence after fistulotomy (FIAF).

Research frontiers

The lack of a universal and effective treatment for FI highlights the need to investigate the underlying mechanisms and develop new therapeutic targets. These mechanisms can be assessed by an electronic barostat, measuring the visceral afferent sensation, and by anorectal manometry, measuring motor function in patients with FI after anorectal surgery.

Innovations and breakthroughs

FI is generally considered to be primarily related to the dysfunction of the anal sphincters. In the current study, the finding that internal anal sphincter function is preserved in FIAF patients but rectal tone and compliance is impaired compared with healthy subjects suggests that the rectum is more involved than the internal anal sphincter in the genesis of FIAF. Furthermore, based on these results, we can infer that FI that appears after fistulotomy is not due to alterations in rectal sensitivity. Therefore, surgeons should be careful to preserve the rectal anatomy and neural networks as much as possible when performing anorectal surgery.

Applications

The present findings may be useful for the understanding the mechanisms of FIAF and to develop a treatment for it.

Terminology

Rectal visceral sensitivity is evaluated with an electronic barostat that is a tool that keeps a constant pressure in an air-filled bag through a feedback mechanism. Barostat studies assess rectal sensitivity, tone, compliance and capacity.

Peer-review

The manuscript written by Richard Alexander Awad *et al* analyzed the mechanism of fecal incontinence observed after fistulotomy. They found that fecal incontinence is not due to alterations in rectal sensitivity and that the rectum is more involved than the anal sphincters in the genesis of the incontinence. Because fecal incontinence greatly affects the QOL of the patients, this study is important for the therapeutic strategy of the patients. The study is well-organized and the manuscript is well-written.

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ORIGINAL ARTICLE

Observational Study

Interferon-λ-related genes and therapeutic response in Chinese hepatitis C patients

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Abstract

AIM: To determine the association between rapid viral response and *IL28B*, *IL28RA*, *IL10RB* and *MxA* polymorphisms in the Chinese Han population.

METHODS: The study cohort consisted of 238 chronic hepatitis C patients treated with interferon (IFN)-α-2b and ribavirin. Six single nucleotide polymorphisms were genotyped using the ABI TaqMan allelic discrimination assay. Biochemical indices were measured at baseline. Serum hepatitis C virus (HCV) RNA was detected at weeks 0, 4, 12 and 24 of therapy.

RESULTS: Only *IL28B* rs12980275 was associated with treatment response in the Chinese Han population. Patients carrying AG/GG genotypes had a reduced rapid viral response compared with patients carrying the AA genotype (additive model: adjusted OR = 0.43, 95%CI: 0.24-0.75). It took less time for patients with the AA genotype to achieve a viral load < 500 copies/mL (log-rank test, $P = 0.004$). In addition, the protective effect of genotype AA was independent of baseline viral load. HCV genotype, and baseline white blood cell count, α-fetoprotein and viral load might also help predict treatment response. The area under the receiver-operating characteristic curve was 0.726.

CONCLUSION: *IL28B* rs12980275 AA genotype is a strong predictor of positive response to IFN therapy in Chinese Han patients with hepatitis C.

Core tip: The association between *IL28B* rs12980275 and viral response to pegylated-interferon (IFN) plus ribavirin treatment has been observed in Japanese patients, but rarely in Chinese patients. Because pegylated-IFN is more expensive, non-pegylated instead of pegylated IFN-α is more commonly used for chronic hepatitis C treatment in Chinese primary hospitals. Therefore, the role of IFN-λ-related genes in the response to non-pegylated IFN-α treatment should be established to help guide clinical decisions and improve cost-effectiveness.

INTRODUCTION

Hepatitis C virus (HCV) poses a serious global health problem due to its adverse clinical outcomes, such as cirrhosis and hepatocellular carcinoma. The estimated prevalence of HCV is 1%-1.9% in the general population of Mainland China, with 75%-80% of those chronically infected[1,2]. The treatment for chronic hepatitis C (CHC) consists of interferon (IFN) plus ribavirin (RBV) and protease inhibitors such as telaprevir and boceprevir. Sustained virological response (SVR), which refers to a negative HCV-RNA test 6 mo after cessation of therapy, is defined as a positive treatment response. Rapid viral response (RVR; negative HCV-RNA test 4 wk after treatment) is thought to be a powerful on-treatment predictor of SVR[3,4]. Patients who achieve RVR are more likely to achieve SVR. The treatment response likely depends on a complex host-virus interaction. Many studies have suggested a range of factors that are associated with RVR and SVR, including HCV genotype, viral load, liver function, and host immune status.

The influence of host gene polymorphisms has drawn attention in recent years. Genome-wide association studies (GWASs) have demonstrated that polymorphisms near the *IL28B* gene, which codes for IFN-λ3, affect the response of CHC to pegylated (PEG)-IFN-α/RBV therapy[5-7]. IFN-λ acts through binding to *IL28RA* and *IL10RB* genes, which subsequently activates the Janus kinase-signal transducer and activator of transcription pathway to up- or down-regulate hundreds of genes, such as *MxA*, *OAS1* and

PKR, and it is then involved in the immune response pathways[8]. *IL10RB* and *IL28RA* gene polymorphisms can predict the natural outcomes of HCV infection in the Chinese population[9]. According to our previous meta-analysis, *MxA* gene polymorphisms may also be associated with virological response to IFN in the Chinese population[10].

Given that the cost of PEG-IFN treatment is higher than non-PEG-IFN treatment, many patients in Chinese primary hospitals cannot afford PEG-IFN treatment. As a result, non-PEG IFN-α is more commonly used in the treatment of chronic hepatitis C. The previous GWASs were based on observations in Australian, European, African-American and Japanese, but not Chinese populations. Therefore, we aimed to establish pre-treatment predictors for response to non-PEG IFN-α/RBV in Chinese patients to help guide clinical decisions and improve cost-effectiveness. We investigated HCV kinetics during non-PEG IFN-α/RBV therapy, clarified the association of *IL28B*, *IL10RB*, *IL28RA* and *MxA* gene polymorphisms with RVR to non-PEG IFN-α-2b/RBV therapy, and determined the predictors of RVR in CHC.

MATERIALS AND METHODS

Patient cohort

Two hundred and fifty-six patients with CHC from Jurong Peoples' Hospital, China were enrolled in this study, fulfilling the following criteria: (1) treatment naïve; (2) positive for HCV antibody (anti-HCV) and HCV RNA for > 6 mo; and (3) without hepatitis B virus (HBV) or HIV co-infection, or other liver diseases.

All patients were treated for 48 wk with non-PEG IFN-α-2b/RBV and treatment was discontinued according to standard guidelines[11]. Blood samples for biochemical analysis, SNP determination, and HCV genotyping were collected prior to antiviral therapy. HCV-RNA viral load was determined at weeks 0, 4, 12 and 24 of therapy.

Ethical approval was obtained from the participating hospital and the study was carried out in accordance with the guidelines of the International Conference on Harmonization for Good Clinical Practice[12]. All patients gave signed informed consent for DNA genotyping before enrollment.

Viral testing

Serum hepatitis B surface antigen and anti-HCV were measured using an ELISA (Beijing Wantai Biological Pharmacy Engineering Co. Ltd., Beijing, China). Serum HCV RNA and HCV genotype were determined by reverse-transcriptase polymerase chain reaction (TaKaRa Biotechnology, Dalian, China)[13,14].

SNP genotyping

IL28B rs12980275, *IL28RA* rs10903035 and rs11249006, *MxA* rs2071430 and rs17000900, and *IL10RB* rs2834167

Table 1 Primer and probe of SNPs		
SNPs		**Primer and probe (5'-3')**
rs10903035	Forward primer	TTGCCACCCTTGACCTCAG
	Reverse primer	GAGGTTTTGTTTAGAGGGATCCAC
	Probe-FAM	TAGCAAACCACTCCTT
	Probe-HEX	TTAGCAAATCACTCCTT
rs11249006	Forward primer	AACTGGAAGGGAGAATGGGACT
	Reverse primer	GTAACATGGCAGGAATCGGACT
	Probe-FAM	CCACAACAGTCAACCA
	Probe-HEX	CACAACGGTCAACCA
rs2834167	Forward primer	TACCACCTCCCGAAAATGTCA
	Reverse primer	GGTGCGTTCCTGCCAATAGT
	Probe-FAM	TTCCCTTTGGCAAAAG
	Probe-HEX	TTCCCTTCGGCAAAA
rs2071430	Forward primer	CCGAGAACCTGCGTCTCC
	Reverse primer	CGCGAAGAAATGAAACTCACAGAC
	Probe-FAM	CGTTTCTGCGCCCG
	Probe-HEX	CGTTTCTGCTCCCG
rs17000900	Forward primer	CCGAGAACCTGCGTCTCC
	Reverse primer	CGCGAAGAAATGAAACTCACAGAC
	Probe-FAM	CAAGTGCTGCAGGTG
	Probe-HEX	CAAGTGCTGAAGGTG
rs12980275	Forward primer	TGAGGTGCTGAGAGAAGTCAAATT
	Reverse primer	CGCTACCCCGGCAAATATT
	Probe-FAM	CTAGAAACGGACGTGTC
	Probe-HEX	CTAGAAACAGACGTGTCT

were chosen for genotyping. These SNPs are possibly associated with treatment or natural clearance of HCV[5-7,9,10]. Genomic DNA was isolated from peripheral blood mononuclear cells using protease K digestion and phenol-chloroform purification according to a standard protocol[15]. Genotyping was performed using the ABI TaqMan allelic discrimination assay on the ABI 7900HT sequence Detection System (Applied Biosystems, San Diego, CA, United States)[16]. The primers used for genotyping are listed in Table 1.

Statistical analysis

The statistical methods were reviewed by Zhao Yang, Department of Epidemiology and Biostatistics, School of Public Health, Nanjing Medical University. The distribution of patient characteristics and clinical features at baseline between the RVR and non-RVR groups were analyzed by χ^2 test. The association of genotypes with RVR were estimated by odds ratio (OR) and 95%CI using univariate and multivariate logistic regression analysis, with adjustment for sex, HCV genotype, HCV-RNA viral load at baseline, alanine aminotransferase (ALT), white blood cell (WBC) count, and α-fetoprotein (AFP). Receiver-operating characteristic (ROC) curves and areas under the curve (AUC) were calculated for the predictive model. Statistical significance between the genotypes and the time of first virus inhibition rates were analyzed using Kaplan-Meier curves and the log-rank test[17]. All statistical analyses were carried out using Stata version 10.0, and $P < 0.05$ in a two-sided test was considered statistically significant.

Table 2 Baseline characteristics of participants *n* (%)			
Variables	**RVR** (*n* = 133)	**NRVR** (*n* = 105)	**P value**
Age (yr)			
≤ 50	51 (38.35)	40 (38.10)	
> 50	82 (61.65)	65 (61.90)	0.968
Sex			
Male	41 (30.83)	21 (20.00)	
Female	92 (69.17)	84 (80.00)	0.059
HCV genotype			
1a/1b	93 (69.92)	70 (66.67)	
3	14 (10.53)	5 (4.76)	
Mixed	26 (19.55)	30 (28.57)	0.102
ALT (U/L)			
≤ 40	64 (48.12)	28 (26.67)	
(40-80]	38 (28.57)	42 (40.00)	
> 80	30 (22.56)	35 (33.33)	0.003
ALB (g/L)			
[40-55]	99 (74.44)	82 (78.10)	
< 40	33 (24.81)	23 (21.90)	0.577
WBC (× 10^9/L)			
[4-10]	75 (56.39)	73 (69.52)	
< 4	57 (42.86)	31 (29.52)	0.035
> 10	1 (0.75)	1 (0.95)	0.985
PLT (× 10^9/L)			
[100-300]	90 (67.67)	68 (64.76)	
< 100	41 (30.83)	37 (35.24)	0.522
AFP (ng/mL)			
≤ 7	95 (71.43)	68 (64.76)	
> 7	19 (14.29)	30 (28.57)	0.016
HGB (g/L)			
[110-150]	99 (74.44)	89 (84.76)	
< 110	13 (9.77)	8 (7.62)	
> 150	21 (15.79)	8 (7.62)	0.116
Baseline RNA (lg, IU/mL)	6.02 ± 0.97	6.19 ± 0.74	0.138

ALB: Albumin; HGB: Hemoglobin; PLT: Platelet; RVR: Rapid viral response; HCV: Hepatitis C virus; ALT: Alanine aminotransferase; WBC: White blood cell; AFP: α-fetoprotein.

RESULTS

The baseline characteristics of the 256 enrolled patients are described in Table 2. Four patients withdrew because of intolerable side effects and 14 were lost to follow-up. A total of 238 patients were screened for analysis. After 4 wk of treatment, 133 patients achieved RVR (55.88%). Treatment response was not related to patient age, sex, or HCV genotype ($P > 0.05$). Among the tested biochemical indices, levels of ALT, WBCs and AFP differed between the RVR and non-RVR groups. Patients with high ALT/AFP and low WBC levels at baseline were more likely to achieve a worse treatment response.

Association of polymorphisms of IFN-λ-related genes with RVR

To examine the effects of *IL28B* rs12980275, *IL28RA* rs10903035 and rs11249006, *MxA* rs2071430 and rs17000900, and *IL10RB* rs2834167 on RVR, each SNP was analyzed in four genetic models (co-dominant, dominant, recessive, and additive). The results for

Table 3 Interferon-λ-related genes polymorphisms and rapid viral response *n* (%)

Genotype	RVR	NRVR	Crude OR (95%CI)	Adjusted OR (95%CI)
rs11249006	*n* = 133	*n* = 105		
AA	51 (38.35)	49 (46.67)	1	1
AG	62 (46.62)	49 (46.67)	1.22 (0.71-2.09)	0.86 (0.45-1.63)
GG	20 (15.04)	7 (6.67)	2.75 (1.07-7.07)	2.33 (0.84-6.48)
Dominant			1.41 (0.84-2.36)	1.06 (0.58-1.94)
Recessive			2.48 (1.01-6.11)	2.52 (0.96-6.64)
Additive			1.47 (0.99-2.19)	1.27 (0.82-1.98)
rs12980275	*n* = 133	*n* = 105		
AA	118 (88.72)	70 (66.67)	1	1
AG	8 (6.02)	28 (26.67)	0.17 (0.07-0.39)	0.11 (0.04-0.30)
GG	7 (5.26)	7 (6.67)	0.59 (0.20-1.76)	0.54 (0.17-1.74)
Dominant			0.25 (0.13-0.50)	0.19 (0.09-0.43)
Recessive			0.78 (0.26-2.29)	0.82 (0.26-2.56)
Additive			0.46 (0.28-0.76)	0.43 (0.24-0.75)
rs2834167	*n* = 133	*n* = 105		
AA	37 (27.82)	28 (26.67)	1	1
AG	79 (59.40)	62 (59.05)	0.96 (0.53-1.74)	0.90 (0.45-1.78)
GG	17 (12.78)	15 (14.29)	0.86 (0.37-2.01)	0.66 (0.23-1.88)
Dominant			0.94 (0.53-1.68)	0.85 (0.44-1.66)
Recessive			0.88 (0.42-1.86)	0.71 (0.28-1.83)
Additive			0.93 (0.62-1.41)	0.83 (0.51-1.36)
rs10903035	*n* = 133	*n* = 105		
AA	43 (32.33)	27 (25.71)	1	1
AG	56 (42.11)	48 (45.71)	0.73 (0.40-1.36)	0.68 (0.34-1.37)
GG	34 (32.28)	30 (28.57)	0.71 (0.36-1.42)	0.91 (0.41-2.02)
Dominant			0.72 (0.41-1.28)	0.76 (0.40-1.44)
Recessive			0.86 (0.48-1.53)	1.14 (0.58-2.25)
Additive			0.84 (0.60-1.19)	0.94 (0.63-1.40)
rs2071430	*n* = 127	*n* = 96		
GG	63 (49.61)	49 (51.04)	1	1
GT	49 (38.58)	40 (41.67)	0.95 (0.54-1.67)	0.94 (0.50-1.80)
TT	15 (11.81)	7 (7.29)	1.67 (0.63-4.40)	2.13 (0.66-6.85)
Dominant			1.14 (0.68-1.91)	1.26 (0.70-2.28)
Recessive			1.78 (0.70-4.54)	2.28 (0.73-7.08)
Additive			1.15 (0.77-1.72)	1.21 (0.76-1.94)
rs17000900	*n* = 130	*n* = 99		
AA	97 (74.62)	69 (69.70)	1	1
AG	29 (22.31)	27 (27.27)	0.76 (0.42-1.40)	0.79 (0.39-1.61)
GG	4 (3.08)	3 (3.03)	0.95 (0.21-4.38)	0.68 (0.13-3.70)
Dominant			0.83 (0.46-1.47)	0.83 (0.43-1.62)
Recessive			1.05 (0.23-4.82)	0.77 (0.14-4.16)
Additive			0.84 (0.51-1.38)	0.80 (0.45-1.42)

Logistic regression analyses adjusted for sex, ALT, WBC, AFP, HCV genotype and baseline viral load. RVR: Rapid viral response.

all six SNPs are shown in Table 3. *P* values of all the adjusted factors were < 0.2 in the univariate analysis. Statistical significance in any model was considered to show a potential relationship with treatment response. As shown in Table 4, the distribution of two SNPs appeared to be associated with different treatment responses. In the co-dominant genetic model, mutant G allele of *IL-28RA* rs11249006 increased RVR (crude OR = 2.75, 95% CI: 1.07-7.07). However, there was no significant difference after adjusting for multiple variables (adjusted OR = 2.33, 95%CI: 0.84-6.48). Mutant G allele of *IL28B* rs12980275 was associated with decreased RVR in the co-dominant, dominant, and additive models. The adjusted OR was 0.11 (95%CI: 0.04-0.30), 0.19 (95%CI: 0.09-0.43), and 0.43 (95%CI: 0.24-0.75), respectively. The association of *IL28B* rs12980275 with RVR to IFN-α-2b/RBV therapy was still significant after Bonferroni correction. The results of genetic analyses suggested that *IL28B* rs12980275 is an indicator of response to IFN therapy.

Predictive factors for RVR

Stepwise regression analysis showed that *IL28B* rs12980275, WBC count, AFP level, HCV genotype, and HCV-RNA viral load at baseline were independent predictors of RVR (Table 5). In addition, the ROC of these variables covered an AUC of 0.726 (Figure 1). The probability of RVR can be predicted using the following formula: log odds (RVR) = 4.13 + 0.67 × WBC (abnormal *vs* normal) - 0.98 × AFP (abnormal *vs* normal) -0.39 × HCV-genotype1-0.46 × log (base viral load) - 1.05 × rs12980275AG/GG.

The predictive value of *IL28B* rs12980275 was further analyzed in stratified analyses. The treatment response in patients with HCV genotype AA was not

Table 4 Polymorphisms of *IL28RA/IL28B* and rapid viral response *n* (%)

Genotype	RVR (*n* = 133)	NRVR (*n* = 105)	Crude OR (95%CI)	*P* value	Adjusted OR (95%CI)	*P* value
rs11249006						
AA	51 (38.35)	49 (46.67)	1		1	
AG	62 (46.62)	49 (46.67)	1.22 (0.71-2.09)	0.048	0.86 (0.45-1.63)	0.637
GG	20 (15.04)	7 (6.67)	2.75 (1.07-7.07)	0.036	2.33 (0.84-6.48)	0.104
Dominant			1.41 (0.84-2.36)	0.197	1.06 (0.58-1.94)	0.852
Recessive			2.48 (1.01-6.11)	0.049	2.52 (0.96-6.64)	0.061
Additive			1.47 (0.99-2.19)	0.055	1.27 (0.82-1.98)	0.282
rs12980275						
AA	118 (88.72)	70 (66.67)	1		1	
AG	8 (6.02)	28 (26.67)	0.17 (0.07-0.39)	< 0.001	0.11 (0.04-0.30)	< 0.001
GG	7 (5.26)	7 (6.67)	0.59 (0.20-1.76)	0.347	0.54 (0.17-1.74)	0.301
Dominant			0.25 (0.13-0.50)	< 0.001	0.19 (0.09-0.43)	< 0.001
Recessive			0.78 (0.26-2.29)	0.648	0.82 (0.26-2.56)	0.736
Additive			0.46 (0.28-0.76)	0.002	0.43 (0.24-0.75)	0.003

Logistic regression analyses adjusted for sex, HCV genotype, baseline levels of ALT, WBC, AFP, and viral loads. RVR: Rapid viral response.

Table 5 Results of multivariate stepwise regression analysis on rapid viral response

Variable	Coef	OR (95%CI)	*P* value
rs12980275	-1.05	0.35 (0.20-0.62)	< 0.001
WBC-group	0.67	1.94 (1.08-3.50)	0.027
AFP-group	-0.98	0.38 (0.19-0.76)	0.006
HCV genotype	-0.39	0.67 (0.48-0.96)	0.014
Baseline RNA(lg)	-0.46	0.63 (0.44-0.91)	0.027

Coef: Coefficient of variation. WBC-group: WBC was divided into three groups. 1: 4×10^9-10^{10}/L; 2: $< 4 \times 10^9$/L; 3: $> 10^{10}$/L. AFP-group: AFP was divided into two groups. 1: $\leqslant$ 7 ng/mL; 2: > 7 ng/mL. RVR: Rapid viral response; HCV: Hepatitis C virus.
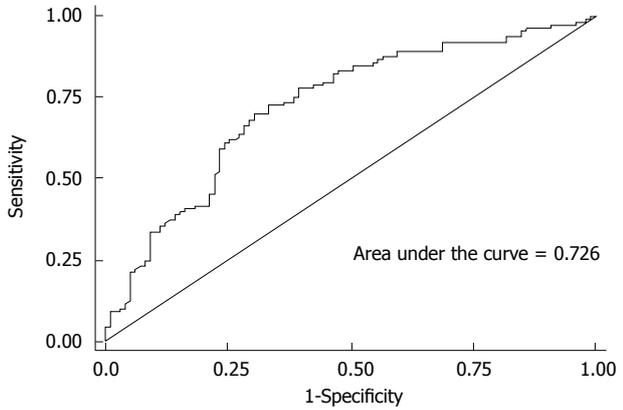
Figure 1 Prediction of rapid viral response. Receiver-operating characteristic (ROC) curve for prediction of RVR using all significant variables. areas under the curve (AUC) was 0.726. RVR: Rapid viral response.

Figure 2 Stratified analysis of baseline hepatitis C virus-RNA viral load and rapid viral response. A: Box plots of baseline hepatitis C virus (HCV)-RNA levels on RVR for rs12980275 AA group. Mean log HCV-RNA viral load was 6.28 ± 0.75 lg(copies/mL) and 6.10 ± 0.95 lg(copies/mL) for the non-RVR and RVR groups, respectively (*t* = 1.47, *P* = 0.143); B: Box plots for rs12980275 AG/GG group. Mean log HCV-RNA viral load was 5.97 ± 0.67 lg(copies/mL) and 5.37 ± 1.01 lg(copies/mL), respectively (*t* = 2.44, *P* = 0.018). The error bars indicate standard deviations. RVR: Rapid viral response.

affected by baseline HCV-RNA viral load. The mean HCV-RNA viral load (log value ± SD) in the non-RVR and RVR groups was 6.28 ± 0.75 lg(copies/mL) and 6.10 ± 0.95 lg(copies/mL), respectively (Figure 2A; *P* = 0.143). For patients carrying mutant G allele, lower baseline viral load was favored for RVR. The mean viral load in the non-RVR and RVR groups was 5.97 ± 0.67 lg(copies/mL) and 5.37 ± 1.01 lg(copies/mL), respectively (Figure 2B; *P* = 0.018).

Effect of IL28B rs12980275 on time of initial virus inhibition

The Kaplan-Meier method and log-rank test were conducted to examine the association of *IL28B* rs12980275 (dominant model) with the time of initial virus inhibition (time of reaching HCV-RNA viral load < 500 copies/mL after therapy) in CHC patients.
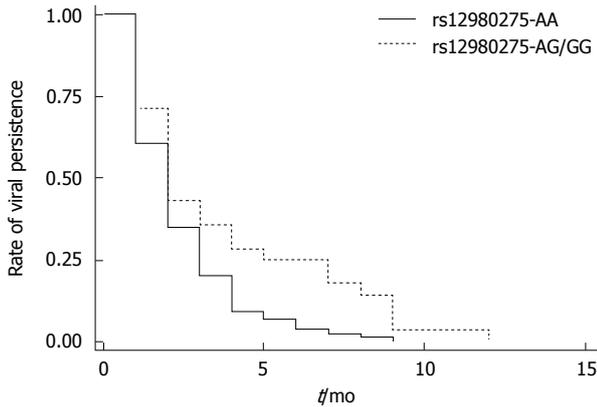
Figure 3 Kaplan-Meier plots of viral persistence rate by *IL28B* rs12980275 (log-rank *P* = 0.0041).

Figure 3 shows that the median time of initial inhibition response was 2 mo (95%CI: 1.72-2.28) for the rs12980275 AA group and 2 mo (95%CI: 1.36-2.64) for the AG/GG group. Although similar, the difference was significant (log-rank test, *P* = 0.004). Also the viral inhibition trends indicated that the inhibition rates were achieved faster in the AA group than in the AG/GG group.

Viral kinetics during therapy

Patients carrying the *IL28B* rs12980275 AA genotype achieved a greater reduction in HCV-RNA viral load at 1 mo (B-1), 3 mo (B-3) and 6 mo (B-6) than those carrying the AG/GG genotype (B-1: 6.18 ± 0.87 *vs* 5.77 ± 0.82 log IU/mL, *P* = 0.003; B-3: 6.18 ± 0.90 *vs* 5.79 ± 0.80 log IU/mL, *P* = 0.01; B-6: 6.27 ± 0.86 *vs* 5.89 ± 0.79 log IU/mL, *P* = 0.021), respectively (Figure 4A). Considering the confounding effect of baseline HCV-RNA levels, patients were further divided into four groups (baseline HCV RNA < 10^5 IU/mL, 10^5-10^6 IU/mL, 10^6-10^7 IU/mL, and ⩾ 10^7 IU/mL). *IL28B* rs12980275 AA carriers dropped to a similar viral load at 1 mo regardless of the baseline HCV-RNA levels (*F* = 2.11, *P* = 0.1) (Figure 4B). Meanwhile, the viral kinetics in the non-AA group were associated with baseline HCV-RNA levels (*F* = 17.64, *P* < 0.001). Viral load declined faster in patients with lower baseline level of virus (Figure 4C). The results of viral kinetics were consistent with the stratified analyses (Figure 2).

DISCUSSION

GWAS studies have identified *IL28B* rs12980275 as a strong SNP associated with HCV treatment in various populations[5-7,18]. Consistent with those studies, we also found that rs12980275 AA was a strong positive response predictor of non-PEG IFN-α/RBV treatment in the Chinese Han population. In addition, patients carrying the AA genotype were likely to achieve faster virological suppression compared with those carrying non-AA loci. The earliest difference among *IL28B* rs12979860 genotypes can occur at week 2[19].
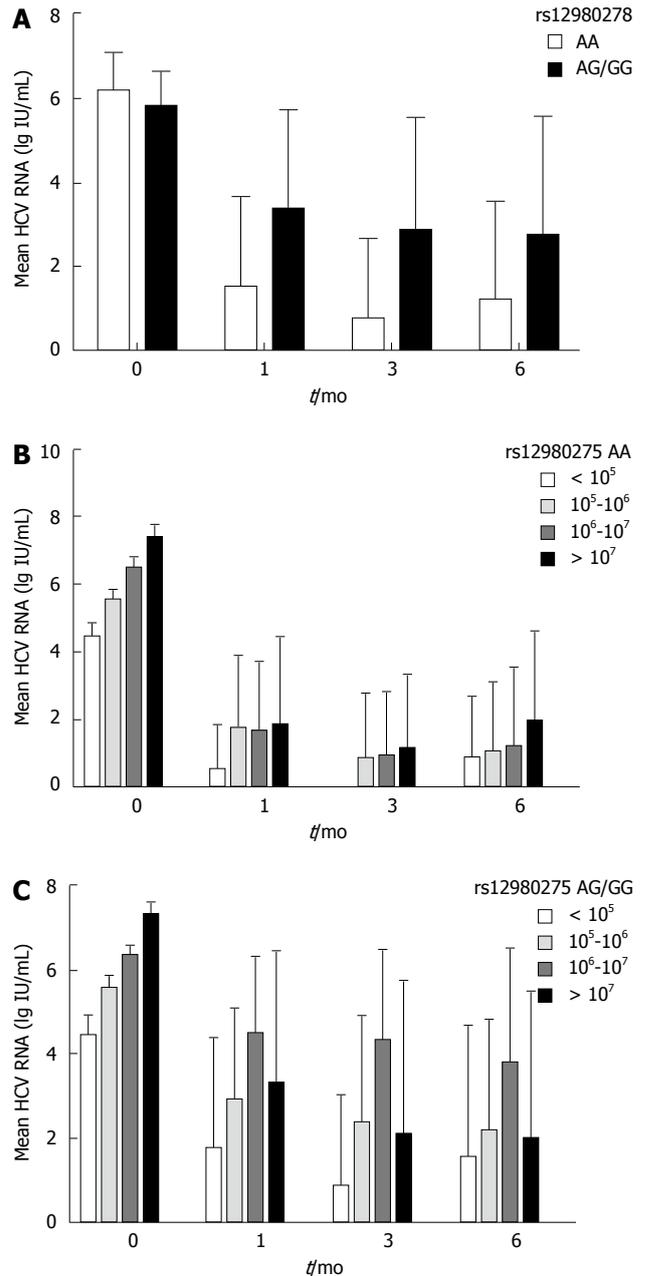
Figure 4 Viral kinetics during therapy. Mean log hepatitis C virus (HCV)-RNA levels at baseline, 1 mo, 3 mo and 6 mo. A: Stratified by *IL28B* rs12980275 (AA *vs* AG/GG); B: Stratified by baseline HCV-RNA levels (< 10^5 IU/mL, 10^5-10^6 IU/mL, 10^6-10^7 IU/mL, ⩾ 10^7 IU/mL) in rs12980275 AA group; C: Stratified by baseline HCV-RNA levels in rs12980275 AG/GG group.

The change in viral load seemed to vary in different rs12980275 genotypes (Figure 4). Unlike AG/GG genotypes, the protective effect of the AA genotype was not affected by baseline viral load. These results suggest that the AA genotype is a strong predictor of HCV treatment. The biological reason might be interpreted by another study of HBV infection. Serum *IL28B* level is higher in patients with the AA genotype and may reduce HBV viral load and liver inflammation[20]. However, the current study did not reveal a significant association between treatment response and polymorphisms in the selected downstream genes of *IL28B*.

Our results suggest that *IL28B* rs12980275 is the most important single predictor of RVR by the Random Forest Model (data not shown). In addition, including other viral and host factors, such as baseline viral load, HCV genotype, WBC count and AFP level, improved the accuracy of the predictive model (Figure 1). The predictive model in our study was similar to that in another Japanese study[21].

After 1 mo of treatment, 55.88% of the patients achieved RVR. Since 256 patients (92.02%) were infected with HCV genotype 1, this low rate of efficacy was understandable. The response rates to IFN therapy are usually higher among patients with HCV genotype 2/3, ranging from 75% to 94%, while patients with HCV genotype 1/4 have poorer response rates of about 50%[22,23]. The fact that HCV genotype 1 was the major strain in Jurong was consistent with a previous study[24].

In conclusion, our findings imply that the genetic variants of *IL28B* rs12980275 may play an important role in determining the response to non-PEG IFN-α-2b/RBV in the Chinese Han population.

ACKNOWLEDGMENTS

COMMENTS

Background
Hepatitis C virus (HCV) poses a serious global health problem due to its adverse clinical outcomes, such as cirrhosis and hepatocellular carcinoma. Treatment for chronic hepatitis C consists of interferon (IFN) plus ribavirin (RBV) and protease inhibitors such as telaprevir and boceprevir. The treatment response likely depends on a complex host-virus interaction. The influence of host gene polymorphisms has attracted attention in recent years. Therefore, establishing calculable pre-treatment predictors for response to IFN-α/RBV in the Chinese population should guide clinical decisions and improve cost-effectiveness.

Research frontiers
Many studies have suggested a range of factors associated with treatment response, including HCV genotype, viral load, liver function, and host immune status. Previous genome wide association studies (GWASs) have demonstrated that polymorphisms near the *IL28B* gene, which codes for IFN-λ3, affect the response to pegylated (PEG)-IFN-α/RBV in CHC.

Innovations and breakthroughs
Previous GWASs were based on observations in Australian, European, African-American, and Japanese, but not Chinese populations. In addition, because PEG-IFN is more expensive, non-PEG-IFN-α is more commonly used for chronic hepatitis C (CHC) in Chinese primary hospitals. The authors in their previous studies showed that *IL10RB* and *IL28RA* gene polymorphisms could predict the natural outcomes of HCV infection in the Chinese population. The present study aimed to clarify the association of IFN-λ-related genes with Rapid viral response to non-IFN-α-2b/RBV therapy in the Chinese Han population.

Applications
The results suggest that IL28B rs12980275 AA genotype is a strong predictor of positive response to IFN therapy in the Chinese Han population with CHC, and HCV genotype, baseline levels of white blood cells, α-fetoprotein, and viral load may help predict treatment response.

Peer-review
It is important to know new predictive factors in the treatment of this disease.

The study is innovative in nature. The original study conducted on large groups of patients is very valuable.

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ORIGINAL ARTICLE
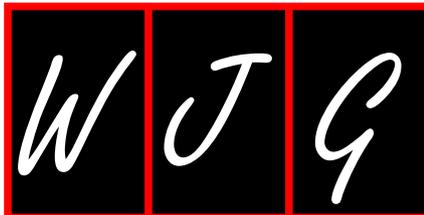
Prospective Study

Endoscopic ultrasound elastography strain histograms in the evaluation of patients with pancreatic masses

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Abstract

AIM: To investigate the accuracy of the strain histogram endoscopic ultrasound (EUS)-based method for the diagnostic differentiation of patients with pancreatic masses.

METHODS: In a prospective single center study, 149 patients were analyzed, 105 with pancreatic masses and 44 controls. Elastography images were recorded using commercially available ultrasound equipment in combination with EUS linear probes. Strain histograms (SHs) were calculated by machine integrated software in regions of interest and mean values of the strain histograms were expressed as Mode 1 (over the mass) and Mode 2 (over an adjacent part of pancreatic tissue, representing the reference area). The ratio between Mode 2 and Mode 1 was calculated later, representing a new variable, the strain histogram ratio. After the final diagnosis was established, two groups of patients were formed: a pancreatic cancer group with positive cytology achieved by fine needle aspiration puncture or histology after surgery (58 patients), and a mass-forming pancreatitis group with negative cytology and follow-up after 3 and 6 mo (47 patients). All statistical analyses were conducted in SPSS 14.0 (SPSS Inc., Chicago, IL, United States).

RESULTS: Results were obtained with software for strain histograms with reversed hue scale (0 represents the hardest tissue structure and 255 the softest). Based on the receiver operating characteristics (ROC) curve coordinates, the cut-off point for Mode 1 was set at the value of 86. Values under the cut-off point indicated the presence of pancreatic malignancy. Mode 1 reached 100% sensitivity and 45% specificity with overall

accuracy of 66% (95%CI: 61%-66%) in detection of pancreatic malignant tumors among the patients with pancreatic masses. The positive and negative predictive values were 54% and 100%, respectively. The cut-off for the new calculated variable, the SH ratio, was set at the value 1.153 based on the ROC curve coordinates. Values equal or above the cut-off value were indicative of pancreatic malignancy. The SH ratio reached 98% sensitivity, 50% specificity and an overall accuracy of 69% (95%CI: 63%-70%). The positive and negative predictive values were 92% and 100%, respectively.

CONCLUSION: SH showed high sensitivity in pancreatic malignant tumor detection but disappointingly low specificity. Slight improvements in specificity and accuracy were achieved using the SH ratio.

Key words: Endoscopic ultrasound; Pancreatic cancer; Pancreatic mass; Elastography; Histogram; Pancreatic tumor; Mass-forming pancreatitis

Core tip: Endoscopic ultrasound quantitative elastography strain histograms (SHs) are a recently developed method for non-invasive differentiation of pancreatic masses. In a prospective single center study, 105 patients with pancreatic masses and 44 controls were evaluated. For SH measurements, Hitachi software was used and applied for the first time in a clinical study as far as we know. We determined the accuracy of the method and a cut-off value between malignant pancreatic tumors and mass-forming pancreatitis. A new variable, the SH ratio, is calculated in an attempt to improve the sensitivity, specificity and accuracy of the method.

INTRODUCTION

Despite numerous imaging techniques, differential diagnosis of focal pancreatic masses is still a diagnostic problem in a significant number of patients.

In this setting, endoscopic ultrasound-guided fine needle aspiration biopsy (EUS-FNA) has high accuracy and traditionally represents the gold diagnostic standard[1-3], but it is not without risks and complications[4,5].

Multiple pathological processes including cancer can induce alterations of tissue stiffness, changing the elastic properties in comparison with surrounding tissues. Elasticity imaging and measurement provide a significant adjunct to current imaging methods[6]. The elastic properties of tissue are assessed by comparing color images superimposed over B mode scans before and after compression, yielding the local index of tissue elasticity[7]. This method was implemented and used in endosonography with the goal of differentiating tissue changes without risks related to FNA.

Modules for qualitative EUS elastography were incorporated into the first generation of commercially available ultrasound platforms in combination with adequate EUS probes. Tissue stiffness analysis for pancreatic masses using a three-point scoring system was developed by Giovannini *et al*[8,9] and refined later to a five-point system, reaching better results with higher accuracy. A similar four-point system was proposed by Iglesias-Garcia[10]. The method demonstrated promising results in the evaluation of pancreatic masses[9,10], although poor accuracy was reported in one study[11] and the differentiation of chronic pancreatitis from hard tumors was not satisfactory in another[12].

Second-generation EUS elastography instruments allowed quantitative analysis of tissue stiffness using the strain ratio, numerically expressed elasticity in the target area relative to a reference soft tissue area. Several investigations have shown high but variable sensitivity and specificity of the strain ratio with a wide span of cut-off values delineating inflammatory form malignant pancreatic masses[13-18].

A quantitative technique based on artificial neural network processing of EUS elastography digitalized videos was first applied by Săftoiu *et al*[19], obtaining average value of the hue histograms with a special computer program. Published results have shown very good sensitivity with satisfactory accuracy[19-21].

Some ultrasound platforms of the latest generation have integrated software for strain (hue) histogram measurement, automatically calculating the graph in real time.

The aim of this study was to evaluate the diagnostic value of the strain histogram in the patients with pancreatic masses and to determine the cut-off value between pancreatic malignancy and focal pancreatitis using software integrated into the commercially available ultrasound platforms.

As the next step we intend to calculate a new variable, the strain histogram ratio, by analogy to the strain ratio, calculating the ratio between the SH over an adjacent part of pancreatic tissue outside the mass and the SH over the mass.

MATERIALS AND METHODS

The investigation was organized as a prospective single center study from January 2011 to March 2013 in the gastroenterology department of the University Hospital Centre Zagreb, an academic center. The
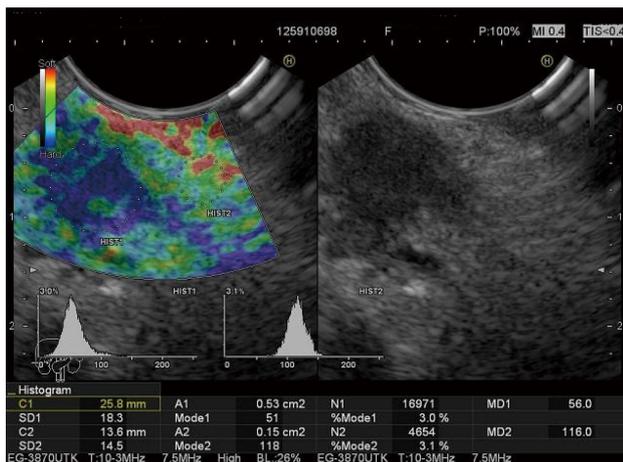
Figure 1 Strain histogram of a malignant pancreatic tumor.
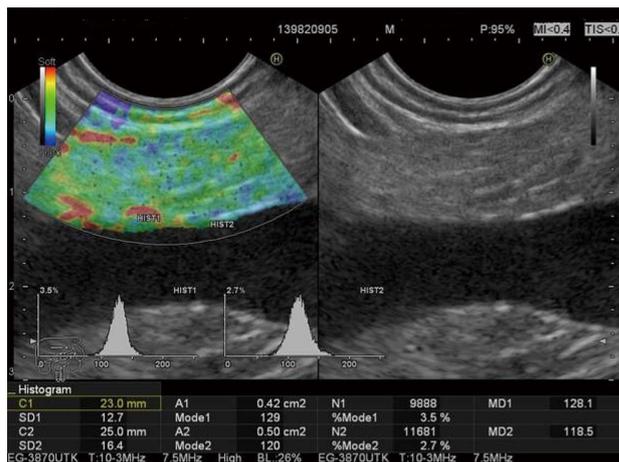
Figure 2 Normal strain histogram values of the pancreas.

study was approved by the hospital ethics committee. Written informed consent for participation in the study was obtained from all patients included in the study. Two experienced EUS examiners performed all endosonographic procedures and elastography measurements. Elastography images were recorded using Pentax FG-38 UX and EG-3870 UTK EUS linear probes in combination with Hitachi 8500 and Hitachi Avius ultrasound machines.

The inclusion criteria were: patients of both sexes over 18 years of age with solid pancreatic masses diagnosed with imaging techniques who accepted participation in the study. The exclusion criteria were: tumors with cystic or liquid components. In total 149 patients were included in the data analysis, 105 with pancreatic masses and 44 controls. Strain histograms were calculated automatically by integrated software in regions of interest manually selected by the endosonographer and expressed as the mean values (Mode 1 over the mass, and Mode 2 over an adjacent part of homogenous pancreatic tissue representing a reference area) (Figure 1). In the control group, two neighboring areas of pancreatic tissue were selected for histogram measurements (Figure 2). Three measurements were performed on the new elastography Figure recorded during the examination, and the average values of Mode 1 and Mode 2 for all three measurements were used for the statistical analysis.

The new variable, the strain histogram ratio, was calculated later by dividing the value of Mode 2 with the value of Mode 1 for each patient.

After the elastography measurements FNA was performed (Echotip, Cook Endoscopy) in all patients with pancreatic masses except in patients with lesions smaller than 3 cm without extrapancreatic or vascular invasion but with strong suspicion of malignancy. In these patients, histology was obtained after surgery. In the patients with negative cytology, follow-up FNA was performed after 3 mo and if necessary after 6 mo. Finally, the patients with pancreatic masses

were divided into two groups according to cytology/ histology and clinical course after a follow-up period of six months. The first group represents patients with verified pancreatic malignancy and the second group represents patients with mass-forming pancreatitis.

All statistical analyses were conducted in SPSS 14.0 (SPSS Inc., Chicago, IL, United States).

The diagnostic validity analysis included receiver operating characteristics (ROC) curve analysis, with specific values for the area under the curve, the significance of the area, and confidence intervals. Sensitivity (SS), specificity (SP), positive and negative predictive values, and positive and negative likelihood ratios are also expressed in the analysis. The calculation of the overall accuracy (ACC) of the prediction was based on previous parameters. The cut-off point for measurement the calculation of which is based on the coordinates of the ROC curve is also presented in the analysis. The positive likelihood ratio is calculated as sensitivity/1 - specificity. The negative likelihood ratio is calculated as 1- specificity/sensitivity. The overall accuracy is calculated as the sum of the positive and negative predictive values.

Statistical significance is set to $P < 0.05$, with all confidence intervals expressed at the 95% level.

RESULTS

The median age of the examined group of patients was 63 years (interquartile range: 54-70 years), of whom 76 were female.

Analyzing the sample according to cytology or histology after surgery and a follow-up period, 58/149 (39%) were finally considered to have pancreatic malignant tumors (53 with pancreatic carcinomas, 2 with neuroendocrine malignant tumors, 3 with metastatic carcinomas), 47/149 (31.5%) were classified as patients with pseudotumoral inflammatory masses, and 44/149 (29.5%) patients were control subjects without any clinical or endosonographic evidence of pancreatic disease.

Table 1 Analysis of the diagnostic validity of the Mode 1 histogram measure in the differentiation of pancreatic masses

	Malignant pancreatic tumor		
	Yes	No	
Mode 1			
Indicative < 86	True positive 58/58	False positive 50/91	Positive predictive value 54% (50%-54%)
Non indicative ⩾ 86	False negative 0/58	True negative 41/91	Negative predictive value 100% (90%-100%)
	Sensitivity 100% (93%-100%)	Specificity 45% (41%-45%)	
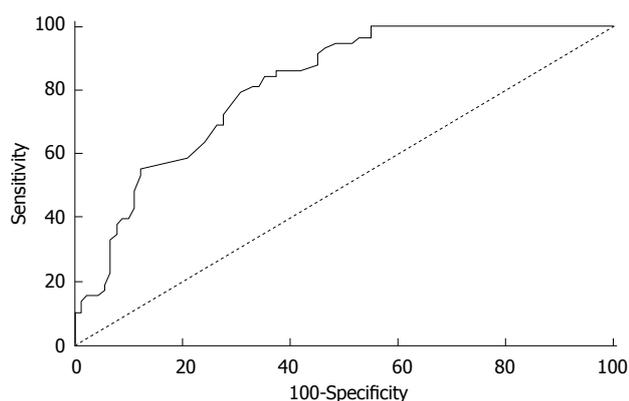
Figure 3 **Coordinates of the receiver operating characteristics curve for the strain histogram variable (Mode 1).**
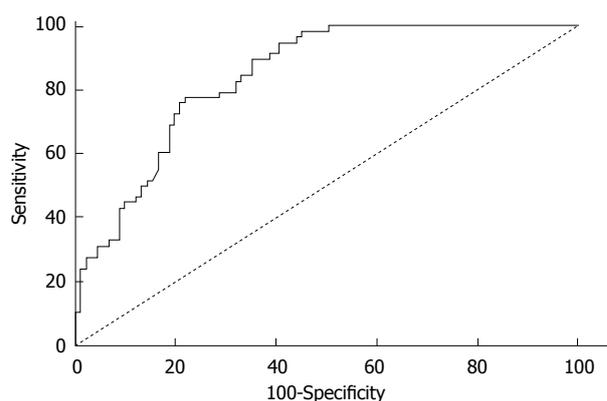
Figure 4 **Receiver operating characteristics curve for the Mode 2/Mode 1 variable.**

The mass lesion diameter was 31.7 mm; SD = 13.2 mm. Focal lesions were most frequently located in the head of the pancreas (51.5%), less frequently in the body (39.5%), whereas only 9% of them were located in the pancreatic tail.

The Mode 1 variable representing the average strain histogram measure achieved the following diagnostic validity parameters: AUC = 0.815; *P* < 0.001; 95%CI: 0.749-0.881.

Based on the ROC curve coordinates in Figure 3, the cut-off point for Mode 1 was set at 86. Considering the direction of the variable, values under the cut-off point indicate the presence of malignancy.

Consequently, using this cut-off point, all tumors were correctly identified as such, but as many as 55% of the non-malignant pancreatic masses were incorrectly identified as malignant (Table 1). Using this cut-off value, the overall accuracy achieved with the Mode 1 measure was 66% (95%CI: 61%-66%).

According to the ROC curve coordinates in Figure 4, the cut-off point for the Mode 2/Mode 1 ratio was set at 1.153. We determined the significance of the diagnostic validity for the new measure derived from the Mode 2/Mode 1 ratio (AUC = 0.843; *P* < 0.001; 95%CI: 0.782-0.903). All values above the determined cut-off point were classified as malignant. The variable therefore achieves high sensitivity where only 2% of patients having a malignant tumor would not be recognized, but as much as 50% of patients would be

incorrectly classified as having a malignant tumor. The overall accuracy achieved with the Mode2/Mode 1 ratio is 69% (95%CI: 63%-70%) (Table 2).

DISCUSSION

The strain ratio and strain histogram quantitative elastography methods are based on different principles[22], as mentioned previously, and that is the reason why we did not compare the sensitivity, specificity and accuracy obtained in our study with the results of published quantitative elastography studies using the strain ratio.

In the three published studies where strain histograms were used as the quantitative elastography method, post-processing software analysis of the elastography video was performed, and the average value of the strain histogram was calculated by a special computer program. A cut-off value was computed initially to distinguish malignant pancreatic masses from pseudotumoral or normal pancreatic tissue[19-21].

Hue histograms were calculated within manually selected regions of interest (ROIs) using a qualitative elastography image. The X-axis in a hue histogram represents tissue elasticity expressed numerically, and the y-axis represents the number of pixels in the ROI. The mean histogram value corresponds to the global hardness of the lesion expressed on a scale from 0

Table 2 Analysis of the diagnostic validity of the new combined histogram measure (Mode2/Mode1) in classifying malignancies		
	Malignant pancreatic tumor	
	Yes	**No**
Mode 2/Mode 1		
Indicative ≥ 1.153	True positive 57/58	False positive 46/91	Positive predictive value 92% (87%-92%)
Non indicative < 1.153	False negative 1/58	True negative 45/91	Negative predictive value 100% (96%-100%)
	Sensitivity 98% (91%-100%)	Specificity 50% (45%-50%)	
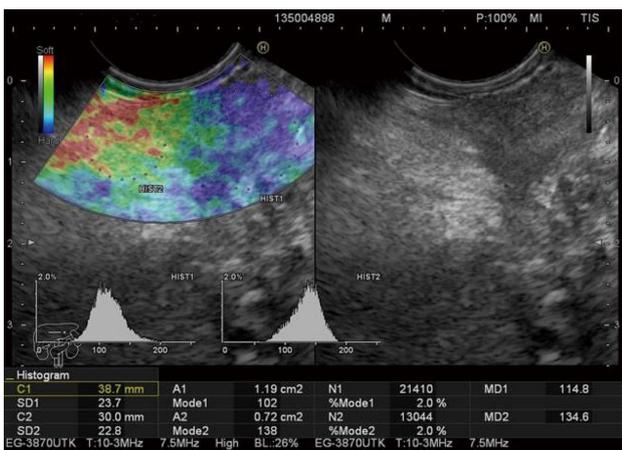
Figure 5 Inconclusive histogram measurement results comparing the strain histogram and the strain histogram ratio.

(softest) to 255 (hardest)[21].

In these studies, based on a cut-off of 175 sensitivity reached 91.4%, 84.8% and 93.4%, the specificity was 87.9%, 76.2% and 66.0%, with overall accuracy of 89.7%, 81.5% and 85.4%[19-21]. Data from the latest study[21] were analyzed in extended neural network analysis with automatic differentiation of benign from malignant lesions, and it achieved better sensitivity and overall accuracy (SS = 87.59%, SP = 82.4%, ACC = 91.4%)[23].

Our investigation represents quantitative elastography analysis with strain histograms of pancreatic masses using commercially available integrated ultrasound machine software for the first time. This software also uses a scale from 0 to 255, but the scale is reversed with 0 representing the hardest tissue structure and 255 the softest. The results were obtained as an average value of measurements from the three Figures selected by the endosonographer.

It is possible to compare the cut-off value from the previous studies with the cut-off value computed in our study. Expressed in the same way, the mean value from the previous studies[19-21] becomes 80, which is very close to 86, the value from our study.

The Mode 1 histogram measured over the tumor in our study showed high sensitivity in cancer detection, but also disappointingly low specificity, with modest overall accuracy (Figure 1: Patient with Mode 1 = 51,

and Mode2/Mode1 = 2.31. Both values suggesting a malignant tumor confirmed by surgery). Slight improvements in specificity and overall accuracy were achieved using the new variable, the Mode2/Mode 1 ratio (Figure 5. Patient with Mode 1 = 102 suggesting inflammatory mass and Mode2/Mode1 = 1.35 suggesting malignant mass. Malignancy was confirmed by EUS-FNA).

Explanations for the significant difference in specificity and accuracy achieved in our study in comparison with the results of previous studies using strain histograms as the measurement method could be just speculative at the moment. More studies on Hitachi ultrasound machines with integrated software are needed, comparing whether other analyses possible with the data processing method of Săftoiu *et al*[19-21] on the same group of patients.

Background

A few recent studies have shown that use of endoscopic ultrasound-based quantitative hue histogram methodology represents a useful tool in the differentiation of pancreatic malignancies from mass-forming pancreatitis.

Research frontiers

Previous studies used artificial neural network processing of digitalized endoscopic ultrasound elastography videos, obtaining average values for hue histograms with a special computer program. This method has shown very good sensitivity with satisfactory accuracy, but a limitation of this type of analysis is its availability to other investigators.

Innovations and breakthroughs

In this study, commercially available integrated ultrasound machine software was used for hue histogram analysis for the first time as far as we know. The authors' determined the accuracy of the method and a cut-off value distinguishing between pancreatic malignant tumors and mass-forming pancreatitis. With the new calculated strain histogram ratio variable, the sensitivity, specificity and accuracy of the method were all improved.

Applications

In hospitals with adequate equipment this method could assist in distinguishing malignant from benign pancreatic lesions.

Peer-review

The study is very interesting. In this paper, a new evaluation approach named the strain histogram ratio was established. The cut-off, sensitivity and specificity for this method were calculated. It is a remarkable step forward. Good work.

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ORIGINAL ARTICLE

Prospective Study

PERFACT procedure: A new concept to treat highly complex anal fistula

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Abstract

AIM: To check the efficacy of the PERFACT procedure in highly complex fistula-in-ano.

METHODS: The PERFACT procedure (proximal superficial cauterization, emptying regularly fistula tracts and curettage of tracts) entails two steps: superficial cauterization of mucosa at and around the internal opening and keeping all the tracts clean. The principle is to permanently close the internal opening by granulation tissue. This is achieved by superficial electrocauterization at and around the internal opening and subsequently allowing the wound to heal by secondary intention. Along with this, all the tracts are curetted and it is ensured that they remain empty and clean in the postoperative period until they heal completely. The latter step also facilitates the closure of the internal opening by preventing collected fluid in the tracts from entering the internal opening and thus not letting it close. Objective incontinence scoring was done preoperatively and 3 mo after the operation.

RESULTS: Fifty-one patients with complex fistula-in-ano were prospectively enrolled. The median follow-up was 9 mo (5-14 mo). The mean age was 42.7 ± 11.3 years. Male:female ratio was 43:8. Fistula was recurrent in 76.5% (39/51), horseshoe in 50.1% (26/51), had multiple tracts in 52.9% (27/51), had an associated abscess in 41.2% (21/51), was anterior in 33.3% (17/51), the internal opening was not found in 15.7% (8/51) and 9.8% (5/51) of fistulas had a supralevator extension. Seven patients were excluded (5 lost to follow up, 2 with tuberculosis leading to/associated with fistula-in-ano). The success rate was 79.5% (35/44) and the recurrence rate was 20.5% (9/44). Out of these recurrences, three underwent reoperation (2 PERFACT procedure, 1 fistulotomy) and all three were successful. Thus, the overall success rate was 86.4%. The only complication was a non-healing tract in 9.1% (4/44) of patients. There was no significant change in objective incontinence scores three months after the operation. The pain was minimal, with all patients resuming their normal activities within 72 h of the operation.

CONCLUSION: The PERFACT procedure is a new effective method for complex fistula-in-ano, effective even in fistula associated with abscess, supralevator fistula-in-ano and where the internal opening is non-localizable.

Key words: Anal fistula; Fistula-in-ano; Incontinence;

Recurrent; PERFACT; Procedure; Abscess; Supralevator

Core tip: The PERFACT procedure is a simple, economical and novel method to cure complex fistula-in-ano. It is associated with little pain, low morbidity and minimal risk of incontinence as both the anal sphincters are completely preserved. It is quite effective in complex fistula cases where other methods do not have a high success rate, like in horseshoe fistula, fistula with multiple tracts, recurrent fistula and fistula with supralevator extension. The PERFACT procedure is also quite successful in cases where the internal opening cannot be localized and in patients presenting with perianal/ischiorectal abscess where it can be done as a definitive procedure at the initial presentation.

INTRODUCTION

There is no satisfactory treatment of complex fistula-in-ano to date. A fistula-in-ano is termed "complex" when the track crosses > 30%-50% of the external sphincter (high-transsphincteric, suprasphincteric and extrasphincteric), is anterior in a female, is recurrent, has multiple tracks, or the patient has preexisting incontinence, local irradiation or Crohn's disease[1-4]. In spite of several new procedures, such as anal fistula plug[5,6], ligation of intersphincteric fistula tract (LIFT)[7], video assisted anal fistula treatment (VAAFT)[8], laser-FiLaC[9] and the OTSC proctology procedure[10] tried in the last decade, the challenge of successfully treating complex anal fistula remains intact. The two main issues in managing such fistulas are to minimize the recurrence rate and prevent any deterioration in continence levels.

Proximal superficial cauterization, emptying regularly fistula tracts and curettage of tracts (PERFACT procedure) is a novel concept in the management of complex fistula-in-ano. In this procedure, the fistula healing entails two components closure of the internal opening and healing of the tract/tracts. The aim is to use body's natural healing tissue (granulation tissue) to close the internal opening. This is done by electrocauterizing the internal opening and the area around it in the anal canal. The tract/tracts are thoroughly curetted and the infected tract lining (epithelium) of the tracts is taken out. To ensure proper healing, it is important to keep the anal canal

wound clean and the tracts empty in the postoperative period.

MATERIALS AND METHODS

Patients with complex fistula-in-ano were enrolled in a prospective study over a period of one and a half years. The institutional ethics committee reviewed and approved the study. Informed written consent was taken from every patient.

Inclusion criteria

All types of complex fistula-in-ano including: (1) fistula associated with multiple tracts; (2) horse shoe fistulas; (3) recurrent fistulas; (4) anterior fistula in females; (5) fistula with long tracts (any tract length > 10 cm); (6) fistula with supralevator blind extension (not with high rectal opening); (7) fistula where internal opening cannot be localized; and (8) fistula associated with abscess/pus collections. It was used as a first line definitive procedure in patients with anal fistulas presenting with ischiorectal or perianal abscess.

Exclusion criteria

(1) Simple low fistula; and (2) fistula with supralevator rectal opening (on MRI and/or examination on the operating table).

Vaizey objective incontinence scoring was done preoperatively and at 3 mo after the operation[11]. On a scale of 0-24, a score 0 implied perfect continence and a score of 24 meant total incontinence.

A pre-operative MRI scan was done in every case to accurately map all the fistula tracts (Figure 1). A schematic diagram consisting of coronal and transverse sections (Figure 1) was made based on the MRI.

The PERFACT procedure had three steps (Figure 2): (1) proximal superficial cauterization: the area around the internal opening was freshened and de-epithelized by electrocautery (Figure 3) and the wound was encouraged to heal by secondary intention (granulation tissue). This usually closed the internal opening in about 10-12 d; (2) curettage of tracts: all the tracts were thoroughly curetted and debrided of their lining with a curette; and (3) emptying regularly fistula tracts: the curetted tracts were kept clean and empty of any serous fluid so as to ensure that the tracts healed (closed) by granulation tissue. Keeping all the tracts clean until they healed completely was a challenging task and the most demanding step of the procedure. It took 4-8 wk (occasionally even longer) for all the tracts to heal fully. Until that time, regular cleaning of the tracts was done.

To ensure proper cleaning of the tracts, the following steps (one or multiple depending upon the requirement and fistula characteristics) could be done in a patient: (1) multiple holes were made along the straight or the
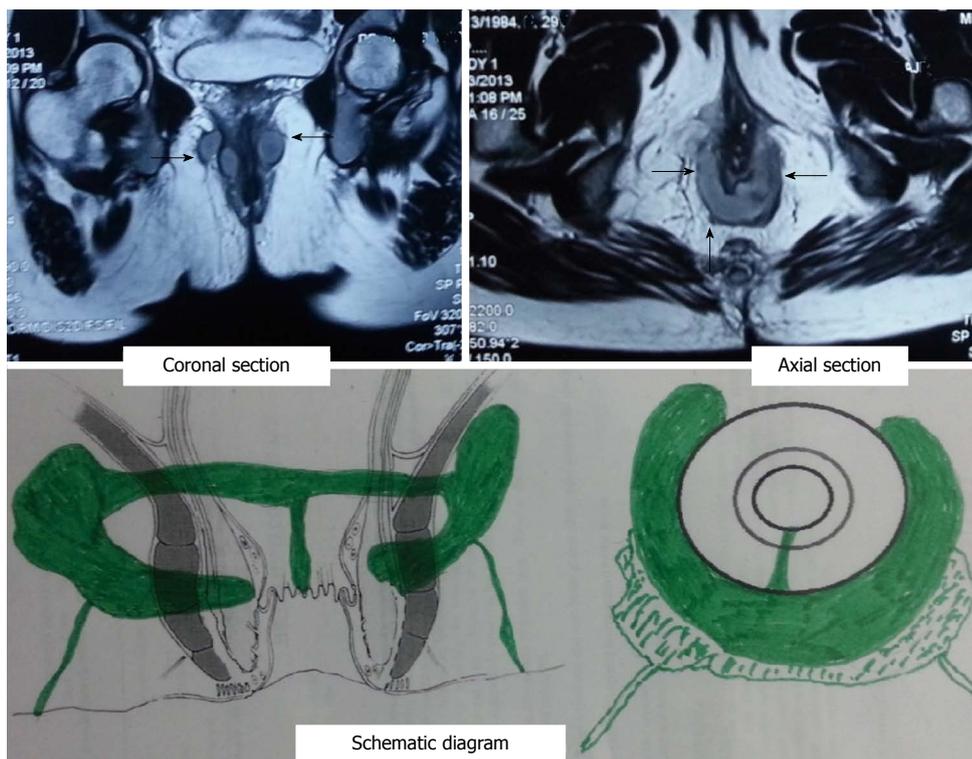
Figure 1 Preoperative magnetic resonance imaging of the perianal region and its schematic diagram showing a recurrent horseshoe abscess and fistula from 2 to 10 o'clock position in a 29-year-old female patient. There was no external opening and the internal opening was at the posterior midline. Arrows in the upper pictures show the position of the horseshoe abscess.
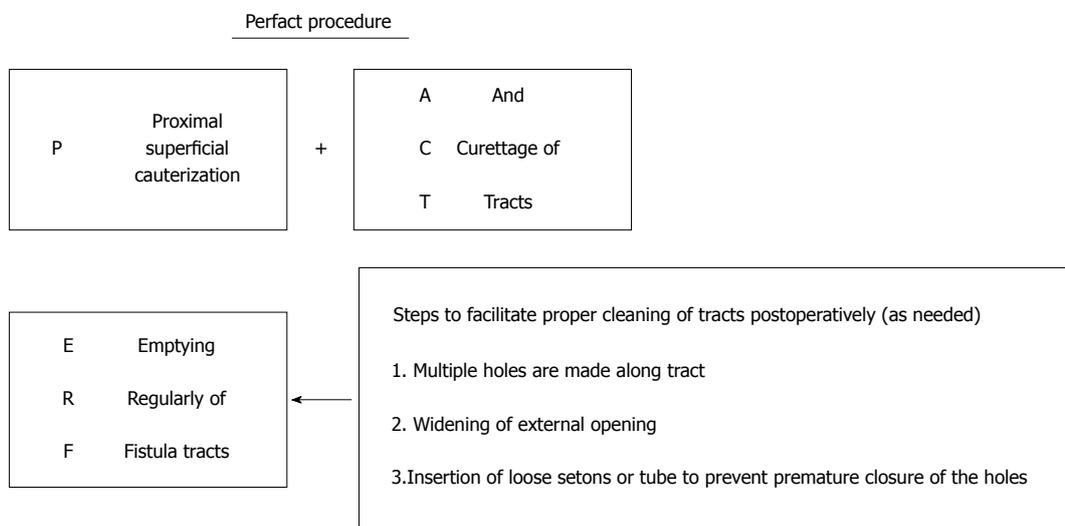
Figure 2 PERFACT procedure - an overview.

horseshoe tract (Figures 4, 5, 6, 7 and 8) in such a way that the farthest corner of the tract could be cleaned with ease; (2) the external opening was widened and the scarred puckered skin (if present) was excised. The aim was to make the opening bigger than 1 cm × 1 cm (Figure 3). This facilitated cleaning of the tracts for a longer duration; and (3) loose seton or tube were put in the tracts to prevent the premature closure of the external opening. These were removed 10-12 d after the operation (Figures 4, 5, 6, 7 and 8).

Intraoperative
A saddle block (spinal anesthesia) or a short general anesthesia was given. The patient was positioned in a lithotomy or a prone jack-knife position. The internal opening was localized. This was facilitated by injecting saline, povidine iodine or hydrogen peroxide through the external opening.
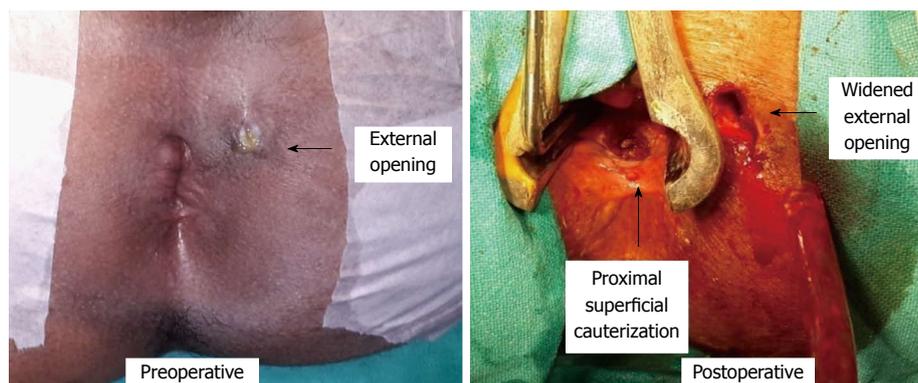
Figure 3 Management of a 45-year-old male patient by PERFACT procedure. He had a recurrent transsphincteric fistula with external opening at 2 o'clock and internal opening at 6 o'clock posterior midline. Shows proximal superficial cauterization and widening of the external opening. Horizontal arrows show the external opening - preoperative in the left picture and postoperative in the right. The vertical arrow in the right picture shows the position of proximal superficial cauterization.
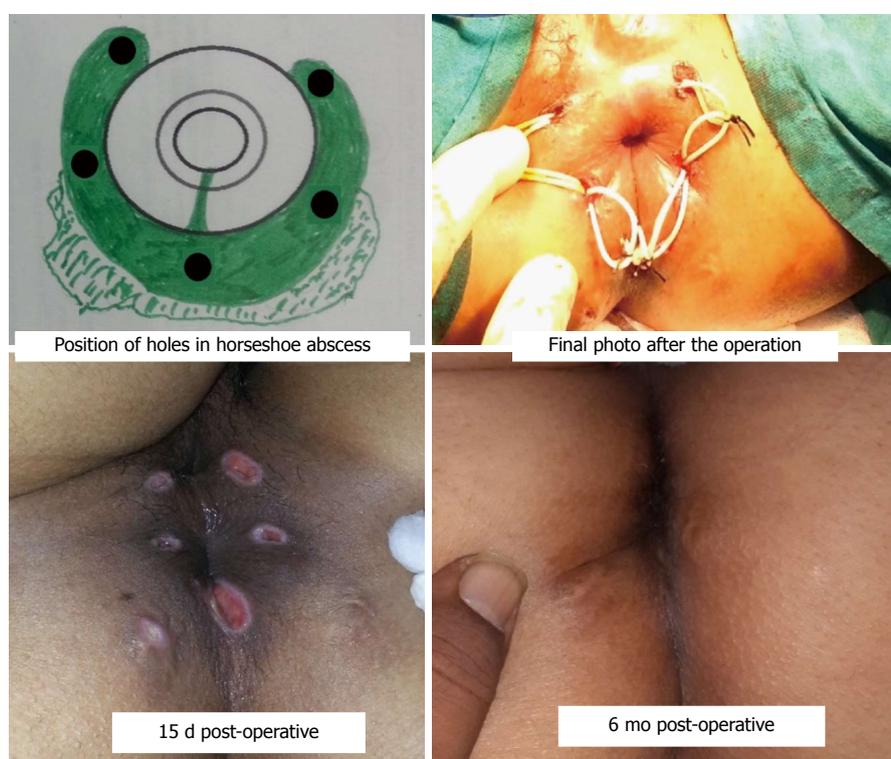
Figure 4 Management of a 29-year-old female patient by PERFACT procedure. She had a horseshoe abscess and fistula from 2 to 10 o'clock. There was no external opening and the internal opening was at the posterior midline. The right bottom picture shows complete healing of the fistula (MRI and diagram of this patient shown in Figure 2).

Proximal superficial cauterization (Figure 3) was carried out with electrocautery around the internal opening, cauterizing only the mucosa and superficial part of the internal sphincter. The crypt glands, the internal opening and the tissue around it were cauterized. This usually resulted in an oval area, approximately 1 cm (wide) and 2 cm (long), with the internal opening at the center of the wound (Figure 3). After cauterization, the wound was left as such and no attempt was made to close the internal opening with any suture, stapler, glue or plug.

After this, the tracts were curetted in accordance with the MRI diagram and the tract lining was scraped out as much as possible with a blunt curette. While doing so, a finger was kept in the rectum so as to ensure that the curette did not accidentally perforate the rectum.

The patient was discharged on the operation day (if done under short general anesthesia) or the first postoperative day (if done under saddle or spinal anesthesia). He/she could resume all his/her normal activities on the same day. The patient was encouraged to walk briskly for 5 km every day. This helped to keep the tracts empty.
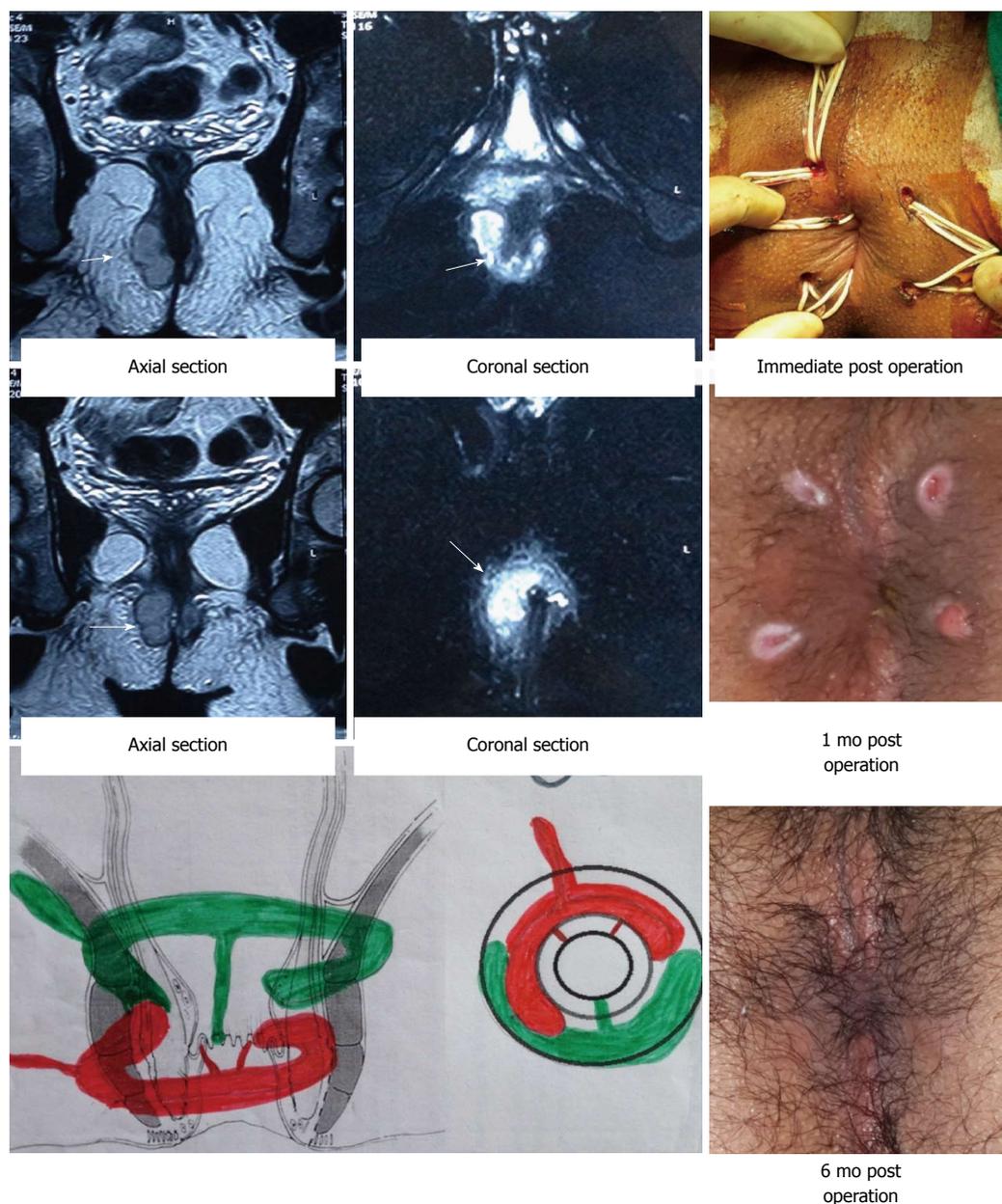
Figure 5 Management of a 22-year-old male patient by PERFACT procedure. He had a double horseshoe intersphincteric abscess and fistula. External opening is at 11 o'clock and the internal opening was not traceable intraoperatively. Electrocauterization was done at both anterior and posterior midline. The left upper picture shows the MRI and the right bottom picture was taken after the final cure (posterior horseshoe abscess shown by green color was at a higher level). Arrows in the upper left pictures show the position of the horseshoe abscess and tracts.

Postoperative cleaning aimed at healing two areas: the cauterized wound in the anal canal (around the internal opening) and the curetted tracts. The former was pivotal as the closure of the internal opening depended upon it and generally took about 10-12 d to heal. The latter was also needed for the complete closure of the fistula and took a variable time (4-8 wk) depending on the fistula characteristics (number, length and complexity of the tracts) and the patient co-morbidities (diabetes, anemia, hypoproteinemia *etc.*).

The cleaning process entailed cleaning the cauterized wound in the anal canal and regular cleaning and emptying of the curetted tracts. The former was done by gentle rubbing of the wound by doing a per rectal finger insertion. The latter was done by a cotton swab mounted on an artery forceps. No povidine iodine, hydrogen peroxide or any liquid was injected in to the tract during the cleaning process as this would have prevented the internal opening from closing. The cleaning was done by a trained nurse, a medical attendant or a relative. In our setting, teaching a relative was an economical and preferred option.

The cleaning process was done four times a day. For the first 10 d, the patient was called to the outpatient clinic for supervised cleaning once or twice a day depending upon the complexity of the fistula. After this, the patient could do the cleaning process at home.
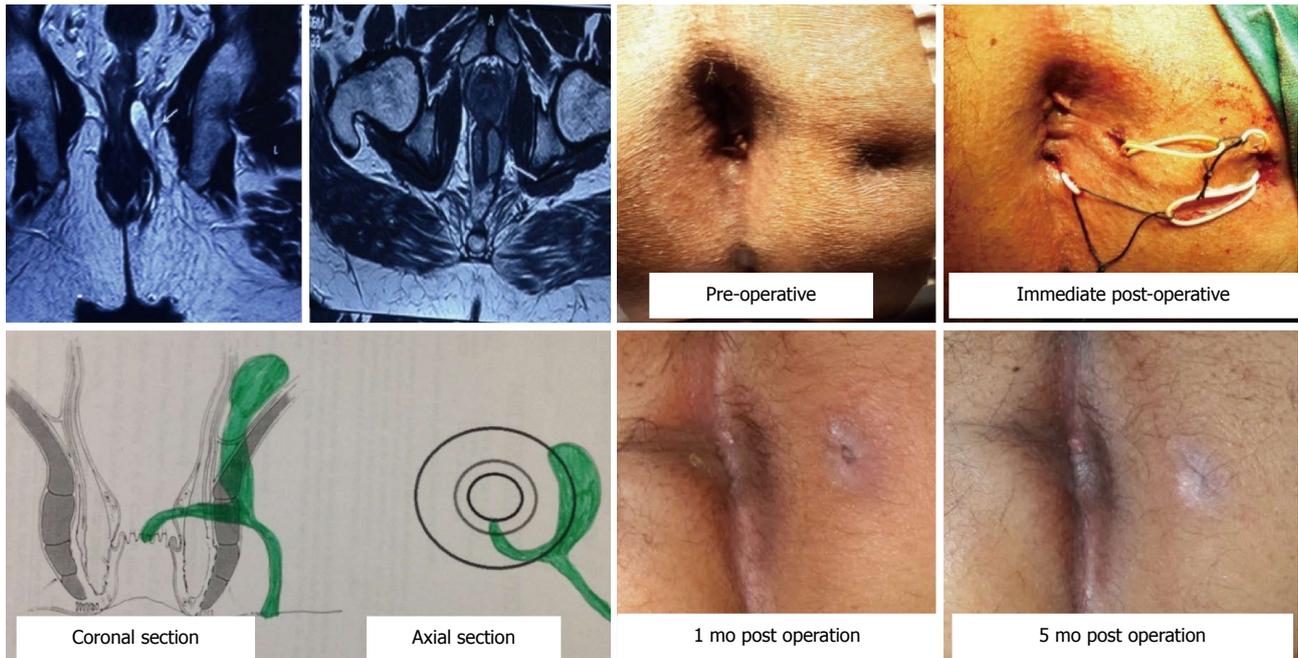
Figure 6 Management of a 36-year-old male patient by PERFACT procedure. He had a supralevator extension at 3 o'clock. External opening is at 3 o'clock and internal opening at 6 o'clock posterior midline. The left upper picture shows the MRI (arrows show the supralevator extension) and the right bottom picture was taken after the patient was fully cured.
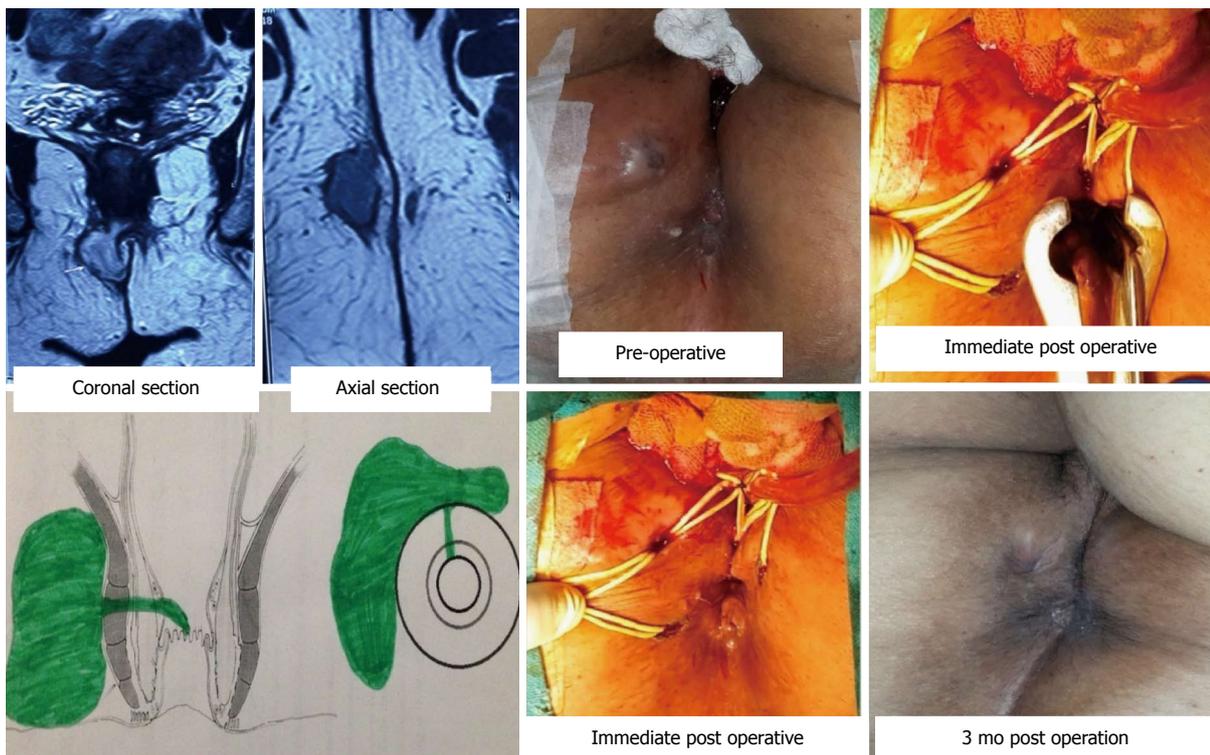
Figure 7 Management of a 55-year-old female patient by PERFACT procedure. She had a large anterior abscess and a fistula. External opening is at 11 o'clock and internal opening at 12 o'clock anterior midline. The left upper picture shows the MRI and the right bottom picture is taken after the final cure.
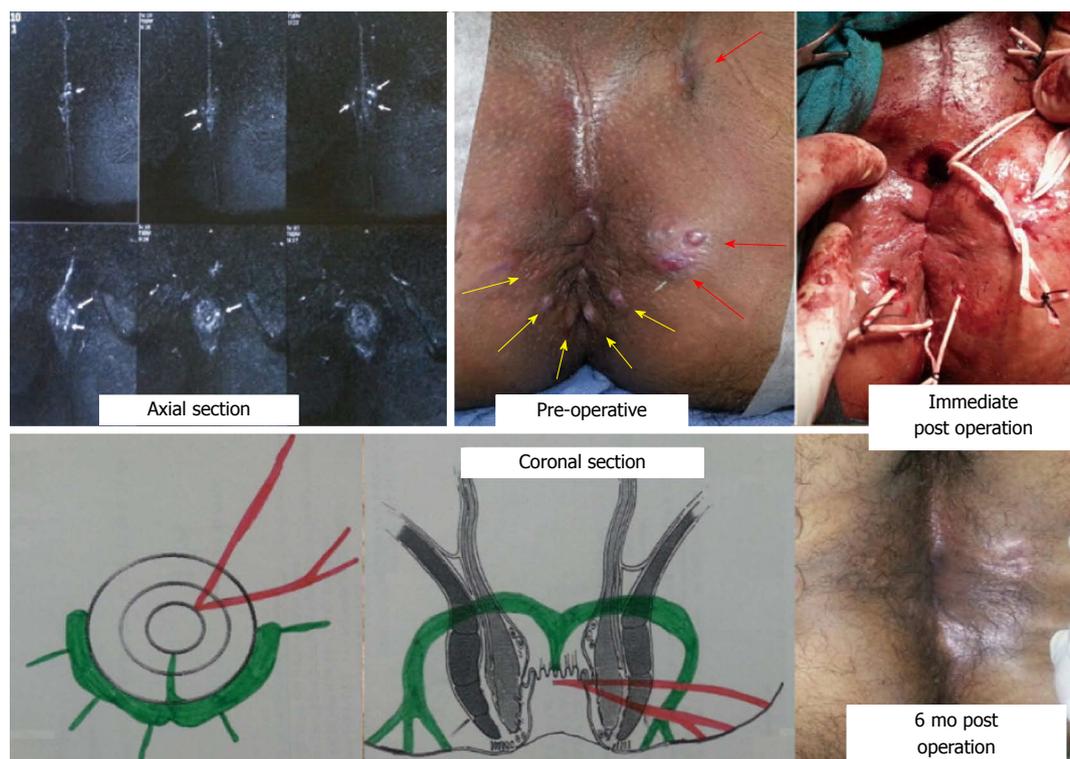
Figure 8 Management of a 38-year-old male patient by PERFACT procedure. He had a total of eight external openings and tracts (including a horseshoe one). The left upper picture shows the MRI and the right bottom picture is taken after the final cure. Arrows in the upper left picture show the position of the multiple tracts and the upper middle picture shows the multiple external openings.

Table 1 Fistula characteristics *n* (%)	
Fistula characteristics	***n* = 51**
Recurrent	39 (76.5)
Multiple tracts	27 (52.9)
Horseshoe fistula	26 (50.1)
Associated/presented with an abscess	21 (41.2)
Anterior tract	17 (33.3)
Internal opening not found	8 (15.7)
Supralevator extension (blind)	5 (9.8)

Statistical analysis

Comparison of categorical variables was performed by χ^2 analysis or Fisher's exact test where appropriate. The significant cut off point was set at $P < 0.05$.

RESULTS

Fifty-one patients with complex fistula-in-ano were prospectively enrolled. The median follow-up was 9 mo (5-14 mo). The mean age was 42.7 ± 11.3 years. Male:female ratio was 43:8. The fistula characteristics were recurrent in 76.5% (39/51), horseshoe in 50.1% (26/51), multiple tracts in 52.9% (27/51), associated abscess in 41.2% (21/51) and anterior fistula in 33.3% (17/51). The internal opening could not be definitely traced intraoperatively in 15.7% (8/51) and there was associated supralevator extension in 9.8% (5/51) (Table 1). Seven patients were excluded from the analysis (5

lost to follow-up, 2 had biopsy proven mycobacterium tuberculosis). The fistula and all the associated tracts healed completely in 79.5% (35/44) of patients and there was recurrence of symptoms in 20.5% (9/44) of patients. Out of these, three underwent reoperation (two PERFACT procedure, one fistulotomy) and all three were successful (Table 2). The subgroup analysis showed that although the presence of multiple tracts and an abscess reduced the cure rate, it was not statistically significant (Fisher exact test $P > 0.05$) (Table 2). The only complication was a non-healing tract in 9.1% (4/44) of patients. There was no significant change in objective incontinence scores after the operation. The pain was minimal, with all patients resuming their normal activities within 72 h of the operation.

DISCUSSION

The PERFACT procedure is a novel concept to treat complex fistula-in-ano. It is simple to perform and easy to reproduce. The results (initial 79.5%, overall 86%) are quite impressive considering that all these patients had highly complicated fistula-in-ano (Table 1).

The concept behind the PERFACT procedure was very simple. It aimed to close the internal opening by proximal superficial cauterization in the anal canal (Figure 3). In the postoperative period, it was ensured that the wound healed by secondary intention so that the internal opening was sealed by granulation tissue.

Table 2 Results - subgroups and overall *n* (%)				
Parameter		Number (*n* = 44)	Healed	*P* value (Fisher's exact test)
Recurrent	Recurrent	33	26 (78.8)	0.2 (Not sig.)
	Non-recurrent (primary)	11	9 (81.8)	
Multiple tracts	Multiple tracts	21	15 (71.4)	0.16 (Not sig.)
	Single tract	23	20 (86.9)	
Horseshoe fistula	Horseshoe	21	16 (76.2)	0.17 (Not sig.)
	Non-horseshoe	23	19 (82.6)	
Associated/presented with abscess	Abscess	18	13 (72.2)	0.17 (Not sig.)
	No abscess	26	22 (84.6)	
Anterior tract	Anterior	15	13 (86.6)	0.18 (Not sig.)
	Non-anterior	29	22 (75.8)	
Internal opening not found		8	7 (87.5)	
Supralevator extension (blind)		4	3 (75)	
Overall	After 1 procedure	44	35 (79.5)	
	After 2nd procedure in 3 patients	44	38 (86.4)	

The second step was curettage of the tracts. This ensured that the infected epithelium was removed and the freshened raw wound in the tracts led to the generation of the granulation tissue which would facilitate the closure of the tracts. However, the serous discharge of the granulation tissue needed to be thoroughly cleaned/removed from the tracts as otherwise the stagnant discharge would become infected, leading to a collection. The latter would not only lead to the rapid re-epithelialization of the tracts but would also flow into internal opening, preventing its closure.

The postoperative management was quite significant. It had two components: to keep the cauterized anal wound clean and to keep the tracts clean and empty. Any inadequacy in this care was detrimental to the final outcome.

The cauterization of the internal opening had been tried earlier without much success. The reason for the success of the same step in the PERFACT procedure needs explanation. Undoubtedly, the internal opening is the prime culprit in a fistula-in-ano by allowing ingress of the bacteria from the anal canal into the fistula tracts. However, once the tracts are formed and are lined by the infected epithelium, then it is a mutually propagating situation. The patent internal opening keeps the tracts infected and the infected collection in the tracts keep the internal opening patent. Therefore, an isolated attempt to close the internal opening would fail until it is accompanied by the meticulous cleaning, emptying and healing of all the associated tracts. This perhaps explains the rigorous need for regular tract cleaning in the postoperative period.

The concept behind this procedure was undoubtedly simple but to achieve good results in complex anal fistulas, it required detailed analysis of the MRI scan, careful planning and mapping of the tracts (preoperatively), meticulous curettage and cleaning of all the tracts (intraoperatively), and disciplined postoperative care (postoperatively). The main benefit of this procedure was minimal morbidity and the least risk of incontinence. The morbidity was minimal as no extensive tissue cutting was done. Apart from a small superficial wound in the anal canal, the external opening was widened (Figure 3) or a few holes were made in the perianal region (to drain accessory tracts) (Figures 4, 5, 6, 7 and 8). The anal wound was usually small and low as the internal opening was located mostly at the dentate line (Figure 3). So, the resultant wound was usually about 2 cm long and 1 cm wide. Due to the small wound and little pain, the patients were able to resume all their normal daily activities from the first postoperative day. The patients were encouraged to walk briskly for 4-5 kilometers from the first postoperative day as it facilitated keeping the tracts empty. Second major advantage was that as the external sphincter was completely spared, the negative impact on incontinence was minimal.

The procedure worked quite well in all types of complex fistula: fistula associated with multiple tracts, horse shoe fistulas, recurrent fistulas, anterior fistula in females, fistula with long tracts, fistula with supralevator blind extension (not with high rectal opening), fistula associated with abscess/pus collections and fistula where no definite internal opening could be localized intraoperatively (Figures 4, 5, 6, 7 and 8).

The PERFACT procedure was quite effective in horseshoe fistula and fistula with multiple tracts. About half of the fistula (50.1%) in our series had a horseshoe fistula and the cure rate was 76.2% (16/21) (Table 2) (Figures 4 and 5). In fact, one of the patients presented with a double horseshoe intersphincteric abscess which encircled the rectum circumferentially. This patient was also cured by this procedure (Figure 5). About 53% of patients had multiple tracts and the success rate in this subgroup was 71.4% (15/21). One of the patients had eight external openings and he underwent this procedure successfully (Figure 8).

In fistula with an associated abscess, the abscess was drained and the PERFACT procedure was carried out as described. There was no need to make a large incision as the setons and regular cleaning of the cavity in the postoperative period ensured that there

was no recollection and good healing ensued. In our series, 41.2% patients presented with an abscess or had an associated significant abscess (Table 1). The PERFACT procedure was done as the definitive first line procedure and the cure rate was 72% (Table 2; Figures 4, 5 and 7).

The PERFACT procedure was effective in fistula cases where no definite internal opening could be localized intraoperatively. Failure to identify the internal opening during the operation perhaps happens because of the temporary closure of the internal opening due to debris or the oblique course of the collapsible tract through the sphincters. As in the literature, this can happen in up to 15-20% of cases[8]. In our series, this happened in 15.7% (8/44) of cases (Table 1). This procedure worked quite successfully in 87.5% (7/8) of such cases in our series (Figure 5) (Table 2). As the MRI was done preoperatively in every case, it helped to localize the tracts in the majority of cases and gave a reasonable idea of where the tract was coursing towards the rectum. This information along with the intraoperative examination findings (induration of the sphincter complex in the region of internal opening) helped to determine the possible site of the internal opening. At that place, the superficial cauterization was done. In two patients, the MRI picture created doubt that the tracts could be going both anteriorly and posteriorly and hence superficial cauterization was done at both places (Figure 5). Superficial cauterization was a safe step to do. Although it created a wound, it was not associated with any risk of incontinence as the wound was quite superficial. Therefore, in case of confusion/doubt, superficial cauterization can be done at two places.

This procedure was also effective in fistula with supralevator extension (blind). The procedure was carried out as described. The position of the supralevator tract was carefully assessed on MRI and intraoperatively this tract was carefully curetted while keeping a finger in the rectum (to avoid injuring the rectal wall). During the postoperative dressings, the supralevator tract was regularly cleaned for at least 2-3 wk (or as needed). While doing so, a finger was inserted in the rectum to avoid any injury. In our series, it was effective in providing cure in 75% of patients (3/4) with supralevator extension (Figure 6).

With careful postoperative management, most of the fistulas healed between 4-10 wk. In 9 (20.5%) patients, the procedure failed. The internal opening did not close and one or multiple tracts failed to heal. The likely reason was inability to regularly clean all the tracts postoperatively, leading to a collection in one of the tracts. This perhaps prevented the tracts as well as the internal opening from healing. Four (9%) of the patients had persistent serous/watery discharge for a prolonged period (10-16 wk). This happened in cases with long fistula tracts. The cauterized wound in the anal canal and the internal opening healed quite well in these cases, leading to the cessation of pus formation.

However, the serous drainage was perhaps due to the re-epithelialization of the outer portion of the tract. We did gentle curettage of the tract in the office under topical anesthesia (lidocaine gel) and it helped to close the recalcitrant tract. However, multiple curettings were needed in two cases.

There are certain patients in whom the internal opening is enlarged/widened due to previous surgical interventions (like tightening setons). In these patients, proximal superficial cauterization fails or takes much longer to heal. In this subgroup, an advancement flap plus the intensified mechanical cleaning of the fistula tract could be a better option.

The PERFACT procedure adds a potentially useful treatment option to our armamentarium against complex fistula-in-ano. It complements the mucosal advancement flap, anal fistula plug, OTSC proctology, LIFT, VAAFT and glue procedures. The PERFACT procedure is simple and associated with lower morbidity and minimal risk of incontinence. Compared to a mucosal advancement flap, the PERFACT procedure is technically less demanding. Unlike an anal fistula plug, laser-FiLaC and OTSC proctology procedure[9,10], the PERFACT procedure can be done as a definitive procedure in fistula patients presenting with an acute abscess or collection. Unlike other existing procedures, the PERFACT procedure can be done in patients where the internal opening cannot be definitely localized. Lastly, unlike fistulotomy and cutting tightening setons, the PERFACT procedure is associated with a minimal risk of incontinence.

The PERFACT procedure has certain distinct advantages. It is associated with the least risk of incontinence, morbidity is minimal, pain is not much and the patient is able to resume normal activities within 1-2 d of the operation. It has a high success rate in all types of complex fistula-in-ano, including horseshoe fistula, recurrent fistula and fistula with multiple tracts. It is effective in highly complicated cases where the other procedures do not work well, such as fistula with supralevator extension, fistula with associated abscess and fistulas where the internal opening cannot be localized. Moreover, the PERFACT procedure can be done as the first line definitive procedure in fistulas presenting with an anorectal or ischiorectal abscess (rather than doing an incision and drainage initially and a definitive procedure later). Another advantage of this procedure is its cost effectiveness. No expensive equipment/gadget is required, operation duration is 15-30 min and hospital stay is only 12-24 h (can be done as a day care procedure). As there is minimal incision/cutting, there is very little scarring and distortion of the anatomy. Last but not the least, this procedure is quite simple to do and reproduce.

The procedure has its limitations. The PERFACT procedure is not effective in cases where a supralevator tract has a high rectal opening. It is also not indicated in low fistula where there is no sphincter

involvement. Secondly, meticulous postoperative care is required, especially for the first two weeks. Although most of the patients are back to their normal routine the first day after the operation, they need to come for twice daily follow-up for at least ten days. Active participation/cooperation is needed from a relative/acquaintance. In our country, teaching a relative (spouse in the majority of cases) was an economically viable and acceptable option. Thirdly, the problem of prolonged serous discharge adds to the morbidity in few patients. Lastly, the long term follow-up (> 3 years) results are awaited.

To conclude, the PERFACT procedure is a simple novel method to treat complex and highly complex anal fistula. This includes fistula-in-ano with multiple tracts, horse shoe fistulas, recurrent fistulas, anterior fistula in females, supralevator fistula, fistula where internal opening cannot be localized and as a first line definitive procedure in patients with fistula-in-ano presenting with ischiorectal or perianal abscess. However, long term multicenter trials are needed with larger numbers of patients to substantiate these findings.

COMMENTS

Background
Complex anal fistula is difficult to treat because of the high risk of recurrence of the disease and the danger of incontinence (losing control over the bowel motions). Apart from regular pus discharge and pain, a complex anal fistula on a long term basis also increases the risk of ano-rectal cancer.

Research frontiers
Apart from several existing methods, many new procedures have been developed to cure this dreaded illness. However, achieving a high success rate (low recurrence) and a low incontinence rate at the same time remains a challenge. The procedures which have a low recurrence rate (high cure rate) have a high incidence of incontinence and the procedures which have a low risk of incontinence have high recurrence rates.

Innovations and breakthroughs
The PERFACT procedure is a new procedure that involves no cutting of anal sphincters (muscles which control bowel motions). Hence, the risk of incontinence is negligible after this procedure. At the same time, this procedure is associated with a high success rate (low recurrence rate). The morbidity of this procedure is also quite low as the patient can resume his/her normal activities within 1-2 d of the operation.

Applications
The medium term follow-up results are quite encouraging. If these results are replicated on a larger scale, then this method has the potential to become the procedure of choice for complex anal fistulas.

Terminology
"PERFACT" in the PERFACT procedure does not mean perfect (ideal). Here, PERFACT is a mnemonic which stands for proximal superficial cauterization,

emptying regularly fistula tracts and curettage of tracts. In proximal superficial cauterization, proximal implies that only the lower part of the anal canal is cauterized (as the internal opening is usually located at the dentate line) and superficial implies that only the mucosa and few medial fibers of the internal sphincter are cauterized, leaving the majority of the internal sphincter and complete external sphincter intact.

Peer-review
This is an interesting description of a novel method of anal fistula closure, including cauterization of the internal opening, curettage of the fistula tract and mechanical debridement of the tract.

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ORIGINAL ARTICLE

Prospective Study

Lower gastrointestinal bleeding: Role of 64-row computed tomographic angiography in diagnosis and therapeutic planning

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Ethics approval: The study was reviewed and approved by the First Affiliated Hospital of Zhengzhou University Institutional Review Board.
Clinical trial registration: We declare that we have no clinical trial registration for this study.
Informed consent: All study participants, or their legal guardian, provided informed written consent prior to study enrollment.
Conflict-of-interest: We declare that we have no conflict of interest in this study.
Data sharing: Technical appendix, statistical code, and dataset available from the corresponding author at rjzjrk@126.com. Participants gave informed consent for data sharing. No additional data are available.
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Abstract

AIM: To determine the value of computed tomographic angiography (CTA) for diagnosis and therapeutic planning in lower gastrointestinal (GI) bleeding.

METHODS: Sixty-three consecutive patients with acute lower GI bleeding underwent CTA before endovascular or surgical treatment. CTA was used to determine whether the lower GI bleeding was suitable for endovascular treatment, surgical resection, or conservative treatment in each patient. Treatment planning with CTA was compared with actual treatment decisions or endovascular or surgical treatment that had been carried out in each patient based on CTA findings.

RESULTS: 64-row CTA detected active extravasation of contrast material in 57 patients and six patients had no demonstrable active bleeding, resulting in an accuracy of 90.5% in the detection of acute GI bleeding (57 of 63). In three of the six patients with no demonstrable active bleeding, active lower GI bleeding recurred within one week after CTA, and angiography revealed acute bleeding. The overall location-based accuracy, sensitivity, specificity, positive predictive value (PPV)

and negative predictive value (NPV) for the detection of GI bleeding by 64-row CTA were 98.8% (249 of 252), 95.0% (57 of 60), 100% (192 of 192), 100% (57 of 57), and 98.5% (192 of 195), respectively. Treatment planning was correctly established on the basis of 64-row CTA with an accuracy, sensitivity, specificity, PPV and NPV of 98.4% (248 of 252), 93.3% (56 of 60), 100% (192 of 192), 100% (56 of 56), and 97.5% (192 of 196), respectively, in a location-based evaluation.

CONCLUSION: 64-row CTA is safe and effective in making decisions regarding treatment, without performing digital subtraction angiography or surgery, in the majority of patients with lower GI bleeding.

Key words: Gastrointestinal bleeding; Digital subtraction angiography; Surgical resection; Computed tomography angiography; Embolization

Core tip: The best modality for the initial diagnosis of acute lower gastrointestinal bleeding (GI) bleeding is controversial. We determined the clinical value of computed tomography angiography (CTA) for diagnosis and therapeutic planning in patients with lower GI bleeding. Sixty-three consecutive patients with acute lower GI bleeding underwent CTA before endovascular or surgical treatment. We found a high overall location-based accuracy, sensitivity, and specificity for the diagnosis and therapeutic planning of acute GI bleeding. We suggest that 64-row CTA is safe and effective in diagnosis and therapeutic planning, without performing digital subtraction angiography or surgery, in patients with lower GI bleeding.

INTRODUCTION

Lower gastrointestinal (GI) bleeding, defined as a bleeding source below the ligament of Treitz in the duodenum, is responsible for approximately 20% of all GI bleeding[1]. Although up to 90% of acute lower GI hemorrhage stops spontaneously without any intervention, a small proportion of these patients will experience exsanguinating hemorrhage and will require invasive procedures to control the bleeding[2,3]. Lower GI bleeding is a common surgical or interventional emergency requiring immediate diagnosis to determine the site and cause of bleeding in order to provide optimal treatment. However, there is considerable controversy regarding the best modality for the initial diagnosis of acute lower GI bleeding.

The current literature suggests that colonoscopy is usually preferred, but the procedure in most cases is technically difficult due to fresh blood or feces; moreover, it is more often a diagnostic rather than a therapeutic modality[4,5]. Over the past few decades, mesenteric angiography and embolization of the bleeding vessels have slowly been accepted as an integral part in the management of patients with acute lower GI hemorrhage[6-10]. However, these procedures are more invasive and require a longer time to perform compared with investigations such as computed tomography (CT). Moreover, colonoscopy and angiography can be relatively inaccessible outside of normal working hours, particularly in some countries or regions due to lack of trained personnel or infrastructure.

Recently, helical computerized tomography has increasingly been used to compare the sensitivity and specificity of computed tomography angiography (CTA) to digital subtraction angiography (DSA) as screening tools for the detection of acute GI bleeding[11-15]. However, the use of CTA in the diagnosis and preoperative planning of acute GI bleeding has received little attention. Therefore, the purpose of the present study was to determine the clinical value of 64-row CTA in the diagnosis and therapeutic planning of patients with acute lower GI bleeding compared with conventional angiography.

MATERIALS AND METHODS

Study design

The institutional review board approved the study protocol, and patients or qualifying family members provided informed consent before participation. From June 2010 to June 2014, 63 consecutive patients with suspected acute lower GI bleeding detected by CTA underwent interventional embolization, surgical resection or conservative treatment. DSA or surgery, regarded as the gold standard, was performed after CTA to confirm the diagnosis of lower GI bleeding.

The patients included 37 men and 26 women aged 18-89 years (median age, 66 years). Acute GI bleeding was defined as hematemesis, melena, or hematochezia that occurred 24 h prior to CTA. Massive bleeding was considered to have occurred if either of the following two criteria was met: the patients required transfusion of at least 4 units of blood during a 24-h period in the hospital, or they were hemodynamically unstable (hypotension with systolic blood pressure < 90 mmHg). The causes of lower GI tract bleeding (*n* = 63) were stress ulceration (*n* = 7), diverticulum (*n* = 18), trauma (*n* = 26), arteriovenous malformation (AVM, *n* = 4), angiodysplasia (*n* = 1), stromal tumor of the GI tract (*n* = 1), Crohn's disease (*n* = 3), colon cancer (*n* = 1), nonspecific colitis (*n* = 1), and unknown (*n* = 1).

Image acquisition

All CTA examinations were performed using a 64-row CT scanner (LightSpeed VCT or Discovery CT750 HD, GE Healthcare, United States). The smart preset scan technique was used for enhancement CTA with the following parameters: 0.625 mm × 64 slice, 120 kVp, 100-600 mA, 1.375:1 helical pitch, and an acquisition time of 30.6 s. The standard dosage for enhancement CTA was 0.7 mL/kg body weight in addition to 40 mL normal sodium, and the contrast material was administered using a power injector at a rate of 4 mL/s through a 22-gauge needle into the antecubital vein. When the concentration of contrast medium in the ascending aorta was 100 HU, the CT scanner automatically scanned from the diaphragmatic dome to the pubic symphysis and collected images. The acquired image data sets were then transferred to a workstation (GE AW4.3 or GE AW4.3, GE Medical, United States), where 3D image reconstruction, including oblique, coronal and sagittal maximum-intensity projection (MIP), multiplanar reconstruction (MPR), and three-dimensional volume-rendered (VR) images of the GI tract and abdominal vascular structures, were performed with a 732 × 732 matrix.

With arterial phase 64-row CT, the following two features were considered diagnostic of lower GI bleeding: (1) the presence of contrast material extravasation in the bowel lumen; and (2) extravasated contrast material with an attenuation level greater than 90 HU.

Image review

Three observers were blinded to all clinical, DSA and surgical data. They independently analyzed all CTA datasets on an offline workstation from multiple on-screen viewing angles. The source images, MIPs, MPR, and VR were presented on-screen, thus allowing adjustment of the appropriate threshold of the window width and level. In the presence of interobserver discrepancies in the detection of lower GI bleeding, a consensus or a majority decision was obtained. Two radiologists (K.Z. and G.H.H.) with 7 and 2 years of experience of abdominal CT, respectively, analyzed the CT images. Final decisions regarding the CT findings were made by consensus. In 56 patients, both observers independently reached the same interpretation. In the remaining seven patients, a decision was reached by consensus.

For this assessment, the location of lower GI bleeding was recorded in the following anatomic locations: jejunum, ileum, ascending colon, transverse colon, descending colon, and rectum. The locations of active bleeding were individually recorded by two authors.

Treatment planning with 64-row CTA

After diagnostic CTA, treatment decisions regarding interventional embolization with coil and/or gelatin sponge or glue, surgical resection or conservative treatment were made by consensus by the attending surgeon and interventional radiologists. There were four treatment options: (1) embolization with coils or glue; (2) surgery; (3) unsuccessful attempt to place coils or surgical resection, no further therapy; and (4) conservative treatment. Treatment methods and treatment criteria based on CTA are shown in Table 1.

In our hospital, surgical resection is considered first-line treatment for lower GI bleeding. Surgical resection was considered when lower GI bleeding was confirmed by CTA and associated with diverticular disease, angiodysplasia or AVM, malignancy or bowel ischemia. In patients with massive bleeding, endovascular embolization was often needed to stop bleeding with subsequent radical surgical resection of the lesions.

Patients were considered for endovascular embolization when lower GI bleeding was confirmed by CTA and associated with stress ulceration or trauma.

Patients were considered suitable for conservative treatment when they had no lower GI bleeding on CTA, had lower GI bleeding that was too difficult for endovascular treatment or surgical resection, or when the treatment had been unsuccessful.

Statistical analysis

The categorical demographic and basic characteristic variables, expressed as numbers and percentages, were compared using the χ^2 test. Continuous variables were expressed as mean ± SD and compared using an unpaired t test, if normally distributed. The calculation of sensitivity, specificity, accuracy, PPV and NPV for the detection of acute lower GI bleeding with 64-row CTA was performed on the basis of a per location analysis in relation to results at angiography or surgery. For the purposes of statistical analysis, a true-positive finding was defined as depiction on 64-row CTA of the presence of contrast material extravasation when the results of angiography were positive for active bleeding. A false-positive finding was defined as depiction on 64-row CTA of the presence of active bleeding that was not detected at angiography. A true-negative finding was defined as the lack of identification of a bleeding focus on 64-row CTA

Table 1 Summary of 64-row computed tomographic angiography findings according to anatomic location

Location	Acute lower GI bleeding present[1] (n = 60)		Acute lower GI bleeding absent (n = 192)	
	True-positive findings	False-negative findings	True-negative findings	False-positive findings
Jejunum	15	1	47	0
Ileum	29	2	32	0
Colon	12	0	51	0
Rectum	1	0	62	0
Total (n = 252)	57	3	192	0

[1]According to the results of conventional angiography or surgery. Data are the numbers of anatomic locations. GI: Gastrointestinal.

Figure 1 Images from a 36-year-old woman with a history of abdominal trauma (hemoglobin, 7.1 g/dL; hematocrit, 24.5%). A: 64-row computed tomography angiogram in arterial phase showed a focus of extravasation in the ileum (arrow); B: Active bleeding site was found on conventional angiography; C, D: Post-embolization angiography confirmed complete occlusion of the active gastrointestinal bleeding with coils (arrow).

images when the results of angiography were negative for active bleeding. A false-negative finding was defined as depiction on 64-row CTA of the absence of active bleeding despite detection of active bleeding at angiography. The diagnostic performance parameters of CTA for the diagnosis of lower GI bleeding compared with those of DSA or surgery (that is, accuracy, sensitivity, specificity, PPV and NPV) were expressed as percentages.

A good correlation from the prospective 64-row CTA protocol was defined as treatment planning by radiologists or surgeons based on CTA that correlated with the actual treatment decision or treatment performed by the interventional radiologists or surgeon based on CTA. A deviation from the protocol was defined as treatment planning by the radiologists or surgeon based on CTA that was changed or differed from the actual treatment decision or procedures performed by the interventional radiologists or surgeon based on DSA. On the basis of this dichotomization, accuracy, sensitivity, specificity, PPV and NPV were also calculated. Statistical analyses were performed using SPSS (version 13.0, SPSS Inc., Chicago, IL, United States).

RESULTS

Diagnostic performance of CTA

64-row CTA detected active extravasation of contrast material in 57 patients and six patients had no demon-

strable active extravasation of contrast material. During 64-row CTA, contrast material extravasation was identified in the jejunum in 17 patients, in the ileum in 33 patients, in the colon in 12 patients, and in the rectum in one patient. Of these 57 patients with contrast material extravasation depicted on 64-row CTA, findings at angiography or surgery confirmed acute GI bleeding in all 57 patients. In three patients, angiography revealed acute duodenal bleeding that was not detected on 64-row CTA (false-negative 64-row CTA findings). Thus, the overall patient-based accuracy of 64-row CTA in the detection of acute GI bleeding was 90.5% (57 of 63). In 57 patients in whom 64-row CTA depicted extravasation of contrast material, the mean attenuation level was 276 HU (attenuation range, 115-378 HU).

We evaluated 252 anatomic locations in 63 patients for the presence or absence of acute GI bleeding (Table 1). The overall location-based accuracy, sensitivity, specificity, PPV and NPV for the detection of GI bleeding by 64-row CTA were 98.8% (249 of 252), 95.0% (57 of 60), 100% (192 of 192), 100% (57 of 57), and 98.5% (192 of 195), respectively. Of the 252 locations evaluated, 57 had evidence of acute GI bleeding on both 64-row CTA and angiography or surgery (true-positive CTA findings) (Figure 1). In three cases without evidence of acute GI bleeding on 64-row CTA, findings were positive at angiography (false-negative findings).

64-row CTA had an accuracy of 100% for localization
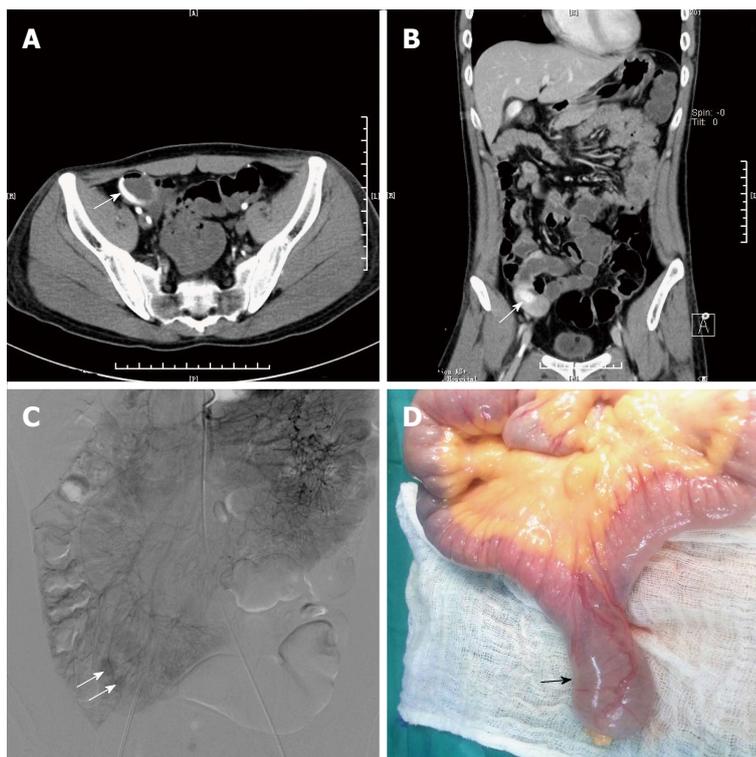
Figure 2 Images from a 64-year-old man with acute ileal bleeding (hemoglobin, 7.4 g/dL; hematocrit, 23.7%). A, B: Axial and coronal computed tomography angiograms in arterial phase showed a focus of active extravasation (arrow) at the distal ileum; C: Superior mesenteric angiography prior to surgical resection confirmed the active extravasation (arrow); D: Emergency surgery was required 2 d after bleeding, and the resected specimen confirmed a diverticulum (arrow) at the distal ileum.

Table 2 Assessment of treatment planning with computed tomographic angiography and actual treatment performed based on computed tomographic angiography in patients with lower gastrointestinal bleeding

Treatment method	Treatment planning by CTA	Actual treatment performed
Endovascular treatment	32	36
Surgical resection	25	24
Conservative treatment	6	3

CTA: Computed tomographic angiography.

of acute GI bleeding. The site of contrast material extravasation on 64-row CTA images corresponded exactly with the angiographically or surgically depicted site of bleeding in all patients in whom a focus of bleeding was detected on 64-row CTA.

Treatment planning following 64-row CTA

When actual treatment decisions were compared with treatment performed based on DSA or surgery, a good correlation with the prospective CTA protocol was obtained in all patients (Table 2, Figure 2). Of the 63 patients with acute lower GI bleeding on 64-row CTA, treatment planning in 32 patients consisted of coils and/or gelatin sponge or glue, 25 patients were managed with surgical resection, and the remaining 6 patients with no acute lower GI bleeding received

conservative treatment. In three of 6 patients who received conservative treatment, active lower GI bleeding recurred within one week after CTA examination, and angiography revealed acute lower GI bleeding that was not detected on 64-row CTA (false-negative findings). Embolization was performed in three patients. In one patient with colon cancer, surgical resection could not be performed due to massive bleeding and endovascular embolization was carried out (false-negative finding). Treatment planning was correctly established on the basis of 64-row CTA with an accuracy, sensitivity, specificity, PPV and NPV of 98.4% (248 of 252), 93.3% (56 of 60), 100% (192 of 192), 100% (56 of 56), and 97.5% (192 of 196), respectively, in a location-based evaluation (Table 3).

DISCUSSION

Lower GI bleeding may involve the small bowel, colon, and rectum, and carries a mortality rate of 3.6%[16]. Lower GI bleeding is less common than upper GI bleeding and accounts for approximately 30% of all GI bleeding[17,18]. Lower GI bleeding tends to affect more elderly patients than young patients[19]. Common causes of lower GI bleeding are diverticular disease, angiodysplasia, neoplasms, colitis, and benign anorectal lesions[16,17,20].

Many diagnostic approaches are available to detect and locate the source of lower GI bleeding, each with

Table 3 Summary of actual treatment performed according to anatomic location

Location	Endovascular or surgical treatment (*n* = 60)		Conservative treatment (*n* = 192)	
	True-positive findings	False-negative findings	True-negative findings	False-positive findings
Jejunum	15	1	47	0
Ileum	29	2	32	0
Colon	11	1	51	0
Rectum	1	0	62	0
Total (*n* = 252)	56	4	192	0

its own advantages and weaknesses[17]. Although endoscopy is considered to be the first-line diagnostic modality for lower GI bleeding, endoscopy often fails to depict the exact focus of bleeding when excessive blood or clots impair visualization[21]. Capsular endoscopy is a relatively new method for establishing the cause of small bowel bleeding, but is not useful in an urgent situation[22]. Although colonoscopy is frequently used in lower GI bleeding, its value in the diagnosis of massive bleeding remains controversial, and appears to be most efficacious only when massive bleeding has stopped, allowing time for a bowel preparation[23]. Radionuclide imaging is noninvasive, simple to perform, sensitive, and has the ability to carry out delayed scans up to 24 h after radioisotope injection to detect re-bleeding, but it is a time-consuming method with a high false localization rate of up to 22%[24]. Catheter-directed angiography is considered accurate in the diagnosis of acute GI bleeding, and may be employed advantageously for immediate therapeutic transcatheter embolization[25]. However, it is an invasive procedure, and negative results are common in patients with a stable hemodynamic status, slower GI bleeding or no active bleeding present at the time of contrast material administration[26]. Therefore, fast and accurate detection, and localization of the bleeding source are crucial for effective hemostatic treatment in patients with rapid acute massive lower GI bleeding.

Recent technical advances in CT have led to the increased use of CTA as a first-line modality in the detection and localization of GI bleeding, particularly within the lower GI tract[27-30]. 64-row CTA allows thinner collimation, faster scanning times, greater anatomic coverage and better multi-planar reformatted (MPR) images, which have greatly expanded the diagnostic role of CTA for various pathologic processes. CTA provides several distinct advantages over nuclear scintigraphy and catheter angiography: CTA is readily available at all times and has been shown to detect rates of bleeding greater than the threshold of first-order selective mesenteric angiography[11-15]. When positive, 64-row CTA provides precise anatomic localization of the bleeding site as well as an exquisite map of the mesenteric vasculature. Preoperative knowledge of the bleeding site and the patient's mesenteric vascular

anatomy facilitates rapid catheterization of the source mesenteric trunk, directs subselective mesenteric catheterization, and obviates the need for aortography. Evaluation with CTA may also demonstrate the etiology of the bleed or additional unsuspected pathology that may require deviation from customary management. Conversely, when active hemorrhage is excluded by CTA, temporarily deferring angiography and continuing supportive care appears safe with no adverse events directly attributable to angiographic deferment in our series. This may prevent unnecessary invasive tests or mobilization of the surgical or interventional radiology team. Therefore, we propose that CTA should be adopted in the clinic as the first-line examination for the evaluation of acute lower GI bleeding.

This prospective study was based on our hypothesis that CTA could replace DSA or surgery as a reliable diagnostic and pretreatment planning tool for patients with lower GI bleeding. The results of our study indicated that arterial phase 64-row CTA is highly accurate for both detection and localization of acute massive lower GI bleeding. For the detection of acute GI bleeding, 64-row CTA had a sensitivity of 95.0% and a specificity of 100%, and for treatment planning, 64-row CTA had a sensitivity of 93.3% and a specificity of 100%. Although there were three false-negative diagnoses, we found that 64-row CTA not only accurately identified the presence of lower GI bleeding, but also demonstrated a good correlation with DSA or surgery in treatment planning for lower GI bleeding. It appears that treatment planning on the basis of CTA, before DSA or surgery, is a feasible and effective option for patients with lower GI bleeding.

Although there have been many reports in the literature assessing the validity of CTA as a diagnostic technique for the detection of lower GI bleeding[11-15], few have evaluated the clinical implications of a protocol that uses CTA instead of DSA or surgery as a diagnostic and pretreatment planning tool. If CTA is to serve as a non-invasive replacement for DSA or surgery in pretreatment planning, it must provide precise visualization of the location of the lower GI bleeding and its surrounding structures. In addition, it is essential to identify the cause of bleeding and the bleeding artery before performing endovascular treatment or surgical resection. A combination of the patient's history and CTA results showed that the cause of bleeding in most patients was clearly known prior to surgical or interventional management. For instance, four patients with AVM, one with angiodysplasia, one with stromal tumor of the GI tract, two with Crohn's disease, one with colon cancer, and 16 with diverticular bleed were treated surgically without angiographic or endoscopic intervention.

The overall sensitivity and specificity of CTA in the detection of GI hemorrhage in our series was 95% and 100%, respectively. These values are similar to the recently published data by Yoon *et al*[12] (sensitivity, 90.9%; specificity, 99%). Yoon *et al*[12] demonstrated

that arterial phase CTA performed well in patients with massive GI hemorrhage in their prospective series of 26 patients. These authors found that the results of their study indicated that arterial-phase 64-row CTA is highly accurate for both the detection (sensitivity of 90.9% and specificity of 99%) and localization (accuracy of 100%) of acute massive GI bleeding as compared with angiograms performed in all patients with GI bleeding. In addition, 64-row CTA had an accuracy of 100% for localization of acute GI bleeding. The site of contrast material extravasation on 64-row CTA scans corresponded to the site of bleeding identified on angiograms or surgical resection in patients with acute GI bleeding. This agreement is of particular importance in the performance of angiography and subsequent embolization procedures in critically ill patients with acute massive lower GI bleeding[12].

The present study had some limitations. Firstly, this was a single-center study, and the patient population was relatively small. The small sample prevents us from generalizing the results. Secondly, the risks of CTA scanning are minor and arise from exposure of the patient to ionizing radiation and the administration of intravenous contrast material. Thirdly, CT artifacts can obscure contrast material extravasation in the bowel lumen which may be misdiagnosed as acute GI bleeding. Lastly, intermittent GI hemorrhage, even in cases of massive bleeding, is not uncommon, and CTA may fail to depict any abnormalities during periods of quiescence[12]. This phenomenon of intermittent hemorrhage may account for the false-negative CT angiograms reported in this study. Therefore, repeated CTA or catheter angiography is often necessary before a bleeding source is definitively localized. In addition, there was a lack of a true gold standard for comparison in three negative CTA cases, as most were successfully observed without additional intervention and the exact site of hemorrhage was never determined.

In conclusion, our findings suggest that 64-row CTA could act as an accurate first-line screening method for the detection and localization of acute lower GI bleeding sites. The procedure is safe and effective for making decisions regarding treatment, without performing DSA or surgery, in the majority of patients with lower GI bleeding.

COMMENTS

Background
Lower gastrointestinal (GI) bleeding is a common surgical or interventional emergency requiring immediate diagnosis to determine the site and cause of bleeding in order to provide optimal treatment. However, there is controversy regarding the best modality for the initial diagnosis of acute lower GI bleeding.

Research frontiers
Computerized tomography has increasingly been used to compare the sensitivity and specificity of computed tomographic angiography (CTA) with digital subtraction angiography (DSA) as screening tools for depicting acute GI bleeding. However, the use of CTA in the diagnosis and preoperative planning of acute GI bleeding has received little attention. The authors determined the clinical value of CTA for diagnosis and therapeutic planning in patients with lower GI bleeding.

Innovations and breakthroughs
CTA was used as a diagnostic and preoperative planning tool in patients with lower GI bleeding. The results of 64-row CTA were compared with those of DSA and surgical resection. This is the first study to determine the value of CTA for diagnosis and therapeutic planning in lower GI bleeding.

Applications
64-row CTA could act as an accurate first-line screening method for the detection and localization of acute lower GI bleeding sites. The procedure was safe and effective for making decisions regarding treatment, without performing DSA or surgery, in the majority of patients with lower GI bleeding.

Terminology
GI bleeding, defined as a bleeding source below the ligament of Treitz in the duodenum, is responsible for approximately 20% of all GI bleeding.

Peer-review
The authors present a nonrandomized prospective trial evaluating the value of CTA for diagnosis and therapeutic planning in lower GI bleeding. The results reveal a high overall location-based accuracy, sensitivity, specificity, and positive and negative predictive values for the diagnosis and therapeutic planning in patients with acute GI bleeding by 64-row CTA. These results suggest 64-row CTA is safe and effective in making decisions regarding treatment on the basis of CTA, without performing DSA or surgery, in the majority of patients with lower GI bleeding and could act as an accurate first-line screening method for detection and localization of acute lower GI bleeding sites.

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META-ANALYSIS

Osteoporosis and bone fractures in alcoholic liver disease: A meta-analysis

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Abstract

AIM: To evaluate the association between alcoholic liver disease (ALD) and bone fractures or osteoporosis.

METHODS: Non-randomized studies were identified from databases (PubMed, EMBASE, and the Cochrane Library). The search was conducted using Boolean operators and keywords, which included "alcoholic liver diseases", "osteoporosis", or "bone fractures". The prevalence of any fractures or osteoporosis, and bone mineral density (BMD) were extracted and analyzed using risk ratios and standardized mean difference (SMD). A random effects model was applied.

RESULTS: In total, 15 studies were identified and analyzed. Overall, ALD demonstrated a RR of 1.944 (95%CI: 1.354-2.791) for the development of bone fractures. However, ALD showed a RR of 0.849 (95%CI: 0.523-1.380) for the development of osteoporosis. BMD was not significantly different between the ALD and control groups, although there was a trend toward lower BMD in patients with ALD (SMD in femur-BMD: -0.172, 95%CI: -0.453-0.110; SMD in spine-BMD: -0.169, 95%CI: -0.476-0.138). Sensitivity analyses showed consistent results.

CONCLUSION: Current publications indicate significant associations between bone fractures and ALD, independent of BMD or the presence of osteoporosis.

Key words: Alcoholic liver diseases; Bone fractures; Osteoporosis

Core tip: Excessive alcohol consumption is a well-established risk factor for osteoporosis and bone fractures. However, light amounts of alcohol ingestion is known to be associated with higher bone mineral density (BMD) and low fracture rates. This study evaluated the current evidence regarding osteoporosis and bone fractures in alcoholic liver disease (ALD). In this meta-analysis, there was significant associations between bone fractures and ALD, independent of BMD or the presence of osteoporosis. Although the

mechanism of bone fractures in ALD is not totally understood, further research utilizing a homogenous population and controlling for confounding risk factors for fractures could elucidate the mechanism.

INTRODUCTION

Chronic excessive alcohol consumption is a well-established risk factor for low bone density and bone fractures[1]. This is included in the fracture risk assessment tool (FRAX), which estimates the 10-year probability of bone fractures combined with other clinical risk factors and the bone mineral density (BMD) of the femoral neck[2,3]. It is assumed that the decreases in bone mass and strength resulting from heavy alcohol use are due to an imbalance between bone formation and resorption[4]. However, ingestion of light or moderate amounts of alcohol is known to be associated with higher BMD and decreased fracture rates[5-15], although conflicting results exist because of inconsistent standards of classification of light, moderate, or heavy alcohol consumption[13,16].

Osteoporosis and bone fractures are frequently overlooked complications in patients with chronic liver disease that could result in serious outcomes[17]. However, the exact prevalence or mechanism of osteodystrophy in patients with alcoholic liver disease (ALD), the deleterious outcome of chronic alcohol abuse, have not been described. The aim of this study was to evaluate the association between ALD and bone fractures or osteoporosis.

MATERIALS AND METHODS

Literature search

MEDLINE (through PubMed), EMBASE, and the Cochrane Central Register of Controlled Trials (CENTRAL) in the Cochrane Library were searched using common keywords related to ALD, osteoporosis, and bone fractures (from inception to April 2014). Medical Subject Headings (MeSH) terminology was used because all 3 databases permit searching using MeSH terminology. The keywords used included "alcoholic liver diseases", "osteoporosis", or "bone fractures" using Boolean operators. Only publications on human subjects were searched, and the bibliographies of relevant articles were also reviewed to identify additional studies. The language of publication was not restricted.

Selection criteria

Due to a lack of randomized-controlled studies relevant to this topic, we included case-control or cohort studies meeting all of the following criteria: (1) designed to evaluate ALD in the target or control group; and (2) included at least one outcome (prevalence of any bone fractures, prevalence of osteoporosis, or BMD) that enabled comparisons of osteodystrophy between patients with ALD and the control group. The exclusion criteria were as follows: (1) incomplete data; (2) review article; or (3) abstract only (study not published as full-text article).

Selection of relevant studies

Two of the authors (C.S.B. and G.H.B.) independently evaluated the eligibility of all studies retrieved from the databases based on the predetermined selection criteria. The abstracts of all identified studies were reviewed to exclude irrelevant articles. Full-text reviews were performed to determine whether the inclusion criteria were satisfied by the remaining studies. Disagreements between the two evaluators were resolved by discussion or by consultation with a third author (D.J.K.).

Assessment of methodological quality

The methodological quality of the enrolled studies was assessed using the Newcastle-Ottawa Scale[18,19]. This tool is categorized into three parameters: the selection of the study population, the comparability of the groups, and the ascertainment of the exposure or outcome. Each parameter consists of subcategorized questions: selection ($n = 4$), comparability ($n = 1$), and exposure or outcome ($n = 3$). Stars awarded for each item serve as a quick visual assessment for the methodological quality of the studies. A study can be awarded a maximum of nine stars, indicating the highest quality[18,19]. The included studies were classified as high-quality ($\geqslant$ 7 stars) or low-quality (< 7 stars). Sensitivity analyses were performed according to the criteria described above.

Main and modifier-based analyses

We investigated the relationship between ALD and bone fractures or osteoporosis using adjusted risk ratios (RRs) and standardized mean difference (SMD). The prevalence of any fractures or osteoporosis assessed by radiologic examinations and BMD assessed by dual-energy X-ray absorptiometry (DXA) or dual-photon absorptiometry (DPA) were extracted and analyzed. Osteoporosis was defined as a value for BMD that was 2.5 standard deviations or more below the reference range[20]. We also performed sensitivity analyses based on methodological quality (high or low), the area measured to determine BMD (femoral neck or spine), the type of control group (normal, healthy control or chronic liver diseases other than
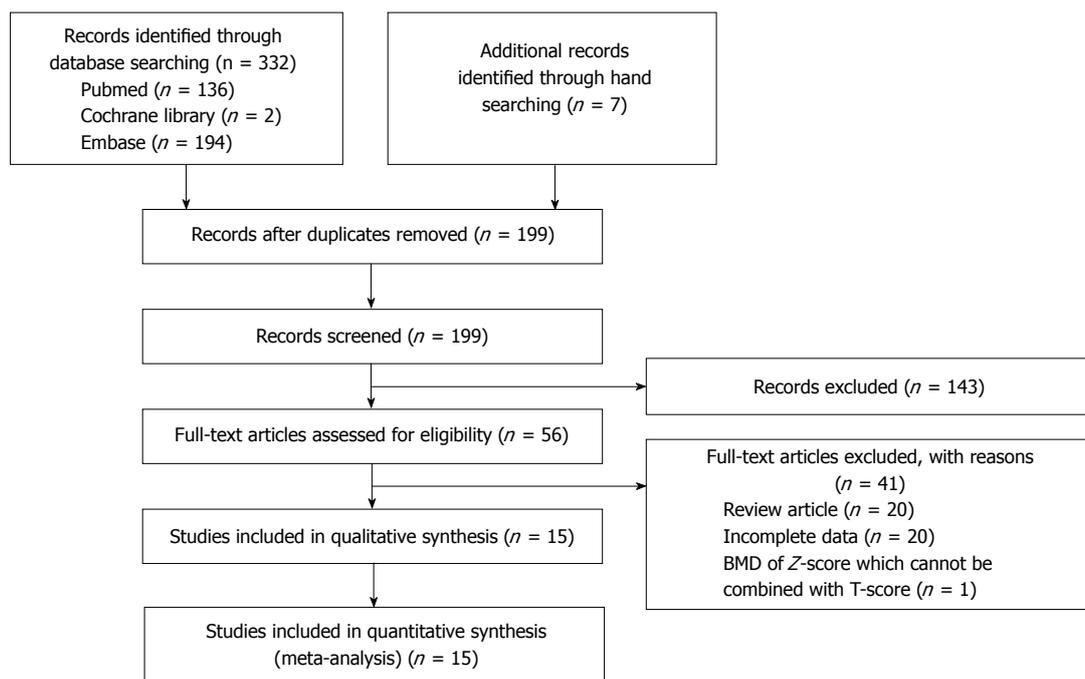
Figure 1 Flow diagram for identification of relevant studies.

ALD), and the effect size, excluding outliers whenever possible. Both a cumulative analysis and a one-study-removed analysis were also performed.

Unit of analysis

For the present evaluation, the independent study was the primary unit of analysis. Thus, for the studies that had multiple control groups (reported multiple outcomes)[21-25] or multiple BMD measured at the femoral neck or spine simultaneously[23,26], an approach with a shifting unit of analysis was used for the determination of an independent estimate of the effect[27]. In these studies, the biggest effect sizes of the control groups or the BMD were used.

Statistical analysis

Comprehensive meta-analysis software (version 2.2.064, Borenstein M, Hedges L, Higgins J and Rothstein H. Englewood, NJ: Biostat; United States) was used for this meta-analysis. We calculated the RRs with 95%CIs using 2×2 tables from the original articles whenever possible to reveal the relationship between ALD and bone fractures or osteoporosis. To compare the BMD directly between patients with ALD and the control groups, the SMD was also used as another effect size calculation. SMD is the difference in mean value between groups divided by the SD among the populations. Therefore, even if the actual scales for BMD are different across the articles, individual data can be combined. SMD was calculated as follows: SMD = (M_1 - M_2) / pooled SD, where M_1 is the mean BMD in the ALD group and M_2 is the mean BMD in the control group[28]. A negative value of SMD means the ALD group has a lower BMD than the control group. Heterogeneity was tested using the I^2 test, which measures the percentage of total variation across studies[29]. I^2 was calculated as follows: I^2 (%) = 100 × (Q-df)/Q, where Q is Cochrane's heterogeneity statistic and df means the degree of freedom. Negative values for I^2 were set to zero, and an I^2 value over 50% was considered to be of substantial heterogeneity (range of 0-100%)[30]. Pooled RRs with 95%CIs were calculated using a random effects model and the method of DerSimonian and Laird because of methodological heterogeneity[31]. These results were confirmed again by the I^2 test. A fixed effects model using the inverse variance-weighted (Woolf's) method was used in the sensitivity analyses, including cumulative and one-study-removed analyses, based on the assumption of a common effect size shared by the subgroup studies[32,33]. Significance was set at $P = 0.05$ in both models. Publication bias was evaluated using Begg's funnel plot, Egger's test of the intercept, Duval and Tweedie's trim and fill, and Begg and Mazumdar's rank correlation test[34-38].

RESULTS

Identification of relevant studies

Figure 1 shows a flow diagram of how relevant studies were identified. A total of 339 articles was identified by a search of 3 core databases and a manual search of relevant bibliographies. In all, 140 duplicate studies and an additional 143 studies were excluded during the initial screening through a review of the titles and abstracts. The full texts of the remaining 56 studies were thoroughly reviewed. Among these studies, 41

Study name	Statistics for each study					Risk ratio and 95%CI
	Risk ratio	Lower limit	Upper limit	*Z* value	*P* value	
Ninkovic 2000	1.698	1.165	2.474	2.757	0.006	
Carey 2003	1.664	0.911	3.042	1.656	0.098	
Diamond 1990	1.875	0.929	3.784	1.755	0.079	
Wibaux 2011	1.377	0.822	2.305	1.215	0.224	
González-Reimers 2011	33.725	2.144	530.617	2.502	0.012	
Lindsell 1982	3.725	1.813	7.653	3.580	0.000	
	1.944	1.354	2.791	3.605	0.000	

Heterogeneity: $\chi^2 = 9.462$, df = 5 ($P = 0.092$); $I^2 = 47.157\%$
Test for overall effect: $Z = 3.605$ ($P < 0.001$)

0.1 0.2 0.5 1 2 5 10
Control   Alcoholic liver diseases

Figure 2 Association between alcoholic liver diseases and bone fractures. The size of each square is proportional to the study's weight. Diamond is the summary estimate from the pooled studies with 95% CI. ALD: Alcoholic liver diseases;CI: Confidence interval (random effect model).

were excluded from the final analysis. The reasons for study exclusion during the final review were as follows: review article ($n = 20$), incomplete data ($n = 20$), or use of Z-score for BMD, which cannot be combined with T-score ($n = 1$). The remaining 15 non-randomized studies were included in the final analysis.

Characteristics of studies included in the final analysis

Within the 15 studies, we identified a total of 726 participants (313 patients with ALD *vs* 413 controls) in the analysis of bone fractures, 470 participants (260 patients with ALD *vs* 210 controls) in the analysis of osteoporosis, and 769 participants (391 patients with ALD *vs* 378 controls) in the analysis of BMD. The clinical characteristics of the enrolled studies are shown in Table 1.

The enrolled studies were published between 1982 and 2011. Eight studies were conducted in Europe[21,25,39-44], whereas the remaining studies were conducted in the United States ($n = 3$)[22,23,45], Canada ($n = 1$)[24], Australia ($n = 1$)[46], India ($n = 1$)[26], and Korea ($n = 1$)[47]. All 15 articles were written in English. The study format of each study was as follows; cohort study (10)[21-26,39,40,42,45] and case-control study (5)[41,43,44,46,47]. Three studies included a normal healthy population as the control group[40,41,44], although the remaining studies included patients with chronic liver diseases other than alcoholic etiologies as the control groups[21-26,39,42,43,45-47].

Among the included studies, 4 studies[22,25,26,42] had ALD groups that consisted only of cirrhotic patients, whereas 2 studies[44,47] included no alcoholic cirrhotic patients, and in 5 studies[23,24,39,45,46], the presence or absence of cirrhosis was not specified.

As for the measurement of BMD, only 1 study used DPA using Prodigy: DPX-NT /DPX-MD + (General Electric, Milwaukee, WI)[22], and the remaining studies[21,24,25,40,41,43,45,47] used DXA.

In terms of the methodological quality, the authors classified the included studies as high-quality ($\geqslant 7$ stars) or low-quality (< 7 stars). Six studies[26,39,40,45-47] were classified as high-quality, whereas the remaining 9 studies[21-25,41-44] were classified into the low-quality group (Table 2).

Association between ALD and bone fractures or osteoporosis

The overall association of ALD and bone fractures was evaluated by a random effects model-based meta-analysis of 6 studies[39,41,42,44-46]. Overall, ALD showed a RR of 1.944 (95%CI: 1.354-2.791, $P < 0.001$) for the development of bone fractures (Figure 2).

The relationship between ALD and osteoporosis was assessed by a random effects model-based meta-analysis of 5 studies[23,26,43,45,47]. ALD showed a RR of 0.849 (95%CI: 0.523-1.380, $P = 0.509$) for the development of osteoporosis (Figure 3).

To compare the BMD directly between patients with ALD and the control groups, the authors performed a random effects model-based meta-analysis of 7 studies[21,22,24,25,40,43,47] in which the BMD was measured at the femoral neck and 9 studies[21,22,24,25,40,41,43,45,47] in which the BMD was measured at the spine. BMD was not significantly different between the ALD and control groups, although there was a trend toward lower BMD in patients with ALD (SMD in the femoral neck BMD: -0.172, -0.453-0.110, $P = 0.233$; SMD in the spine BMD: -0.169, -0.476-0.138, $P = 0.282$).

Sensitivity meta-analysis

The cumulative meta-analysis of the enrolled studies in the order of published year showed a decreasing trend of RRs, but a consistent and statistically significant increase in bone fractures. With regard to osteoporosis, the cumulative meta-analysis of the enrolled studies showed an increasing trend of RRs that was consistently non-statistically significant. For the measurement of BMD, cumulative meta-analyses of enrolled studies showed a decreasing trend in SMD, although there was still no difference in BMD *vs* the control group. The one-study-removed meta-analyses of the enrolled studies also showed consistent results.

In the sensitivity analyses of high-quality[39,45,46] and low-quality[41,42,44] studies for bone fractures, consistent results were noted (RR = 1.719, 95%CI: 1.285-2.299, $P < 0.001$; RR = 2.058, 95%CI: 1.360-3.114, $P = 0.001$). Consistent results were also noted in the

Table 1 Clinical data of included studies

Ref.	Population characteristics	LC in ALD	Bone fractures					Osteoporosis				ALD BMD	Control BMD
			Fracture site	ALD fracture	Total ALD	Control fracture	Total control	ALD osteoporosis	Total ALD	Control osteoporosis	Total control		
Lindsell et al[44]	149 CLD (72 ALD, 77 non-alcoholic CLD), 149 normal controls	0	Rib or clavicle	18	72	10	149 (normal control)						
Diamond et al[46]	115 CLD (M: 72, F: 43), 113 Controls (M: 52, F: 61), Mean age: 51.1 ± 10.9)	Unidentifiable	Peripheral bone	12	40	12	75 (other CLD)						
Bonkovsky et al[23]	133 CLD (M: 70, F: 63, Mean age: 47 ± 1.1)	Unidentifiable	Unidentifiable					5 (L), 4 (FN)	32	15 (L), 5 (FN)	48 (other LC)		
Ninkovic et al[39]	37 CLD (6 ALD, 31 other CLD) (M: 20, F: 17, Mean age: 51.3, range: 32-65)	Unidentifiable	Vertebra	6	6	17	31 (other CLD)						
Ninkovic et al[21]	243 CLD (M: 128, F: 115, Mean age: 51.1 ± 10.9)	Predominantly LC			46		55 (HCV)					T (L1-4): -0.94 ± 1.38, T (FN): -1.49 ± 1.15	T (L1-4): -1.59 ± 1.6, T (FN): -1.66 ± 1.27
Carey et al[45]	207 CLD (66 ALD, 73 HCV + ALD, 68 HCV) (M: 131, F: 76, Mean age: 51, range: 32-68)	Unidentifiable	Overall fractures	21	66	13	68 (HCV)	12	66	19	68 (HCV)	T (L1-4): -0.87 ± 1.61	T (L1-4): -1.43 ± 1.68
Kim et al[47]	18 ALD (Mean age: 50.2 ± 9.5), 18 normal controls (Mean age: 51.2 ± 14)	0						4	18	3	18 (normal control)	T (L2-4): 1.04 ± 0.14, T (FN): 0.844 ± 0.12	T (L2-4): 1.131 ± 0.22, T (FN): 0.908 ± 0.15
Sokhi et al[22]	104 CLD (M: 54, F: 50, Mean age: 54.4 ± 12.9)	All (17)			17		69 (LC-HCV)					T (L2-4): -1.17 ± 0.16, T (FN): 0.97 ± 0.18	T (L2-4): 1.23 ± 0.25, T (FN): 0.98 ± 0.16
Alvisa-Negrin et al[43]	77 ALD (M: 68, F: 9, Mean age: 50.43 ± 10.81), 28 normal controls (M: 25, F: 3, Mean age: 49.83 ± 9.24)	41 ALC, 30 Non-LC						9	77	0	28 (normal control)	[1]T (L): -1.2 ± 1.16, T (FN): -1.1 ± 1.28	[1]T (L): -0.49 ± 0.89, T (FN): -0.5 ± 1.23
González-Reimers et al[40]	124 ALD (Mean age: 50.5 ± 11.23), 38 Controls (Mean age: 48.47 ± 11.08)	51 ALC, 61 Non-LC			124		38 (normal control)					[2]T (L5): -1.17 ± 1.22, T (FN): -1.24 ± 1.38	[2]T (L5): -0.5 ± 0.86, T (FN): -0.36 ± 1.34
Mitchell et al[24]	117 CLD (M: 74, F: 43, Mean age: 50.4 ± 10.5)	Unidentifiable			20		46 (viral CLD)					T (L1-4): -1.51 ± 1.35, T (FN): -1.31 ± 1.02	T (L1-4): -1.08 ± 1.35, T (FN): -1.11 ± 1.07
Mahmoudi et al[25]	109 CLD (M: 72, mean age: 55.3 ± 11.4, F: 37, mean age: 65.2 ± 11)	All (31)			31		43 (LC-HCV)					T (L1-4): -0.34 ± 1.46, T (FN): -0.07 ± 1.45	T (L1-4): -0.83 ± 1.66, T (FN): -0.56 ± 1.49
Wibaux et al[42]	99 CLD (M: 79, F: 20, mean age: 55 ± 8)	All (39)	Vertebra	17	39	19	60 (other CLD)						
Choudhary et al[26]	115 LC (M: 107, F: 8, mean age: 49 ± 5.5)	All (67)						16	67	10	48 (viral LC)		
González-Reimers et al[41]	90 ALD (Mean age: 50.14 ± 10.49), 30 normal controls (Mean age: 50.11 ± 10.4)	40 ALC, 48 Non-LC	Overall fractures	49	90	0	30 (normal control)					[3]T (L): -1.15 ± 1.18	[3]T (L): -0.56 ± 0.9

[1]Measured only in 23 patients for each group; [2]Measured in 117 patients with ALD and 24 patients in control group; [3]Measured in 85 patients with ALD and 28 patients in control group. LC: Liver cirrhosis; ALD: Alcoholic liver disease; BMD: Bone mineral density; CLD: Chronic liver disease; Other CLD: CLD of etiology other than alcohol; L: Lumbar spine; FN: Femoral neck bone; Other LC: Liver cirrhosis of etiology other than alcohol; HCV: Chronic hepatitis C.

Study name	Risk ratio	Lower limit	Upper limit	Z value	P value
Carey 2003	0.651	0.344	1.232	-1.319	0.187
Bonkovsky 1990	0.500	0.202	1.240	-1.496	0.135
Choudhary 2011	1.146	0.570	2.303	0.383	0.701
Kim 2003	1.333	0.347	5.127	0.419	0.675
Alvisa-Negrín 2009	7.064	2.425	117.532	1.363	0.173
	0.849	0.523	1.380	-0.660	0.509

Heterogeneity: $\chi^2 = 5.281$, df = 4 ($P = 0.26$); $I^2 = 24.261\%$
Test for overall effect: $Z = -0.66$ ($P = 0.509$)
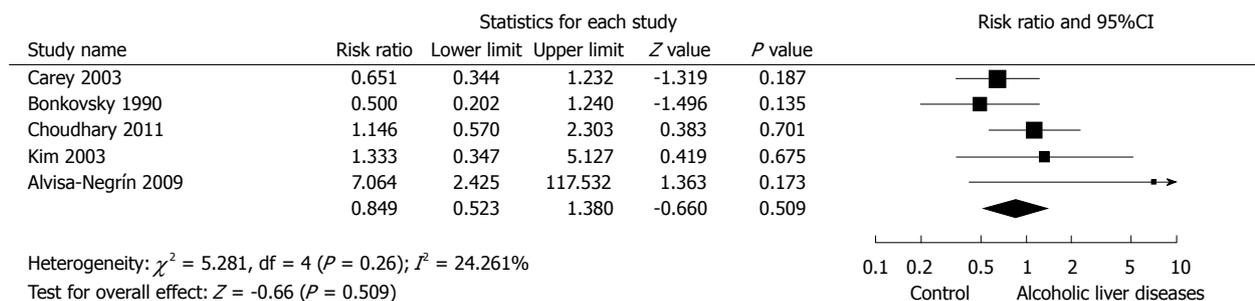
Figure 3 Association between alcoholic liver diseases and osteoporosis. The size of each square is proportional to the study's weight. Diamond is the summary estimate from the pooled studies with 95%CI. ALD: Alcoholic liver diseases; CI: Confidence interval (Random effect model).

Table 2 Methodological quality of included studies measured by Newcastle-Ottawa scale				
Study	**Selection**	**Comparability**	**Exposure or outcome**	**Total**
Lindsell *et al*[44]	2		3	5
Diamond *et al*[46]	3	1	3	7
Bonkovsky *et al*[23]	3		2	5
Ninkovic *et al*[39]	4		3	7
Ninkovic *et al*[21]	3		3	6
Carey *et al*[45]	3	1	3	7
Kim *et al*[47]	3	2	3	8
Sokhi *et al*[22]	3		3	6
Alvisa-Negrín *et al*[43]	3	1	2	6
González-Reimers *et al*[40]	3	1	3	7
Mitchell *et al*[24]	3		3	6
Mahmoudi *et al*[25]	3		3	6
Wibaux *et al*[42]	3		3	6
Choudhary *et al*[26]	3	2	3	8
González-Reimers *et al*[41]	3	1	2	6

sensitivity analysis of osteoporosis in both the high-quality[26,45,47] and low-quality[23,43] studies (RR = 0.885, 95%CI: 0.568-1.381, $P = 0.592$; RR = 0.642, 95%CI: 0.271-1.523, $P = 0.315$).

When analyzing the included studies by the characteristics of the control, the association between ALD and bone fractures was statistically significant both in studies utilizing the normal healthy population as the control arm[41,44] (RR = 7.379, 95%CI: 1.001-54.391, $P < 0.05$) and studies with CLD (etiology other than alcohol) serving as the control arm[39,42,45,46] (RR = 1.629, 95%CI: 1.265-2.099, $P < 0.001$).

In the analysis of the association between ALD and bone fractures, the outlier[41] was noted to have an effect size of 33.725 (RR) (Figure 2). After excluding this outlier[41] in the analysis of the association between ALD and bone fractures, the result was unchanged and was statistically significant (RR = 1.811, 95%CI: 1.370-2.395, $P < 0.001$).

For the association between ALD and osteoporosis, studies using a normal, healthy control-arm[43,47] showed a RR = 1.908 (95%CI: 0.498-7.300, $P = 0.346$), and studies with CLD (etiology other than alcohol) as a control arm[23,26,45] showed a RR = 0.751 (95%CI: 0.474-1.188, $P = 0.221$), which did not differ from the general analysis (Figure 3).

Currently, DXA is recommended for the measurement or monitoring of BMD[48]. A T-score measured by DPA, a different tool for the measurement of BMD, could result in significant bias. Thus, an analysis excluding the study[22] that utilized DPA for the measurement of BMD was performed. The sensitivity analysis for BMD excluding the study that measured BMD by DPA demonstrated results consistent with the full analysis (SMD in femoral neck BMD: -0.157, -0.357-0.043, $P = 0.123$; SMD in spine BMD: -0.073, -0.234-0.088, $P = 0.375$).

Analysis of publication bias
A funnel plot for the enrolled studies was asymmetrical. Egger's regression test revealed that the intercept was 2.442 [95%CI: -0.282-5.167, *t*-value: 2.489, df: 4, $P = 0.034$ (1-tailed) and $P = 0.068$ (2-tailed)]. A trim and fill analysis showed that 1 study was missed or trimmed. The rank correlation test showed a Kendall's tau of 0.800 with a continuity correction [$P = 0.012$ (1-tailed) and $P = 0.024$ (2-tailed)].

A funnel plot for the enrolled studies of the analysis of the association between ALD and osteoporosis is also asymmetrical. However, Egger's regression test revealed that the intercept was 1.644 [95%CI: -2.081-5.368, *t*-value: 1.405, df: 3, $P = 0.127$ (1-tailed) and $P = 0.255$ (2-tailed)]. A trim and fill analysis showed that 2 studies were missed or trimmed. The rank correlation test showed a Kendall's tau of 0.300 with a continuity correction [$P = 0.231$ (1-tailed) and $P = 0.462$ (2-tailed)].

In the evaluation of the BMD measured at the femoral neck between the ALD and control groups, the resulting funnel plot was of a symmetrical shape. Egger's regression test revealed that the intercept was -3.701 [95%CI: -12.537-5.135, *t*-value: 1.076, df: 5, $P = 0.165$ (1-tailed) and $P = 0.331$ (2-tailed)]. A trim and fill analysis showed that no study was missed or trimmed. The rank correlation test showed a Kendall's tau of -0.19 with a continuity correction [$P = 0.274$ (1-tailed) and $P = 0.548$ (2-tailed)].

In the evaluation of the BMD measured at the spine between the ALD and control groups, the funnel plot showed a symmetrical shape. Egger's regression test revealed that the intercept was -6.231 [95%CI:

-12.759-0.296, *t*-value: 2.257, df: 7, *P* = 0.029 (1-tailed) and *P* = 0.059 (2-tailed)]. A trim and fill analysis showed that no study was missed or trimmed. The rank correlation test showed a Kendall's tau of -0.25 with a continuity correction [*P* = 0.174 (1-tailed) and *P* = 0.348 (2-tailed)].

Overall, publication bias was detected in the analysis of bone fractures and osteoporosis. However, there was no evidence of publication bias in the analysis of BMD.

DISCUSSION

In this meta-analysis, bone fracture was associated with ALD, whereas osteoporosis was not associated with ALD. This signifies that the fractures occurring in patients with ALD could be BMD-independent fractures. This result is consistent with the findings of a previous meta-analysis by Kanis *et al*[49]. This study concluded that high intake of alcohol confers a substantial risk for fractures and that this risk is largely independent of BMD[49]. However, there are several considerations when interpreting the results of these studies. The meta-analysis by John A. Kanis *et al*[49] aimed to quantify the fracture risk associated with alcohol consumption in the normal population. As demonstrated in previous studies, light to moderate amounts of alcohol use decrease fracture rates and increase bone density, which is contrary to the results of heavy alcohol consumption[5-15]. This is known as the threshold effect, indicating a J-shaped relationship between alcohol consumption and fracture risk, which was confirmed by another meta-analysis[15]. However, studies in the normal population have the problem of spectrum bias. The proportion of heavy alcoholics in the cohort dictates the threshold, and studies with a low proportion of heavy alcohol users lack the power required to examine this effect[49]. Moreover, these studies have the limitation of timing for alcohol consumption measurement and self-reported alcohol consumption, which could be inaccurate[15]. Contrary to the studies consisting of the normal population, our study enrolled articles utilizing the ALD population. Considering that ALD is the deleterious outcome of chronic alcohol abuse, these limitations could be minimized in our study.

In the present study, however, there was also substantial methodological heterogeneity between the enrolled studies, which had a potential effect on the risk estimates. This phenomenon was evaluated by sensitivity analyses for the confirmation of the robustness of this meta-analysis. The most noticeable modifier was the different populations used as control groups among the enrolled studies. Previous studies have suggested that metabolic bone disease is prevalent in CLD, especially in cholestatic liver disease[17,50]. Thus, studies with CLD as a control group run the potential risk of reporting an underestimated

effect size (*vs* ALD). Moreover, CLD is a complex and vague terminology that cannot include specific populations. ALD may include alcoholic fatty liver, alcoholic hepatitis, and liver cirrhosis of alcoholic origin. Despite the anticipated problems described above, the main analysis and the sensitivity analyses divided by control-group (normal population *vs* CLD) showed consistent results regarding bone fractures, osteoporosis, and BMD. The proportion of liver cirrhosis in ALD was unidentifiable in most of the enrolled studies. Future studies using homogenous populations are needed to determine the applicability of these results to the subpopulations of ALD or CLD.

Another modifier was the quality of the enrolled studies. The included studies were classified as being either in the high-quality ($\geq$ 7 stars) or the low-quality group (< 7 stars). This standard was decided by the authors. The average number of stars awarded in 15 studies was 6.4 (Table 2). Of note, 8 studies were awarded zero stars in the section of comparability. Despite the pitfalls of methodological quality, the sensitivity analyses divided by study quality showed consistent results. High-quality studies are needed for the wide application of these results.

During the main and sensitivity analyses, a significant outlier[41] was observed. In the analysis of the association between ALD and bone fractures, the effect size of the outlier (RR = 33.725) was more than 10 times the adjusted effect size (RR = 1.944) (Figure 2). The analysis excluding the outlier showed consistent results. The reason for the large effect size of the outlier is postulated to be a methodological problem. In this study, the presence of fracture was recorded by anamnesis and chest X-ray film[41]. This inaccurate methodology could overestimate the rate of bone fractures. Moreover, the quality measured by NOS was low (6 stars) in this study.

In terms of the main mechanism behind the pathophysiology of bone fractures and osteoporosis in ALD, it is inferred that alcohol causes an imbalance in bone remodeling with a predominant decrease in bone formation[4,15,51]. Alcohol is known to cause direct effects on the numbers and activity of osteoblasts and osteoclasts[4]. In addition to the direct effects of alcohol on the osteoblasts and osteoclasts, many indirect effects have also been reported. These indirect effects are mainly linked to impaired nutrition, which leads to weight loss, decreased fat and lean body mass, and hormone alterations, which may change in bone cell activity[4,15,51]. More recent studies indicated the effects that alcohol have on bone mass may be due to a suppression of the Wnt/DKK1 signaling pathway through the stimulation of oxidative stress[52,53]. With an accumulating body of evidence regarding the effects alcohol has on the skeletal system, a more detailed pathophysiology could be confirmed in the near future.

This study is the first meta-analysis examining the association between ALD and bone fractures

or osteoporosis. The strength of this study is the evaluation of 3 effect sizes. The main result was confirmed, compared, and interpreted with regard to the other effect sizes. Another strength of this analysis is the selection of ALD for the study population. As described above, biases from various standards of alcohol consumption (light, moderate, heavy, or binge drinking), inaccurate amounts of alcohol consumption from self-reporting, and inconsistent timing for the measurement of alcohol consumption could be minimized. Potential modifiers were detected whenever possible from the articles and evaluated through the sensitivity analyses.

Despite its strengths, there are several limitations within the present study. First, there was no consideration given to any potential confounders, which could be influential to bone fractures and osteoporosis. These include sex, age, menopausal status, bone fracture history, family history of bone diseases or fractures, smoking, medications such as corticosteroids and body composition. However, most the important confounder is assumed to be trauma, such as frequent falls in patients with ALD. As noted in many studies, this powerful but easily overlooked confounder should be included *via* a thorough history in the studies relevant to this topic[4,15]. Second, the Newcastle-Ottawa scale was used to assess the methodological quality of the studies. This scale has been criticized for its low agreement between authors and reviewers[54]. Alternative tools such as the risk of bias table by the Cochrane group have been proposed as an alternative approach. However, this particular tool has strength in the evaluation of randomized studies, and poor inter-rater agreement was also noted[55]. Additional validated and commonly used tools are needed.

In conclusion, current publications indicate a significant association between bone fractures and ALD, independent of BMD or the presence of osteoporosis. Due to the qualitative and quantitative heterogeneity among studies, further studies utilizing homogenous populations and controlling for confounding risk factors for fractures are needed to elucidate the underlying mechanism of bone fractures in ALD.

COMMENTS

Background
Chronic excessive alcohol consumption is a well-established risk factor for low bone density and bone fractures. However, ingestion of light or moderate amounts of alcohol is known to be associated with higher bone density and decreased fracture rates. Osteoporosis and bone fractures are frequently overlooked complications in patients with chronic liver disease that could result in serious outcomes. However, the exact prevalence or mechanism of osteodystrophy in patients with alcoholic liver disease (ALD), which is the deleterious outcome of chronic alcohol abuse, have not been described

Research frontiers
Meta-analysis by John A. Kanis concluded that high intake of alcohol confers a substantial risk for fractures and that this risk is largely independent of bone mineral density (BMD). However, this study included normal population and there has been no such analysis in the ALD population.

Innovations and breakthroughs
From the fifteen non-randomized studies, ALD demonstrated a RR =1.944 (95%CI: 1.354-2.791) for the development of bone fractures. However, ALD showed a RR = 0.849 (95%CI: 0.523-1.380) for the development of osteoporosis.

Applications
Current publications indicate significant associations between bone fractures and ALD, independent of BMD or the presence of osteoporosis. Although the mechanism of bone fractures in ALD is not totally understood, further research utilizing a homogenous population and controlling for confounding risk factors for fractures could elucidate the mechanism.

Terminology
ALD: Excessive alcohol consumption is associated with a spectrum of hepatic manifestations, including alcoholic fatty liver, alcoholic hepatitis, and cirrhosis. These diseases share the core cause of hepatic injury which is an excessive alcohol consumption and generally refers to alcoholic liver disease.

Peer-review
The study addresses an interesting topic and merit. However, it has some intrinsic methodological limitations which decrease its potential clinical impact.

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CASE REPORT

Resolution of Crohn's disease and complex regional pain syndrome following treatment of paratuberculosis

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Abstract

A cohort of family members with various chronic diseases including Crohn's disease, asthma, complex regional pain syndrome, hypothyroidism, type 1 diabetes mellitus, and lymphangiomatosis and/or evidence of infection by *Mycobacterium avium* subsp. *paratuberculosis* (MAP) are described in this series of case reports. MAP was cultured from the blood of three members affected by the first five diseases and there was accompanying elevated anti-MAP IgG in two members. The patient affected by the sixth disease has a markedly elevated anti-MAP titer. The two patients affected by the first four diseases have been treated with a combination of anti-MAP antibiotics and ultraviolet blood irradiation therapy with resolution of the disease symptomatology and inability to culture MAP in post treatment blood samples. These case reports of patients with MAP infections provide supportive evidence of a pathogenic role of MAP in humans.

Key words: Crohn's disease; Complex regional pain syndrome; Lymphangiomatosis; *Mycobacterium avium paratuberculosis*; Cure

Core tip: Five patients with multiple diseases of unknown etiology were found to have evidence of infection by *Mycobacterium avium* subsp. *paratuberculosis* (MAP) including positive blood cultures (except in case 4). Two of the cases (case 1 with Crohn's disease and asthma and case 2 with complex regional pain syndrome, hypothyroidism and Raynaud's phenomenon) have been treated with a combination of anti-MAP antibiotics and ultraviolet blood irradiation therapy with resolution of the disease symptomatology and inability to culture MAP in post treatment blood samples. These case reports of patients with MAP infections provide supportive evidence of a pathogenic role of MAP in humans.

INTRODUCTION

In 1998, David Relman described features of a number of poorly understood clinical syndromes that strongly indicate a microbial etiology. His list of chronic inflammatory diseases with possible microbial etiologies included sarcoidosis, inflammatory bowel disease, rheumatoid arthritis, systemic lupus erythematosus, Wegener granulomatosis, diabetes mellitus, primary biliary cirrhosis, tropical sprue and Kawasaki disease[1]. He noted that molecular methods of microbial identification offer an alternative when culture based microbial detection methods fail. His prediction regarding the emerging importance of molecular methods has proven correct since the combination of molecular methods of microbial detection and improvements in culture methods has led to advances in the field of paratuberculosis.

Mycobacterium avium subsp. *paratuberculosis* (MAP) is a bacterium that causes Johne's disease, a chronic diarrheal wasting disease in cattle[2] and sub-human primates[3] and a chronic wasting disease in sheep and goats[2]. In Johne's disease, it is well documented that once an animal is infected with MAP, the MAP bacterium grows and multiplies inside the macrophages of the immune system. The organism is excreted in the feces, and to a lesser extent in milk[2]. Outside the host animal, MAP multiplies poorly, but can survive for extended periods in the environment because of its resistance to heat, cold and the effect of drying[2]. This slow-growing bacterium affects the ileum and causes diarrhea and cachexia. There are anecdotal reports of Johne's disease in which prolonged administration of antibiotics resulted in suppression but not cure of the disease[4].

The viable bacterium has been found in commercially available pasteurized milk[5,6]. Ellingson *et al*[6] reported that 2.7% of retail pasteurized milk samples purchased in Wisconsin, Minnesota and California contained viable MAP. Because of the presence of this organism in the food supply, it would not be surprising if MAP is widespread in the environment and the human population. The first mass screening study for evidence of MAP infection in humans was done in North India on serum, blood and stool samples submitted from patients with multiple medical conditions including diabetes, liver disorders, anemia, thyroid, tuberculosis, typhoid, abdominal disorders, inflammatory illness and ion imbalance. Singh *et al*[7] reported that 34% of 23196 serum samples had anti-MAP antibodies (a comparison with normal subjects was not included). The same study showed that 12.7% of 1246 blood samples from normal healthy individuals had IS900 PCR evidence of MAP in their blood and 8.4% of 3093 blood samples from patients with the above listed medical conditions had PCR evidence of MAP.

It has been suggested for years that there may

be an association between Crohn's disease (CD) and Johne's disease. Dalziel first speculated in 1913 that chronic enteritis, now known as CD, might be caused by MAP[8] and Chiodini first reported the culturing of mycobacteria from the intestinal tissues of CD patients[9]. For many years, the data were conflicting[2,10,11] and the theory that MAP causes CD remains controversial[12-14]. Later on, Hermon-Taylor and others described a case of a boy with cervical lymphadenitis caused by MAP who later developed CD[15]. Recent studies show an increase in the detection and isolation of MAP in adult Crohn's patients[16] and in children with newly diagnosed CD[17] Meta-analyses by Feller *et al*[18] and Abubakar *et al*[19] have concluded that a majority of studies on the association of MAP and CD show that most patients with CD have MAP infection. In 2004, Naser *et al*[20] reported culturing MAP from the blood of 50% of patients with CD and this work was confirmed in four laboratories including the Centers for Disease Control and Prevention[21,22].

In addition, a large, randomized, double-blind, placebo-controlled study from Australia showed a significant but not lasting response of individuals with CD who were treated with antibiotics against MAP[23] Apparently unaware that antibiotics fail to cure a majority of patients with *Mycobacterium avium* complex infection (MAC)[24], the authors incorrectly concluded that because they failed to cure patients, CD could not be caused by mycobacterial infection. This study and the conclusions of its authors were significantly flawed[25,26]. The editorial which accompanied the article acknowledged that, "subtherapeutic doses of rifabutin (450 mg), clarithromycin (750 mg) and clofazimine (50 mg) per day were used, whereas the optimal dose of rifabutin, clarithromycin and clofazimine for treatment of *M avium* complex infections is 600 mg/d, 1000-2000 mg/d, and 100 mg/d, respectively"[27]. A recent meta-analysis of antibiotic trials in CD conducted by Feller and coworkers concludes that a substantial benefit was evident in trials using nitroimidazoles, clofazimine and ciprofloxacin and that a combination of clarithromycin and rifamycin and ciprofloxacin should be studied[28].

Most research attention in inflammatory bowel disease has focused on the genetics of CD rather than the association of the disease with paratuberculosis infection. However, these two areas of research are probably complementary because the genetic mutations, which have been described, may indicate increased susceptibility to MAP infection. Also in cattle, NOD2 mutations are associated with susceptibility to MAP infection[29], and this same mutation has been linked to patients with CD. A 2009 study from China showed that patients with another mycobacterial infection, leprosy, and patients with CD have higher rates of the NOD2, TNFSF15 and IL12B mutations than healthy controls[30,31]. A large meta-analysis genome-wide association study concluded that there is considerable overlap between the susceptibility loci for IBD and mycobacterial infection[32]. In the only simulated human-challenge trial, Israeli researchers showed that fetal human small intestine explants in mice with severe combined immunodeficiency and then inoculated with MAP intraluminally, showed invasion of the goblet cells, tissue damage and inflammation[33].

In 2006, Dow postulated that MAP may be the trigger for type I diabetes mellitus (T1DM)[34] because of the association of T1DM with mutations of the SLC11a1 gene[35]. This gene encodes a membrane protein of the lysozymes of monocytes and macrophages. Mutations in this gene have been associated with susceptibility to infectious diseases including tuberculosis and leprosy and lead to a more hospitable host environment for bacterial survival and replication[36]. Subsequently, Sechi and others reported an association of MAP and T1DM[37-39]. Recently, Naser *et al*[40] showed that there is a high degree of homology between GAD65 and Hsp65 which supports a mycobacterial role in the immune destruction of the beta cells of the pancreatic islets through molecular mimicry.

Additional findings in T1DM also present in other mycobacterial infections include elevated angiotensin converting enzyme (ACE) levels[41,42] and elevated vascular endothelial growth factor (VEGF)[43]. VEGF has been reported to be elevated in active pulmonary tuberculosis and to decline following successful treatment[44]. Some 24.5% of patients with T1DM have a positive *Saccharomyces cerevisiae* Antibody (ASCA) test which is similar to the frequency of ASCA positivity in Crohn's disease[45]. Consumption of milk is a risk factor for the development of T1DM[46,47].

Frau and others have also reported an association of MAP and multiple sclerosis (MS)[48]. Consumption of milk is also a risk factor for the development of MS[49].

The following case reports demonstrate an association of MAP with several of the above described diseases as well as with two diseases which have not yet been linked to MAP. In addition, the diseases which were treated with anti-MAP therapy resolved.

Assays for evidence of MAP infection

Three assays were performed on EDTA blood samples from each patient (Figure 1). The plasma was assayed for antibodies to MAP by ELISA using culture filtrate antigens of MAP strain UCF-4 as described[50].

Peripheral blood leukocytes were harvested and used for DNA extraction followed by IS*900* PCR as described[51]. The remainder of the leukocytes were inoculated into BACTEC MGIT ParaTB medium with supplements but without antibiotics and incubated for 6 mo at 37 ℃. After incubation, the culture pellets were harvested and subjected to DNA extraction, followed by nested IS*900* PCR as described[22]. Subcultures were done on all PCR-positive MGIT cultures to attempt recovery of MAP in pure culture.
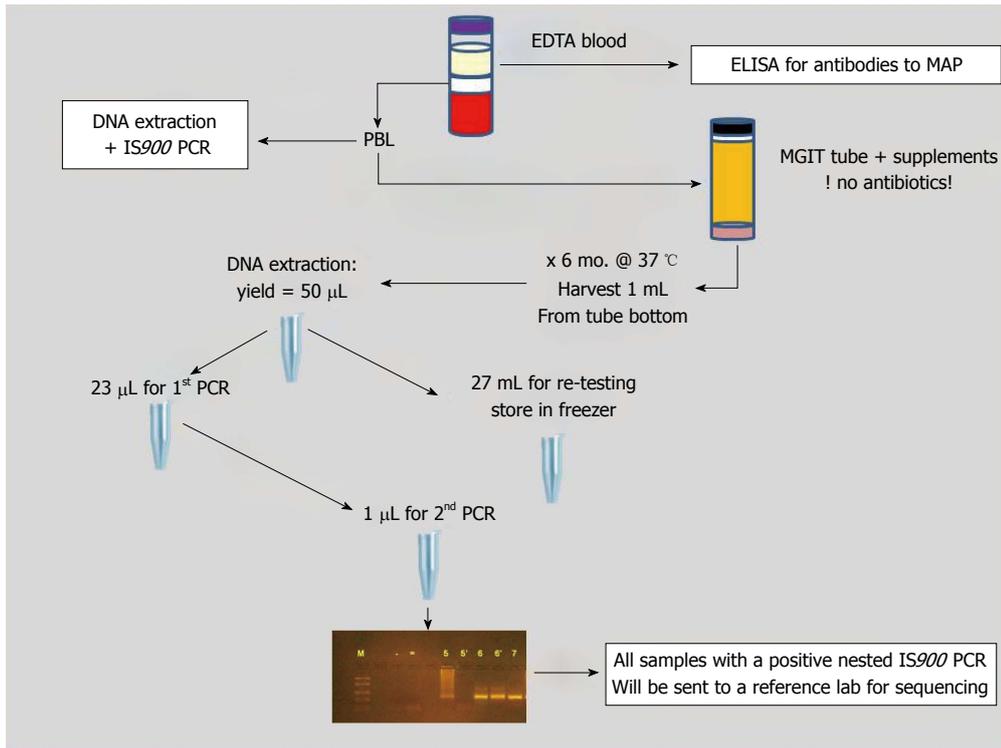
Figure 1 Schematic of sample processing and testing methods.

Figure 2 Terminal ileum with multiple ulcers.

Assay for evidence of leprosy

One assay was performed to detect antibodies to *M. leprae* based on phenolic glycolipid-1 antigen. The assay was performed as described earlier for Para-LP-01 based lipid-ELISA for Johne's disease[52]. Wells were coated with 100 ng PGL-1 dissolved in iso-propanol and dried. Plates were blocked for one hour at room temperature with 100 μL 3% BSA (in PBS, pH 7.4). ELISA was then performed[52]. One hundred μL of subject serum diluted 1:20 in 10% FBS/PBS (pH 7.4) was added to the wells and incubated for 30 min at room temperature. Plates were washed three times with PBS followed by adding secondary conjugated antibody (sheep anti-human IgH-h+1 HRP conjugated antibody diluted 1:2000 in 10% FBS/PBS). 100 μL of secondary

antibody solution were added per well and plates were incubated at room temperature for 30 min. Plates were washed as before and 100 μL of room temperature 3,3',5,5'-tetramethylbenzidine were added per well. Plates were incubated at room temperature for 10 min. The reaction was stopped by adding 100 μL of 2 mol/L sulfuric acid per well. Plates were read at 450 nm using an iMark Microplate Reader (BioRad).

CASE REPORT

Case 1

At the Children's Hospital of Wisconsin, Dr. Grzegorz Telega began following a 9-year-old boy who was diagnosed with CD in June 2004. He initially presented in 2004 with persistent diarrhea, weight loss and unexplained fever. His linear growth had slowed considerably. Colonscopy and upper gastrointestinal endoscopy showed multiple aphthous ulcers in the colon, terminal ileum and stomach (Figure 2) and biopsies obtained in the colon and gastric antrum contained the granulomas of CD (Figure 3). His erythrocyte sedimentation rate (ESR) and C reactive protein (CRP) were increased.

MAP testing was performed on the patient's blood. The initial sample showed mildly elevated antibody titers to one of the MAP antigens, p35, and after several months of incubation, MAP was grown from the patient's blood. The second sample drawn more than 3 mo later showed greater elevations of antibodies to

Table 1 Summary of *Mycobacterium avium* subsp. *Paratuberculosis* antibody, PCR and culture results for cases 1 through 5

	Case 1	Case 2	Case 3	Case 4	Case 5
MAP Ab	5/11/04 p35-0.25 p36-0.16	11/6/12 ELISA S/P 1.24	1/14/13 ELISA S/P 0.49	3/14/13 ELISA S/P 1.72	1/10/13 ELISA S/P 0.15
MAP PCR	5/11/04 negative	11/6/12 negative	1/14/13 negative	3/14/13 negative	1/10/13 negative
MAP culture	5/11/04 positive	11/6/12 negative	1/14/13 negative	3/14/13 negative	1/10/13 positive
MAP Ab	8/18/04 p35-0.5 p36-0.3	11/20/12 ELISA S/P 1.31		Month/year-pending	
MAP PCR	8/18/04 negative	11/20/12 negative		Month/year-pending	
MAP culture	8/18/04 positive Anti-MAP therapy started 8/19/04	11/20/12 positive Anti-MAP therapy started 12/15/12	1/18/13 positive	Month/year-pending	
MAP Ab	9/20/04 p35-0.33 p36-0.22	4/17/13 ELISA S/P 1.20			
MAP PCR	9/20/04 negative	4/17/13 negative			
MAP culture	9/20/04 positive	4/17/13 negative			
MAP Ab	7/9/07 negative	5/7/14 ELISA S/P 1.69			
MAP PCR	7/9/07 negative	5/7/14 negative			
MAP culture	7/9/07 negative	5/7/14 negative			
MAP Ab	5/27/14 ELISA S/P 0.67				
MAP PCR	5/27/14 negative				
MAP culture	5/27/14 negative				

MAP: *Mycobacterium avium* subsp. *Paratuberculosis*.
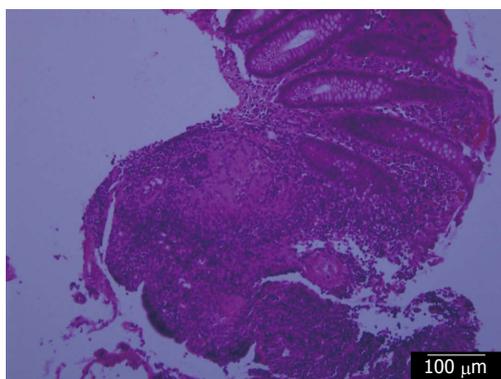
Figure 3 Biopsy from the colon showing a granuloma of Crohn's disease.

both p35 and p36 antigens and also grew MAP (See Table 1 for the summary of MAP testing in this and the subsequent 4 cases). During the 3 mo between the initial and second sample, the patient's clinical condition steadily worsened with increasing abdominal pain and frequency of diarrhea. At the time of the initial diagnosis, the patient was 4 feet 8.75 inches or in the 95th percentile in stature and weighed 71.8 pounds (75th percentile). Prior to the onset of illness, his weight had previously reached 80 pounds (90th percentile). Initially, in August 2004, the patient received azathioprine and steroids with concurrent antibiotic therapy including clarithromycin and rifabutin, in low doses similar to those used in the Australian trial[23]. Dr. Telega, the pediatric gastroenterologist, prescribed the antibiotics and received consultative advice initially from Dr. Hermon-Taylor and later additionally from Drs. Chamberlin and Borody. The patient also took daily probiotics, which were administered at mid-day. After 7 d of antibiotic therapy, as predicted by Dr. John Hermon-Taylor the patient developed a mild fever that lasted for several days, which Dr. Hermon-Taylor had previously observed in other patients and compared to a Jarish Herxheimer reaction. Because of an elevated ALT and AST, the azathioprine was discontinued in
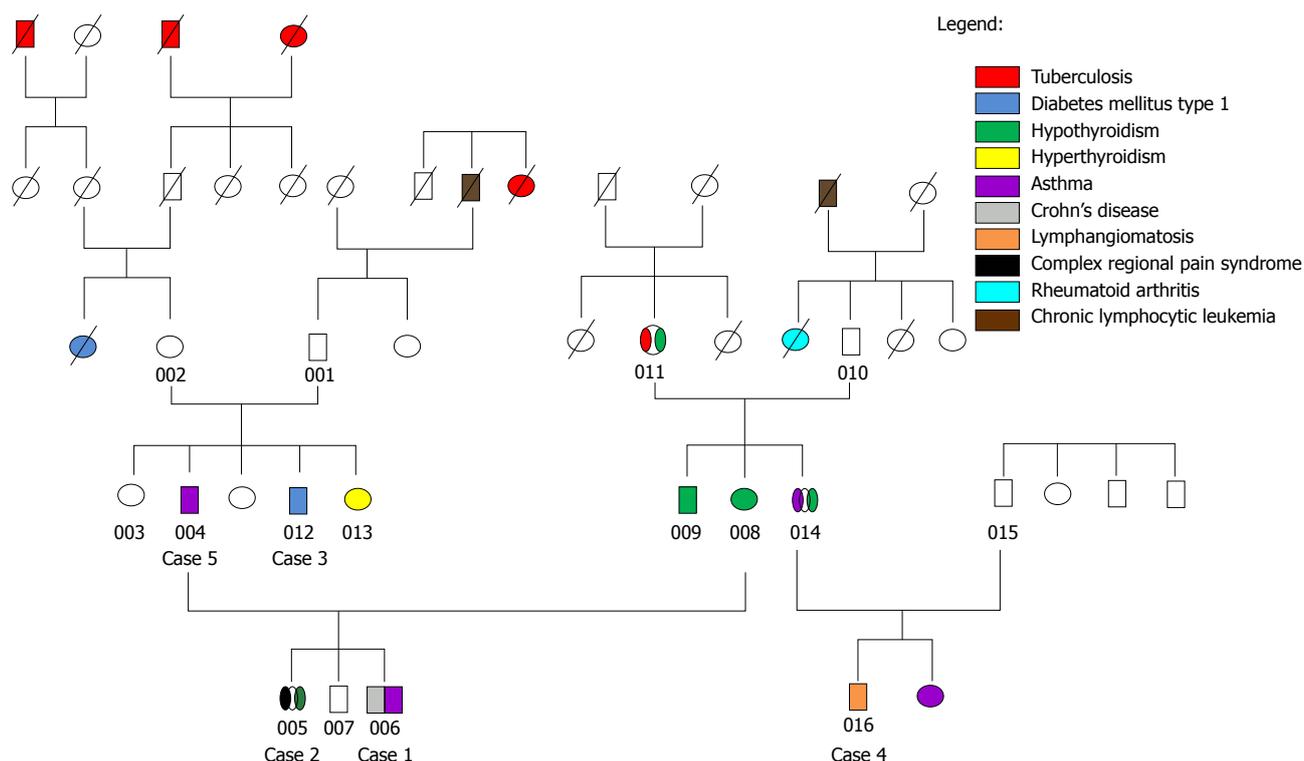
Figure 4 Family pedigree summarizing history of mycobacterial infection and other diseases of cases 1 through 5 and additional family members.

December 2004. The patient responded favorably to the antibiotics for about 8 mo, but by June 2005, he became symptomatic and relapsed (a finding similar to that of the Australian trial). The period of relapse lasted from June 2005 to March 2006 and during this time he remained on low dose antibiotics. A colonoscopy on January 11, 2006 showed multiple aphthous ulcers in the colon and his weight on that day was 77.3 pounds (35 kg). On January 13th a short course of prednisone was initiated at a dose of 10 mg/d. On January 15th, the dose of prednisone was increased to 20 mg/d. By February 11th, his weight was 90 pounds (41 kg).

In late 2005, in addition to receiving antibiotics, over the course of a three-month period, the patient received a total of 11 once weekly ultraviolet blood irradiation (UVBI) treatments which were performed by Dr. Mitchell Kurk at his office in Long Island, New York. A similar UVBI device has been successfully advanced through phase II clinical trials at the FDA.

In addition to UVBI therapy, on advice from experts, the doses of clarithromycin and rifabutin were increased and ciprofloxacin was added to the regimen. On January 13, 2006, the patient was started on ciprofloxacin at a dose of 125 mg taken twice per day (7 mg/kg per day) and two weeks later this dose was increased to 250 mg taken twice per day (14 mg/kg per day). On February 12, 2006, when the patient weighed 90 pounds (41 kg) the dose of clarithromycin was increased to 750 mg, 500 mg taken in the am and 250 mg taken in the pm (18 mg/kg per day) and the dose of rifabutin was increased to 450 mg taken

150 mg in the am and 300 mg in the pm (11 mg/kg per day). In May 2006, after the patient was in clinical remission, clofazimine (an antibiotic with restricted use in the United States which is used for the treatment of leprosy and *Mycobacterium avium* complex) was added at a dose of 50 mg taken once daily. The clofazimine was obtained from a source in Australia.

The patient had a history of seasonal (triggered by pollen) asthma beginning at age 3 years and the last episode of asthma he has experienced was in April 2006.

These antibiotics have been used in many prior studies to treat MAP in humans. The doses in this patient were adjusted over time. He received over 4 years of continuous antibiotic therapy until January 2009. From January 2009, he was on cycled therapy of rifabutin, ciprofloxacin and clarithromycin until May 2011. The patient has been in complete remission since April 2006.

Since May 2011, he has received no medications of any type and he has been without any signs or symptoms of CD and is now 5 feet 10.5 inches and 185 pounds (84.1 kg). A follow-up blood culture for MAP in July 2007 failed to recover MAP by culture or detect MAP DNA by PCR and he tested negative for anti- MAP antibody. Currently, he has a normal blood count and is negative for inflammatory markers including ESR and CRP. A colonoscopy and upper gastrointestinal endoscopy in August, 2014 were normal. There are many reports in the literature of patients with CD who have responded favorably to antibiotic therapy[28,53-55].

Case 2

In early 2012, the sibling of case 1, a 23-year-old female began experiencing symptoms initially thought to be carpal tunnel syndrome and by August 2012, developed Raynaud's phenomenon in both hands. She had a several year history of hypothyroidism and was on thyroid hormone replacement. The symptoms of neuralgia and paresthesia progressively advanced and involved her bilateral hands, elbows, shoulders, neck, legs and feet. By the time she was seen at the Cleveland Clinic Neurological Center for Pain in late November, the physician who examined her noted Raynaud's phenomenon in both hands and described the purple color change and cold temperature as profound.

Her workup included a normal EMG study, normal CT scan of the brain, and normal values for procalcitonin, ESR, CRP, IL-6, ASCA IgA and IgG, rheumatoid factor, ANA, SS-A/RO, SS-B/LA, SCL, RNP, SM, CCP, JO-1, Centromere antibodies, Anti-Hu TTG-IgA, lyme serology, gliadin peptide IgA and IgG, anti-endomysial IgA, serum MPA IgG, MPA IgA, MPA IgM, MPA kappa, MPA lambda, MPA kappa/lambda ratio, glutamic acid decarboxylase antibody and ganglioside antibody studies. Because of a history of travel to Guatamala 5 years prior to the onset of her illness, the patient's blood was tested for antibody to *M. leprae*. The PGL-1 assay was negative. The initial diagnosis at the Cleveland Clinic was hypersensitivity syndrome and the patient was referred to the Cleveland Clinic Neurological Center for Pain where she received the diagnosis of thoracic outlet syndrome with probable evolving complex regional pain syndrome (CRPS).

Recommendations for therapy included physical therapy, muscle relaxants and gabapentin. Gabapentin at the lowest recommended dose made her very dizzy and therefore, she discontinued this medication. The patient obtained multiple sessions of physical therapy which were beneficial and engaged in gradually increasing regular exercise including walking and swimming as tolerated. In December 2012, she could only walk 300 feet or tread water wearing a floatation device for 5 min. The cause of this condition is unknown.

Due to suspicion that CRPS could be a manifestation of a MAP infection, blood samples were tested for evidence of MAP infection; the first blood sample was obtained November 6, 2012 and the second on November 20, 2012. The results of the MAP ELISA assays from both samples showed significantly elevated titers, S/P values of 1.24 and 1.31 respectively, where the positive control serum was from a veterinarian who had accidentally injected himself with the MAP vaccine. The MAP PCR tests were both negative. MAP was detected by culture from the second blood sample. There was rapid progression of clinical disease between November 6, 2012 when her MAP antibody titer was 1.24 and the organism could not be cultured while she had monocytosis and lymphocytosis and November 20, 2012 when her antibody titer increased to 1.31 and the organism could now be cultured while she no longer had monocytosis and lymphocytosis. During this two week period she developed generalized extreme hypersensitivity to minor tactile stimuli. MAP experts were consulted and appropriate antibiotics were prescribed.

Other diagnostic test results included elevated cryoglobulins of 57 (normal 0-50 ug/mL) and ACE level of 59 (normal 8-53 U/L). Cryoglobulins [57-59] and ACE[60,61] are elevated in other mycobacterial infections including tuberculosis and leprosy. Prior to the onset of disease and the initiation of therapy, the patient had persistent relative lymphocytosis and eosinophilia which was present as early as 1997. Relative lymphocytosis has been described in tuberculosis[62].

Neurologic findings are not uncommon in CD[63]. In addition, siblings of patients with CD are at much higher risk of developing CD than the general population[64].

In mid December 2012, the patient was placed on anti-MAP therapy and supplementary Vitamin A and Vitamin D similar to that administered to her brother. Her height and weight are 5 feet 9.5 inches and 150 pounds (68.2 kg), respectively and her antibiotic doses were as follows: Clarithromycin 500 mg twice daily (15 mg/kg per day), rifampin 300 mg twice daily (9 mg/kg per day), levofloxacin 500 mg per day (7 mg/kg per day) and clofazimine 100 mg 3 times per week (4 mg/kg per week). Four days after the initiation of therapy she experienced a mild fever which lasted two days. Dr. Stuart Weg performed 12 UVBI treatments at weekly intervals for 3 mo from January through early April 2013. Previously, Weg speculated that CRPS is due to an infection caused by a cell wall deficient bacterium[65].

Dr. David Haas of the University of Charleston Chemistry Department, confirmed by gas chromatography, mass spectroscopy, ultraviolet absorption spectroscopy and infrared spectroscopy that the clofazimine, which was imported from India, was not a counterfeit drug.

Following the initiation of therapy, she developed monocytosis and the relative lymphocytosis persisted. Since that time, she has shown marked clinical improvement including disappearance of the generalized hypersensitivity, disappearance of the previously grossly visible Raynaud's phenomenon in her hands, and improved ability to perform motor skills with a reduction in reported pain. By the fall of 2014, she could swim one mile or walk five miles per day. Although her general condition has greatly improved including absence of the generalized extreme hypersensitivity, she still experiences episodes of migratory pain. With treatment of leprosy, reversal reactions and prolonged neuralgia have been observed[66].

Six weeks after beginning the anti-MAP therapy, while still taking supplemental thyroxine, she began experiencing palpitations and it was noted that her TSH had dropped to the low normal range. On the

presumption that the palpitations indicated that her thyroid function was recovering, in January 2013, she stopped supplemental thyroxine, has not experienced symptoms of hypothyroidism, and her TSH is now in the normal range. A TSH from May 7, 2014 was 4.06 μIU/mL (reference range 0.350-5.55 μIU/mL). An ACE level from May 7, 2014 was still elevated at 58 U/L and a complete blood count from the same day was normal except for mild monocytosis of 9.3 % (reference range 0%-8%) and eosinophilia of 9.5% (reference range 0%-4%). By October 14, 2014, a complete blood count and differential were normal.

After four months of therapy (April 7, 2013), a follow-up blood culture for MAP showed a minimally decreased MAP ELISA S/*P* value of 1.2, the MAP PCR test was negative and MAP could not be cultured from this sample. A follow-up cryoglobulin study obtained from April 17, 2013 was negative after 4 h and positive after 72 h. In early January 2014, the patient consulted Dr. Kuruvilla John who has since that time followed her case.

Case 3

Since two siblings had evidence of MAP infections and responded to anti-MAP therapy, other relatives were tested. The paternal uncle of cases 1 and 2, who has longstanding T1DM, is also infected with MAP. In addition, the uncle was found to have elevated ASCA IgA, a serologic marker, which is present in T1DM and CD[45]. The uncle's MAP serum antibody S/*P* value was 0.49 (negative). The patient has declined treatment for MAP.

Case 4

The nephew of the mother of cases 1 and 2 has lymphangiomatosis, a disease of unknown etiology. His blood showed a MAP ELISA antibody S/*P* value of 1.72 (exceptionally high). His MAP PCR and MAP culture results were negative. He had a very elevated VEGF of 506 pg/mL (reference range of 31-86 pg/mL), mild monocytosis of 992 (reference range of 200-950 cells/uL) and a normal neopterin test, ASCA IgA and IgG, and ACE tests. The MAP ELISA study, VEGF and monocytosis in this case suggest a possible mycobacterial causation of lymphangiomatosis and further study is indicated.

Case 5

The father of cases 1 and 2 was tested for MAP infection. After 6 mo of incubation, MAP was grown from his blood. His MAP PCR on PBMCs was negative and his MAP ELISA antibody S/*P* value was 0.15 (negative). He is healthy but suffered from seasonal asthma (triggered by pollen) at age 12 years and also while living in Germany from 1986 to 1989. In addition, he has rosacea, which was diagnosed by clinical signs and a skin biopsy showing non-caseating granulomas. This condition is treated with a topical ointment containing azelaic acid. In 2004, his blood was found positive for antibodies to p35 and p36 MAP antigens.

Because of the devastating nature of the diseases in case 1 and case 2 and the poor record of efficacy, standard therapies were eschewed. Institutional review board (IRB) approval was not sought since the law allows off label use of FDA approved drugs and also allows the administration of UVBI in New York. IRB approval is generally not required in the care of individual patients. In cases 1 through 3, infectious disease specialists were consulted and informed about the elevated MAP ELISA antibody titers and/or positive MAP cultures but declined to make recommendations regarding therapy.

Additional family members were tested for evidence of MAP infection as well. The mother of case 1 and case 2 was negative for MAP by PCR on PBMC and culture, and had an ELISA S/P of 0.08 (negative). The brother of case 1 and case 2 had a negative MAP PCR and negative culture and ELISA S/P of 0.59 (slightly elevated). The maternal grandfather of case 1 and case 2 had a negative MAP PCR on PBMCs and negative culture and an ELISA S/P of 0.13 (negative). The maternal grandmother (with hypothyroidism) of case 1 and case 2 had a negative MAP PCR on PBMCs and negative MAP culture and an ELISA S/P of 0.0 (negative).

The families of both parents of case 1 and case 2 have a history of susceptibility to mycobacterial infection. Figure 4 which is a family pedigree summarizing the cases and the mycobacterial infection and other disease history in other members.

DISCUSSION

The presence of viable MAP in the blood of a majority of CD patients is an important finding which has been previously reported by Naser[20,22]. Some observers ascribe this phenomenon to the "leaky bowel" resulting from mucosal disruption in CD[67]. In case 1, the recovery of the viable organism in the setting of two diseases and the failure to recover the viable organism in the absence of these two diseases argues in favor of a pathogenic role of MAP in these patients. Similarly, in case 2, the recovery of the viable organism in the setting of two other diseases and the failure to recover the organism in the absence of these two other diseases also argues in favor of a pathogenic role of MAP. Furthermore, the recovery of the viable organism in case 2 in which the patient suffered from CRPS cannot be explained by the leaky bowel hypothesis since this patient has not experienced bowel related symptoms. In addition, a pathogenic role of MAP in the human host is likely, considering the zoonotic capacity of slow-growing mycobacteria and because this organism is an obligate pathogen, *i.e.*, one which does not propagate in the environment[68].

A second possible interpretation of the findings in these case reports is that the diseases were not caused by MAP and went into remission spontaneously. In the consideration of the probable events in case 1 and case 2, the percentage of patients who experience long term remissions in CD, CRPS, hypothyroidism and Raynaud's phenomenon is 10%[69], 74%[70], 62%[71], and 64%[72], respectively. With the assumption that case 1 and case 2 resolved spontaneously, the outcome follows the likelihood function. The probability of spontaneous resolution in case 1 is 0.10 and in case 2 is 0.30 (0.74 × 0.62 × 0.64 = 0.30) and the probability of spontaneous resolution in both patients is 0.10 × 0.30 or 0.03 which is very unlikely. Controlled clinical trials of anti-MAP therapy are necessary to determine whether these case reports are reproducible. Clinical trials have been designed and funding is being sought.

A third possible interpretation of the recovery of MAP from the blood samples in case 1 and in case 2, is that the MAP organism is a contaminant from specimen processing. This interpretation is unlikely since in both cases there are increased antibodies directed against MAP indicating a host response to the organism. The presence of elevated serologic markers which are associated with mycobacterial infection, including CRP in case 1 and ACE in case 2, also weighs against this possibility.

We believe that the profound long lasting remission in case 1 resulted from anti-MAP therapy and is unlikely due to steroid administration, since such remissions rarely result from steroid administration alone. Based on these anecdotal reports, three additional cases of children with CD and MAP infection treated successfully with combination anti-MAP antibiotics and UVBI (personal communication), open label trials in CD, and controlled trials in MAC infection, we recommend the use of three antibiotics including clarithromycin, rifampin and levofloxacin (at 15, 9 and 7, respectively, mg/kg per day) for at least two years in combination with periodic UVBI (if available). However, at this time, the optimal antibiotic combination is unknown.

In cases 1 and 2, the rapid progression of the disease accompanied by an increase in antibodies to MAP antigens between the first two specimens may mirror Johne's disease in dairy cattle in which the progression in the severity of disease and the degree of mycobacterial colonization coincides with a switch from the TH1 to TH2 type immune response[73].

The presence of the viable bacterium in the blood of an apparently healthy host (case 5) is an interesting finding. Apparently healthy individuals may have less virulent forms of disease such as transient childhood asthma or rosacea as noted in case 5. In addition, if MAP-infected people are followed over a long enough period of time, some may eventually develop one of the diseases traditionally considered autoimmune.

It would not be surprising if there is a population of individuals who are MAP-infected but never develop disease. *Mycobacterium tuberculosis*, causes active disease in only 10% of infected humans[74]. A similar situation probably pertains to human paratuberculosis, *i.e.*, most MAP infected individuals may never develop disease. Clinically normal cattle with known MAP infection are common suggesting a parallel in the human population[75].

Any theory of causation of the autoimmune diseases must explain two consistent observations for most of these diseases: (1) the north south gradient in geographical distribution of the disease (in the northern hemisphere)[76]; and (2) the predominant female to male ratio in most of these diseases. The first observation is concordant with the worldwide distribution of Johne's disease[77], the lower levels of Vitamin D in the human host at northern latitudes[78], and the role of Vitamin D in the clinical course of patients with CD and T1DM[79,80]. A possible explanation for the second observation includes reduced host immunity due to the effects of estradiol and/or progesterone[81-83]. Future work may shed light on the immunology of gender differences with these diseases. Because of the known risk of disease progression in CD from birth control medication, women who have been diagnosed and treated for a MAP infection should consider non-hormonal birth control methods.

The optimal hosts for MAP are ruminants; cattle, sheep, and deer, in which, a higher burden of bacteria are generally found than in humans. These animals have a higher body temperature than humans ranging from 100.4 to 102.8, 100.9 to 103.8 to 104 F, for cattle, sheep and deer, respectively[84,85]. These differences in body temperature suggests that the growth of MAP in laboratory culture may be accelerated by raising the incubation temperature to 104 F. Further investigation of this issue is warranted.

If controlled trials of MAP related illnesses confirm the findings of these case reports and the autoimmune diseases can be cured, because the bacterium is present in the food supply, will treated patients redevelop disease on re-exposure to the organism? The precautionary principle should apply and improved food safety and public health measures are necessary to limit human exposure to MAP. Until improved measures are in place, treated and cured patients should probably avoid known sources of MAP which include pasteurized milk and milk products such as yoghurt, cheese and ice cream and undercooked beef.

Open label trials of long-term antibiotic therapy in CD have a significant relapse rate. Adjunctive therapy such as UVBI combined with appropriate antibiotics may be a way to improve therapeutic outcomes. UVBI was developed by Knott[86]. In his article on the development of ultraviolet blood irradiation, he refers to the work of European investigators who "believed that most of the systemic reactions observed following exposure of the skin to ultraviolet rays were due to the influence of the rays upon the blood"[86]. Knott was most likely aware of the work of Finsen who received the Nobel Prize in 1903, for his work showing

the beneficial effects of ultraviolet treatments of the skin in patients with lupus vulgaris, *i.e.*, tuberculosis of the skin[87]. The Knott device was used for the treatment of many infections[88-90]. and while exact figures are unavailable, probably thousands of patients were treated with this therapy throughout the United States. Several studies[91,92] as well as three controlled trials from Russia have shown beneficial effects in the treatment of tuberculosis[93-95]. Because the Knott hemo-irradiator predated the advent of the FDA, this device was never FDA approved. However, in recent years, UVBI was advanced successfully through phase Ⅱ clinical trials for the treatment of hepatitis C infection.

Various studies on UVBI that may explain the benefit of this therapy include the following. Ultraviolet light in the C region (UVC) inactivates bacterial and viral pathogens, present in the blood, which is irradiated. In the case of bacteria and DNA viruses, UVC induces the formation of thymine-thymine dimers, which prevents replication[96]. In the case of RNA viruses, UVC induces the formation of uracil-uracil dimers which also prevents replication[97]. Bacteria including *Mycobacterium tuberculosis* have UV repair mechanisms and normal lymphocytes also have UV repair mechanisms[98,99].

Because only 200 cc of blood in an average adult (or 4% of the total 5.0 liter blood volume) is treated during a single session, factors other than pathogen inactivation are likely to explain the potential benefit. Ultraviolet light shined on murine fibroblasts results in the formation of hydrogen peroxide and hydroxyl radicals which are also bactericidal and virucidal[100]. Ultraviolet light in the A region and at higher doses and exposure durations causes immune suppression, but ultraviolet light in the B (UVB) region and UVC have been shown to stimulate dendritic cells[101-103]. Hemoglobin which has been irradiated with UVB and UVC wavelengths exhibits fluorescence[104] and the wavelength of light which is emitted, 365 nm, causes the formation of DNA or RNA adducts in riboflavin and other chromophores and these adducts are bactericidal and virucidal[105]. It is now known that in spite of long term treatment of tuberculosis by antibiotics, there are persisters, which are not killed by the drugs[106,107]. Also *Mycobacterium avium* complex organisms can resist the bactericidal activity of clarithromycin within the phagosomes of macrophages[108]. Viable MAP organisms which have survived the antibiotics by either of these routes and which are within macrophages may not survive ultraviolet irradiation[68]. An *in-vitro* study showed that monocytes which are irradiated with UVB and then infected with *Mycobacterium avium intracellulare* (MAI) organisms, efficiently inhibit the intracellular replication of MAI[109]. The authors in this work speculated that the intracellular inhibition of MAI replication in the UV treated macrophages may be due to the induction of intracellular vitamin D production by the UVB.

Vitamin D has been shown to play an important role in the host immune response to mycobacterial infection[110]. Vitamins A and D have been shown to inhibit the growth of MAP *in vitro*[111]. Vitamin D has also been shown to reduce the proliferation of *M. tuberculosis* in macrophages[112]. Activated dendritic cells are known to produce Vitamin D[113] and Vitamin D induces the intracellular production of cathelicidin, which is an antimicrobial protein[114]. High levels of Vitamin D have been correlated with a reduced risk of developing multiple sclerosis, and Vitamin D intake is inversely associated with rheumatoid arthritis (another autoimmune condition) and the severity of this latter disease also correlates with Vitamin D levels[113].

Finally, many types of cells including leukocytes and, in particular, monocytes, exposed to ultraviolet light secrete heat shock proteins and these proteins play an important role in the response to infection[115-117].

A small open label trial of UVBI in 4 patients with severe Raynaud's syndrome showed clinical improvement that lasted for 3 mo in all of the patients and a reduction of mycobacterial heat shock protein antibodies in one of the patients[118].

These case reports support a pathogenic role of MAP in humans. Large controlled trials are indicated for many of the autoimmune diseases associated with MAP infection including CD, T1DM, MS and CRPS using anti-MAP therapy combined with UVBI (perhaps substituting ethambutol for ciprofloxacin)[119] in one arm and combination infliximab and azathioprine in the control arm to determine whether properly dosed anti-MAP therapy is more effective than currently available therapies. RedHill Biopharma Ltd., Tel Aviv, Israel, has initiated phase Ⅲ clinical trials in Europe and North America to treat CD and MS using a combination therapy of clarithromycin, rifabutin and clofazimine[120]. MAP prevalence studies are indicated in lymphangiomatosis, ankylosing spondylitis, rheumatoid arthritis, hypothyroidism, hyperthyroidism, adrenal insufficiency, systemic sclerosis, Sjogren syndrome, systemic lupus erythematosus, dermatomyositis, psoriasis, sarcoidosis, celiac disease, rosacea, asthma, fibromyalgia, amyotrophic lateral sclerosis, myasthenia gravis, Parkinson's disease and Alzheimer's disease.

The results of therapeutic trials should be evaluated with consideration of the success rate in treating *Mycobacterium avium* complex infections, a mere 42%[27] and that the treatment of *Mycobacterium leprae* is associated with an absolute relapse rate of 3% and that relapses may occur more than 10 years after multiple drug therapy has concluded[121].

Researchers who explore the role of MAP in the autoimmune diseases should be aware that our current diagnostic tests are crude. The ELISA for serum antibodies to MAP[46] was adapted from the cattle assay which has a sensitivity of only 30% to 40% in cattle which are known to be MAP-infected[122]. The suboptimal sensitivity and the variation between current serologic assays for MAP make the diagnosis

of MAP infection difficult. However, in the presence of otherwise unexplained autoimmune disease, the occurrence of positive blood cultures for MAP should be a significant finding.

Furthermore, pre-existing therapies for these conditions may hinder culture recovery methods. Many of the currently used immunomodulators have demonstrated bacteriostatic effects on MAP *in-vitro*[123,124]. When possible, MAP diagnostic testing should be conducted on newly diagnosed patients prior to instituting immunosuppressive therapies which can inhibit the growth of MAP in cultures. While the blood culture method of Naser has been a great advance in the field of human paratuberculosis research, it is positive in 55% of patients with inflammatory bowel disease and in 22% of non-inflammatory bowel disease patients[22], and therefore cannot by itself serve as a discriminator for the presence or absence of disease. Concurrent detection of antibodies directed at MAP will probably be helpful in this regard[50]. MAP cultures should be performed in laboratories with expertise. Parrish *et al*[125] failed to replicate the blood culture study of Naser *et al*, but their method did not include egg yolk in the medium (regarded by many as vital for MAP growth) and the cultures were only held for 18 wk (MAP cultures for humans are usually held for at least 6 mo and up to one year).

The current MAP ELISA reacts to both host IgM and IgG. Modifying the assay into its isotype components, *i.e.,* IgM and IgG, may permit a better determination of whether the host response reflects active disease or remote exposure. Further research is necessary in this area.

Should MAP be proven to cause many of the auto-immune diseases, a potential role in carcinogenesis should be explored. *Helicobacter pylori* is now recognized as playing a major role in the pathogenesis of primary gastric MALT lymphoma and gastric carcinoma[126]. CD patients are known to have increased risk of bowel cancer and lymphoma. Whether this increased risk is due to the immunosuppressive therapies used in this disease or due to infection by MAP is unknown and should be investigated further[127]. In summary, much more must be learned about this elusive and enigmatic organism and about the human diseases with which it is associated.

COMMENTS

Case characteristics
Please summarize main symptoms in one sentence. Case 1 had Crohn's disease (CD) and experienced abdominal pain, diarrhea and weight loss while case 2 had complex regional pain syndrome (CRPS) and experienced generalized hypersensitivity, neuralgia, paresthesias and Raynaud's phenomenon.

Clinical diagnosis
Please summarize main clinical findings in one sentence. Case 1 had CD while case 2 had CRPS.

Differential diagnosis
Please summarize thoughts and methods for differential diagnosis in one sentence. The differential diagnosis in case 1 included celiac disease and food allergy while the differential diagnosis in case 2 included multiple sclerosis.

Laboratory diagnosis
Please summarize laboratory testing methods and major findings in one sentence. Case 1 had anemia, elevated erythrocyte sedimentation rate, CRP and WBC while case 2 had elevated angiotensin converting enzyme, cryoglobulins, lymphocyte and eosinophil count and TSH and initially, both patients had blood cultures positive for *Mycobacterium avium* subsp. *paratuberculosis* (MAP).

Imaging diagnosis
Please summarize imaging methods and major findings in one sentence. Case 1 had multiple aphthous ulcers on upper gastrointestinal endoscopy and on colonoscopy while case 2 had an unremarkable EMG study.

Pathological diagnosis
Please summarize pathological methods and major findings in one sentence. Case 1 had granulomas in the gastric and colonic biopsies while case 2 had no biopsies.

Treatment
Please summarize treatments and drugs used in one sentence. Both case 1 and case 2 received a combination of periodic ultraviolet blood irradiation (UVBI) and antibiotics which included clarithromycin, rifampin and ciprofloxacin for at least 2 years.

Related reports
Please provide other contents related to the case report to help readers better understand the present case.

Term explanation
Please explain uncommon terms present in the case report. UVBI is ultraviolet blood irradiation which consists of periodic irradiation of approximately 200 cc of patient blood using ultraviolet light in the B and C regions.

Experiences and lessons
Please summarize experiences and lessons learnt from the case in one sentence. Both CD and CRPS, when caused by MAP, resolve when the organism is eradicated from the host.

Peer-review
Please summarize the strengths and weaknesses of the article based on the reviewers' comments so that readers can obtain objective knowledge from the article. This study is anecdotal and it will be necessary to study large numbers of patients in a controlled trial setting to determine whether these results are reproducible.

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CASE REPORT

Urea cycle disorders: A case report of a successful treatment with liver transplant and a literature review

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Abstract

The urea cycle is the final pathway for nitrogen metabolism. Urea cycle disorders (UCDs) include a variety of genetic defects, which lead to inefficient urea synthesis. Elevated blood ammonium level is usually dominant in the clinical pattern and the primary manifestations affect the central nervous system. Herein, we report the case of a 17-year-old girl who was diagnosed with UCD at the age of 3. Despite a controlled diet, she was hospitalized several times for acute attacks with recurrent life risk. She came to our attention for a hyperammonemic episode. We proposed an orthotopic liver transplant (OLT) as a treatment; the patient and her family were in complete agreement. On February 28, 2007, she successfully received a transplant. Following the surgery, she has remained well, and she is currently leading a normal life. Usually for UCDs diet plays the primary therapeutic role, while OLT is often considered as a last resort. Our case report and the recent literature data on the quality of life and prognosis of traditionally treated patients *vs* OLT patients, support OLT as a primary intervention to prevent life-threatening acute episodes and chronic mental impairment.

Key words: Urea cycle disorders; Hyperammonemia; Diet; Liver transplantation; Quality of life

Core tip: Urea cycle disorders (UCDs) include a variety of genetic defects which lead to inefficient urea-synthesis with hyperammonemia. The liver is the main site of urea cycle's enzymatic activity. Diet has traditionally been the primary therapy; orthotopic liver transplantation (OLT) is often considered as a last resort. This case report presents a 17-year-old girl with UCD who was successfully treated with OLT. Her recovery and a recent literature review support OLT as a primary intervention for UCDs patients to prevent life-threatening acute episodes and chronic mental impairment, with an approximately 90% survival rate and a better quality of life.

INTRODUCTION

The urea cycle is the final pathway for nitrogen metabolism, which occurs primarily through amino acid catabolism with urea production and subsequent excretion (Figure 1). Urea cycle disorders (UCDs) include a variety of genetic defects in ammonia metabolism in which one of the urea-synthesis passages is defective, leading to hyperammonemia. The genetic defect can involve one of the enzymes involved in the urea cycle or a transporting protein related to the metabolic process. The liver is the most important site of this enzymatic activity.

Although data from population studies are lacking, the prevalence of this type of genetic defect is approximately 1: 30.000-46.000 live births[1].

The cycle consists of the action of cytoplasmic and mitochondrial enzymes and some transporting proteins, leading to the production of urea molecules from nitrogen atoms. The different metabolic blocks determine the accumulation of nitrogen and intermediate substances, with a variety of corresponding clinical features.

The classification of urea cycle defects is summarized in Table 1.

In patients with normal hepatic function and hyperammonemia, the diagnosis of UCD should be considered after excluding other causes of elevated blood ammonemia, such as medications (valproate and chemotherapeutic agents), portosystemic shunt, urea producing organisms and hyperalimentation[2]. The diagnosis of UCD is based on clinical, biochemical and molecular data[3,4].

Elevated blood ammonium levels are usually dominant in the clinical pattern and the main manifestations affect the central nervous system[5]. The presentation of UCDs can be quite variable ranging from agitation to coma in acute attacks (common cause of neonatal exitus in these patients) and different degrees of mental impairment chronically (even with correct dietary intake).

Usually, patients with this type of metabolic defect develop a tolerance to high ammonium levels. Therefore, they can be completely asymptomatic even with levels of approximately 200 μg/dL. These episodes are not rare even in mild forms of UCD because many factors (*e.g.,* infections, fever, fasting, surgery) can break down the metabolic equilibrium. Moreover, the long-term compliance of these young

patients is usually difficult. The central nervous system damage is at least in part reversible when ammonium levels do not exceed 200-400 μg/dL. Nevertheless, mild neurological damage in the presence of lower ammonium levels cannot be completely ruled out[6,7]. The consequent neurological deterioration is inversely proportional to the frequency of acute episodes and to the ammonia levels. Additional disease-specific symptoms are related to the particular metabolic defect, which are often due to an excess or lack of specific amino acids[8].

Herein, we discuss the case of a girl with UCD caused by carbamyl phosphate synthetase (CPS Ⅰ) deficit (Table 1) who underwent an orthotopic liver transplantation (OLT) 14 years after her diagnosis.

CASE REPORT

A 17-year-old girl came to our attention in November 2007. She was diagnosed with a CPS Ⅰ defect in a Pediatric Hospital when she was 3 years old after evidence of lethargy attacks, sight disturbances and growth retardation. Despite a strict dietary regimen, the symptoms control was partial and recurrent acute attacks developed. These attacks were characterized by sudden somnolence, ataxia, coordination defects and temporal cognition distortion (never completely resolved). These often required hospitalization to reduce the ammonia levels with parenteral therapy infusion.

She came to our hospital for an acute attack with lethargy and a blood ammonia concentration of 305 μg/dL. The episode was resolved with a 72-h infusion of a parenteral complex containing sodium benzoate and arginine. Her cerebral magnetic resonance imaging results were normal.

Considering the constant risk of death and the poor quality of life, even with otherwise normal liver parameters, we proposed OLT as a treatment. The patient and her family were in complete agreement.

After a complete work-up, the patient was admitted to the Pediatric list of Bologna Liver Transplantation Center and after two months she underwent OLT. The post-operative course was free from complications with rapid ammonia blood level normalization. After a 7-d recovery period, she was discharged. Currently, her clinical conditions are good; she leads a normal life without dietary restrictions and is free from lethargy episodes and hospital admissions, except for immunosuppressive therapy and routine follow-up visits at the Transplant Center.

DISCUSSION

The treatment for UCDs is a dietary intervention consisting of severe protein restriction[5]. Additionally, citrullin and arginine supplementation (except in cases of argininemia) and sodium benzoate and sodium phenylacetate administration (useful to
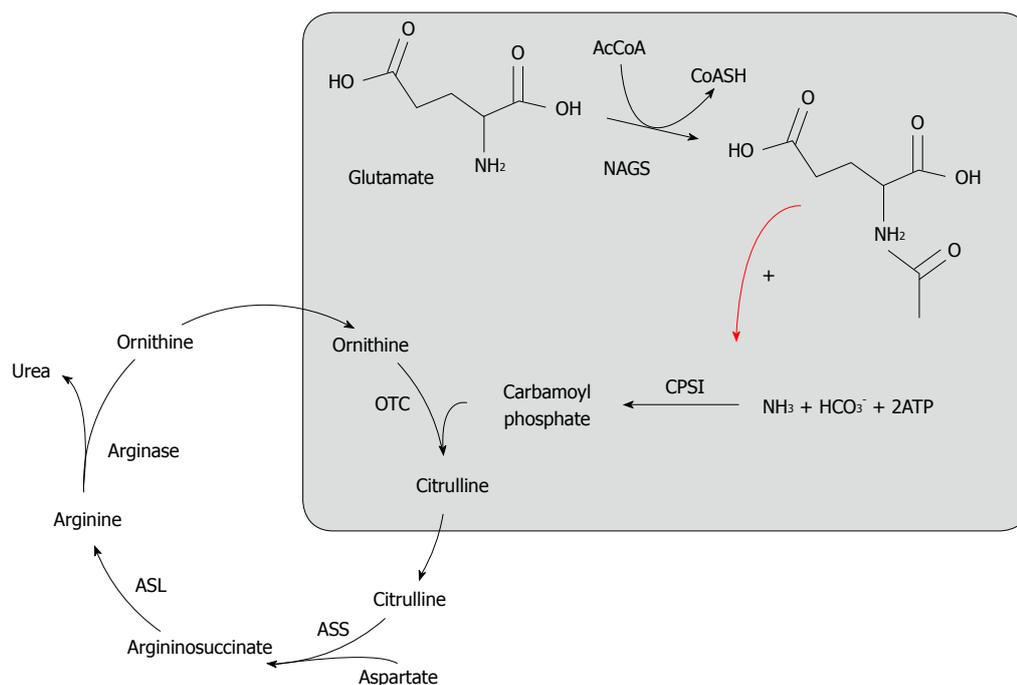
Figure 1 Urea cycle. NAGS: N-acetylglutamate synthetase; NAG: N-acetylglutamate; CPS I: Carbamyl phosphate synthetase 1; OTC: Ornithine transcarbamylase; ASS: Argininosuccinate synthetase; ASL: Argininosuccinate lyase.

Table 1 Main causes of urea cycle defects	
CPS I deficiency	Genetic defect of carbamoyl phosphate synthetase, inherited as autosomal recessive mutation
	Block of the first passage of nitrogen in urea cycle
	Neonatal onset when there is a severe enzyme deficiency and adult onset when the effects of mutation are mild
OTC deficiency	X-linked disorder, the most common among UCDs
	Mutation in ornithine transcarbamylase (second passage in urea cycle)
	Clinical presentation very similar to CPS I deficiency
ASS deficiency/citrin deficiency (citrullinemia)	Citrullinemia type Ⅰ: autosomal recessive defect caused by a deficit of argininosuccinate synthetase, with consequent very high citrulline levels in blood
	Citrullinemia tipe Ⅱ: caused by a deficit of citrin, a mitochondrial carrier protein of glutamate and aspartate who consequently fail to shuttle to and from the mitochondrion with increase of citrulline levels in blood
ASL deficiency (argininosuccinic aciduria)	Autosomal recessive mutation
	Loss of argininosuccinate lyase with accumulations in body fluids (also in cerebrospinal fluid)
	Seems to lead to mental retardation in all the affected, even when dietary ammonium control is well performed
Arginase deficiency (argininemia)	Arginase I defect
	Peculiar manifestations such as paraplegia and intractable mental retardation
N-acetylglutamate synthetase deficiency	Very rare and clinically overlapped to CPS I deficiency

UCDs: Urea cycle disorders; CPS I: Carbamyl phosphate synthetase 1; ASL: Argininosuccinate lyase; OTC: Ornithine transcarbamylase.

excrete nitrogen excess) are often used to treat acute hyperammonemia. Nocturnal enteral nutrition and prompt recognition and treatment of intercurrent illnesses are also necessary to prevent catabolism[9]. In cases of acute hyperammonemia attacks, the treatment is based on ammonium removal by dialysis or hemofiltration and on reversing the catabolic state by caloric and arginine supplementation[9]. This strategy, in the actual daily life of teenage patients, could be hindered by the difficulty in maintaining dietary restrictions; daily monitoring helps to control the trigger causes (infections, fasting and other conditions often make the metabolic imbalance

unpredictable) of hyperammonemia. In patients whose symptoms cannot be controlled by diet, OLT is a treatment option because the liver is the principal site of urea cycle activity.

In absence of viral reinfection, tumor recurrence and alcohol relapse (typically affecting patients who underwent OLT for more frequent indications), transplants in patients with metabolic disorders (*e.g.,* hemochromatosis, Wilson's disease, familial amyloidosis, cystic fibrosis) have significantly better outcomes compared with primary liver diseases. The 5 year survival rate is nearly 90% with better results when structural liver disease is absent[10]. Moreover,

the consequent metabolic correction is associated with a considerably improved quality of life and elimination of specific death risks.

After OLT, dietary restriction in our patient was no longer needed. Consequently, the other medications for amino acid supplementation and urea excretion were discontinued with no further encephalopathy episodes. Furthermore, our patient has demonstrated increased intellectual performance leading to maturation of personality and behavior comparable with her peers; these improvements are not to be generalized since other authors reported cases of interrupted neurological deterioration without significant intellectual improvements[7,11,12]. A possible explanation could be the timing of OLT, which was performed prior to the development of irreversible brain damage.

The exact number of OLTs for patients with UCDs is unknown; a United States survey in 1998[12] described 16 cases of OLTs for UCD patients. Kyoto University[1] refers to 13 patients who underwent liver transplant for urea cycle defects, and a recent article describes 2 UCD patients who were subjected to OLT in China[13]. A European review[11] reported of 59 transplants with a 93% survival after 3 years. Of these transplant recipients, 6 patients had a CPS I deficit and all of the patients were alive after the 3-year follow-up. In Italy, the Sicilian transplant group[14] reported one case of a living-donor liver transplant (LDLT) for OTC deficiency.

Recently, the United Network for Organ Sharing (UNOS) described 293 patients who underwent liver transplantation for UCDs and organic acidemias with a 5-year graft survival rate of 78% for children < 2 years old at the time of transplant and 88% for children ≥ 2 years old at the time of transplant[15]. Finally, Kasahara *et al*[16] described 40 patients with UCDs who underwent LDLT, in which the patient and graft survival rate was 96.1% after 15 years.

Despite these good results, currently the univocally accepted indications for OLT in patients with UCDs are very severe disease with poor prognosis; progressive liver disease with prospect of liver failure; severe complications that cannot be avoided by other interventions; or when diet is insufficient to maintain normal ammonia blood levels[1,11]. A recent UNOS review highlighted that eight children died while they were waiting for transplantation, thus confirming the lethal nature of this disease. The optimal timing for transplantation remains an important question because younger patients have a higher risk of graft loss due to increased liver transplant complications. Recent expert consensus conference guidelines recommend OLT between 3 and 12 mo of age if the child weighs more than 5 kg[17].

An important aspect highlighted by Hadzic and Vara is that children with these rare conditions are followed by specialists in metabolic units that often have no direct contact with the transplant center. Considering the positive results of transplantation for metabolic diseases, there should be closer contact between these sectors[18].

Moreover, the periodic lack of compliance in these children and adolescents, facing this long-term life restriction, that can cause recurrent hyperammonemia episodes is another factor favoring OLT.

Of course, all patients require an individual evaluation for risk and benefit options. However, when considering the significant improvements in transplant techniques, the overall results and the positive and generally better outcomes following OLT in patients with metabolic defects compared to primary liver diseases, we support the idea of liver transplant as an option for every single patient. Furthermore, liver transplant should no longer be a last resort. In particular, we agree with some authors, such as Meyburg *et al*[19], who have strongly promoted OLT as the first and only definitive cure for UCDs given that liver transplant is associated with the best survival rate in the whole field of solid organ transplantation. OLT should be performed as soon as possible to prevent irreversible brain damage.

The OLT indications are not different for the different UCDs types, except NAGS deficiency for whom the definitive therapy is N-carbamyl-L-glutamate, a NAG analogue that is taken up enterally and replaces NAG for the activation of CPS1[17]. For all the other UCD forms, liver transplantation should be considered in all patients before irreversible neurological damage and/or repeated crises occur[17].

In the literature, OLT has been reported for patients with all UCD types, except NAGS deficiency. Wakiya *et al*[20] described twelve children with OTC deficiency who underwent living donor liver transplantation with satisfactory outcomes. Kimura *et al*[21] reported two cases of adult-onset type Ⅱ citrullinemia successfully treated with OLT. Nagamani *et al*[22] report OLT as a long-term correction for patients with ASL deficiency. Silva *et al*[23] described two cases of children with argininemia successfully treated with OLT. Morioka *et al*[1] reported 13 patients with UCDs who underwent OLT at Kyoto University and reviewed 38 OLTs for patients with UCDs reported in the worldwide English literature of whom 28 patients had an OTC deficiency; 4 patients had a CPSI deficiency; 4 patients had citrullinemia type Ⅰ; 27 patients had citrullinemia type Ⅱ; 1 patient had an arginase deficiency.

Regarding our patient, in our opinion, the transplant should have been performed long before she came to our hospital, which would have prevented more than 10 years of mental impairment and life-threatening episodes during her childhood. The improved quality of life that OLT offers, despite the immunosuppressive therapy, to these young people and the great benefit for their social development should be taken into account.

Therefore, our present experience, together with the results obtained worldwide in patients with this type of metabolic disease, supports OLT as soon as

possible. The advantages for these children are an approximate 90% survival rate with normal mental development and a better quality of life.

COMMENTS

Case characteristics
A 17-year-old girl diagnosed with urea cycle disorder (carbamyl phosphate synthetase deficiency diagnosed when she was 3) presented with an acute attack characterized by lethargy.

Clinical diagnosis
Drowsiness, ataxia, coordination defects and temporal cognition distortion.

Differential diagnosis
Urea cycle disorders (UCDs), other metabolic disorders, encephalitis, hepatic encephalopathy, cerebral mass, drug intoxication.

Laboratory diagnosis
The patient's blood ammonia concentration was 305 µg/dL; the liver function tests were within the normal limits.

Imaging diagnosis
The cerebral magnetic resonance imaging results were normal.

Pathological diagnosis
No pathological investigations were performed.

Treatment
The acute episode was resolved with a 72-h infusion of a parenteral complex containing sodium benzoate and arginine; shortly afterwards, after a complete work-up, the patient underwent orthotopic liver transplantation.

Term explanation
The urea cycle is the primary nitrogen disposal pathway in humans. The cycle requires the function of six enzymes to catalyze the conversion of ammonia and bicarbonate into urea. Ammonia is toxic, but urea is relatively inert, is soluble in water and is readily excreted by the kidney into the urine. The liver is the most important site of this enzymatic activity. Urea cycle disorders are metabolic inborn errors caused by a deficiency in one of the enzymes of the cycle. Ammonia (and other toxic intermediates) accumulation leads to predominant neurologic sequelae, ranging from mild cognitive deficits to deep coma and death.

Experiences and lessons
This case report, together with the recent literature data, supports liver transplantation as a primary intervention in patients with UCDs to prevent life-threatening acute episodes and chronic mental impairment.

Peer-review
This case report focuses on the issue of liver transplantation for patients with urea cycle disorders, which is relevant and not thoroughly discussed in the transplant literature.

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CASE REPORT

Gastroenterology case report of mesalazine-induced cardiopulmonary hypersensitivity

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Ethics approval: The study was reviewed and approved by the Central University Hospital of Asturias research ethics committee.

Informed consent: All study participants, or their legal guardian, provided informed written consent prior to study enrollment.

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Abstract

Mesalazine is a 5-aminosalicylic acid derivative that has been widely used to treat patients with inflammatory bowel disease. Accumulating evidence indicates that mesalazine has a very low rate of adverse drug reactions and is well tolerated by patients. However, a few cases of pulmonary and cardiac disease related to mesalazine have been reported in the past, though infrequently, preventing clinicians from diagnosing the conditions early. We describe the case of a 32-year-old man with ulcerative colitis who was admitted with a two-month history of persistent fever following mesalazine treatment initiated 14 mo earlier. At the time of admission, mesalazine dose was increased from 1.5 to 3.0 g/d, and antibiotic therapy was started with no improvement. Three weeks after admission, the patient developed dyspnea, non-productive cough, and chest pain. Severe eosinophilia was detected in laboratory tests, and a computed tomography scan revealed interstitial infiltrates in both lungs, as well as a large pericardial effusion. The bronchoalveolar lavage reported a CD4/CD8 ratio of 0.5, and an increased eosinophil count. Transbronchial biopsy examination showed a severe eosinophilic infiltrate of the lung tissue. Mesalazine-induced cardiopulmonary hypersensitivity was suspected after excluding other possible etiologies. Consequently, mesalazine treatment was suspended, and corticosteroid therapy was initiated, resulting in resolution of symptoms and radiologic abnormalities. We conclude that mesalazine-induced pulmonary and cardiac hypersensitivity should always be considered in the differential diagnosis of unexplained cardiopulmonary symptoms and radiographic abnormalities in patients with inflammatory bowel disease.

Key words: Eosinophilia; Mesalazine; Pericardial effusion; Lung hypersensitivity; Ulcerative colitis

Core tip: We report a case of lung and cardiac hypersensitivity caused by mesalazine therapy in a patient

with ulcerative colitis. Despite a few previously reported mesalazine-induced cardiac and pulmonary hypersensitivity cases, both entities are extremely infrequent making it difficult for the clinician to recognize these conditions during their early stages. An early diagnosis of these entities is extremely important, as the treatment consists of mesalazine suspension, usually resulting in a complete resolution of symptoms.

INTRODUCTION

Mesalazine, a 5-aminosalicylic acid derivative, is a medication widely used in the management of inflammatory bowel disease (IBD). The precise mechanism of mesalazine action remains poorly understood. However, it has been proposed that the drug acts locally on the colonic mucosa reducing inflammation through a variety of anti-inflammatory processes. These processes include the inhibition of proinflammatory cytokines (interleukin-1, -2, and -8 and tumor necrosis factor-α), the induction of the proliferator activated receptor-γ gene expression, or mesalazine acting as a potent antioxidant and free-radical scavenger[1]. The use of sulfasalazine in the treatment of IBD has been limited by the side effects, most of them secondary to the sulfapyridine component[2]. On the other hand, the use of mesalazine is usually well tolerated by patients, due to its favorable safety profile. Due to a limited number of cases of mesalazine-induced pulmonary disease and pericardial effusion, it has been difficult for clinicians to diagnose these diseases early. We describe the case of a patient with ulcerative colitis (UC) who, due to mesalazine treatment, simultaneously developed lung disease, pericardial effusion, and severe eosinophilia.

CASE REPORT

A 32-year-old non-smoking man with a 16-mo history of extensive UC treated with mesalazine (1.5 g/d) since the initial UC diagnosis and azathioprine (150 mg/d) for the last 13 mo was admitted to the hospital with a 2-mo history of asthenia, fever and night sweats. Prior to the appearance of the symptoms, UC was in clinical remission. Laboratory tests showed microcytic hypochromic anemia, a normal WBC count, and an increase in the erythrocyte sedimentation rate (91.0 mm/h) and the C-reactive protein level (10.3 mg/dL). Both chest radiograph and electrocardiogram

were normal. At the admission, mesalazine dose was increased to 3 g/d. Blood, urine and stool samples were collected for culture prior to a 10-d course of intravenous antibiotic treatment with ciprofloxacin and metronidazole. Nevertheless, the patient continued to be febrile resulting in termination of the antibiotic therapy. Cultures drawn at admission, as well as serologic testing for human immunodeficiency virus, were all negative. A rectosigmoidoscopy showed no evidence of disease activity. A computed tomography (CT) scan of the chest revealed the presence of centrilobular pulmonary nodules in the left lower lobe and lingula, as well as mediastinal and axillary lymphadenopathy.

After a few days of hospitalization, a progressive increase in the WBC and eosinophil counts were detected in peripheral blood. Three weeks after admission, a blood test showed a WBC count of 12.6 $\times$ 10^9/L and a severe eosinophilia of 7.8 $\times$ 10^9/L (62.3%). Immunoglobulins (IgA, IgG and IgM) and complement levels were normal. Rheumatoid factor, anti-citrullinated peptide antibodies, and anti-nuclear antibodies were all negative, while the anti-neutrophil cytoplasmic antibody exhibited a positive cytoplasmic staining pattern (titer, 1:160). During this time, our patient developed clinical symptoms of dyspnea, a non-productive cough, and thoracic pain. A second CT scan was performed, revealing the presence of a patchy ground glass opacification, centrilobular pulmonary nodules extending to both inferior lobes, and a 33.6-mm pericardial effusion not previously present (Figure 1A and B). An echocardiogram showed a large pericardial effusion with no signs of hemodynamic instability. Additionally, pulmonary function testing revealed a marked decrease of 66.8% in the diffusion capacity for carbon monoxide (DLCO). The tuberculin skin test revealed no induration, and the QuantiFERON TB-Gold test was also negative. Bronchoscopy findings reported an inflammatory stenosis of the left principal bronchia. The bronchoalveolar lavage (BAL) showed an eosinophilia of 72.0%, with CD4 and CD8 counts of 29.0 and 56.0%, respectively (CD4/CD8 ratio: 0.52). Transbronchial biopsy examination demonstrated the presence of a dense eosinophilic infiltrate throughout the interstitium, alveolar spaces, and capillaries, consistent with eosinophilic pneumonia, but no indication of necrosis or granulomas (Figure 2).

One month after admission, mesalazine-induced eosinophilic pneumonia, pericardial effusion and severe eosinophilia were suspected. Consequently, mesalazine was withdrawn, and therapy with prednisone was initiated. A few days after discontinuation of mesalazine, our patient had a quick and significant clinical improvement as indicated by normalization of the hemoglobin level and eosinophil count. In addition, a chest radiograph and a CT scan of the thorax revealed a complete resolution of the mediastinal and axillary lymphadenopathy, pericardial effusion,
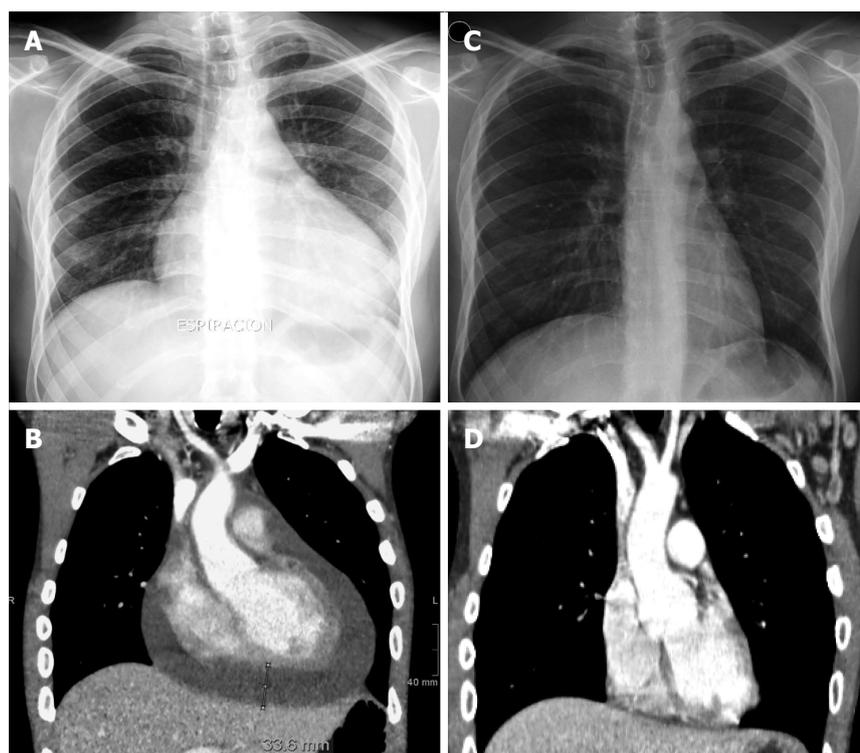
Figure 1 Radiograph of the chest and computed tomography scan before and after mesalazine suspension. A: Chest radiograph showing an enlarged cardiac silhouette due to a cardiac effusion during mesalazine treatment; B: Computed tomography (CT) scan of the chest revealing cardiomegaly due to a large pericardial effusion (maximum width of 33.6 mm) during mesalazine therapy; C: Normal chest radiograph after mesalazine withdrawal; D: CT scan of the chest showing a complete resolution of the pericardial effusion after suspension of mesalazine.
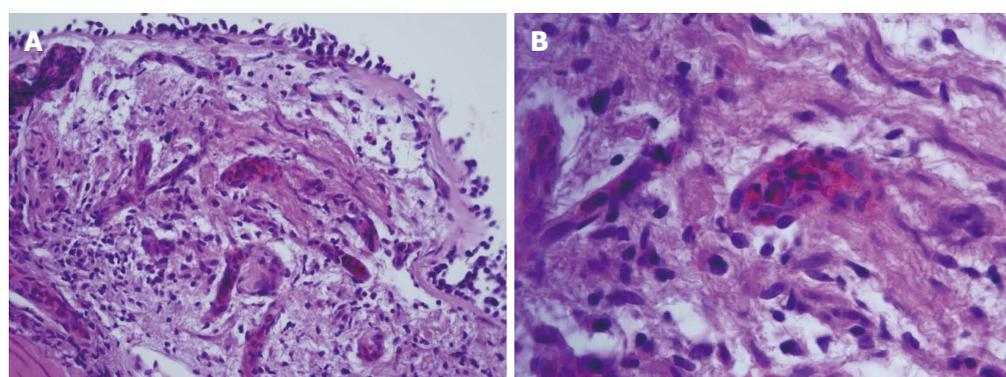
Figure 2 Transbronchial biopsy of the left inferior pulmonary lobe. A: An eosinophilic infiltrate throughout the alveolar septa, alveolar spaces and capillaries (HE stain, × 40); B: An eosinophilic infiltrate in the interior of capillaries (HE stain, × 100).

pulmonary nodules and infiltrates (Figure 1C and D). Despite continuous reduction in the DLCO (73.5%) 6 mo after discontinuation of mesalazine, our patient remained completely asymptomatic and capable of conducting a fully active life.

DISCUSSION

Clinicians treating patients with IBD exhibiting pulmonary symptoms face multiple causes for the onset of these symptoms. A respiratory involvement as an extraintestinal manifestation of UC (serositis, sarcoidosis, interstitial lung disease, or pulmonary embolism) should be taken into consideration. Furthermore, lung infections, triggered or worsened by the immunosuppressive medication, as well as pulmonary adverse reactions induced by the drugs used to treat the underlying disease, should also be considered as the underlying cause of the pulmonary symptoms in IBD patients[3].

The use of sulfasalazine has been related to multiple adverse drug reactions (ADRs), hence, mesalazine has become the first-line treatment for the induction and maintenance of remission of mildly to moderately active UC[2]. Although mesalazine is usually well tolerated by patients, some serious ADRs like

hepatitis, blood dyscrasias, pancreatitis, and interstitial nephritis have been reported[4]. Mesalazine-induced pulmonary side effects are rare, and its pathogenesis is not well understood. Nevertheless, it is thought that two mechanisms may be responsible for these side effects: (1) a toxicity mechanism that could be dose-dependent; and (2) an immunologic mechanism that might be dose-independent[5,6].

Typical mesalazine-related side effects, which include fatigue, non-productive cough, fever, dyspnea and chest pain[7], usually appear after 1-6 mo of treatment. In very rare cases, patients treated with mesalazine exhibited side effects as early as a few days or several years after the treatment[6]. Laboratory tests may reveal peripheral eosinophilia while pulmonary nodules and an interstitial infiltrate with a ground glass pattern are usually seen in the chest radiograph or CT scan. If performed, the BAL frequently shows an elevated count of eosinophils or lymphocytes, and a reduction of the CD4/CD8 ratio. In addition, pulmonary function tests usually demonstrate a reduced DLCO. Histopathologic findings include interstitial lymphocytic infiltrates, alveolar eosinophilic infiltrates, alveolar fibrosis, and non-necrotizing granulomas[5].

Differentiating mesalazine-induced lung disease from IBD-related pulmonary manifestation, as well as establishing a diagnosis, is challenging. It is known that mesalazine-induced lung disease usually affects the lung parenchyma[8], whereas the IBD-related pulmonary manifestations typically involve the upper respiratory tract[9]. If the diagnosis remains unclear, a lung biopsy should be considered in order to exclude other conditions. Preferably, the presence of eosinophilia in peripheral blood, BAL, or lung tissue should be used as indicators for diagnosing mesalazine-induced lung disease. Otherwise, diagnosis should be based on clinical presentation, exclusion of other causes of lung disease, and a trial of drug discontinuation. One case reported a complete resolution of the symptoms after the reduction of the mesalazine dose[6]. In patients with severe respiratory symptoms or with a lack of improvement after mesalazine withdrawal, glucocorticoid therapy with prednisone (1 mg/kg per day) should be considered. Reintroduction of mesalazine is not usually recommended. Nonetheless, cases have been reported where rechallenge did not produce recurrence of pulmonary symptoms[10-12].

Thirty-eight cases of mesalazine-induced pulmonary disease were found in the literature. The principal characteristics of those cases are summarized in Table 1. There were 18 men (46.2%) and 21 women (53.8%) with a mean age of 42 years (range: 10-72 years) at the onset of symptoms. Thirty-three of them were diagnosed with UC (84.6%) and six had Crohn's disease (15.4%). Mesalazine dose at the onset of symptoms varied from 750 mg to 4.8 g/d. The time between initiation of mesalazine treatment and the onset of pulmonary symptoms ranged from two days to 4-5 years. The most

common symptoms were non-productive cough, fever and dyspnea, which were present in 74.0%, 72.0% and 64.0% of the patients, respectively. Eosinophilia in peripheral blood was reported in 18 patients (46.0%), and pulmonary infiltrates with an interstitial pattern were the most frequent radiologic finding, appearing in 73.0% of patients. Similarly, eosinophilic pneumonia was the most common histologic finding, appearing in 41.0% of the biopsies. Mesalazine was suspended in 38 patients (97.4%), and 23 of them (60.5%) received systemic glucocorticoids as part of the treatment. Rechallenge was tried in seven patients, but recurrence was only seen in four of them (57.1%).

Cardiac disease as an extraintestinal manifestation of IBD is very rare. When it does occur, acute pericarditis is the most frequent form of presentation[13], but myocarditis, pericardial effusion, and cardiac tamponade have also been described[14,15]. Conversely, most cases of cardiac disease in patients with IBD are drug induced and, even when its pathogenesis is unclear, the consideration is that an idiosyncratic hypersensitivity reaction and a drug-induced lupus-like syndrome mechanism are related[16]. Although most cases of mesalazine-induced cardiac hypersensitivity in IBD patients are not severe, life-threatening complications have been reported[17]. The treatment for the condition consists of mesalazine suspension and administration of non-steroidal anti-inflammatory drugs or corticosteroids, taking into consideration that the former may exacerbate the underlying IBD in some patients[18]. Table 2 summarizes the principal characteristics of previously published cases of mesalazine-induced cardiac hypersensitivity in patients with IBD.

The Naranjo algorithm scale[19] was used to assess the probability of ADRs. The Naranjo algorithm scale is a questionnaire designed to determine the likelihood of whether ADRs are secondary to a drug rather than the result of other factors. ADRs of $\geq$ 9 points were considered to be definite, probable at 5-8 points, possible at 1-4 points, and doubtful at 0 points. Our patient scored 8 points, suggesting probable ADRs. However, due to the presence of eosinophilia in the peripheral blood, BAL and lung biopsy, the patient's deteriorating condition following mesalazine treatment followed by a significant improvement after discontinuation of mesalazine therapy, all strongly support our diagnosis, especially since other causes of cardiopulmonary disease were excluded. Azathioprine-induced pulmonary disease has also been described[20], nevertheless, it is by far less frequent and in this case it did not seem to contribute to the patient's symptoms as he was receiving this medication at same dose for 13 mo prior to admission, throughout his entire hospitalization, and after being discharged. As clinical improvement was documented at the time mesalazine was stopped, we conclude this to be the cause.

Cardiopulmonary toxicity related to mesalazine is extremely infrequent making it difficult for clinicians to

Table 1 Summary of previously published cases of mesalazine-induced pulmonary hypersensitivity in patients with inflammatory bowel disease

Ref.	Age (yr)/sex	Disease	Daily dose	Duration of therapy[1]	Symptoms	EOS	RP	HF	CD4/CD8	BAL	DLCO	Steroid therapy	Rechallenge /recurrence
Le Gros et al[21]	54/F	UC	750 mg	5 d	T, R	ND	I	ND	Ratio: 0.95	Mo: 63% L: 35% E: 1.5%	53%	No	No
Welte et al[22]	67/M	UC	1 g IR	10 d	D, DC, R	ND	I	ND	ND	ND	ND	Yes	No
Reinoso et al[3]	64/F	UC	3.6 g	2 yr	T, D, DC	ND	I	ND	ND	ND	↓	No	No
Lagler et al[23]	66/M	UC	1.5 g	3.5 mo	D, DC	No	I	LP	ND	Mo: 30% L: 67%	54%	Yes	No
Honeybourne et al[24]	30/F	UC	1.6 g	7 mo	T, D, DC, CP	Yes 16%	ND	EP	ND	ND	ND	No	No
Declerck et al[25]	45/F	UC	3 g	3 mo	D	No	I	ND	ND	Mo: 45% L: 38% E: 11%	ND	No	No
Muzzi et al[10]	60/F	CD	2.4 g	ND	T, D, DC	No	I	ND	CD4: 56% CD8: 31% Ratio: 1.80	Mo: 40% L: 55% E: 3%	ND	No	Yes/No
Bitton et al[26]	32/F	UC	4 g	9 mo	T, D, DC	Yes 8.9%	ND	LP/IF	ND	ND	ND	Yes	No
Sviri et al[27]	49/M	CD	3 g	3.5 mo	T, D, DC	No	I	LP/IF	ND	L: 60% E: 10%	80%	Yes	Yes/Yes
Lázaro et al[28]	60/M	UC	ND	4 wk	T, D, DC	Yes	I	IP	ND	Mo: 80% L: 8% E: 10%	67%	No	No
Pascual-Lledó et al[29]	64/F	CD	3 g	2 mo	D, DC, CP	No	I	NL	ND	ND	ND	No[2]	No
Sesin et al[30]	72/F	UC	1.6-2.4 g[3]	2 mo	T, D, CP, PC	No	ND[4]	ND	ND	ND	ND	No	No
Tanigawa et al[31]	35/F	UC	1.5 mg	40 d	T, DC	Yes	I	EP	CD4: 44% CD8: 34% Ratio: 1.3	Mo: 44% L: 49% E: 7%	ND	No	No
Guslandi et al[32]	29/F	UC	3 g	2 d	D, CP	No	ND	ND	ND	ND	ND	No	Yes/Yes
Facchini et al[33]	15/M	UC	2.8 g	4 mo	D, DC	No	A	ND	ND	ND	ND	Yes	No
Zamir et al[34]	23/F	UC	ND	6 wk	F, DC	Yes	ND	ND	ND	ND	ND	Yes	No
Saltzman et al[35]	53/F	UC	ND	4 mo	T, DC, R	Yes 27%	A	EP	ND	E: 79%	ND	Yes	No
Haralambou et al[36]	18/F	UC	1.6 g P.O. + 4 g (enema)	2 mo	T, D, DC, CP	Yes 88%	I	BO	ND	ND	ND	Yes	No
Sossai et al[6]	70/F	UC	2.4 g	3 mo	D, DC	No	I	IP	Ratio: 0.39	Mo: 40% L: 60%	ND	No	N/A[5]
Pérez et al[37]	50/M	UC	4 g	2 mo	T, D, DC	ND	ND	EP	ND	ND	ND	Yes	No
Foster et al[5]	44/M	CD	2.4-4.8 g	15 mo	T, D, DC	No	A	IP/IF	ND	N: 73%	ND	Yes	No
	30/F	UC	4.8 g	2 yr	T, D, DC, CP	No	A	IP/IF	ND	N: 43%	ND	No	No
	29/F	UC	3.6 g	8 mo	T, D, DC	No	A	IP	ND	ND	19%	Yes	No
Hakoda et al[38]	30/M	UC	2.25 g	4 wk	T, DC	Yes	ND	EP	ND	ND	ND	Yes	No
Actis et al[39]	57/M	UC	ND	2 yr	T, D, DC, CP	No	A	ND	ND	ND	↓	Yes	No
Kohli et al[40]	10/F	UC	3.2 g	2 wk	T, D, DC	Yes 12%	I	IP	ND	ND	ND	Yes	Yes/Yes
Katsenos et al[41]	18/M	UC	ND	1 yr	T, PC	Yes	I	ND	ND	Mo: 15% L: 20% E: 60%	ND	Yes	No
Price et al[11]	28/F	UC	1.2 g	4-5 yr	T, PC	Yes	B	ND	ND	N: 95%	ND	Yes	Yes/No[6]
Iannone et al[42]	32/F	UC	ND	4 mo	T, DC	No	I	ND	ND	ND	74%	Yes	No
Cilloniz et al[43]	14/M	CD	3 g	8 mo	CP	No	I	IP	ND	Mo: 68% L: 27%	93%	Yes	No

Ref.	Age/sex	Disease	Daily dose	Duration	Symptoms	EOS	RP	HF	BAL	DLCO	Rechallenge	Recurrence
Park *et al*[44]	35/M	CD	4 g	3 mo	T, DC	Yes 32%	I	EP	CD4: 54% CD8: 41% Ratio: 1.3	L: 31% E: 41%	ND	No	No
Shimizu *et al*[45]	50/F	UC	ND	4 wk	T, DC	ND	ND	ND	ND	L: 58% E: 20%	ND	No	No
Sposato *et al*[46]	42/M	UC	3.2 g	8 d	T, CP	Yes 14%	I	ND	ND	Mo: 13% E: 47% N: 34%	ND	Yes[7]	No
Lamsiah *et al*[47]	57/F	UC	ND (enemas)	3 mo	T, D, DC	Yes	I	ND	ND	ND	ND	Yes	Yes/Yes[8]
Kevans *et al*[48]	17/M	UC	4 g	3 mo	D, DC, CP	Yes 23%	A	ND	ND	ND	ND	Yes	No
Abraham *et al*[49]	65/M	UC	4.8 g	2 wk	T, D, DC[9]	No	I	LP/IF	ND	ND	ND	Yes	No
Kim *et al*[50]	30/F	UC	1 g IR	19 d	PC	Yes 24%	I	EP	ND	Mo: 73% N: 19%	ND	Yes	No
Michy *et al*[12]	72/M	UC	ND	4 mo	D	Yes	ND	EP	ND	L: 23% N: 28% E: 14%	ND	Yes	Yes/No[10]
Current case	32/M	UC	1.5-3 g[3]	14 mo	T, D, DC, CP	Yes 62%	I	EP	CD4: 29% CD8: 56% Ratio: 0.51	E: 72%	67%	Yes	No

[1]Time under mesalazine treatment before symptoms appeared; [2]Patient was on low-dose Deflazacort during all the course of the pulmonary disease; [3]Mesalazine dose was increased after patient was admitted; [4]Bilateral pleural effusion was also noted; [5]Patient improved after reducing mesalazine dose; [6]Rechallenge was intended with olsalazine 1.5 g/d, with no recurrence; [7]Patient had no response to glucocorticoid therapy but showed improvement after mesalazine withdrawal; [8]After mesalazine removal a second rechallenge with sulfasalazine was intended with no recurrence of symptoms; [9]Patient required endotracheal intubation for severe respiratory insufficiency; [10]No recurrence was noted after the reintroduction of mesalazine enemas. A: Alveolar pattern; B: Bronchiectasis; BAL: Bronchoalveolar lavage; BO: Bronchiolitis obliterans; CD: Crohn's disease; CP: Chest pain; CT: Corticoid therapy; D: Dyspnea; DC: Dry cough; DLCO: Diffusion capacity for carbon monoxide; E: Eosinophils; EOS: Eosinophilia; EP: Eosinophilic pneumonia; F: Female; HF: Histopathologic findings; I: Interstitial pattern; IF: Interstitial fibrosis; IP: Interstitial pneumonitis; IR: Intrarectal; L: Lymphocytes; LP: Lymphocytic pneumonitis; M: Male; Mo: Monocytes; N: Neutrophils; N/A: Not applicable; NC: No change; ND: No data available; NL: Normal; PC: Productive cough; PE: Pleural effusion; R: Rash; RP: Radiologic pattern; T: Fever; UC: Ulcerative colitis.

Table 2 Summary of previously published cases of mesalazine-induced cardiac hypersensitivity in patients with inflammatory bowel disease

Ref.	Age (yr)/sex	Disease	Daily dose	Duration of therapy	Symptoms	Cardiac disease	Pharmacologic treatment	Rechallenge/recurrence
Vayre *et al*[51]	53/M	CD	500 mg	8 yr	T, CP	AP, PE	Prednisolone	No
Ishikawa *et al*[16]	17/M	UC	1.5 g	2 wk	T, CP	AP, PE	Prednisolone	Yes/Yes
Doganay *et al*[52]	21/M	UC	2 g	10 d	T, D	AM	Budesonide	No
García-Morán *et al*[17]	39/M	UC	4 g P.O. + 2 g (enemas)	2 d	T, CP	AM, AMI	Methylprednisolone	No
Martín *et al*[53]	22/M	UC	3 g	ND	CP	AM	Corticosteroids	No
Cappell *et al*[15]	32/M	UC	ND	10 yr	T, CP, D	Chronic pericarditis, PT	Prednisone	No
Bernal-Sprekelsen *et al*[54]	54/M	UC	1.5 g	3 wk	T, CP	AP, PE	ASA	Yes/Yes[1]
Freeman *et al*[55]	26/M	UC	1.6 g	3 wk	T, CP	AM	Hydrocortisone	Yes/No[2]
Sierra Ausín *et al*[56]	47/M	UC	3 g P.O. + 1 g (enemas)	3 wk	T, CP	AP	NSAIDs	No
Park *et al*[57]	26/M	UC	2.4 g	1 mo	T, CP	AM, PE	ASA, prednisolone	Yes/Yes
Calafat *et al*[13]	37/M	UC	1 g IR	1 mo	CP	AP, PE	ASA	No
	37/F	UC	3g	2 wk	CP	AP, PE	Analgesics	No
Sonu *et al*[58]	20/F	UC	Sulfasalazine 2 g + mesalazine (enemas)[3]	3 wk	CP	AM, PT	Ibuprofen and colchicine	Yes/Yes[4]
Current case	32/M	UC	1.5-3 g[5]	14 mo	T, CP, D	AP, PE	Prednisone	No

[1]After mesalazine suspension a rechallenge with mesalazine 500 mg IR was intended with recurrence of symptoms; [2]After mesalazine suspension a rechallenge with sulfasalazine was intended without recurrence of symptoms; [3]Mesalazine dose was not specified; [4]After both sulfasalazine and mesalazine enemas were suspended, and low-dose balsalazide was initiated with recurrence of symptoms; [5]Mesalazine dose was increased after patient was admitted. AP: Acute pericarditis; AM: Acute myopericarditis; AMI: Acute mitral insufficiency; ASA: Acetylsalicylic acid; CD: Crohn's disease; CP: Chest pain; D: Dyspnea; F: Female; IR: Intrarectal; M: Male; ND: No data available; NSAIDs: Nonsteroidal anti-inflammatory drugs; PE: Pericardial effusion; PT: Pericardial tamponade; T: Fever; UC: Ulcerative colitis.

recognize and diagnose it in the regular practice. The drug-induced pulmonary and cardiac hypersensitivity should be considered in any IBD patient who develops unexplained lung or cardiac disease while on mesalazine. Early recognition of these ADRs may lead to prompt cessation of the drug, most likely resulting in a complete resolution of the symptoms and radiologic abnormalities.

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COMMENTS

Case characteristics
A 32-year-old man with ulcerative colitis presented with fever, dyspnea, non-productive cough, and chest pain 14 mo from the initiation of mesalazine treatment.

Clinical diagnosis
Mesalazine-induced eosinophilic pneumonia and pericardial effusion.

Differential diagnosis
Cardiorespiratory involvement as an extra-intestinal manifestation of ulcerative colitis (serositis, sarcoidosis, interstitial lung disease or pulmonary embolism); lung infections; and drug-induced adverse reactions.

Laboratory diagnosis
Microcytic hypochromic anemia, WBC count of 12.6×10^9/L, eosinophilia of 7.8 x 10^9/L (62.3%), diffusion capacity for carbon monoxide of 66.8%, and a bronchoalveolar lavage that reported an eosinophilia of 72.0%, with CD4 and CD8 counts of 29.0% and 56.0%, respectively (CD4/CD8 ratio: 0.51).

Imaging diagnosis
Computed tomography scan showed the presence of a patchy ground glass opacification, centrilobular pulmonary nodules that extended to both inferior lobes, and a large pericardial effusion of 33.6 mm. An echocardiogram confirmed the presence of a large pericardial effusion without evidence of hemodynamic instability.

Pathological diagnosis
A transbronchial biopsy examination showed the presence of a dense eosinophilic infiltrate throughout the interstitium, alveolar spaces, and capillaries, consistent with eosinophilic pneumonia.

Treatment
Mesalazine was suspended, and therapy with prednisone was initiated. Azathioprine therapy was continued at the same dose, before, during and after hospitalization.

Related reports
Mesalazine-induced pulmonary and cardiac hypersensitivity are extremely infrequent entities, making it difficult for clinicians to recognize them. This diagnosis was supported by the presence of eosinophilia in the peripheral blood, bronchoalveolar lavage, and lung biopsy, the deterioration of our patient after an increment of the mesalazine dose, as well as the improvement of the patient after discontinuation of mesalazine therapy. Nevertheless, elimination of other causes is required prior to establishing mesalazine-induced pulmonary and cardiac hypersensitivity diagnosis.

Experiences and lessons
An early recognition and an extensive diagnostic workup are essential in recognizing mesalazine-induced lung and cardiac hypersensitivity in inflammatory bowel disease patients as drug withdrawal may result in a favorable outcome for the patient.

Peer-review
The authors described an interesting case of a patient with ulcerative colitis who developed lung and cardiac hypersensitivity related to mesalazine therapy. This article highlights the importance of considering drug-induced pulmonary and cardiac hypersensitivity in all inflammatory bowel disease patients who develop unexplained lung or cardiac disease while receiving mesalazine treatment.

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CASE REPORT

Treatment of Crohn's disease and familial Mediterranean fever by leukopheresis: Single shot for two targets

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about this clinical situation is limited with a few case reports in the literature. The treatment of both diseases depends on their individual therapies. However, it is very hard to deal with this coexistence when CD is refractory to standard therapies. Ongoing activity of CD triggers the clinical attacks of FMF and the symptoms like abdominal pain interfere with both disease presentations which can cause problems about diagnostic and therapeutic approach. The main therapeutic agent for FMF is colchicine and diarrhea is the most common side effect of this drug. This side effect also causes problems about management of these diseases when both of them are clinically active. Here we report probably the first case in the literature with coexisting CD and FMF who was successfully treated by leukopheresis since he was refractory to conventional therapies for CD.

Key words: Crohn's disease; Familial Mediterranean fever; Leukopheresis

Core tip: Management of coexisting autoimmune diseases is sometimes problematic because of resistance to standard therapies. Coexistence of Crohn's disease and familial Mediterranean fever is a rare condition, so the experience about their treatment is lacking. In our report we are sharing our treatment experience of a patient with this coexistence, where the case is refractory to conservative therapies and apheresis was applied as the last choice of treatment. This treatment modality seems to be the first in the literature for these diseases.

Yuksel M, Saygili F, Coskun O, Suna N, Kaplan M, Kuzu UB, Kilic ZMY, Ozin YO, Kayacetin E. Treatment of Crohn's disease and familial Mediterranean fever by leukopheresis: Single shot

Abstract

Coexistence of Crohn's disease (CD) and familial Mediterranean fever (FMF) is a rare condition and knowledge

INTRODUCTION

Crohn's disease (CD) is a chronic inflammatory disorder which can affect any part of the alimentary tract, tending to attack in a transmural manner[1]. Perianal involvement, abscesses, strictures and fistula formation are the different complications that can be faced during the active stage of the disease. Evolving technology and growing knowledge are bringing new therapeutic modalities for this progressive and sometimes problematic illness. However, no curable treatment has been developed due to poorly understood disease pathology. Induction and maintenance of remission are the goal of therapy. Budesonid, 5-aminosalicylic acid (5-ASA) derivatives, systemic steroids, thiopurines and anti-tumor necrosis factor (anti-TNF) agents are the main medications that are currently available and effective for induction and maintenance of remission all over the world. Surgical intervention is frequently needed for patients with abscess, fistula and strictures or those who are refractory to standard therapies. However, surgical therapy is not often satisfactory as we can use in ulcerative colitis.

During the active stage of CD, extravasation of granulocytes and monocytes/macrophages and their migration to the intestinal wall play a crucial role in transmural inflammation as they are the main sources of inflammatory cytokines, especially TNF[2]. Depleting activated granulocytes and inflammatory mononuclear cells by using apheresis to induce remission is the main goal for granulocyte and monocyte adsorptive apheresis (GMA). GMA is an effective and safe therapeutic option for patients with inflammatory bowel diseases refractory to pharmacological therapy[3].

Familial Mediterranean fever (FMF) is the most prevalent monogenic autoinflammatory disease, affecting more than 100000 subjects worldwide, mainly in the Mediterranean region. Despite novel advances in genetic testing of mutations, the diagnosis of FMF is still based on clinical criteria that include symptoms such as abdominal and thoracic pain, family history and response to treatment with colchicine[4]. Nevertheless, according to our recent knowledge the finding of mutations in the *MEFV* gene is mandatory for a definitive FMF diagnosis.

Coexistence of FMF and CD is a rare condition although a limited number of publications suggest that *MEFV* mutations are more frequent among CD patients when compared to healthy controls[5]. The treatment for both diseases mainly consists of using colchicine combined with 5-ASA derivatives, corticosteroids, thiopurines and biological agents.

Here we report the first case in the literature with coexisting FMF and refractory CD successfully treated by using GMA for both diseases.

CASE REPORT

A 20-year-old male patient was admitted to our clinic with complaints of abdominal pain, weight loss and diarrhea more than 20 times a day. Diarrhea was not bloody but contained mucus. The patient had a long and confusing medical history. He had complaints of chronic diarrhea when he was only 1-year-old. He has received medical therapy against amebiasis in a children's hospital. He had temporary improvement but mainly had the same complaints for more than two years. After an attack of bloody diarrhea and suspicion of perforation he underwent left hemicolectomy when he was 3-year-old. The pathological investigation of hemicolectomy specimen revealed chronic active colitis without any definitive diagnosis. The patient had same complaints after the first postoperative month. Mesalazine 50 mg/kg and prednisolon 2 mg/kg were initiated. Under medical therapy to induce remission he had flares of bloody diarrhea. The patient was reported to be mainly steroid dependent up to 9 years old. When he was 9 under mesalazine therapy, corticosteroids were initiated again to induce remission but he had a generalized tonic clonic convulsion. Cerebral evaluation by computed tomography (CT) magnetic resonance imaging, single-photon emission CT and as well as cerebrospinal fluid investigation revealed no abnormality. An electroencephalogram was consistent with pseudotumor cerebri. Corticosteroids were withdrawn and valproate sodium was initiated. After another flare of colitis azathioprine was added to mesalazine and valproate sodium treatment at the age of 10. The patient showed growth retardation and was still steroid dependent for colitis. When he was 10 as he had involvement of total residual colon and distal ileitis, total colectomy + endorectal anastomosis (straight) + end ileostomy + Hartman procedure was performed. Pathological assessment showed transmural involvement of chronic mononuclear inflammation which suggests the diagnosis of CD. Under medical therapy with mesalazine and azathioprine the patient complained of fever, arthralgia and pleuretic chest pain after the first year of extended surgical operation. *MEFV* gene mutation analysis was then performed and he was found to have a homozygous M694V mutation. Familial Mediterranean fever was diagnosed and colchicine therapy was added to his treatment regimen. Despite an effective dose of colchicine he had fever attacks, and due to increase in stool number he had to discontinue colchicine sometimes. While he was 15 years old, he had perianal complaints which were diagnosed as perianal involvement of CD. As he was resistant to all therapies, adalimumab (ADA) 40 mg/2 weeks was

Figure 1 Perianal swelling and inflammation which reflect activity of perianal Crohn's disease.

Figure 2 Computed tomography appearance of diffuse symmetric thickening at the site of rectal anastomosis (arrows).

initiated. He had 9 cycles of ADA with dose modification but remission induction could not be accomplished. ADA therapy was discontinued and he was on mesalazine, AZA and steroids when he was admitted to our clinic. Physical examination revealed growth retardation, dehydration, malnutrition and perianal swelling and inflammation (Figure 1). Colonoscopy with biopsies up to distal 30 cm revealed ileal and rectal ulcerations and inflammation. Abdominal CT showed intestinal thickening in distal ileal and anastomotic regions (Figure 2). CD activity index (CDAI) was calculated to be 397. He also had fever and chest pain attacks during hospitalization. A total of 12 cycles of GMA by using Cellsorba filters, twice weekly, were performed as the patient still had medical therapy resistant luminal and perianal CD with coexisting FMF which has not been possible to be treated properly. The ileal and rectal biopsy specimens showed findings of active CD without any sign of amyloidosis. After 12 cycles of GMA, the clinical symptoms and laboratory findings improved and CDAI was found to be 139. The patient did not have fever and chest pain attacks since GMA therapy. AZA and mesalazine were continued to sustain remission. The patient is still in remission after 3 mo of follow-up and is still attack free in terms of FMF.

DISCUSSION

Coexistence of CD and FMF is a rare condition. Combination of individual therapies of both diseases is the main choice of treatment in this situation. However, the resistance of CD to standard therapies brings difficulties to the management of both diseases as colchicine itself induces diarrhea as a side effect. GMA is available in Europe and Japan for the treatment of patients with active inflammatory bowel disease (IBD) that may have become refractory to standard drug based medication, including TNF-α blockers[6]. GMA was first used for CD in the literature by Fradji *et al* and Gonzalez Carro *et al* showed in a case report that weekly GMA when added to scheduled infliximab (IFX) maintenance therapy was effective in a CD patient while disease was active despite IFX therapy[7]. In another case report form Japan, Fukunaga *et al*[8] also showed similar successful induction of remission in an IFX refractory patient with additional weekly GMA therapy. Further, intensive GMA involving two sessions per week has been recently shown to be more effective than conventional weekly GMA for refractory IBD[9]. In addition, GMA does not have any high risk of serious adverse events. The use of GMA in IBD has been increasing during the last decade and new reports are emerging about efficacy and safety of this procedure[10,11].

In our case we have reported a patient with refractory CD and coexisting FMF with fever and pleural attacks. GMA was used mainly for induction of remission for CD but it was also found to be beneficial for FMF attacks. Our patient seems to be the first patient in the literature who was successfully treated by GMA for both CD and FMF. It is a novel finding because we do not have any data about the use of apheresis among FMF patients. Positive response of FMF to GMA therapy may be a result of depletion of systemic inflammatory state. Also, the beneficial effect can be coincidential, but it is still promising and needs to be evaluated carefully. The coexistence of these two inflammatory diseases is a challenging problem and GMA may be a new and encouraging answer. As the patient could not use colchicine properly due to side effects, he had limited choices of treatment for FMF. Luckily, rectal specimens did not show amyloid deposits which is the most important complication of this disease, although the patient had FMF for more than 9 years. Long term maintenance with GMA for both CD and FMF is not possible, but during remission of CD the patient would have a chance to initiate colchicine again which will be a definitive therapy for FMF. We have already known the beneficial effect of GMA in CD from the literature. However, the effect of GMA in FMF still seems to be mysterious. Apart from

the mechanism of action from the patient's point of view, it seems to be a good single shot for two targets.

COMMENTS

Case characteristics
A 20-year-old male with coexisting Crohn's disease (CD) and familial Mediterranean fever (FMF) had complaints of abdominal pain and diarrhea.

Clinical diagnosis
Increased bowel sounds and abdominal tenderness were observed.

Differential diagnosis
Acute abdomen and infectious diarrhea.

Laboratory diagnosis
WBC 16.4 k/μL, HGB 11.8 g/dL, ESR 64 mm/h, and CRP, 44 mg/L.

Imaging diagnosis
An abdominal computed tomography scan showed diffuse thickening of the intestinal wall at the site of previous anastomosis, which revealed recurrence and active disease.

Pathological diagnosis
Ileal and rectal biopsy specimens showed findings of active CD without any sign of amyloidosis.

Treatment
Twelve cycles of granulocyte monocyte adsorptive apheresis (GMA) with Cellsorba filters was performed twice weekly to induce remission in CD and treat FMF attacks.

Related reports
There is not any report on treating both diseases by using apheresis.

Term explanation
GMA is variant of apheresis that aims to deplete granulocytes and monocytes in the circulation to control systemic inflammatory activity.

Experiences and lessons
This report mentions a successful experience of treating coexisting active CD and FMF by using GMA, which is a novel procedure for this situation.

Peer-review
This report suggests a new therapeutic approach for two diseases that are refractory to standard therapies.

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CASE REPORT

Adenocarcinoma arising from heterotopic pancreas at the third portion of the duodenum

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Author contributions: Fukino N, Oida T, Mimatsu K, Kuboi Y and Kita K carried out the operation and were consultants overseeing the patient's care; and Fukino N wrote the manuscript.
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Informed consent: Because a patient dies, the written consent is impossible.
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Abstract

A 62-year-old Japanese man presented to our hospital with a history of weight loss of 6 kg in 4 mo. Imaging examinations revealed a tumor located on the third portion of the duodenum with stenosis. We suspected duodenal carcinoma and performed pancreas-preserving segmental duodenectomy. Adenocarcinoma arising from a heterotopic pancreas at the third portion of the duodenum was finally diagnosed by immunohistochemical staining. Malignant transformation in the duodenum arising from a heterotopic pancreas is extremely rare; to our knowledge, only 13 cases have been reported worldwide, including the present case. The most common location of malignancy is the proximal duodenum at the first and descending portion. Herein, we describe the first case of adenocarcinoma arising from a heterotopic pancreas, which was located in the third portion of the duodenum, with a review of the literature.

Key words: Adenocarcinoma; Heterotopic pancreas; Duodenum; Segmental resection; Weight loss

Core tip: Based on all abdominal surgeries, the incidence of heterotopic pancreas ranges from 0.25% to 1.2%. The frequency of malignant transformation ranges from 0.7% to 1.8% among all heterotopic pancreas cases. Malignant transformation arising from a heterotopic pancreas is extremely rare. The most common location of malignancy is the proximal duodenum at the first and descending portion. In this paper, we report the case of a patient with weight loss who was diagnosed as having an adenocarcinoma arising from a heterotopic pancreas, at the third portion of the duodenum, with a review of the literature.

Fukino N, Oida T, Mimatsu K, Kuboi Y, Kita K. Adenocarcinoma arising from heterotopic pancreas at the third portion of the duodenum. *World J Gastroenterol* 2015; 21(13): 4082-4088 Available from: URL: http://www.wjgnet.com/1007-9327/full/

INTRODUCTION

Heterotopic pancreas is defined as an unusual pancreatic tissue existing in other organs without any connection to the original pancreas. The most frequent locations are the duodenum (9%-36%), stomach (24%-38%), jejunum (0.5%-27%), and Meckel's diverticulum (2%-6.5%)[1]. Heterotopic pancreas has been classified into four types by Heinrich[2,3]. The incidence of heterotopic pancreas ranges from 0.25% to 1.2% among all abdominal surgeries[4]. Malignant transformation arising from a heterotopic pancreas is extremely rare, and has been reported in several cases in the literature. The frequency of malignant transformation ranges from 0.7% to 1.8% among all cases of heterotopic pancreas[5,6]. Malignant tumors arising from a heterotopic pancreas, as well as heterotopic pancreas, commonly appear pathologically as a smooth nodule, and occasionally, as a mass with an irregular surface. These tumors are usually located in the submucosa and only occasionally expand into the muscularis[1]. Since only a small number of cases have been reported, the prognosis of patients with malignant tumors arising from a heterotopic pancreas is not clear. Herein, we describe the first case of adenocarcinoma arising from a heterotopic pancreas, which was located in the third portion of the duodenum.

CASE REPORT

A 62-year-old Japanese man presented to our hospital with a history of weight loss of 6 kg in 4 mo. There were no remarkable findings on the patient's medical history. The patient's physical examination and vital signs were normal. He was admitted to our hospital for examination because of elevated serum levels of the following tumor markers (the normal range for each parameter is in parentheses): carbohydrate antigen 19-9, 500 U/mL (0-37 U/mL); DUPAN-2, 226 U/mL (less than 150 U/mL); and SPan-1, 41 U/mL (less than 30 U/mL). The level of carcinoembryonic antigen was within the normal range. The results of the complete blood count test, coagulation test, and laboratory analysis were normal. Contrast-enhanced computed tomography of the abdomen showed a poorly enhanced mass around the middle section of the third portion of the duodenum and superior mesenteric artery (Figure 1A). Duodenofiberoscopic findings (Figure 1B) and hypotonic duodenography (Figure 1C) revealed a submucosal tumor with a smooth surface and obstruction at the third portion of the duodenum. Biopsies of the submucosal tumor were taken and revealed no evidence of malignancy. We
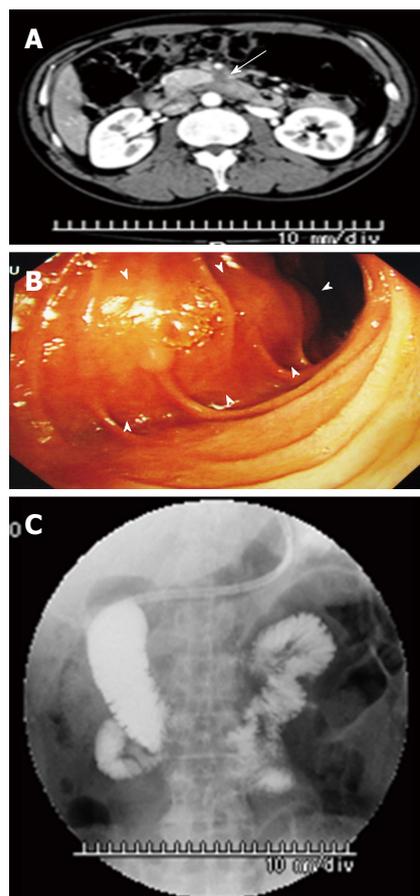

Figure 1 Contrast-enhanced computed tomography, duodenofiberoscopy, and hypotonic duodenography. A: Abdominal CT scan shows a poorly enhanced mass around the third portion of the duodenum (arrow); B: Duodenofiberoscopic findings reveal submucosal tumors at the third portion of the duodenum (arrowheads); C: Hypotonic duodenography reveals stenosis at the third portion of the duodenum. CT: Computed tomography.

suspected a diagnosis of duodenal carcinoma from the examination results and performed open resection with intraoperative frozen section analysis. Intraoperative examination revealed that the stomach and pancreas were normal. The umbilication was located in front of the ligament of Treitz and was completely separable from the superior mesenteric artery (Figure 2A). The intraoperative frozen section analysis revealed adenocarcinoma of the duodenum. We diagnosed primary duodenal carcinoma and performed pancreas-preserving segmental duodenectomy from the third portion to the fourth portion of the duodenum. The intestinal reconstruction was carried out by side-to-side reconstruction using the GIA™ Stapling System (Covidien, Tokyo, Japan). Macroscopic findings were normal at the surface of duodenum (Figure 2B). Microscopically, the tumor was 15 mm in diameter and extended into the submucosa of the third portion of the duodenum. The major part of the tumor was moderately differentiated adenocarcinoma (Figure 3A). Moreover, only islet cells were present within and near the tumor (Figure 3B). In immunohistochemical staining, cytokeratin (CK) 7 (Figure 4A), neural cell
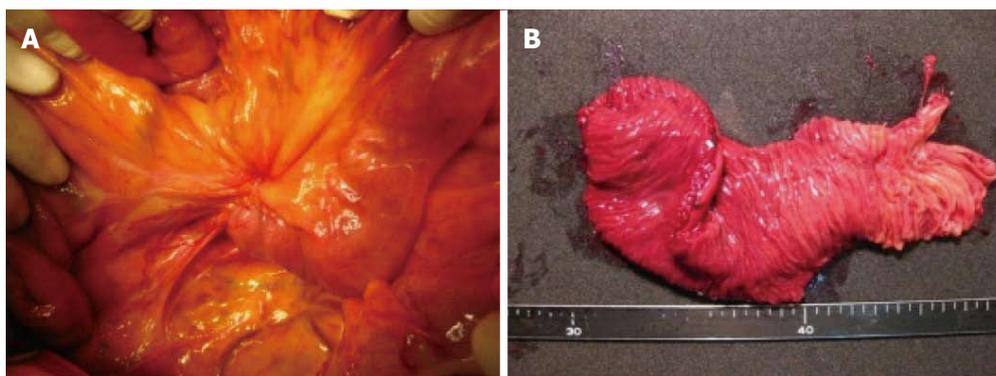
Figure 2 Intraoperative findings. A: During surgery, the tumor with the umbilication was located in front of the Treitz ligament; B: Examination of the resected specimen revealed a normal duodenal surface.
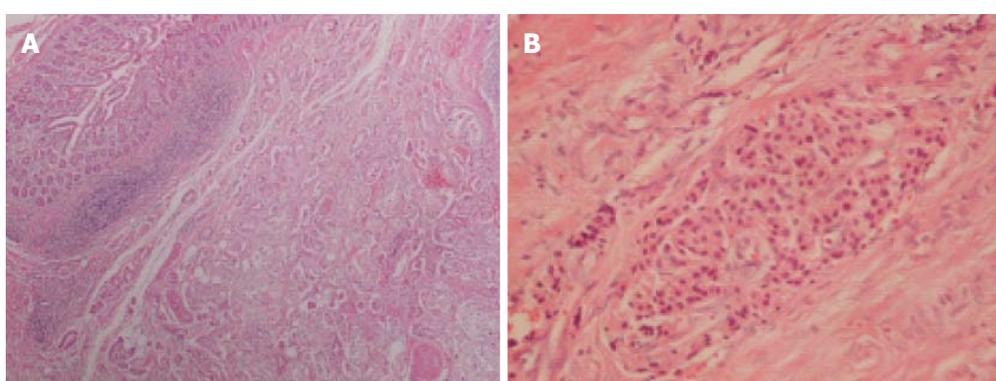

Figure 3 Histological examinations. A: The resected part of the duodenum shows a moderately differentiated tubular adenocarcinoma [Hematoxylin and eosin (HE), × 40]; B: Only the islet cells are present within and near the tumor (HE, × 200). HE: Hematoxylin and eosin.

adhesion molecule (CD56) (Figure 4B), chromogranin A (Figure 4C), synaptophysin (Figure 4D), insulin (Figure 4E), and mucin 1 (MUC1) (Figure 4F) were positive. On the other hand, CK20 (Figure 5A) and MUC2 (Figure 5B) were significantly negative. From the above results, we finally diagnosed adenocarcinoma arising from a heterotopic pancreas at the third portion of the duodenum. The postoperative course was uneventful, and our patient was discharged from our hospital 25 d after surgery. The patient was referred to the oncology department for adjuvant chemotherapy. We monitored the serum level of CA19-9 (Figure 6). On postoperative month 1, our patient underwent 3 courses of oral tegafur-gimeracil-dihydropyrimidine dehydrogenase (S-1) plus cisplatin chemotherapy and S-1 monotherapy, respectively. Unfortunately, serum levels of CA19-9 did not decrease. We diagnosed local recurrence of heterotopic pancreas carcinoma by positron emission and computed tomography (PET-CT) at 8 mo after surgery. After radiation therapy at another hospital (total 54 Gy), gemcitabine (GEM) monotherapy, 1000 mg/m^2, was administered intravenously for 30 min on days 1, 8, and 15 of each 28-d cycle. The serum level of CA19-9 decreased gradually from 271 U/mL to 76 U/mL by GEM monotherapy. We changed the treatment from GEM monotherapy to GEM plus S-1 after administrating 10 courses of GEM monotherapy, because the serum level of CA19-9 increased again. At 24 mo after surgery, a gastrostomy was performed and a central venous access device was implanted for administrating total parenteral nutrition because the patient suffered from intestinal obstruction. We decided to terminate all chemotherapy and provide best supportive care. Our patient died 33 mo after the primary operation.

DISCUSSION

Heterotopic, ectopic, or aberrant pancreas, which is commonly an incidental finding at surgery or autopsy, is defined as a pancreatic tissue existing in other organs without any connection to the original pancreas and has been frequently reported in the gastrointestinal tract, especially in the stomach. The first report of heterotopic pancreas was identified by Shultz in 1727. In 1859, the histological confirmation was reported by Klob[7-9]. The frequency of heterotopic pancreas tissue has been reported to be 0.55%-13.7%, 0.25%, and approximately 1.2% in autopsy material, abdominal surgery, and gastrectomy operation, respectively[7]. In Japan, Tanaka *et al*[4] reported that the incidence of heterotopic pancreas was 0.25% among 6035 patients who underwent laparotomy. Clinical symptoms caused by heterotopic pancreas
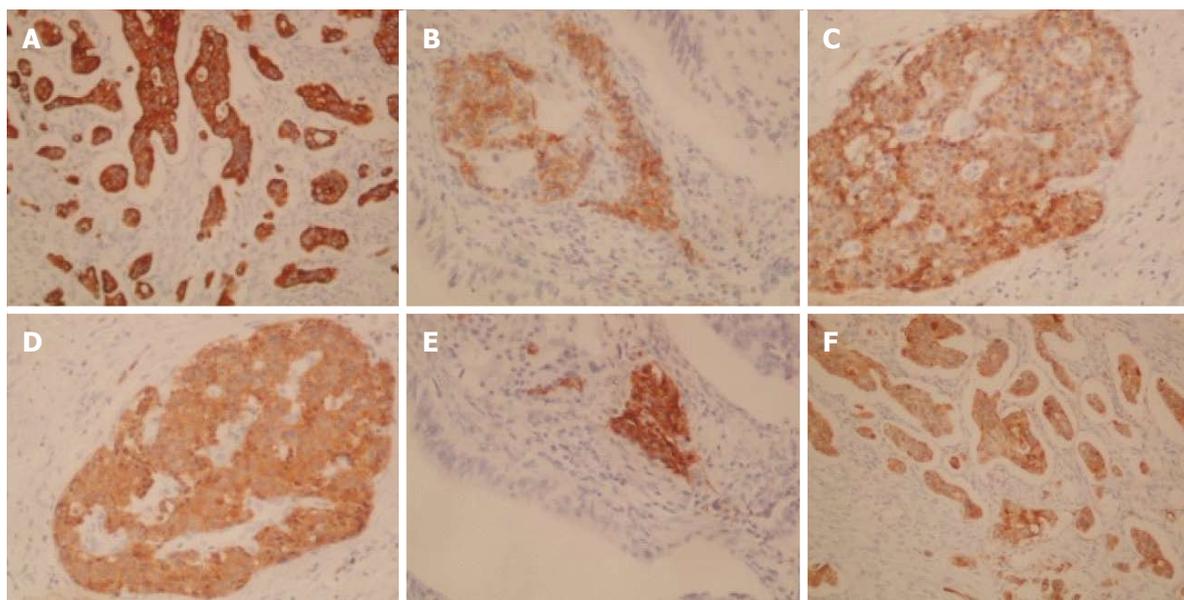
Figure 4 Immunohistochemical staining: positive staining. A: Cytokeratin (CK) 7 (× 100); B: Neural cell adhesion molecule (CD56) (× 200); C: Chromogranin A (× 200); D: Synaptophysin (× 200); E: Insulin (× 200); F: Mucin 1 (MUC1).
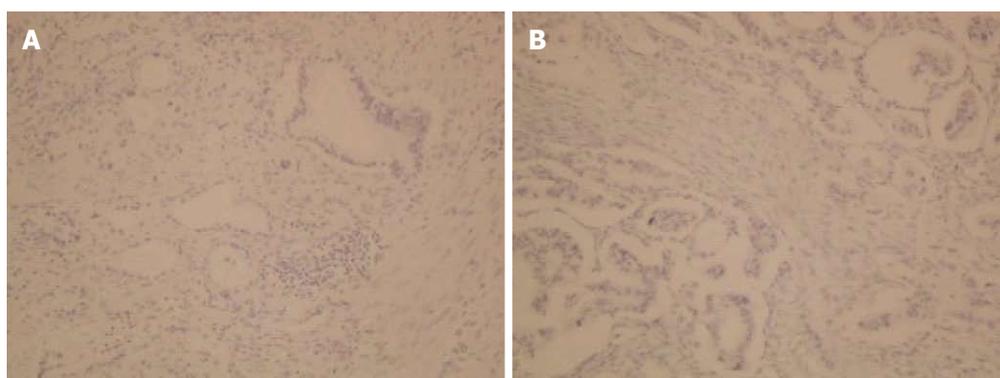

Figure 5 Immunohistochemical staining: negative staining. A: Cytokeratin (CK) 20 (× 100); B: Mucin 2 (MUC2) (× 200).

are not specific. Symptoms such as abdominal pain, vomiting, bleeding, and jaundice appear in conjunction with tumor growth. In the present case, the patient presented weight loss of 6 kg in 4 mo.

Heterotopic pancreas has been classified into 4 types by Heinrich in 1909[2,3]. Type I is characterized by the presence of typical pancreatic tissues with acini, ducts, and islet cells similar to those seen in a normal pancreas. Type II is characterized by the presence of pancreatic ducts and acini, while islet cells are not present. Type III is characterized by ducts with a few acini or dilated ducts only, so called adenomyoma. Type IV characterized by the presence of islet cells only. Based on Heinrich's classification, most heterotopic pancreas cases are Type II. Although the reason is unknown, to date, Type IV heterotopic pancreas cases have not been reported. The present case was classified as Type IV according to Heinrich's classification, since pathological findings revealed only islet cells within and near the tumor.

Guillou *et al*[6] indicated that the incidence of malignancy due to heterotopic pancreas was 0.7% and extremely rare. They studied the frequency of malignant transformations among 146 cases of heterotopic pancreas, including surgical and autopsy specimens, between 1975 and 1991. In 1999, Makhlouf *et al*[5] reported that among 109 patients diagnosed as having pancreatic heteropia in the gastrointestinal tract, the incidence of malignant transformation arising from a heterotopic pancreas was 1.8%. According to their study, there was a slight predominance of women over men; the mean age was 47-49 years. Most commonly, the tumor was located in the upper gastrointestinal tract, from the stomach to the jejunum. The most common symptoms were digestive symptoms such as nausea, vomiting, and jaundice caused by obstruction. There were no specific symptoms. The mean tumor size was 3.5-3.7 cm. Most of these tumors were ductal adenocarcinoma, similar to those seen in the pancreas[5,6]. Jaervi and Lauren[10]
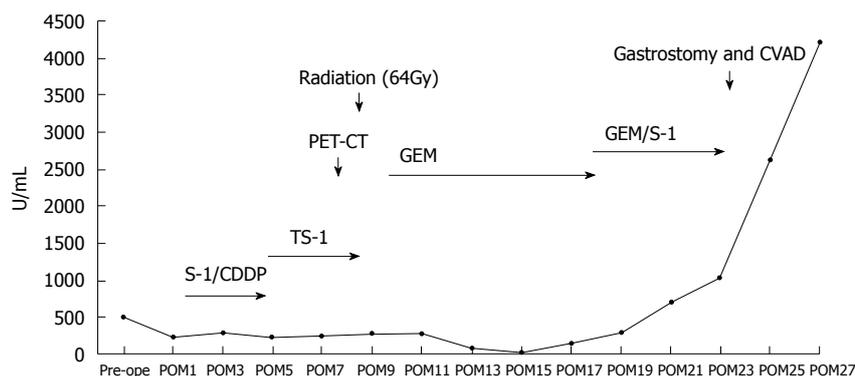
Figure 6 Chronological change of the serum level of CA19-9. Pre-ope: Preoperation; POM: Post operative month; S-1: Tegafur-gimeracil-ditrydropyrimidine dehydrogenase; CDDP: Cisplatin; PET-CT: Positron emission and computed tomography; GEM: Gemcitabine; CVAD: Central venous access device.

have proposed 3 criteria for the diagnosis of carcinoma arising from a heterotopic pancreas: (1) the tumor must be found within or close to the ectopic pancreatic tissue; (2) direct transition must be observed between pancreatic structures and the carcinoma (malignant transformation of an ectopic pancreas must be differentiated from a metastatic deposit or a neoplastic invasion from a neighboring digestive cancer, especially from the stomach, the biliary tract, and the eutopic pancreas); and (3) the non-neoplastic pancreatic tissue must comprise at least fully developed acini and ductal structures. According to those criteria, Guillou *et al*[6] suggested that it was impossible to ascertain the pancreatic origin of the tumor in the absence of acini and/or islets of Langerhans, since ductal structures might have a local origin by means of a metaplastic phenomenon. Therefore, Heinrich's type III, so called adenomyoma, should not be classified as an aberrant pancreas. Carcinomas arising from a heterotopic pancreas have been classified by most authors according to the 3 criteria and the opinion of Guillou *et al*[6]. However, as mentioned above, Heinrich classified the histologic pattern of heterotopic pancreas into 4 types. In the present case, the tumor extended into the submucosa microscopically. The major part of the tumor changed into moderately differentiated adenocarcinoma. Moreover, only the islet cells were present within and near the adenocarcinoma. For all these reasons, we diagnosed the patient as having type IV heterotopic pancreas according to Heinrich's classification.

To our knowledge, 36 cases of malignant transformation arising from a heterotopic pancreas, including the present case, have been reported in PubMed (key words: heterotopic (ectopic, aberrant) pancreas, carcinoma). The incidence according to location is as follows: duodenum, 36%; stomach, 36%; jejunum, 8%, and other (including the mediastinum, liver, esophagus, hiatal hernia, periaorta, and rectum), 19%. We summarized 13 cases of malignant transformation arising from a heterotopic pancreas, in the duodenum (Table 1)[4,9,11-18]. The mean age was 70.9 years (range: 56-86 years). There was no sex predilection.

Most commonly, the tumor was located from the bulb to the descending portion in the duodenum; only in the present case, the tumor was located in the third portion of the duodenum. The mean tumor size was 25 mm (range: 12-50 mm). Approximately 80% and 50% of the tumors were pathologically diagnosed as adenocarcinomas (tubular adenocarcinoma, poorly differentiated adenocarcinoma, papillary adenocarcinoma, and mucinous adenocarcinoma) and classified as Heinrich's type I. The mean tumor size of the malignant transformation is unknown; however, most tumors were bigger than the previously reported mean tumor size of heterotopic pancreas (mean 18 mm; range: 4-5 mm)[4]. In our study, the mean size varied from 12 to 50 mm in maximum dimension with a mean of 25 mm.

Most of these tumors were pathologically diagnosed as ductal adenocarcinomas. In addition, there are noninvasive carcinomas within intraductal papillary mucinous neoplasia and acinar cell carcinoma. It is impossible to diagnose heterotopic pancreas and malignant transformation preoperatively and precisely, because these tumors are located within the submucosal and subserosal layers. Endo *et al*[11] diagnosed ectopic pancreas adenocarcinoma preoperatively by endoscopic ultrasonography-guided fine-needle aspiration (EUS-FNA) in 2 cases. They suggest that EUS-FNA is particularly helpful for the preoperative diagnosis. However, cytological examinations are inconclusive in about 50% of cases[16]. In our study, the accurate preoperative diagnosis rate by EUS-FNA and biopsy was about 40%. We diagnosed adenocarcinoma of the duodenum by pathological diagnosis during surgery, and malignant transformation arising from a heterotopic pancreas of the duodenum by the immunohistochemical staining, similar to previous articles.

The prognosis of patients with adenocarcinoma arising from an ectopic pancreas seems to be somewhat better than that of patients with tumors arising from the pancreas itself, probably because of earlier presentation[19,20]. To our knowledge, long-term survival was reported in a case of invasive ductal

Table 1 Review of cases of malignant formation arising from a heterotopic pancreas in the duodenum

Case	Year	Author	Age (yr)	Sex	Clinial symptoms	Location	Preoperative diagnosis	Diagnostic approach	Size (mm)	Pathology	Heinrich	Outcome
1	1993	Tanaka	72	M	Abdominal pain	D (ND)	ND	Operation	12	Cancer	ND	ND
2	1996	Inoue	81	F	Anorexia	D (ND)	Duodenal cancer	Biopsy	25	pap + muc	I	ND
3	2006	Inoue	75	M	Melena	D (ND)	No evidence of malignancy	Operation	20	tub	III	ND
4	2007	Tison	72	M	Abdominal pain, jaundice	D (2nd; Vater)	Tumor of the ampulla of Vater	Biopsy	ND	Adenocarcinoma, CDHP	ND	Death (16 mo)
5	2007	Kawakami	68	F	Jaundice	D (2nd; Vater)	Carcinoma of the ampulla of Vater	Biopsy	12	Acinar cell carcinoma	ND	Not death (19 mo)
6	2008	Rosok	59	F	No symptom	D (proximal)	No evidence of malignancy	Operation	50	IPMC	ND	Not death (36 mo)
7	2010	Inoue	75	M	Nausea, vomiting	D (2nd)	Cancer, GIST, MTL	Operation	ND	tub (well)	III	Not death (72 mo)
8	2010	Bini	56	M	Vomiting	D (1st)	Adenocarcinoma of uncertain origin	Biopsy	ND	Adenocarcinoma	I	ND
9	2011	Stock	79	F	Abdominal fullness, intermittent abdominal pain	D (4th)	No evidence of malignancy	Operation	30	Adenocarcinoma	I	ND
10	2012	Kinoshita	62	F	Vomiting, epigastralgia	D (1st)	Gastric cancer	Operation	34	tub (mode)	I	Not death (12 mo)
11	2013	Ginori	86	F	Abdominal pain, nausea, vomiting	D (1st)	Acute cholecystitis	Operation	30	well + muc + por	I	ND
12	2014	Endo	75	M	Epigastric pain, tarry stool	D (2nd)	Adenocarcinoma of uncertain origin	EUS-FNA	22	por	I	Not death (60 mo)
13	Present case		62	M	Weihgt loss	D (3rd)	Suspected duodenal cancer	Operation	15	tub (mode)	IV	Death (33 mo)

ND: Not described; GIST: Gastrointestinal stromal tumor; MTL: Malignant lymphoma; EUS-FNA: Endoscopic ultrasonography-guide fine-needle aspiration; tub: Tubular adenocarcinoma; well: Well differentiated type; mode: Moderately differentiated type; por: Poorly differentiated adenocarcinoma; pap: Papillary adenocarcinoma; muc: Mucinous carcinoma; CDHP: Cystic dystrophy in heterotopic pancreas; IPMC: Intraductal papillary-mucinous carcinoma.

carcinoma arising from an ectopic pancreas within the gastric wall, with no recurrence for 11 years[20]. In the present case, our patient died 33 mo after surgery because of local recurrence of the adenocarcinoma.

There is no evidence of the efficacy of chemotherapy for adenocarcinoma arising from a heterotopic pancreas. In the present case, it took time to reach a definite diagnosis. First, we diagnosed primary adenocarcinoma of the duodenum only when the immunohistochemical staining results were available. We selected a regimen of S-1 plus cisplatin as first-line therapy for duodenal cancer, based on the guidelines for the treatment of gastric cancer in Japan. Our case was referred for 3 courses of adjuvant chemotherapy with S-1 plus cisplatin and S-1 monotherapy, respectively. Unfortunately, the serum level of CA19-9 remained high. On postoperative month 8, PET-CT revealed local recurrence. After radiation in another hospital (total 54 Gy), the patient was administered GEM. The serum level of CA19-9 decreased gradually to 76 U/mL. Our patient eventually developed tolerance to chemotherapy and died 33 mo after the primary operation. To the best of our knowledge, there is no literature in PubMed on the efficacy of chemotherapy (including adjuvant and systemic chemotherapy) for adenocarcinoma arising from a heterotopic pancreas. However, our experience suggests that GEM is effective for these tumors, as in cases of primary pancreatic cancer. The study of further cases of adenocarcinoma due to a heterotopic pancreas, including the efficacy of chemotherapy treatment, is necessary.

In conclusion, we herein present the first case of malignant transformation arising from a heterotopic pancreas at the third portion of the duodenum.

COMMENTS

Case characteristics
A 62-year-old Japanese man with a weight loss of 6 kg in 4 mo presented to our hospital.

Clinical diagnosis
The physical examination was normal.

Differential diagnosis
Malignant tumors (primary duodenal adenocarcinoma and metastatic tumors of uncertain origin) and benign neoplasms (submucosal tumors, heterotopic pancreas, and adenoma).

Laboratory diagnosis
The patient had elevated serum levels of carbohydrate antigen 19-9 (500 U/mL) DUPAN-2 (226 U/mL), and SPan-1 (41 U/mL), while there were no remarkable

findings on other laboratory analyses.

Imaging diagnosis
Abdominal enhanced computed tomography, duodenofiberoscopy, and hypotonic duodenography revealed a mass and submucosal tumor located in the third portion of the duodenum.

Pathological diagnosis
Histological examination revealed moderately differentiated adenocarcinoma with islet cells only; therefore, the immunohistochemical staining revealed positive cytokeratin 7, neural cell adhesion molecule (CD56), chromogranin A, synaptophysin, insulin, and mucin 1.

Treatment
The patient underwent pancreas-preserving segmental duodenectomy; therefore, chemotherapy and radiation was administered after surgery.

Related reports
A total of 13 cases of malignant transformation arising from a heterotopic pancreas in the duodenum have been reported in the literature. This is the first report of adenocarcinoma arising from a heterotopic pancreas at the third portion of the duodenum, worldwide, and the chemotherapeutic method is controversial.

Term explanation
Heterotopic, ectopic, or aberrant pancreas, which is commonly an incidental finding at surgery or autopsy, is defined as pancreatic tissue existing in other organs without any connection to the original pancreas and has been frequently reported in the gastrointestinal tract, especially in the stomach.

Experiences and lessons
Commonly, the tumor, which is an adenocarcinoma arising from a heterotopic pancreas, is located from the bulb to the descending portion in the duodenum. This article presents the first case of a patient with an adenocarcinoma arising from a heterotopic pancreas, located at the third portion of the duodenum.

Peer-review
This is a well written and interesting study that addresses an unusual case in pancreatic adenocarcinoma.

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CASE REPORT

Lymphoepithelioma-like cholangiocarcinoma: A mimic of hepatocellular carcinoma on imaging features

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Abstract

Primary lymphoepithelioma-like carcinoma in the liver is extremely rare. A few cases of lymphoepithelioma-like cholangiocarcinoma have been reported, but few radiologic features were described. We reviewed 23 cases of lymphoepithelioma-like cholangiocarcinoma reported between 1996 and 2014 and describe a rare case of a 35-year-old woman in our hospital who was diagnosed with lymphoepithelioma-like cholangiocarcinoma of the liver and was a hepatitis B carrier. The tumor (1.6 cm) in our patient appeared to be hypoechoic in sonographic images and hypodense in computed tomography (CT) images. In addition, it was homogeneous hypointense in T1-weighted magnetic resonance (MR) images (MRI) and hyperintense in T2-weighted MRI. Dynamic gadolinium-enhanced MRI showed typical image pattern of hepatocellular carcinoma (HCC). The patient underwent a laparoscopic left hepatic lobectomy, and the resected tumor consisted of well-differentiated glandular cells with extensive lymphocytic infiltration that were immunoreactive to CK (AE1/AE3), CD3, and CD20. In addition, the tumor was positive for Epstein-Barr virus-encoded RNA *in situ* hybridization. Finally, lymphoepithelioma-like cholangiocarcinoma was diagnosed. In previous studies, the incidence is highest among middle-aged people. Most tumors appeared to be hypodense with either hypovascular or hypervascular patterns in CT images. This case report is the first study to address sonography, CT, and MRI observations and delineate pathologic correlations. We suggest that the imaging pattern of lymphoepithelioma-like cholangiocarcinoma, either the typical cholangiocarcinoma pattern or a mimic of HCC, should be considered in the differential lists for HCC.

Key words: Epstein-Barr virus; Hepatocellular carcinoma; Lymphoepithelioma-like carcinoma; Lymphoepithelioma-like cholangiocarcinoma; Magnetic resonance imaging

Core tip: We report the first case of lymphoepithelioma-

like cholangiocarcinoma observed using sonography, computed tomography, and magnetic resonance images and delineate the pathologic correlations. According to a review of previous studies, lymphoepithelioma-like cholangiocarcinoma may affect more middle-aged woman. We suggest that the imaging pattern of lymphoepithelioma-like cholangiocarcinoma, either a typical cholangiocarcinoma pattern or a mimic of hepatocellular carcinoma, should be considered in the differential lists for hepatocellular carcinoma.

INTRODUCTION

Lymphoepithelioma-like carcinoma (LELC) is a tumor with morphologic features similar to those of undifferentiated nasopharyngeal carcinoma that occurs outside the nasopharynx and is associated with Epstein-Barr virus (EBV) infection. In the liver, this type of tumor is extremely rare, and only 23 cases of lymphoepithelioma-like cholangiocarcinoma have been described in reports focused on histologic and immunohistochemical analyses[1-12]. A recent study that reported seven female cases of EBV-associated lymphoepithelioma-like cholangiocarcinoma demonstrated molecular genetic pathology, including frequent DNA hypermethylation[9]. However, few radiologic features have been described. Therefore, we report a case of lymphoepithelioma-like cholangiocarcinoma in a young patient and review the literature on imaging features. According to our review of relevant research, our case report is the first to describe observations made using imaging procedures such as sonography, computed tomography (CT), magnetic resonance imaging (MRI).

CASE REPORT

This report involves the case of a 35-year-old woman who was a chronic hepatitis B carrier. She had no other systemic diseases, such as hypertension or diabetes mellitus. In 2013, a small hypoechoic hepatic nodule was discovered during regular annual sonographic examination, and an abdominal CT at a local hospital revealed a small hypodense nodule with mild enhancement at the medial segment of the left hepatic lobe (Figure 1). She then visited our center for a second opinion.

During hospitalization, the patient did not complain of abdominal problems, and physical examinations and laboratory tests, including that of her serum alpha-fetoprotein level (1.3), did not indicate any abnormalities. The patient did not meet the criteria for a diagnosis of hepatocellular carcinoma (HCC) according to the American Association for the Study of Liver Diseases Practice Guidelines[13]. Because the CT scan was not dynamic contrast enhanced and the acquisition time was too early to be differentiated as a small HCC or other hepatic tumor. We then evaluated other tumor markers, including carbohydrate antigen (CA19-9; 33 μ/mL) and carcinoembryonic antigen (CEA; 1.4 ng/mL), which were within normal limits. A repeat sonographic examination and dynamic abdominal MRI were scheduled to investigate the possibility of other hepatic neoplasms.

Sonographic examination revealed a small (1.7 cm × 1.2 cm) well-defined and homogeneous hypoechoic nodule protruding into the liver surface at the lateral segment (Figure 2). The MR image depicted a well-defined 1.7-cm nodule at S4b of the liver (Figure 3). The nodule appeared to be homogeneous hypointense on in-phase and out of phase T1-weighted images without decreased signal intensity, meaning no intracellular fat (Figure 3A and B), and hyperintense on axial T2-weighted images (Figure 3C). No calcification, hemorrhaging, or fat components were observed in the lesion. In addition, hepatolithiasis and intrahepatic bile duct dilatation were absent. The main portal vein and its major branches were patent. Dynamic gadolinium-enhanced MRI indicated hypointense on pre-contrast T1-weighted image (Figure 3D), early arterial enhancement in the arterial phase (Figure 3E), washout in portal venous phase and delayed fibrous capsule enhancement (Figure 3F). These are typical imaging characteristics of HCC.

Tentatively diagnosed with HCC, the patient subsequently underwent a laparoscopic left lobectomy. During surgery, a solitary 1.7 cm whitish tumor was located at the medial segment of the liver, immediately below the falciform ligament. The tumor was well defined and gray-white in color with a thick fibrous capsule and without extrahepatic capsular extension (Figure 4A).

Histologically, the tumor consisted of well-differentiated glandular cells with extensive lymphocytic infiltration and scattered lymphoid follicles (Figure 4B). In a few areas, a syncytial growth pattern was observed in the tumor (Figure 4C). The tumor cells were diffusely positive for CK (AE1/AE3) and EBV-encoded RNA (EBER) *in situ* hybridization (Figure 4D). CD3 and CD20 stains indicated mixed B- and T-cell infiltration. The adjacent non-tumorous liver tissue did not reveal significant histopathologic abnormality. The final diagnosis was stage I EBV-related lymphoepithelioma-like cholangiocarcinoma. The patient recovered gradually after surgery and was discharged from our hospital.
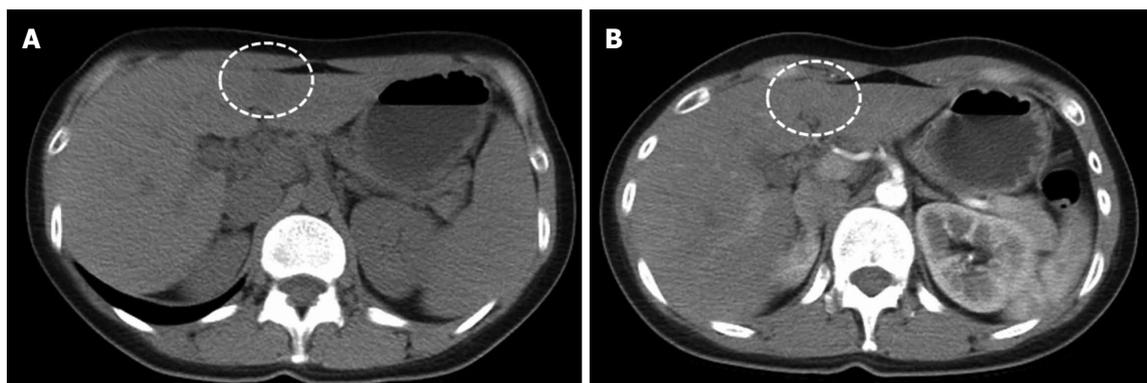
Figure 1 Computed tomography of lymphoepithelioma-like cholangiocarcinoma (dash circle). A: Noncontrast CT showed a small hypodense nodule (38 HU) at the lateral segment of liver; B: The tumor nodule depicted enhancement in early arterial phase of contrast enhanced CT (65HU).

Figure 2 Sonography of lymphoepithelioma-like cholangiocarcinoma.

DISCUSSION

After HCC, intrahepatic cholangiocarcinoma is the most common primary hepatic malignant tumor type, and cholangiocarcinoma is the most prevalent in Southeast Asia. However, radiologists encounter difficulty in diagnosing cholangiocarcinoma because of its wide spectrum of radiologic appearances. Intrahepatic cholangiocarcinoma is classified according to morphology into mass-forming, periductal infiltrating, and intraductal growing types[14]. Typically, CT features of mass-forming cholangiocarcinoma include homogeneous attenuation and irregular peripheral enhancements with gradual centripetal enhancement[15-17]. Intrahepatic duct stones with upper stream duct dilatation are also observed. Sonographic examinations indicate a mostly hyperechoic and heterogeneous mass with an ill-defined margin. Tumors > 3 cm in size are usually hyperechoic, whereas tumors < 3 cm are hypo- or isoechoic[18,19]. In MR images, the mass exhibits hypointensity on T1-weighted imaging and an irregular margin with hyperintensity on T2-weighted imaging. Contrast-enhanced T1-weighted images show peripheral and centripetal enhancement[20-22].

Twelve studies containing 23 cases of lympho-epithelioma-like cholangiocarcinoma were reviewed (7 men, 16 women; mean age, 53.2 y; range, 19-79 y) (Table 1). The incidence is highest among middle-aged people. Chronic hepatitis has been identified as a risk factor for cholangiocarcinoma. In present reviewed study, there were 8 patients with hepatitis B and one patient with hepatitis C. The tumor diameters ranged from 1.7 to 10.0 cm (mean size, 4.7 cm). Larger tumors tended to present with abdominal pain or discomfort (8/23 cases), whereas most of small tumors were discovered incidentally (10/23 cases). Regarding the tumor location, no difference was observed between the right hepatic lobe and the left hepatic lobe. All patients underwent surgical resection for tumor treatment and were pathologically approved. Almost all of the patients were positive for EBER *in situ* hybridization (21/23 cases). In a recent study, 7 cases (all female) of EBV-associated lymphoepithelioma-like cholangiocarcinoma were described with clinical presentations similar to our case[9]. In addition, the results showed that EBV-associated lymphoepithelioma-like cholangiocarcinoma had a favorable overall survival, and was frequently associated with distinctive DNA hypermethylation, which is an important epigenetic mechanism for inactivating tumor suppressor genes involved in tumorigenesis.

The reviewed studies focused on histologic and immunohistochemical findings, and information on imaging features was inadequate. They demonstrated that tumors with hypodense CT images may be either hypovascular or hypervascular. Three small tumors exhibited hypoechoic echogenicity in sonographic examinations. Only two case reports described MRI findings: the typical MRI features (T1 hypointensity, T2 hyperintensity, progressive enhancement pattern) were observed in one case, whereas in the second case, a centrifugal enhancement pattern was observed and the T1 and T2 signals were unknown. In summary, a lymphoepithelioma-like cholangiocarcinoma may present with several typical characteristics of mass-forming cholangiocarcinoma.

Most patients diagnosed with lymphoepithelioma-
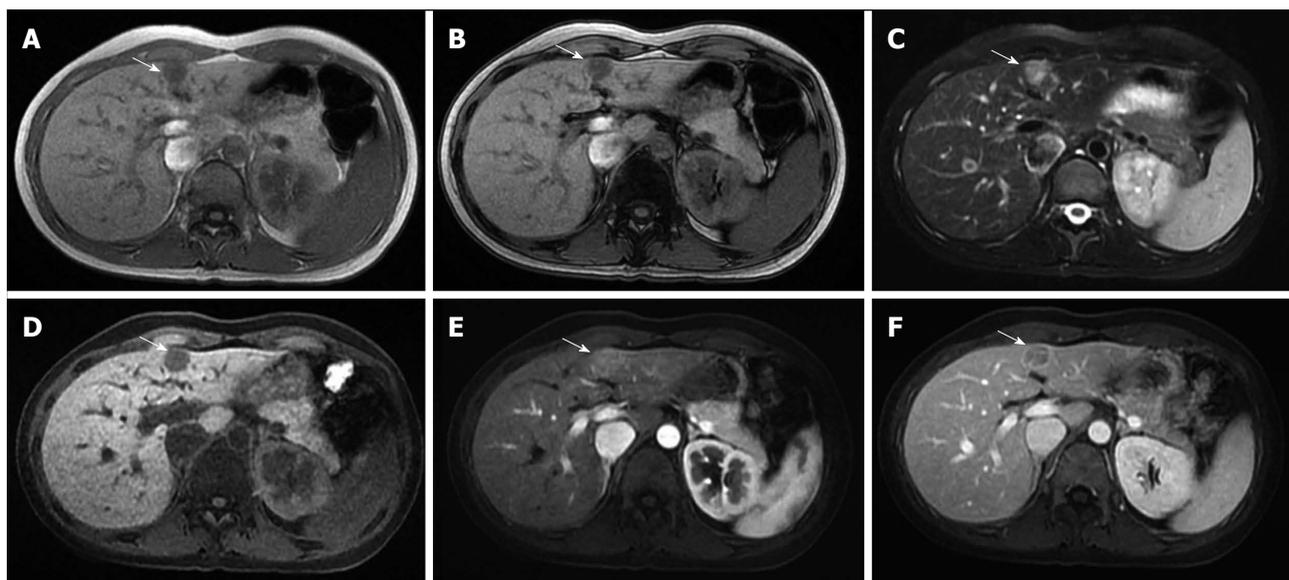
Figure 3 Liver magnetic resonance images demonstrated a small lymphoepithelioma-like cholangiocarcinoma at the medial segment of the liver (arrow). A: In-phase T1-weighted image; B: Out of phase T1-weighted image; C: Axial T2-weighted image with fat suppression; D: Pre-contrast; E: Arterial T1-weighted image; F: Portal venous phase T1-weighted image with fat suppression.
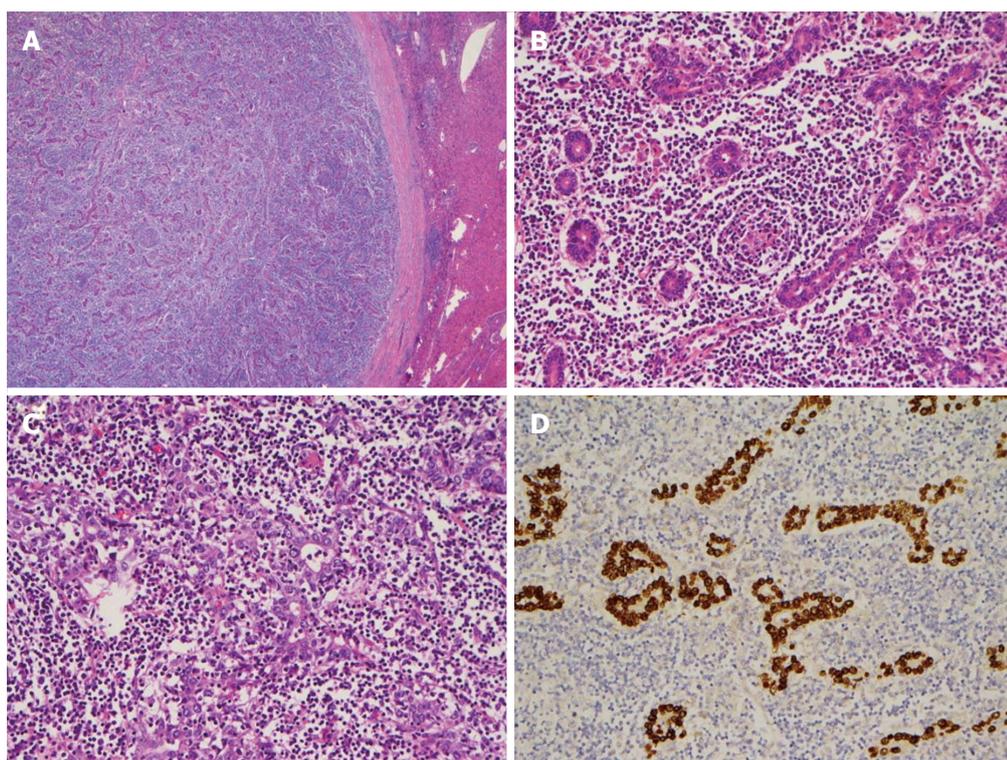

Figure 4 Histochemical findings. A: The tumor is well circumscribed with a thick fibrous capsule (HE stain, × 20); B: The majority of the tumor is composed of well differentiated glandular cells with dense lymphocytic infiltration and scattered lymphoid follicles (HE stain, × 200); high-power view of another area in the tumor; C: The tumor reveals a solid, poorly differentiated growth pattern (HE stain, × 200); D: *In situ* hybridization for Epstein-Barr virus-encoded RNA is diffusely positive in the tumor cells (× 200).

like cholangiocarcinoma had a medical history of chronic hepatitis. Because some intrahepatic tumors contain both elements of cholangiocarcinoma and HCC in the same nodule, the imaging characteristics may overlap[23]. HCC and metastatic tumors should be considered when typical characteristics of mass-

forming cholangiocarcinoma are not observed. Therefore biopsy may be needed for confirmation of the diagnosis before surgery. In addition, [18]fluorodeoxyglucose-positron emission tomography (^{18}F-FDG PET) is of value for the diagnosis and staging of cholangiocarcinoma, and demonstrates

Table 1 Demographics, radiologic manifestations, and treatment of cases of lymphoepithelioma-like cholangiocarcinoma

Ref.	Case	Age (yr)/sex	Site/size (cm)	Hepatitis	EBV	Images	Symptom	Treatment
Hsu *et al*[12]	1	47/F	Left lobe/10.0	Negative	+	Angiography: hypovascular CT: hypovascular	Abdominal fullness	Left lobectomy
Chen *et al*[1]	2	67/F	S8/5.0	C	+	Sonography: mixed echoic Angiography: hypervascular	RUQ pain	Hepatectomy
	3	41/M	S2/3.0	B	+	Angiography: hypervascular	Epigastric pain	Resection
Jeng *et al*[6]	4	47/F	Left lobe/10.0	Unknown	+	CT: hypovascular	Abdominal fullness and a firm epigastric mass	Left lobectomy
	5	42/M	S6 / 3.0	Unknown	+	CT: heterogeneous density	Incidental finding	Segmentectomy
	6	67/F	Lateral segment/3.0	Unknown	+	CT: hypodense	Incidental finding	Left lobectomy
	7	50/M	Left lobe/4.0	B	+	CT: hypodense	Vague epigastric pain	Left lobectomy
	8	50/F	Right lobe/4.0	B	+	Sonography: hypoechoic	Incidental finding	Atypical hepatectomy
Huang *et al*[4]	9	60/F	S5/3.0	B	+	Sonography: hypoechoic CT: hypodense, hypovascular	Incidental finding	Resection
Adachi *et al*[2]	10	64/M	Left lobe/4.0	Unknown	+	CT: heterogeneous density	Fever	Lateral segmentectomy
Ortiz *et al*[11]	11	19/F	Left lobe/5.5	Negative	+	CT: Hypovascular	Abdominal fullness	Left lobectomy
Szekely *et al*[10]	12	60/M	Unknown 6.0	Unknown (non-B, non-C)	-	Sonography: Unknown (no mention)	Incidental finding	Resection
Henderson-Jackson *et al*[3]	13	63/F	Medial segment/3.8 Right lobe/1.6	Unknown	+	CT: hypodense, CT: hypodense, Sonography: hypoechoic	Right flank pain and back pain	Resection Resection
Kim *et al*[7]	14	61/M	S6/2.2	Unknown	+	CT: low density w/o enhancement MR: T1 slightly hypointensity, T2 hyperintensity, progressive enhancement in periphery of the lesion	Unknown	Resection
Lee *et al*[8]	15	79/M	Lateral segment/3.7	B	-	CT: hypervascular	Incidental finding	Left lobectomy
Hur *et al*[5]	16	57/F	S6/2.6	Non-B Non-C	+	MR: centrifugal enhancement	Incidental finding	Segmentectomy
Chan *et al*[9]	17	53/F	Right lobe/1.6	B	+	No report (ultrasound, CT, or MR)	Incidental finding	Resection
	18	40/F	Right lobe/7.5	B	+		Incidental finding	Resection
	19	57/F	Left lobe/7.1	Negative	+		Non-painful vague and abdominal mass	Resection
	20	56/F	Left lobe/6.0	Negative	+		Dyspepsia and reflux symptoms	Resection
	21	59/F	Left lobe/6.0	B	+		Incidental finding	Resection
	22	45/F	Left lobe/3.0	Negative	+		Biliary colic	Resection
	23	57/F	Right lobe/3.0	Negative	+		Incidental finding	Resection
Current study	24	35/F	Medial segment/1.6	B	+	Sonography: hypoechoic CT: hypodense with enhancement MR: peripheral and the centripetal enhancement	Incidental finding	Left lobectomy

EBV: Epstein-Barr virus; F: Female; M: Male; MR: Magnetic resonance; RUQ: Right upper quadrant.

high accuracy for detecting unsuspected distant metastases[24]. However, to the best of our knowledge, the application of this method for discriminating LELC and other histologic types of cholangiocarcinoma has not been studied.

Our patient had a history of chronic hepatitis without jaundice and a normal serum alpha-fetoprotein level. In addition, a typical pattern for HCC (T1 hypointensity, T2 hyperintense, early arterial enhancement, washout on the portal venous phase, and delayed fibrous capsule enhancement) was observed in MR images; therefore, the preoperative diagnosis was HCC.

In conclusion, these previous studies indicated that lymphoepithelioma-like cholangiocarcinoma is a rare variant of cholangiocarcinoma that affects more middle-aged females. This case report and review article is the first study to describe the findings from ultrasound, CT, and MRI. Various atypical patterns of mass-forming cholangiocarcinoma are based on

tumor components. Even though the imaging findings of the liver tumor present a typical pattern of HCC, a lymphoepithelioma-like cholangiocarcinoma still needs to be considered in the differential list in the setting of chronic hepatitis, especially in females with EBV infection. Diagnosing lymphoepithelioma-like cholangiocarcinoma remains a challenge for clinic physicians, surgeons, and radiologists.

COMMENTS

Case characteristics
A 35-year-old woman had a history of chronic hepatitis without jaundice and a normal serum alpha-fetoprotein level.

Clinical diagnosis
A hepatic nodule was incidentally found under surveillance of chronic hepatitis B.

Differential diagnosis
Hepatocellular carcinoma and mass-forming intrahepatic cholangiocarcinoma.

Laboratory diagnosis
The serum alpha-fetoprotein, carbohydrate antigen 19-9, and carcinoembryonic antigen levels were within normal limits.

Imaging diagnosis
The dynamic magnetic resonance imaging (MRI) resembled the typical pattern for hepatocellular carcinoma (T1 hypointensity, T2 hyperintense, early arterial enhancement, washout on the portal venous phase, and delayed fibrous capsule enhancement).

Pathological diagnosis
Histologic examination showed well-circumscribed tumor with a thick fibrous capsule; the majority of the tumor was composed of well-differentiated glandular cells with dense lymphocytic infiltration and scattered lymphoid follicles, a poorly differentiated growth pattern. Tumor cells were positive for Epstein-Barr virus-encoded RNA *in situ* hybridization.

Treatment
A laparoscopic left lobectomy was performed.

Related reports
This case report is the first to describe observations of lymphoepithelioma-like cholangiocarcinoma made using sonography, computed tomography (CT), and MRI.

Term explanation
Lymphoepithelioma-like cholangiocarcinoma is a rare tumor with morphologic features similar to those of undifferentiated nasopharyngeal carcinoma that occurs outside the nasopharynx and is associated with Epstein-Barr virus infection.

Experiences and lessons
Mass-forming intrahepatic cholangiocarcinoma has variable enhancement patterns on dynamic CT and MRI, thus the imaging interpretation should be careful. Biopsy may be needed for confirming the diagnosis if the imaging was not a typical pattern.

Peer-review
The authors reported on the rare imaging characteristics of a lymphoepithelioma-like cholangiocarcinoma and it is of potential interest and relevance.

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CASE REPORT

Segmental small bowel necrosis associated with antiphospholipid syndrome: A case report

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Abstract

Antiphospholipid syndrome is a multi-system disease characterized by the formation of thromboembolic complications and/or pregnancy morbidity, and with persistently increased titers of antiphospholipid antibodies. We report the case of a 50-year-old, previously healthy man who presented with fever and new-onset, dull abdominal pain. A contrast-enhanced computed tomography scan showed segmental small bowel obstruction, for which an emergency laparotomy was performed. Histopathologic examination of resected tissues revealed multiple intestinal and mesenteric thromboses of small vessels. Laboratory tests for serum antiphospholipid (anticardiolipin IgM) and anti-β2-glycoprotein I antibodies were positive. Despite proactive implementation of anticoagulation, steroid, and antibiotic therapies, the patient's condition rapidly deteriorated, and he died 22 d after admission. This case highlights that antiphospholipid syndrome should be suspected in patients with unexplainable ischemic bowel and intestinal necrosis presenting with insidious clinical features that may be secondary to the disease, as early diagnosis is critical to implement timely treatments in order to ameliorate the disease course.

Key words: Anticardiolipin antibodies; Antiphospholipid syndrome; Intestinal necrosis; Mesenteric arteriolar thrombosis; Small bowel obstruction

Core tip: Antiphospholipid syndrome is a multi-organ disease characterized by the presence of thromboembolic complications and/or pregnancy morbidity, and with persistently increased titers of antiphospholipid antibodies. This case report demonstrates that antiphospholipid syndrome should be suspected for cases of unexplainable ischemic bowel and intestinal necrosis with insidious clinical features that may be secondary to the disease, as early diagnosis is critical to amelioration of the disease course.

INTRODUCTION

Antiphospholipid syndrome (APS) is an autoimmune disease characterized by thrombotic microangiopathy, recurrent fetal loss, and moderate thrombocytopenia[1]. APS can affect any organ system, thus the manifestations vary greatly[2]. Hepatic manifestations, such as Budd-Chiari syndrome, small hepatic vein thrombosis, infarction (spleen, pancreas, gallbladder, intestine, *etc*.), ascites, esophageal perforation, and ischemic colitis, are often caused by vascular occlusion[1,3]. However, abdominal manifestations of APS are rare, representing only 1.5% of APS cases[3]. In this report, we describe an unusual case of segmental small bowel necrosis with insidious onset and atypical clinical features that may have been secondary to APS.

CASE REPORT

A 50-year-old, previously healthy man was admitted to our hospital due to fever, dull left lower quadrant pain, and vomiting that had persisted for four days. The fever had first developed three months earlier, reaching 39.0 ℃, and was resolved with antibiotics. The patient worked as a driver and denied alcohol abuse and addiction to drugs, but had a 30-year history of cigarette smoking. He had no family history of autoimmune diseases.

Upon physical examination, the patient had a blood pressure of 210/62 mmHg, 124 beats/min pulse rate, a respiratory rate of 22 breaths/min, and body temperature of 38.6 ℃. The abdomen was soft and mildly tender in the left lower abdomen without organomegaly or abnormal masses. Bowel sounds were hypoactive. There was no malar rash, livedo reticularis, or cardiac murmurs. Laboratory tests showed a leukocyte count of 22.1×10^9/L with 92% neutrophils, 127 g/L hemoglobin, and a platelet count of 52×10^9/L. His liver enzyme levels, creatinine, uric acid, amylase, lipase, electrolytes, coagulation tests, protein C, protein S and Venereal Disease Research Laboratory tests were within normal ranges.

Contrast-enhanced computed tomography (CT) of the abdomen revealed segmental dilated small bowel loops with wall thickening and contrast weakening, which indicated small bowel necrosis (Figure 1). No intestinal perforation or thrombosis was observed in the mesenteric arteries. The patient's working diagnosis was thus considered as segmental small bowel obstruction and necrosis, and an emergency laparotomy was performed. The superior and inferior

Figure 1 Contrast-enhanced computed tomography of the abdomen indicated segmental dilated small bowel loops with wall thickening and contrast weakening.

Figure 2 Gross appearance of resected necrotic small bowel.

mesenteric and celiac arteries appeared normal and pulsatile, however, a 20 cm section of the small bowel that was 60 cm from the ligament of Treitz was entirely necrotic (Figure 2). The necrotic segment was resected, and an end-to-end anastomosis of the small bowel was performed. Histologic examination of resected tissues revealed extensive intestinal and mesenteric mucosal necrosis, congestion, and multiple small vessel thromboses (Figure 3).

The patient was administered piperacillin and tazobactam postoperatively, and remained stable for six days after surgery. The hemogram improved with a leukocyte count of 9.9×10^9/L with 90.3% neutrophils, 103 g/L hemoglobin, and a platelet count of 128×10^9/L. However, the fever returned on day 10 after surgery, and further laboratory tests showed an increased leukocyte count of 27.3×10^9/L with 89.6% neutrophils, 96 g/L hemoglobin, and a platelet count of 121×10^9/L. The antibiotic treatment was supplemented with levofloxacin, and samples were sent to test for lupus anticoagulant and antiphospholipid antibodies. The patient's fever was not reduced despite the addition of imipenem from day 14 through 17 after surgery. Results of the antibody tests indicated elevated IgM-anticardiolipin titers at 54 MPU (normal range: < 5 MPU) and anti-β2-glycoprotein I antibodies at 35 U/mL (positive > 15 U/
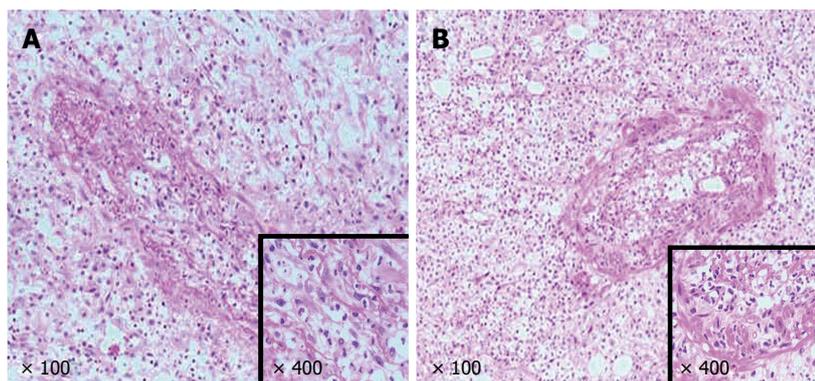
Figure 3 Pathologic findings. Hematoxylin-eosin staining showed necrosis of the mucosae, congestion, and thrombosis of small vessels in the small bowel (A) and mesentery (B).
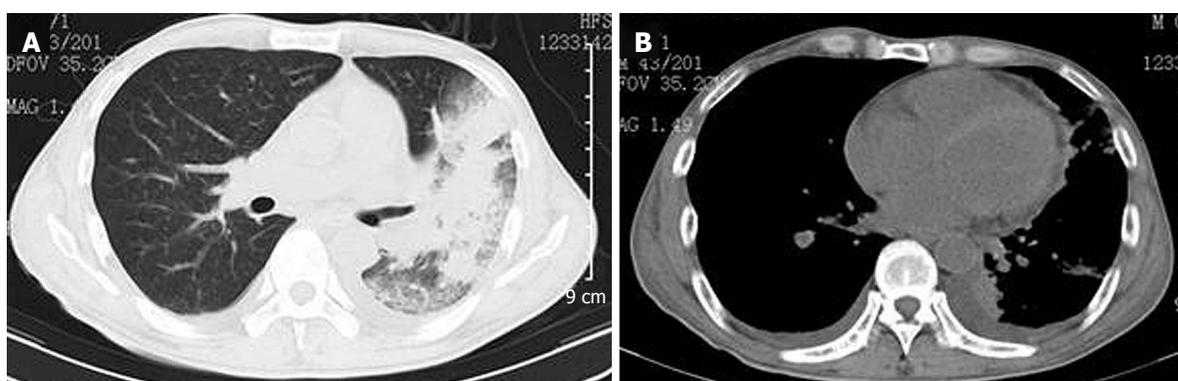

Figure 4 Radiologic findings. Computed tomography showed characteristics of pulmonary infection (A) and hydropericardium (B).

mL), whereas the lupus anticoagulant was within the normal limit. Treatment with intravenous heparin and methylprednisolone was then given. However, on day 19 after the surgery, the patient developed a cough with expectoration, and a CT scan indicated severe pulmonary infection and hydropericardium (Figure 4). At this time, the blood tests showed a leukocyte count of 0.9×10^9/L with 36.2% neutrophils, 52 g/L hemoglobin, and a platelet count of 47×10^9/L; the sputum culture was positive for *Acinetobacter baumannii*. The patient was then transferred to the intensive care unit, but died of respiratory failure on day 22 after the surgery. No post-mortem examination was conducted.

DISCUSSION

The diagnosis for APS is based on the presence of at least one clinical and one laboratory criterion according to the Sapporo statement[4-7], and requires the presence of vascular thrombosis or fetal loss/premature birth associated with a positive lupus anticoagulant or anticardiolipin or anti-β2-glycoprotein I antibodies. For a positive diagnosis, laboratory tests must be verified after at least 12 wk. Catastrophic APS is the most severe form of the disease, for which the diagnostic criteria include multiple-organ system

involvement, aggravation of manifestations within a week, small vessel occlusion in at least one organ or tissue, and presence of antiphospholipid antibodies[8]. As the patient in the case reported here did not survive past 12 wk, a definitive diagnosis of APS could not be obtained. However, the available clinical and laboratory evidence and the rapid progression of the patient's condition indicate APS. Moreover, multiple organs were involved (small bowel, lung, and heart), indicating a fulminant form of APS. A biopsy was not obtained from the lung because of the patient's rapid deterioration.

Bowel involvement is the second most frequently observed condition in APS with abdominal manifestation[9]. Although visceral ischemia occurs from arterial thrombosis in APS, only a few cases of intestinal ischemia have been described in detail[2,10-16]. Consequently, the incidence of ischemic bowel and infarction in APS is likely underestimated. Intestinal ischemia results from reduced flow in the superior and inferior mesenteric and celiac arteries, caused by emboli, atherosclerotic obstruction, thrombosis, or vasospasm. CT is considered a first-line investigation in APS patients with abdominal symptoms[17], which revealed no sign of thrombosis in the large vessels of our patient, but rather segmental small bowel obstruction and necrosis. An emergency laparotomy indicated that the underlying cause for the obstruction

Table 1 Possible etiology for thrombosis

Description	Cause
Ⅰ. Injury of vascular endothelial cells	Mechanical (atherosclerosis), chemical (medication), or biologic (endotoxin)
Ⅱ. Increase in platelet amount and activity	Thrombocytosis, injuries caused by mechanical, chemical, or immune factors
Ⅲ. Increased blood coagulation	Pregnancy, advanced age, trauma, or tumor
Ⅳ. Decreased anticoagulant activity	Decreased antithrombin, protein C, S, or vitamin B12 deficiency, hyperhomocysteinemia
Ⅴ. Decrease of fibrinolytic activities	Abnormality of plasminogen activator release or increased inhibition
Ⅵ. Abnormality of hemorheology	Hyperfibrinogenemia, hyperlipidemia, dehydration, or polycythemia

was thromboses in the small vessels of the intestine and mesentery, which is typical in cases of APS[18,19]. Moreover, other possible etiologies for thrombosis were excluded (Table 1).

Treatment of APS, which includes anticoagulants and corticosteroids, was administered to our patient at an advanced stage, and thus did not provide significant improvement. Recent expert recommendations suggest that plasma exchange should be initiated in patients with catastrophic APS who do not respond well to these treatments[20]. However, our patient's circulatory condition precluded plasma exchange. The insidious onset of thrombosis combined with mild clinical symptoms delayed a diagnosis of APS[12,21], which may have facilitated further progression of vascular occlusion and organ involvement.

The presentation of venous or arterial intestinal thromboses can be non-specific, thus a high index of suspicion is needed for any signs of abdominal involvement in similar cases. In the case presented here, we believe that the dull abdominal pain and fever were due to bowel ischemia that was secondary to APS. Subsequently, the patient developed segmental small bowel necrosis, pulmonary infection, and hydropericardium. This case report highlights that a high level of suspicion for APS is required when patients present with unexplained abdominal pain and fever, as early diagnosis is critical to slow the course of the disease.

COMMENTS

Case characteristics
A 50-year-old, previously healthy man, presented with fever, dull left lower quadrant pain, and vomiting that had persisted for four days.

Clinical diagnosis
The abdomen was soft and mildly tender in the left lower abdomen and bowel sounds were hypoactive.

Differential diagnosis
Appendicitis; gastrointestinal tumor; ileus.

Laboratory diagnosis
WBC, 22.1×10^9/L; hemoglobin, 127 g/L; platelet count, 52×10^9/L; liver enzyme level, creatinine, uric acid, amylase, lipase, electrolytes, coagulation tests and Venereal Disease Research Laboratory tests were within normal range.

Imaging diagnosis
Contrast-enhanced computed tomography of the abdomen indicated segmental dilated small bowel loops with wall thickening and contrast weakening.

Pathological diagnosis
Histologic examination of resected tissues revealed extensive intestinal and mesenteric mucosal necrosis, congestion, and multiple thromboses in the small vessels.

Treatment
The patient was treated with anticoagulation, steroids, and antibiotics (piperacillin/tazobactam, levofloxacin, and imipenem).

Related reports
Gastrointestinal manifestations are observed in only 1.5% of antiphospholipid syndrome (APS) cases.

Term explanation
Acinetobacter baumannii is an emerging nosocomial pathogen that is responsible for infection outbreaks worldwide.

Experiences and lessons
Unexplainable ischemic bowel and intestinal necrosis with insidious clinical features may be secondary to APS, and therefore a high level of suspicion is required, as early diagnosis may ameliorate the course of the disease.

Peer-review
This article suggests that APS should be suspected in cases presenting with unexplained abdominal symptoms, including ischemic bowel and intestinal necrosis.

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LETTERS TO THE EDITOR

Lower folate levels in gastric cancer: Is it a cause or a result?

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Abstract

Folate deficiency and its association with cancer have been studied in the literature, but its clinical impact is still unknown. Folate deficiency and its result on gastric cancer is a mysterious part of oncology, with ongoing studies hopefully clarifying its impact on gastric cancer management. Lee *et al* studied folate deficiency and its impact on staging and clinical results. Here we try to contribute to the field by expressing our own thoughts about the paper.

Core tip: The analysis by Lee *et al* in the paper has some limitations that must be pointed out. The severity of folate deficiency, replacement strategies, and probable vitamin B12 deficiencies should have been discussed. The paper does not do enough to accurately conclude a relationship between folate deficiency and gastric cancer severity.

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TO THE EDITOR

We have read the case control study by Lee *et al*[1] with great interest. Well designed and with good control group selection, the study provided appropriate evaluation of the effects of folate deficiency on gastric cancer. Although the study by Vollset *et al*[2] could not demonstrate folate deficiency as a risk factor for gastric cancer, this paper reminded us of the necessity for further workup.

Folate deficiency as a cause of cancer has been studied before. Its functions in purine metabolism and S-adenosyl methionine production made it an important part of DNA repair and methylation, as well as being necessary for DNA structure stabilization. The importance of low and high levels of folate, which has been demonstrated *via* animal studies, shows the necessity for further clinical studies. However,

folate levels are difficult to interpret in a clinical study. Gastric cancer cases are usually related to *Helicobacter pylori*, pernicious anemia, and atrophic gastritis. These pathologies coexist with vitamin B12 deficiency, which is normally present with folate deficiency; when evaluating folate levels, vitamin B12 must be evaluated as well. Besides gastric premalignant pathologies, gastric cancer patients usually present with dyspepsia and have a history of a few months of decreased oral intake. Irritant vegetables are the most problematic foods for these patients, and so it is not surprising that patients may present with folate deficiency at initial diagnosis. With the increased stage of the disease, more symptomatic patients may present with less oral intake, and as a result, lower folate levels may ensue. So, is folate deficiency a result or a cause of gastric cancer? Unfortunately, it is currently a difficult subject to analyze and, therefore, to form a conclusion on.

Other information not mentioned in the paper was the folate replacement strategy and that medications used during therapies can interfere with folate absorption and metabolism. As concluded in other studies, higher levels of folate can further induce a malignant transformation[3]. Folate replacement strategies and the efficacy of replacement should have been included in the analysis. Drugs that interfere with folate pharmacokinetics are not usually used in gastric cancer therapy, but antiepileptics for platinum-induced neuropathy can decrease absorption of folate. Folate supplementation strategy during therapy may be an important determinant of survival.

Folate and colon cancer association has been widely studied in the literature. Further clinical studies are needed to answer the current questions on the effects of folate metabolism on gastric cancer.

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